Immunity to Parasitic Infection

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Edited by

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Introduction

Immunoparasitology: the making of a modern immunological science

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The field of immunoparasitology has developed from a subspecialty of parasitology into a dynamic immunological discipline with its own unique intellectual territory and conceptual contributions. Much of this evolution has occurred in recent times. Indeed, the word 'immunoparasitology' only came into common usage in the last 40, years appearing in the *Merriam Webster Dictionary* as 'a branch of immunology that deals with animal parasites and their hosts'. It is significant that the lexicographer who provided this definition grasped that immunoparasitology was now in the realm of the immunologist and no longer a discipline practised primarily by parasitologists. In this introductory chapter, I will briefly trace the history of our field and highlight the important influence that research in immunoparasitology has had on modern immunological thought.

Origins

In considering the origins of immunoparasitology, one is immediately confronted with the issue of why the study of parasitology selectively deals with helminths, protozoa and ectoparasites. Although all of these agents were initially classified as eukaryotes, this definition now makes little taxonomic sense, as pathogenic fungi which are also eukaryotes are not referred to as parasites. Moreover, several parasitic unicellular organisms with primitive genomes (e.g. *Giardia* and microporidia) which were formerly thought to be protozoa have either been reclassified as fungi and/or been given the more general designation of 'protists' due to their unclear evolutionary status (see Chapter 2). Clearly, the original classification of protozoa and helminths as parasites was

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a historical invention, which likely reflected not only their unusual taxonomy but also, perhaps, their identity as common and widespread disease agents of the tropics.

Given this artificial taxonomic classification which came into common usage in the 19th century, one can reach back earlier in history for examples of when host resistance phenomena concerning pathogens now known as parasites were first recognised. Human cutaneous leishmaniasis (Chapter 7) is a likely candidate, because it has been known since ancient times that individuals who had healed their skin lesions were protected from further infections. For this reason, Bedouin or some Kurdistani tribal societies traditionally exposed infants to sandfly bites in order to protect them from parasite mediated facial lesions later in life.

Another ancient form of immunisation against leishmaniasis, described in written records from 10th century Persia, is the use of a thorn to transfer infectious material from lesions to uninfected individuals. Ironically, this practice of 'leishmanisation', in which humans are inoculated with live, unattenuated organisms that produce self-healing infections, remains the only truly effective procedure for vaccination against parasitic pathogens, although it is now seldom used (Chapter 25.2). Acquired resistance to 'swamp fever' (malaria) was also recognised by European colonisers of tropical countries, who came to realise their extreme susceptibility relative to that of the indigenous populations. This, too, was an unwitting exercise in immunoparasitological thought.

The era in which most of the major parasitic infections of man were defined (i.e. the second half of the 19th century and the early 20th) coincided with the age in which the role of the immune system in host defence was first elucidated. While others may deserve consideration for the title of first modern immunoparasitologist, Robert Koch, the great German microbiologist, is a prominent candidate (Figure 1). Using newly developed techniques for identifying malaria parasites stained in blood films, Koch compared the frequency and density of parasitaemia in two distinct populations in Java with either high or low endemicity. The conclusion of this cross-sectional analysis was that protection against malaria is acquired only after heavy and repeated exposure to the parasite. These early observations were soon confirmed and extended by other investigators, who described the basic features of naturally acquired immunity to malaria in man, in part through the later use of live malaria infection to treat neurosyphilis. Although recognised earlier than protozoa as human parasites, the existence of acquired immunity to helminths was less obvious and awaited the next phase in the development of the field.

Development of immunoparasitology as an experimental science

As the life cycles of the major parasitic infections of humans and livestock were elucidated, scientists began to question the involvement of the immune response in determining the nature of the host-parasite interaction, as well

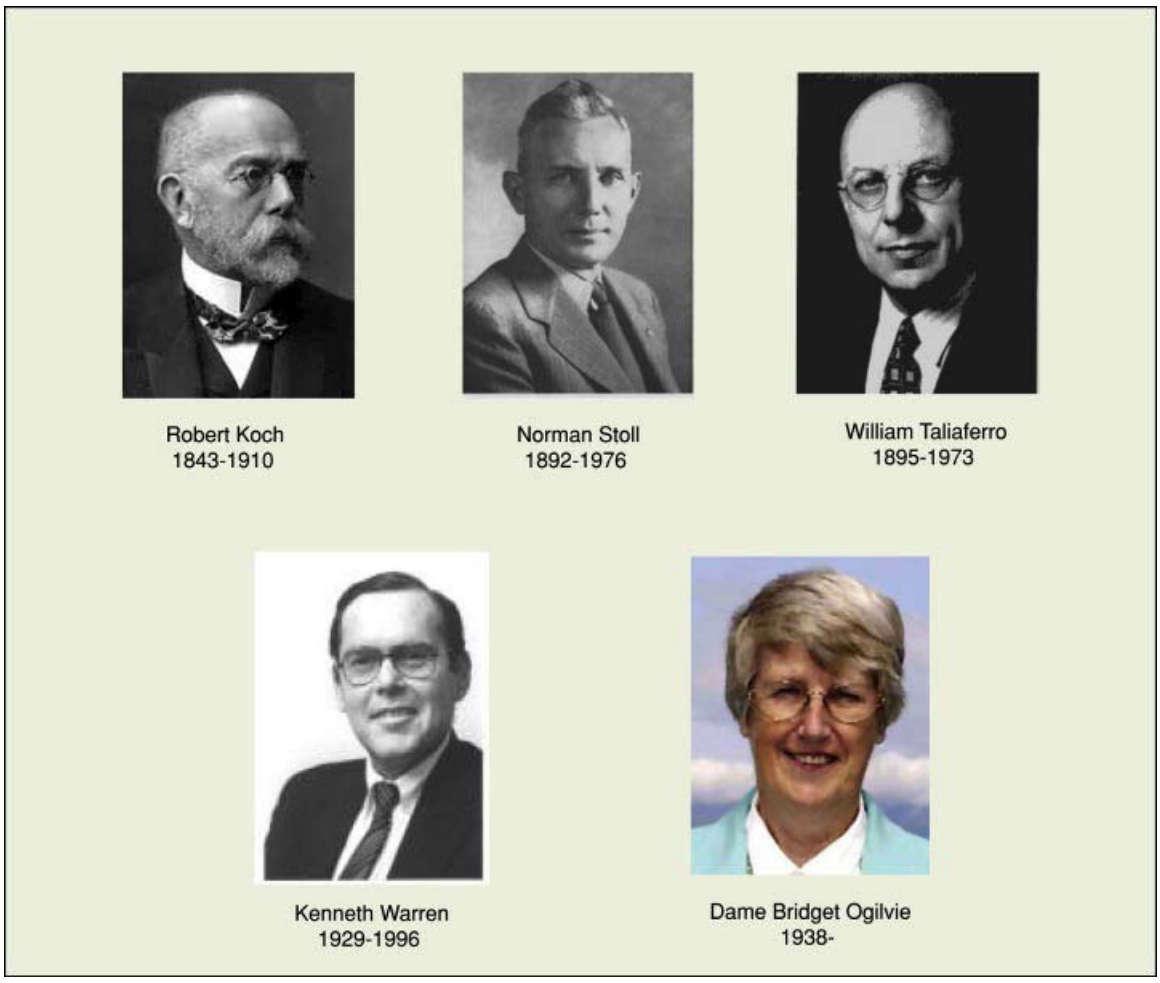

Figure 1 Some pioneers and early champions of the field of immunoparasitology.

as considering the possible use of vaccination as an intervention strategy in parasitic diseases. Two American scientists, Norman Stoll and William Taliaferro, who produced most of their important work in between the two World Wars, were leading figures in this new research effort.

- Stoll (1892–1976), considered by many to be the father of quantitative helminthology, challenged the dogma of his time that argued that worms fail to induce immunity. He did so by documenting the phenomena of self-cure and acquired resistance in farm and experimental animals infected with gastrointestinal helminths, and he went on to perform vaccination trials with parasite extracts.
- Taliaferro (1895–1973) was Stoll's counterpart in protozoan immunology. Together with his wife Lucy, he conducted experimental infections with trypanosomes and malaria in rodents, documenting both acquired immunity and the role of antibodies in protection against parasite variants.

The work of these American pioneers (performed largely at Johns Hopkins, Rockefeller and Princeton) thus demonstrated the existence of immunological resistance to protozoan and helminth infection and revealed the power of animal models in studying the immune response to parasitic pathogens.

The immunological renaissance

The 1960s and 1970s saw an explosion of interest in parasite immunology. This response was fuelled by two important developments of that era. The first was the highly optimistic (and, in retrospect, quite short-sighted) sentiment that most other major infectious disease problems could be contained and even eradicated by the vaccines, drugs, and vector control measures that were being deployed successfully in many settings at that time. This attitude was epitomised in the statement attributed to the then Surgeon General of the United States, William Stewart, that: "It is time to close the book on infectious diseases, and declare the war against pestilence won."

This bold conclusion, however, clearly did not apply to parasitic diseases, which then continued to represent a global scourge, with no vaccines and a limited number of partially effective and often toxic drugs. For this reason, leaders in the infectious disease community advocated new funding initiatives for the development of intervention strategies for parasitic infection, referring to it as the last frontier for the field.

A key figure in this effort was Kenneth Warren, a schistosomiasis researcher, who, at the Rockefeller Foundation, built an influential international network of grantees known as 'The Great Neglected Diseases of Mankind' (or GND for short). Warren also attracted other private foundations (e.g. Edna McConnell Clark, Macy, McArthur) to the field. Warren was himself an immunologist, and many of the investigators he enlisted in the GND programme (e.g. Graham Mitchell, Hans Wigzell, John David, Peter Pearlman, Gus Nossal) were leaders in that field.

The second major development that fuelled the growth of immunoparasitology in the 1970s was the rapid expansion of cellular and molecular immunology that occurred during the same period. The major discoveries made during this era established the cellular basis of the immune response, revealing the fundamentals of immunological specificity and of 'self/non-self discrimination' and providing powerful new tools (e.g. T cell clones and monoclonal antibodies) for identifying antigenic targets of immune reactions.

Of critical importance were those parasitologists who were quick to realise the potential of the 'immunobiological revolution' and to incorporate its new insights and approaches into their own research. In Britain, these scientists included Sydney Cohen and Neal Brown, who established the roles of humoral immunity in malaria and antigenic variation as an immune evasion strategy, and Bridget Ogilvie. The latter was one of the first to investigate the parallels between the immune response to helminths and allergy, and she had enormous vision in seeing the immunological lessons to be learned from studying worm

infections. Later, as Director of the Wellcome Trust, Ogilvie played a key role in promoting the development of modern parasitology research in the UK.

Another important British investigator was James Howard, who, in addition to building a highly innovative parasitology group at the Burroughs Wellcome Research Labs in Beckenham, UK, performed pioneering studies with FY Liew on the function of CD4+ T cells in host resistance and susceptibility to *Leishmania major* in the murine model (Chapter 7). In the USA, Jack Remington played an analogous role in establishing the critical function of cell-mediated immunity in host resistance to *Toxoplasma* infection (Chapter 4). These transitional pioneers helped open up the field of parasitology to modern immunological approaches and laid the groundwork for the major discoveries of the following decades.

Early breakthroughs and disappointments in parasite vaccine development

In addition to the advent of new tools, such as monoclonal antibodies for identifying targets of the immune response, the late 1970s and 1980s saw the development of powerful technology for cloning and expressing pathogen proteins. In the case of parasites, these approaches first came together in the development of protective monoclonal antibodies (mAb), recognising the circumsporozoite protein (CSP) of malaria by Ruth Nussenzweig, and the cloning, sequencing and expression of this major vaccine antigen (Chapter 25.1) by several groups in the early 1980s. This was a major achievement for the field, and it set off a wave of well-funded studies in which the same strategy was used to identify and synthesise potential vaccine immunogens in other major parasitic pathogens. Nevertheless, despite the enormous optimism of these projects, in no case (including malaria CSP itself) was sufficient protection achieved with the recombinant antigens during this era, either in experimental or human trials, to justify further development into operational vaccines.

The widespread failure of this molecularly-based strategy for parasite vaccine development underscored the dearth of information concerning immunological effector mechanisms capable of controlling parasitic infections, and how one administers parasite antigen to specifically trigger them. These important questions had been largely ignored by the molecular vaccine researchers, but were central issues for the new breed of immunoparasitologists studying the cellular immunology of parasitic infection.

Effector mechanisms and effector choice in parasitic infection

The researchers studying the basics of the immune response to parasites had early on focused on the identification of effector mechanisms capable of restricting helminth and protozoan infections. Using *in vitro*-grown parasite stages as targets, both antibodies and/or cells could be demonstrated to mediate pathogen cytotoxicity or growth inhibition.

An important example of such work was a study by Butterworth and colleagues, demonstrating antibody-dependent cytotoxicity against schistosome larvae by human eosinophils (Chapter 16). However, it soon became clear that multiple immune mechanisms were capable of killing parasites or interfering with their growth *in vitro*, and that what was needed was the identification of immune responses capable of controlling parasitic infection *in vivo*.

In early studies, it was recognised that helminths induce immune responses that are quite distinct from those induced by protozoa. Whereas worm infections were associated with elevated levels of IgE, eosinophilia and mastocytosis that are manifestations of immediate-type hypersensitivity, protozoan infections generally lacked these responses and, instead, often displayed cell-mediated immune reactions (e.g. delayed-type hypersensitivity). It was natural to propose that these different immune effectors played important roles in host resistance to the parasites that triggered them.

With the discovery that both immediate-type and delayed-type hypersensitivity responses are mediated by the same T cell subpopulation defined by the presence of the CD4 molecule, the field was presented with an interesting paradox. Moreover, this effector choice dichotomy was not limited to worm versus protozoan infections. Thus, when certain *Leishmania major* strains were used to infect BALB/c and C57BL6 mice, a similar immediate versus delayed type response dichotomy was observed, accompanied by either exacerbation or healing of infection (Chapter 7).

An explanation for this effector dichotomy came with the discovery that CD4+ T helper (Th) cells consist of multiple subsets defined by their cytokine secretion patterns, with Th1 cells producing interferon (IFN)-γ and inducing cell-mediated immunity, while Th2 cells promote immediate hypersensitivity responses through the production of interleukin (IL)-4, IL-5 and IL-13.

Importantly, the immune response dichotomies seen in experimental and human parasitic infection provided the first well-defined demonstrations of the *in vivo* relevance of this concept. Of particular importance was the murine *L. major* model, where healing was linked with Th1 and exacerbation of infection with Th2 induction. At the same time, helminth infection was revealed to be a potent and robustly consistent stimulus for Th2 responses, and IL-4 (and, later, IL-13) were shown to participate in resistance to gastrointestinal nematodes. Thus, work on parasitic infection models contributed enormously to the development of the concept of immunological effector choice.

Parasites define roles for regulatory T cells

An exciting by-product of the work on CD4 T lymphocyte subsets in the *L. major* model was the discovery that Th2 cells promote infection by down-modulating the host protective Th1 response. At that time, the concept that CD4 T cells could regulate immune responses was novel, as CD8+ T cells were thought to be specifically endowed with regulatory ('suppressor') function. Nevertheless, the situations in which Th2 lymphocytes were found to regulate Th1 effector

function *in vivo* in parasitic infection were later shown to be limited to a few models and. in the case of *L. major*, to some – but not all – parasite isolates.

However, with the discovery that CD25+Foxp3 T regulatory (Treg) cells play a major role in dampening immune responses, it became clear that this CD4+ T cell subset has a more generalised function in regulating host resistance to microbes. Once again, many of the pioneering *in vivo* observations supporting this concept were made in parasitic infection models of mice. As discussed below, studies on parasitic infection later led to the discovery that effector cells can, themselves, be induced to display regulatory activity.

Parasites help define the roles of regulatory cytokines and the plasticity of CD4+ T cell subsets

The growing interest in T cell subsets and immune regulation in parasite-infected hosts led naturally to a focus on the cytokine mediators of these functions. Major discoveries were made that helped define the *in vivo* activities of two cytokines in particular: IL-10 and IL-27. Although IL-10 was originally described as a product of Th2 cells, we now know that it can be produced by a variety of T cell subsets, including Tregs, as well as appropriately stimulated myeloid cells. Early work established that IL-10 could dampen immune responses through its down-regulation of antigen presenting cell (principally macrophage and dendritic cell (DC)) effector function.

However, when IL-10 deficient mice were infected with *Toxoplasma gondii*, they became more susceptible, rather than more resistant, to parasite-induced mortality. This was because, in the absence of IL-10, the host mounts an uncontrolled pro-inflammatory cytokine response, resulting in tissue damage and shock. In a contrasting set of studies, treatment with anti-IL-10R antibodies was shown to trigger healing of mice chronically infected with *L. major*. These findings indicated that, while IL-10 is critical in protecting the host against parasite-induced immunopathology, its induction can promote parasite persistence in different settings. Similarly, the discovery of the *in vivo* anti-inflammatory properties of IL-27 (an IL-12 family cytokine that was originally thought to be a Th1-promoting cytokine) came from studies on infection with *Toxoplasma* and other parasites.

While IL-27 is produced largely by myeloid cells, T cells represent one of the major sources of IL-10. As noted above, the suppressive effects of Treg can be attributed to IL-10. However, it was work in protozoan models that established for the first time the role of Th1 cells as a biologically important source of the IL-10 that can suppress both immunopathology in *T. gondii* infection and promote the survival of non-healing strains of *L. major*.

Interestingly, in the former situation, the same IL-10 producing cells can display IFN-γ dependent effector function against the parasite, making them dual effector-regulatory cells. In later work with the *T. gondii* model, the opposite situation was also demonstrated: Treg CD4+ T cells were shown to acquire the ability to produce IFN-γ as an effector cytokine. Thus, work on parasite models

has contributed enormously to our understanding of the plasticity of cytokine expression in CD4+ T cell subsets.

Parasites as triggers of the innate immune response

In approaching the problem of T helper polarisation, immunoparasitologists deduced that events occurring soon after parasite entry must be dictating the subsequent choice of T cell subset induced. These events consist of specific signals delivered by the innate immune system in response to parasite recognition. With the discovery that the cytokines IL-12 and IL-4 potently influence Th1 and Th2 differentiation respectively, it made sense that the selective triggering of one or the other mediator by different parasites would explain their ability to mediate CD4+ T cell polarisation. Indeed, mice deficient in IL-12 or IL-4 showed greatly reduced Th1 or Th2 responses following infection with intracellular protozoa or helminths, respectively.

The above findings prompted a search for the cellular sources of IL-12 and IL-4 in the innate immune system and the receptor on these cells that trigger cytokine production in response to parasite recognition. DCs, macrophages and neutrophils were documented as major sources of the Th1-polarising cytokine IL-12, and Toll-like receptors as the likely NFκ-B trigger of IL-12. However, at the same time, it became clear that, although DCs can promote Th2 responses, this is not the result of their production of IL-4. Instead, more recent work in helminth infection has documented a role for basophils as the source of the IL-4 involved in Th2 differentiation (Chapter 14), although there is debate as to whether the IL-4 derived from this source is actually initiating the Th2 response or merely amplifying a Th2 population already triggered by a signal from another innate cellular source, such as DCs.

The study of helminth Th2 response initiation has also contributed to the recent discovery of novel innate lymphoid populations (collectively designated as ILC2) that produce IL-4 and other Th2 cytokines in response to IL-25 and IL-33 stimulation and participate in worm expulsion. The parasite-host receptor interactions that trigger these innate Th2 signals remain to be defined, and are currently an exciting frontier in this rapidly expanding field.

Lessons from helminth immunology about allergic and fibrotic disease

As noted above, early immunoparasitologists were quick to grasp the commonalities between the immune response to worms and the allergic response and were fascinated with the paradox of why the former is host-beneficial while the latter is host-detrimental. In dissecting the key immunological hallmarks of helminth immunity (IgE, eosinophils and mast cells/basophils), immunoparasitologists have made major contributions to understanding their role in allergic tissue inflammation, along with the cytokines (e.g. IL-10, IL-13, IL-25, IL-33) and the cells (e.g. Treg, Th1) that regulate their function.

The recognition that helminth Th2 responses are carefully modulated by the host and as a consequence trigger minimal pathology has helped promote a version of the 'hygiene hypothesis' that proposes that elimination of helminth infection as a consequence of economic development has led to a worldwide increase in allergic disease (Chapter 23). Indeed, the field of helminth immunology is currently enjoying a resurgence, largely based on the insights it has provided to the role of Th2 responses in tissue sites and the mechanism of their regulation. In this context, the study of the immunopathology induced by schistosome eggs in liver and lung (Chapter 16) has yielded important basic information on Th2-driven granuloma formation and fibrosis. In the case of the latter topic, it has also revealed major roles for IL-13 and other cytokine mediators in the fibrotic process.

Returning from mouse to man

The field of immunoparasitology has contributed to modern immunology largely through discoveries made in murine experimental models. Nevertheless, it is clear that, to be relevant, the concepts that have emerged must be also be valid in humans. Since mechanistic experiments (e.g. the demonstration of passive immunity in malaria by Cohen and colleagues) are feasible only in rare instances, the findings obtained have been gleaned largely from descriptive longitudinal studies on parasitic infection.

However, clever use of ethically acceptable interventional studies, for example those tracking immune responses and reinfection following chemotherapy, have yielded important insights into mechanisms of immunoregulation, host resistance and immunological memory in human parasitic disease. With the advent of powerful new tools, such as multifunctional flow cytometry and gene expression profiling, these studies now offer greatly increased analytical depth and have expanded, particularly in the malaria field, where the animal models for studying human *P. falciparum* and *P. vivax* infections are limited (Chapter 3). The new developments in human cellular immunology have also advanced the interpretation of vaccine trials, providing correlates of both protection and failed protection that can be analysed using systems approaches.

Andrade's challenge and the future of immunoparasitology

At a schistosome vaccine meeting in the 1980s the distinguished Brazilian pathologist, Zilton Andrade made the highly quoted statement: "Schistosomiasis has done more for immunology, than immunology for schistosomiasis." His words were a challenge to the attendees at the conference to translate their knowledge of the immune response directly into vaccine development, rather than merely using parasites to study the immune system. The issue raised by Professor Andrade is complex, since we still lack the understanding required to rationally design effective immunisation strategies protective against complex pathogens.

Milestones in the history of immunoparasitology.

1900	Robert Koch's demonstration of naturally acquired immunity in human malaria.
1920	Basic parameters of acquired immunity in malaria defined in infected populations and neurosyphilis patients receiving 'malariatherapy'.
1924	William Taliaferro describes antibody-mediated control of trypanosome infection in a rat experimental model.
1927	von Wagner-Jauregg awarded Nobel Prize for malariatherapy.
1928	Norman Stoll defines phenomena of self-cure and protection in intestinal nematode infection.
1929	Taliaferro publishes landmark volume, *The immunology of parasitic infections*.
1961	Cohen and McGregor demonstrate passive transfer of resistance to malaria in Gambian children.
1964	Reaginic (IgE) antibodies described in helminth infection by Ogilvie
1965	Discovery of antigenic variation in malaria by Brown and Brown and classic analysis of phenomenon in African trypanosomes by Gray
1967	Nussenzweig demonstrates protection against malaria mediated by irradiated sporozoites
1967	Demonstration of schistosome egg granuloma as manifestation of cell-mediated immunity by Warren, using lung injection model developed by von Lichtenberg (1962).
1975	Eosinophils described as effector cells against schistosome larvae by Butterworth.
1980	Enhanced susceptibility of BALB mice to cutaneous Leishmaniasis demonstrated by Howard and Liew to be form of immunologic suppression mediated by CD4 T cells.
1980	Monoclonal antibodies against circumsporozoite protein (CSP) shown by Nussenzweigs to confer protection against malaria.
1984–1985	Gene encoding malaria CSP cloned and sequenced by McCutchan and Godson.
1986	Th1 and Th2 subsets of CD4+ T lymphocytes identified by Mosmann and Coffman.
1987	Development of RTS,S vaccine initiated at Glaxo Smith Kline and Walter Reed.
1988–9	Opposing roles of Th1 and Th2 subsets in resistance to Leishmania described by Scott and Locksley.
1993–8	Functions of IL-12 and IL-10 in host resistance to parasitic infection elucidated by Gazzinelli, Scott, Wynn and Trinchieri.
1997–8	Initiating roles for dendritic cells in the host response to protozoan infection demonstrated by Reis e Sousa and Kaye.
1999	IL-13 revealed by Wynn to be a major determinant of helminth-induced Th2 tissue pathology and fibrosis.
2002–2007	Demonstration of roles of Treg and IL-10 producing Th1 cells in control of parasitic infection and disease by Belkaid, Sacks and Jankovic.
2003	Function of IL-27 as negative regulator of CD4+ T cell effector function in protozoan infection demonstrated by Hunter.
2010	Innate lymphoid cell subsets discovered as drivers of Th2 responses in helminth infection.
2011	Partial efficacy of RTT,S vaccine against malaria confirmed in large phase three clinical trials.

Nevertheless, through successive refinements to the original concept of the Nussenzweigs of immunisation with CSP, a partially effective malaria vaccine that has now given reproducible results in multiple trials has emerged. This vaccine, known as RTS,S, is a recombinant protein that fuses a part of the *P. falciparum* CSP with the hepatitis B virus surface antigen as a carrier matrix and is administered with a proprietary liposome-based adjuvant system produced

by Glaxo Smith Kline (Chapter 25.1). RTS,S is believed to function through the production of antibodies and T cells that inhibit hepatocyte infection and parasite development, although the contribution of each of these mechanisms is poorly understood. Strikingly, the improving efficacy of RTS,S is very much the product of empirical changes in its formulation, not the malaria Ag construct itself, which was developed over 14 years ago.

The RTS,S story, while a milestone for the field, sends a clear message for future priorities for the field. First, it responds to Andrade's challenge by vindicating the need to understand better the immune effector mechanisms that can act against parasites and how best to induce them – questions that are core issues in cellular immunology. At the same time, it points to the glaring deficiency that we have in immune correlates of protection in humans, as well as the need both for extensive further research and for the development of novel, more powerful tools for carrying out this analysis. Indeed, in the case of malaria vaccination, the RTS,S vaccinees themselves represent a potential goldmine for identifying these correlates within a group uniformly exposed to the same immunogens and adjuvant.

This introductory chapter has chronicled the emergence of immunoparasitology in the latter part of the 20th century, as well its contributions to modern immunology. Host parasite systems will undoubtedly continue to provide key models for the study of immune function and, at the time of writing, they have established themselves as major tools for the study of mucosal immune responses.

While the goal of global vaccines against parasitic pathogens still eludes us, we can now, at last, point to tangible progress. It is a tempting, but perhaps not unreasonable, dream that the young scientists entering this field today may, in their future careers, both directly contribute to and witness the deployment of immunisation campaigns against parasitic diseases.

Acknowledgments

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Section 1

1 Notes on the Immune System

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This chapter provides some background to the immune system, outlining the cells involved in carrying out immune responses, the receptors mediating recognition of foreign antigens (such as those carried by parasitic organisms) and the effector mechanisms activated to destroy parasites and contain infection. This outline is not a comprehensive account of the workings of the immune system; instead, these notes focus on the aspects of the immune system that are most relevant to the chapters which follow on specific parasite infections.

Readers are encouraged to refer to the suggestions for further reading cited at the end of this chapter, or to one of the many comprehensive textbooks published, such as *Janeway's Immunobiology*, for a more detailed account of specific aspects of the immune system.

1.1 The immune system

The body has external physical barriers to prevent infection, such as the skin, the production of sweat containing salt, lysozyme and sebum, and the mucous membranes, which are covered in a layer of mucous that pathogens find hard to penetrate. If these barriers are breached, the body will then mount an immune response and mobilise immune cells to destroy the intruder.

Immune responses are carried out by a variety of different immune cells, all of which initially arise from progenitor stem cells in the bone marrow (Figure 1.1). While most cells mature in the bone marrow, T cells undergo additional development in the thymus. The number of immune cells in the body (homeostasis) is regulated through tight controls on haematopoiesis in the bone marrow, an environment rich in growth factors (such as colony-stimulating factors) and cytokines that support the growth and differentiation of immune cells. The bone marrow and thymus are known as the primary lymphoid organs, because they are the primary sites of immune cell development and maturation.

Immunity to Parasitic Infection, First Edition. Edited by Tracey J. Lamb.

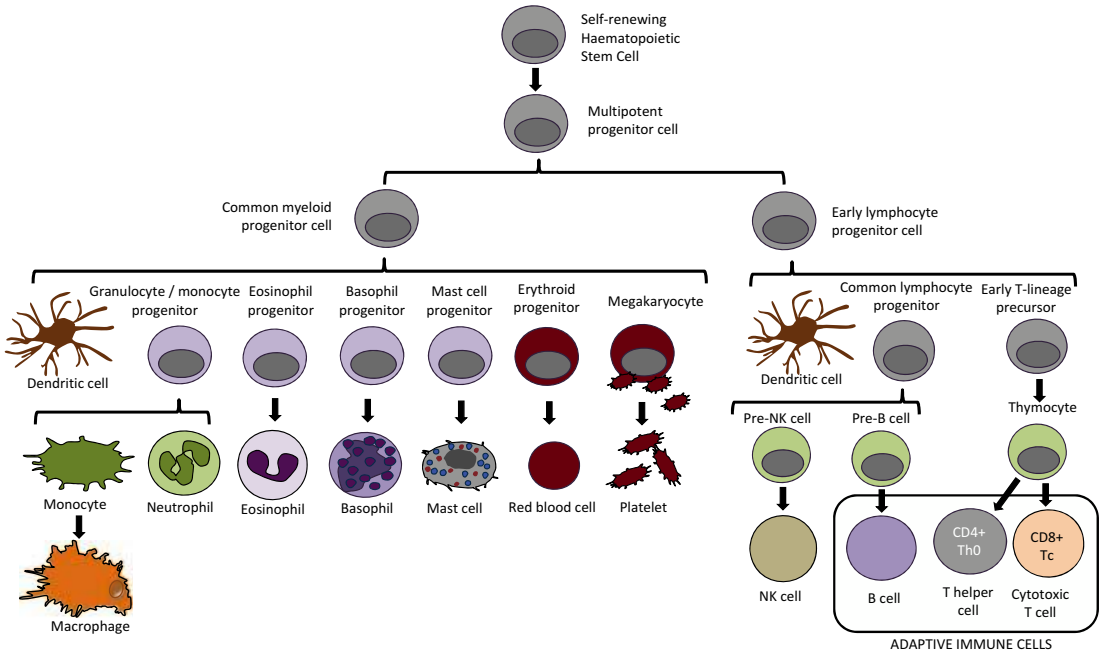


Figure 1.1 Innate and adaptive immune cells of the human body. All cells are derived from self-renewing haematopoietic stem cells in the bone marrow, and they arise from myeloid or lymphoid progenitors. Dendritic cells can develop from both lineages and also differentiate from monocytes (pathway not shown).

Once mature, immune cells exit the bone marrow (or the thymus, in the case of T cells) and take up residence in highly organised structures composed of both immune and non-immune cells, known as the secondary lymphoid organs (Figure 1.2). Although immune responses are initiated at the point where the body's external barrier has been breached, the establishment of an immune

Some of the main lymphoid organs in the human body			
	Organ	Location	Function
SECONDARY	Tonsils	At the back of the throat	Detection of ingested or inhaled pathogens
PRIMARY	Thymus	Above and in front of the heart	Maturation and selection of T cells
SECONDARY	Spleen	Upper left quadrant of the abdomen	Filtration of the blood for invading pathogens
PRIMARY	Bone Marrow	Bone cavities	Main location for haematopoiesis
SECONDARY	Lymph nodes and lymphatics	Widely distributed throughout the body	Detection of invading pathogens at sites distal to other lymphoid organs

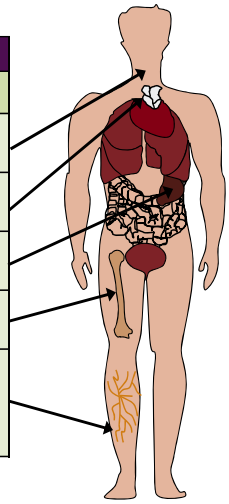


Figure 1.2 Lymphoid organs in the human body. Immune cell development occurs in the primary lymphoid organs, whereas secondary lymphoid organs are the sites where immune responses are coordinated.

Table 1.1 Functions of the innate and adaptive arms of the immune system.

Innate Immune Cells	Adaptive Immune Cells
Immune recognition	
Immune effector mechanisms	
Immune regulation	
	Immunological memory

response – particularly the adaptive arm of the immune response – occurs in the secondary lymphoid organs draining the site of infection.

The immune system has evolved a number of effector mechanisms capable of destroying pathogenic organisms. Immune responses can be classified as innate or adaptive (see Table 1.1). The innate arm of the immune system recognises pathogens non-specifically and generates immediate generic mechanisms of pathogen clearance. The adaptive arm of the immune system is more specific for individual pathogens, and it takes a number of days to develop.

There is a high degree of 'cross-talk' between the innate and adaptive arms of the immune system. In general, an adequate adaptive immune response is only activated after initiation by the cells of the innate immune system; conversely, innate immune effector mechanisms become more efficient by interaction with an active adaptive immune response.

1.2 Innate immune processes

The innate immune system is able to mount an immediate immune response to a foreign pathogen, or to whatever is 'dangerous' to the human body as embodied by Matzinger's 'Danger hypothesis'. Innate immune responses are generic and mounted upon recognition of pathogen-associated molecular patterns (PAMPs) commonly found in molecules that are part of, or produced by, pathogenic organisms. PAMPs are recognised by pattern recognition receptors (PRRs – see Figure 1.3), primarily (but not exclusively) expressed on (and in) phagocytic antigen presenting cells (APCs) such as macrophages, dendritic cells (DCs) and some types of granulocytes. PRRs can also recognise host molecules containing damage-associated molecular patterns (DAMPs) – molecules that are often released from necrotic cells damaged by invading pathogens.

1.2.1 Inflammation

Once recognition of PAMPs or DAMPs occurs, a series of innate immune processes are activated by innate immune cells that contribute to pathogen destruction. 'Inflammation' is a generic term used to describe the dilation and increased permeability of the blood vessels in response to leukotrienes and prostaglandins secreted by phagocytes upon pathogen recognition. Inflammation results in increased blood flow and in the loss of fluid and serum components from capillaries into tissue, as well as the extravasation of white blood cells to the breached area. Superficially, inflammation is responsible for the visible symptoms swelling, pain and redness in infected tissue.

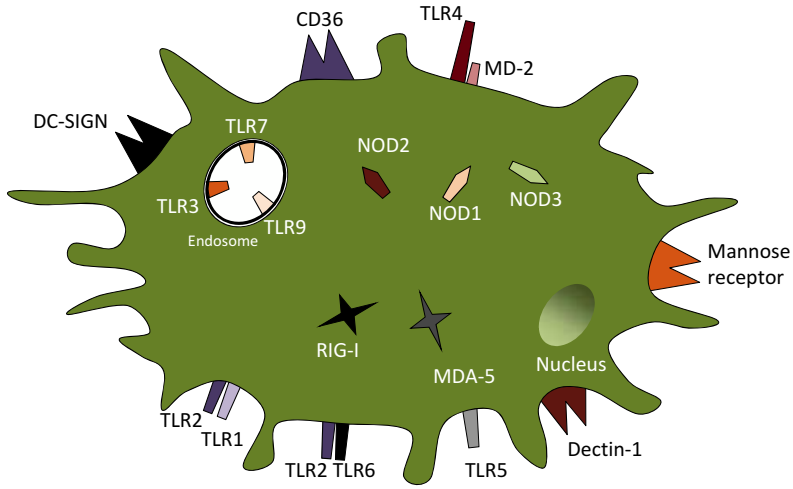


Figure 1.3 Some of the main pattern recognition receptors found on antigen presenting cells.
Abbreviations: DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; MD2, myeloid differentiation factor-2; MDA-5, myeloid differentiation-associated gene-5; NOD, nucleotide-binding oligomerisation domain; RIG-I, retinoic acid inducible gene-I; TLR, Toll-like receptor.

1.2.2 The acute phase response

The acute phase response is initiated by activation of macrophages upon ligation of PRRs with pathogen-associated molecules. This term is used to describe the production of several different proteins which enhance the containment and clearance of invading pathogens.

The production of acute phase cytokines (interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)) are collectively called endogenous pyrogens, because they stimulate the induction of prostaglandin E2, which acts on the hypothalamus to induce fever. Fever is effective in inhibiting the growth of some pathogens and can also enhance the performance of phagocytes. When uncontrolled, however, fever can be damaging to the body.

IL-6 acts on the liver to induce the production of acute phase proteins which include C-reactive protein, serum amyloid protein and mannose binding lectin (MBL). Acute phase proteins opsonise invading pathogens, promoting their phagocytosis and activating the complement pathway to induce pathogen lysis – the latter a particular feature of mannose-binding lectin (MBL) which activates the lectin-pathway of complement (see below).

1.2.3 Anti-microbial peptides

Anti-microbial peptides vary in length from between 12 and 50 amino acids, and are ionically charged molecules (anionically or cationically). In mammals, there are two large families of anti-microbial peptides: defensins and cathelicidins. These peptides can opsonise pathogens, attaching and inserting into the membrane to modify the membrane fluidity and form a pore that lyses and

destroys the pathogen. It has also been suggested that some anti-microbial peptides exert their anti-microbial effects by translocating across the pathogen membrane and inhibiting essential enzymes necessary for nucleic acid and protein synthesis, effectively killing the pathogen by starvation. Anti-microbial peptides are effective against some protozoan pathogens as well as against bacteria.

1.3 The complement cascade

The complement cascade involves several different components activated in sequence leading to the generation of a cytopathic 'membrane attack complex' (MAC). The MAC is a structure that is able to form a pore in the membrane of the invading pathogen, leading to damage and lysis (Figure 1.4). Complement can be activated by three different pathways: the classical pathway, the alternative pathway and the lectin pathway:

- The **classical pathway** is linked to the adaptive arm of the immune system and is activated by antibody recognition of pathogens (specifically IgM or IgG) (Figure 1.4).
- The **alternative pathway** is an innate antibody-independent mechanism activated by a variety of 'danger signals' and spontaneous hydrolysis of C3.
- The **lectin pathway** depends on the binding of MBL to surface proteins of invading pathogens that contain mannose residues.

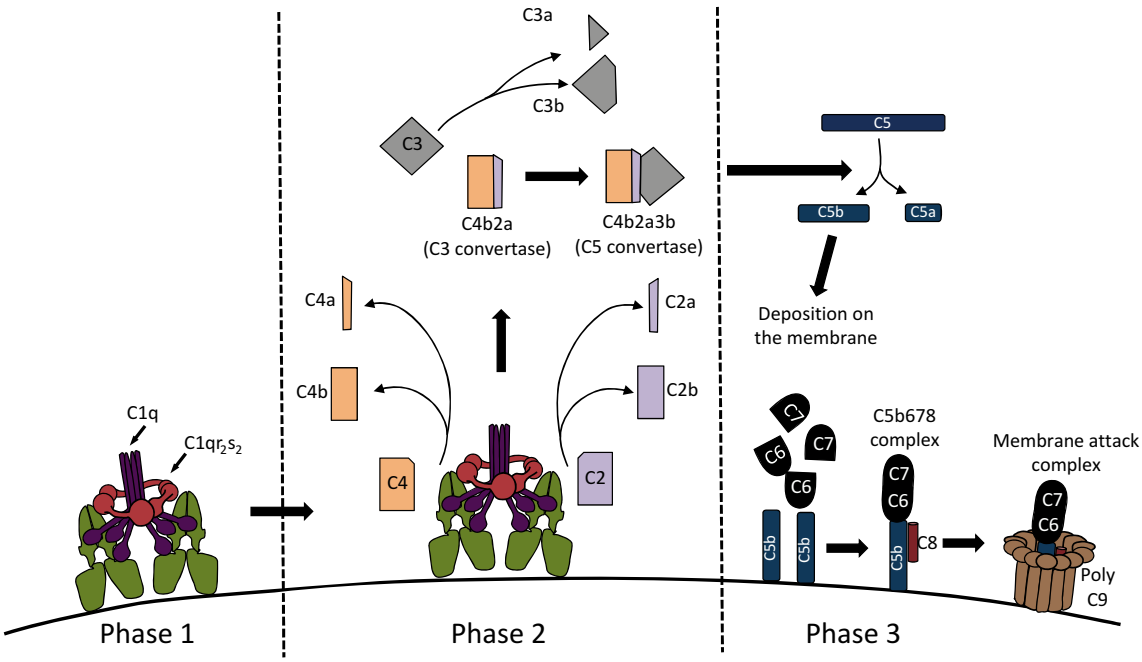


Figure 1.4 The complement cascade. The cascade involves nine components (C1-C9) and can be split into three phases: the first phase involves the attachment of C1 to antibodies opsonising the surface of a pathogen; the second phase leads to cleavage of the C2 and C4 components and the formation of C3 convertase, which in turn cleaves C3 to form C5 convertase; the third phase involves the cleavage of C5 by C5 convertase and the deposition of C5b on the surface of the pathogen. C5b activates the formation of the membrane attack complex (MAC) which creates a pore in the membrane, leading to lysis.

On a molecular level, MBL shares similarity to the complement component C1q, enabling this reaction to occur. Five per cent of the world's population have polymorphisms in the gene encoding MBL which leave people with low levels of MBL. Although the lectin pathway of complement activation has been little studied, it is known to play a role in defence against some protozoan parasites, such as *Cryptosporidium* (see Chapter 5). Although *Cryptosporidium* can activate both the classical and lectin pathways of complement, it is the lectin pathway that is most effective at destroying the parasite.

Upon activation of complement the deposition of C5b on the surface of the invading pathogen leads to lysis via the assembly of the MAC. Other components of complement (in particular C3b) are opsonising agents. Phagocytic cells express complement receptors that can detect pathogens opsonised in complement fragments, promoting phagocytosis and pathogen clearance. The by-products of the cleavage of complement components C3 and C5, C3a and C5a (Figure 1.4) are also called anaphylotoxins, and these are potent inflammatory molecules that induce degranulation of mast cells and basophils, leading to the vasodilatory effects and vascular leakage associated with granule release from these cells.

Unwanted complement activation can be damaging to the host tissues, so it is necessary to regulate the process of complement activation. This regulation is carried out by several soluble and membrane-bound complement regulatory proteins, which regulate different points of activation in the complement pathway. C1 inhibitor (C1-INH) controls activation of complement via the classical and lectin-binding pathways, by associating with the C1 complex and causing the separation of C1r and C1s from C1q (see Figure 1.4). Further down the complement pathway, Factor H is able to hinder the formation of C3 convertase, while carboxypeptidase N inactivates the C3a and C5a fragments from cleavage of C3 and C5 respectively. Membrane-bound complement regulatory proteins include decay-accelerating factor (DAF or CD55), which accelerates the decay of C3 convertases, rendering them ineffective at cleaving C3.

1.4 Innate recognition

Innate immune recognition of pathogens and pathogen-associated molecules occur via several families of pattern recognition receptors (PRRs) (Figure 1.3), as well as receptors that recognise molecules that opsonise pathogens, such as MBL or anaphylotoxins (C3a and C5a). For many pathogens (in particular parasitic organisms), there is no complete picture of PAMP-containing molecules and the PRRs that initiate an innate immune response upon infection. However, it is known that innate immune responses can cross strain-specificity within a species of pathogen and, indeed, also species-specificity, because the patterns recognised by PRRs are often commonly occurring repetitive sequences.

The main PRRs that have been studied with respect to parasitic infection are the Toll-like receptors (TLRs) and some of the C-type lectin receptors, but a role for more recently discovered PRRs cannot be ruled out at present. Although APCs are generally the first type of immune cell to recognise pathogens via PRRs,

many other types of immune and non-immune cells of the body have been found to express PRRs to some extent. The repertoire of PRRs expressed – and, correspondingly, the type of pathogen that can be recognised by individual cell types – varies.

1.5 Pattern recognition receptors

TLRs were first discovered in *Drosophila* fruit flies, and they are thought to be a microbe-detection system conserved widely throughout the animal kingdom. There are now ten described members of this family in humans and twelve in mice. The expression of individual TLRs varies with cell type, and not all TLRs are expressed on the cell surface: some are expressed intracellularly on the endoplasmic reticulum (ER) membrane.

TLRs do not always work individually, but some become activated as dimeric complexes. For example, TLR2 recognises ligands by forming heterodimers with TLR1 or TLR6, and it is also known to act as a co-receptor for the scavenger receptor CD36. Similarly, TLR4 recognises lipopolysaccharide (LPS) when complexed with myeloid differentiation factor 2 (MD2). PAMPs recognised by TLRs are found on a diverse range of molecules, some of which are listed in Table 1.2.

C-type lectins are an important family of PRRs that are likely to play an important, if understudied, role in parasitic diseases. They recognise carbohydrate motifs found on glycoproteins of both protozoan and helminth parasites. Examples of C-type lectins include the mannose receptor, which recognises MBL produced by the liver in response to IL-6 during the acute phase response and Dectin-1, which recognises zymosan.

Other PRRs that have been characterised in viral and bacterial infections and may have some role in parasitic infection, include the cytoplasmic nucleotide-oligomerisation domain (NOD)-like receptor family. The members of this family contain the protein-binding motifs caspase activation and recruitment domain (CARD), a pyrin domain and/or baculovirus inhibitor of apoptosis protein repeat (BIR). Current defined ligands for the NOD receptors include bacterial peptidoglycans and uric acid, an inflammatory by-product that forms upon degradation of hypoxanthine released during shizogeny of malaria-infected red blood cells (see Chapter 3).

1.5.1 Signalling events activated upon ligation of PRRs

Ligation of PRRs with pathogens or pathogen products activates signalling pathways that lead to the transcription of genes encoding products involved in the inflammatory immune response. Signalling pathways emanating from TLR ligation have been particularly well characterised. TLR signalling is mediated by sequential phosphorylation of kinases, brought together via adaptor proteins that bind to the TLR upon activation. The adaptor proteins involved in TLR signalling pathways contain Toll/Interleukin-1 receptor (TIR)-domains. TLR signalling pathways are classified into myeloid differentiation factor of

Table 1.2 Some innate receptors commonly used for recognition of pathogens.

Recognition receptor	Receptor name	Cellular location	Examples of molecules recognised
Toll-like receptors	TLR1	Surface	Triacyl lipoprotein
	TLR2	Surface	Zymosan (glucan from yeast cell wall) GPI anchors (*Plasmodium*, *Trypanosomes*)
	TLR3	Endosomal	Nucleic acids: double stranded RNA
	TLR4	Surface	LPS (gram negative bacteria) Lipoteichoic acid (gram positive bacteria)
	TLR5	Surface	Flagellin
	TLR6	Surface	Diacyl lipoprotein
	TLR7 (human TLR8)	Endosomal	Nucleic acids: single stranded RNA
	TLR9	Endosomal	Nucleic acids: CPG (bacteria) Haemozoin (*Plasmodium*)
	TLR10	Endosomal	Unknown
	TLR11	Surface	Profilin (*Toxoplasma)*
Nod-like receptors NALPS	NOD1	Cytoplasmic	iE-DAP (peptidoglycan component of gram negative bacteria)
	NOD2	Cytoplasmic	MDP (peptidoglycan component of both gram positive and gram negative bacteria)
	NOD3	Cytoplasmic	Uric acid
RIG-I-like receptors	RIG-I	Cytoplasmic	Nucleic acids: short double stranded RNA fragments (<1 kb)
	MDA5	Cytoplasmic	Nucleic acids: long double stranded RNA fragments (>2 kb)
C-type lectin receptors	DC-SIGN	Surface	Mannose-type carbohydrates
	Mannose receptor	Surface	MBL (opsonic protein that binds to carbohydrates of pathogens)
	Dectin-1	Surface	Zymosan (glucan from yeast cell wall)
Co-receptors	CD14	Surface	Co-receptor for LPS
Scavenger receptors	CD36	Surface	Lipids *Pf*EMP-1 (malaria)

Abbreviations: CPG, -C-phosphate-G- DNA; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; GPI, glycosylphosphatidylinositol; iE-DAP, g-D-glutamyl-mesodiaminopimelic acid; LPS, lipopolysaccharide; MBL, mannose-binding lectin; MDA5, melanoma differentiation-associated gene 5; MDP, muramyl dipeptide; NOD, nucleotide-domain oligomerisation domain-containing proteins; *Pf*EMP-1, *Plasmodium falciparum* erythrocyte membrane protein-1; RIG-I, retinoic acid inducible gene-I.

88kD (MyD88)-dependent (all TLRs except TLR3) or TIR-domain-containing adaptor inducing IFN-β (TRIF)-dependent (TLR3 and TLR4).

TLR4 can signal through both MyD88- and TRIF- pathways (Figure 1.5). When MyD88 is recruited to the TLR4 upon ligation, it interacts with IL-1R-associated kinases (IRAKs) – initially IRAK4, then IRAK1 and IRAK2. These then associate with TNFR-associated factor (TRAF)-6, which in turn activates TGF-β-activated kinase 1 (TAK-1) and the transcription of pro-inflammatory genes such as TNF-α (via NF-κB activation) and IL-12 (via MAP kinase activation). Additionally, TLR4 recruits a third adaptor, called TRIF-related adaptor molecule (TRAM), to activate TRIF, which in turn complexes with TRAF3, TANK-binding

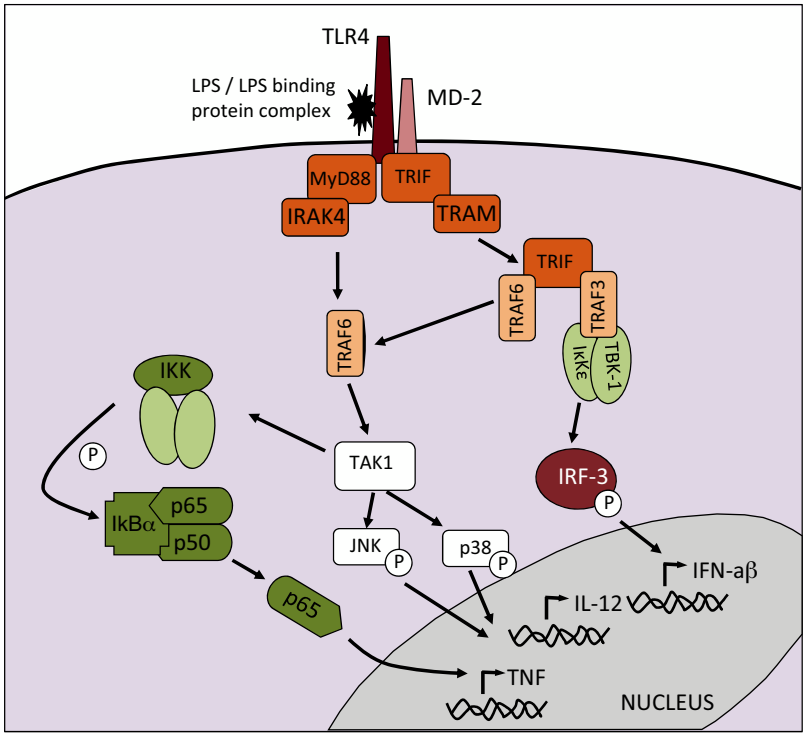


Figure 1.5 Signalling pathways activated by ligation of TLR4. The TLR-4 receptor can signal through both MyD88- and TRIF-dependent pathways to activate the transcription of pro-inflammatory genes in the nucleus.
Abbreviations: IFN, interferon; IKK, IκB kinase; IL, interleukin; IRAK, IL-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response gene (88); TAK, TGF-β-activated kinase; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAM, TRIF-related adaptor molecule; TRAF, TNF receptor-associated factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

kinase (TBK-1) and IKK-ε to facilitate the eventual transcription of genes encoding type 1 interferons (IFN-α and IFN-β – not to be confused with type 2 IFN-γ). Thus ligation of TLR4 leads to the transcription of pro-inflammatory cytokines and the initiation of the innate immune response.

1.6 Innate immune cells

1.6.1 Macrophages

Macrophages are a heterogeneous population of cells that reside in most tissues of the body. They arise from monocyte precursors but are terminally differentiated when resident in a tissue. Tissue-resident cells with macrophage-like properties include alveolar macrophages ('dust cells') in the lungs, Kupffer cells in the liver, histiocytes in the connective tissues and microglial cells in the central nervous system, which may play a role in the pathogenesis of cerebral malaria. The combination of surface markers expressed and the location

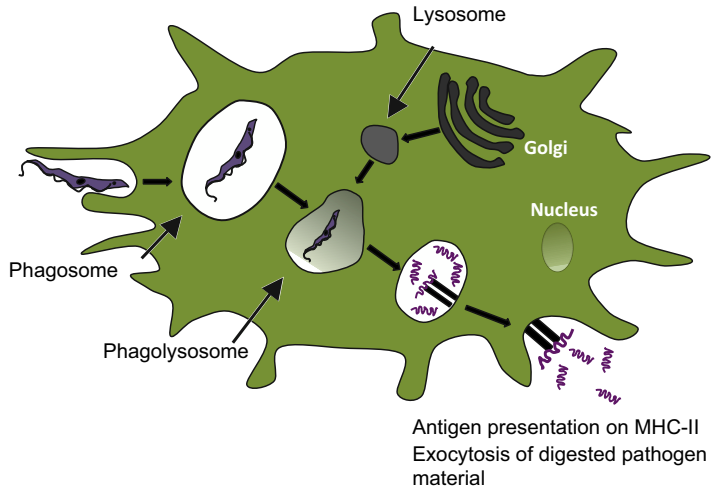


Figure 1.6 Phagocytosis leads to uptake and digestion of extracellular pathogens and their products. The pathogen is engulfed in a phagosome, which then fuses with acidic lysosomes which bud from the Golgi and contain enzymes such as hydrolases for digestion of the endocytosed material. The digested material is expelled from the cell in a process known as exocytosis, but some peptides will be loaded onto major histocompatibility complexes (MHC) for presentation to T cells.

of the macrophages isolated can define the type of macrophages being studied. In mice, some of the generic markers used to define macrophages include F4/80, CD11b and the glycoprotein CD68, the latter found intracellularly in the cytoplasm.

Macrophages are phagocytic cells, and continuously clear senescent erythrocytes and apoptotic cells from the body. The capacity of macrophages to phagocytose, digest and destroy invading pathogens once activated by PRR ligation (Figure 1.6) is aided by a number of different opsonins, notably antibodies, complement fragments and acute phase proteins. Macrophages also play an important immunoregulatory role, both by the secretion of cytokines and chemokines and also as effective APCs that can express peptide-loaded Major Histocompatibility Complex (pMHC) to activate T cells.

The different phenotypes adopted by macrophages is dependent on the molecular cues they receive from the local environment. In parasitic infection, macrophages are often divided into classically activated macrophages (abbreviated to M1 macrophages) and alternatively activated macrophages (abbreviated to M2 macrophages). This division arises after exposure to pro-inflammatory Th1 conditions commonly associated with protozoan infections (M1), or to type 2 inflammation commonly found in helminth infections (M2). M1 and M2 macrophages are quite different, both functionally and on a molecular level (Figure 1.7).

In the context of classical activation, M1 macrophages typically express and up-regulate the receptor for the pro-inflammatory cytokine IFN-γ. They are thus able to respond to the IFN-γ present in a type 1 response. The ability of

Comparison of classically and alternatively activated macrophages

Function	M1 macrophages	M2 macrophages
Cytokine production	TNF, IL-1, IL-6 (acute phase response) IL-8 (neutrophil chemoattractant) IL-12, IL-23 (pro-inflammatory)	IL-4 and IL-13 (down-regulation of type 1 infalmmation)
Distinguishing molecules expressed	iNOS NADPH oxidase IDO	Arignase-1 Chitinase-like enzymes (Ym-1 and Ym-2) RELM-α Upregulated mannose receptor (CD206) and Dectin-1
Phagocytic capacity	Highly phagocytic	Reduced phagocytocytic capacity
Immunoregulatory capacity	Elevated expression of co-stimulatory molecules CD80 and CD86 Able to present antigen to T cells	Up-regulation of MHC-II but poor antigen presentation capacity
Destruction of protozoan parasites	Good producers of toxic nitrogen and oxygen radicals Destruction of intracellular pathogens	Poor at producing toxic nitrogen and oxygen radicals Permissive to infection with *Leishmania* Associated with susceptibility to *Trypanosoma brucei*
Destruction of helminth parasites	Not generally stimulated in helminth infection	Contribute to the immune mechanisms leading to the clearance of parasitic nematodes

Abbreviations: IDO, indoleamine 2,3 dioxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; NADPH, reduced form of nicotineamide adenine dinucleotide phosphate$^+$; TNF, tumour necrosis factor; RELM, resistin-like molecules

Figure 1.7 A comparison of classically activated (M1) and alternatively activated (M2) macrophages.

M1 macrophages to phagocytose protozoan pathogens and digest them is enhanced in response to IFN-γ.

The signalling pathway emanating from the IFN-γ receptor leads to the transcription of a number of IFN-γ-inducible genes that are involved in the destruction of phagocytosed pathogens. These include the up-regulation of enzymes which can generate nitrogen and oxygen derivatives toxic to invading pathogens. This process is known as respiratory burst, and it is mediated by inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO), nicotineamide adenine dinucleotide phosphate (NADPH) oxidase (which generates superoxide) and superoxide dismutase (which generates hydrogen peroxide from superoxide). In M1 macrophages, IFN-γ also up-regulates the expression of indoleamine 2,3-deaminase (IDO), a rate-limiting enzyme in the kynurenine pathway for the degradation of tryptophan, an amino acid essential for the growth of many intracellular pathogens.

M1 macrophages are prolific producers of pro-inflammatory cytokines, including those associated with the acute phase response (TNF, IL-1 and IL-6) and

the neutrophil chemoattractant IL-8. Upon secretion of IL-8, phagocytic neutrophils migrate into the infected tissue and help to clear infection. The secretion of IL-12 by activated M1 macrophages also amplifies the Th1 response due to the polarising effect of this cytokine on CD4+ T cells (see below).

Alternatively activated M2 macrophages are generated in type 2 inflammatory environments and become polarised in response to IL-4. In helminth infections, IL-4 produced by basophils and mast cells in response to chitin (a polymeric component of the body of helminth parasites) may contribute to the development of M2 macrophages. Other type 2 cytokines, such as IL-13 and IL-21, can also polarise macrophages towards an M2 phenotype. Ym-1 (also called chitinase3-like3) and Ym-2 are chitinase-like enzymes secreted by M2 macrophages, but they do not possess chitinase activity. Other molecules associated with an M2 phenotype include increased surface expression of C-type lectins (in particular the mannose receptor and dectin-1 (Table 1.2)) and the expression of resistin-like molecule-α (RELM-α), the latter produced in response to IL-13.

M2 macrophages also produce arginase-1 (Arg1), an enzyme which catalyses the amino acid arginine to ornithine. Since ornithine is a precursor of collagen, a constituent of the extracellular matrix, it has been hypothesised that M2 macrophages may facilitate repair of tissue mechanically damaged by helminths. When uncontrolled, however, excessive deposition of extracellular matrix may lead to fibrosis, a condition that occurs in the liver in the context of strong Th2 responses to trapped Schistosome eggs (Chapter 16). Although associated with helminth infections, M2 macrophages can also be found in protozoan infections. In *Leishmania* (Chapter 7) and trypanosome (Chapter 8) infections, they are associated with susceptibility to infection.

1.6.2 Granulocytes

Granulocytes are composed of a granulated cytoplasm containing granules rich in immunomodulatory molecules. There are four types of granulocytes in the body: neutrophils, eosinophils, basophils and mast cells. With the exception of mast cells, which are largely confined to the tissues (particularly in the gut), granulocytes can be found in the peripheral blood circulation. They form an important part of the body's defence against helminth parasites although, when activated by innocuous antigens, they are responsible for hypersensitivity reactions such as allergic responses (see below).

1.6.2.1 Neutrophils

Neutrophils are the most abundant type of granulocyte in the bloodstream. They are also called polymorphonuclear cells (PMNs), due to their characteristic multi-lobed nucleus. Neutrophil granules stain with both acidic and basic dyes, and they contain a variety of lytic enzymes. Primary granules (azurophilic) contain peroxidase, elastase, lysozyme and hydrolytic enzymes, whereas secondary granules contain collagenase and lysozyme. The bone marrow can release an increased number of neutrophils in response to infection, leading to a transient neutrophil leukocytosis.

Neutrophils are generally one of the first cell types recruited to an area of acute inflammation. They are attracted by a number of chemotactic factors, including IL-8 (also called KC) and leukotrienes secreted by macrophages, and anaphylotoxins derived from the complement cascade. Like macrophages, neutrophils are proficient phagocytic cells. Upon activation, neutrophils degranulate; the release of substances within the neutrophil granules is generally toxic to invading pathogens. Neutrophils can also release neutrophil-extracellular traps (NETs), which are extracellular fibres composed of DNA that can bind to pathogens and more effectively target the delivery of granule contents.

1.6.2.2 Eosinophils

Like neutrophils, eosinophils are motile phagocytic cells that can migrate into the tissue in response to inflammatory stimuli. The granules of eosinophils stain with the acidic dye eosin red ('eosin-ophil'), and contain potent immune mediators such as eosinophil cationic protein, major basic protein and eosinophil peroxidase. Eosinophils express the high-affinity receptor for IgE, FcεRI, and cross-linking of FcεRI by IgE complexed with multivalent antigen leads to eosinophil activation. Eosinophils have long been recognised for their role as effector cells in the anti-helminthic immune response, and releasing the granule contents on the surface of macroparasites such as Schistosomes can damage their surface coat (see Chapter 16).

1.6.2.3 Mast cells

Mast cells are tissue-resident and often defined by their location, either as mucosal mast cells (gastrointestinal tract and lung) or connective tissue mast cells (all other tissues). Mast cells are important mediators of allergic immune responses by virtue of the presence of vasodilator histamine in the granules. Histamine works in concert with other granule substances such as serotonin, prostaglandins and leukotrienes, all of which increase vascular permeability, vasodilation and smooth muscle contraction in the area of release. In addition, mast cell granules are full of chemotactic factors for neutrophils and eosinophils, recruiting these cell types the site of activation.

Mast cells can degranulate in response to binding of anaphylotoxins (the complement fragments C3a and C5a). Like eosinophils, mast cells are also activated by cross-linking of the high affinity receptor for IgE (FcεRI) by IgE/antigen complexes. In addition, mast cells also express FcγRIII, a receptor which can bind to IgG/antigen complexes.

1.6.2.4 Basophils

Mast cells are not the only granulocytes involved in hypersensitivity reactions such as allergic immune responses. Although the least common type of granulocyte in the body, basophils share some similarities with mast cells and are also important contributors to hypersensitivity reactions. The granules of basophils stain with basic dyes ('bas-ophil') and also contain histamine. Although they have previously been considered to be non-phagocytic, this view has been challenged by recent evidence in models of helminth infection (Chapter 14) and in allergy; it is now thought that basophils are important APCs, particularly in the

polarisation of CD4+ T cells towards a Th2 phenotype. Basophils are thought to contain stores of pre-formed IL-4 and they may be an important early source of this cytokine during priming of T cells.

Like mast cells, basophils can express FcεRI (which binds to IgE with high affinity) and the IgG receptors FcγRIII (CD16) and FcγRII (CD32) that bind to IgG. However, although cross-linking of FcεRI by IgE/antigen complexes leads to degranulation, the events following IgG binding on basophils is still unclear, since this can lead to an inhibitory rather than a stimulatory effect. Basophils also harbour a receptor for IgD, and ligation by cross-linked IgD can lead to the release of IL-4. Similar to mast cells, basophils can also degranulate in an antibody-independent manner in response to the binding of anaphylotoxins.

1.6.3 Dendritic cells

Dendritic cells (DCs) are key cells that link the innate and adaptive arms of the immune system. They take their name from the numerous extensions, or 'dendrites', that they possess. Like macrophages, DCs can differentiate from monocytes, and they have the ability to recognise PAMPs on pathogens and pathogen-associated molecules via the expression of PRRs. DCs are 'professional' APCs, whose main function is to process and present antigens to naïve and memory T cells. They provide activating signals such as co-stimulation and secrete cytokines to help shape adaptive immune responses and induce the expansion of clonal polarised CD4+ T cells.

DCs are defined by their expression of the integrin CD11c, but they also express an array of other surface markers. The heterogeneity within DCs has led to the description of a number of different sub-populations of DCs. Myeloid DCs are derived from the myeloid lineage during haematopoiesis (Figure 1.1). In mice, myeloid DCs can be separated by the presence or absence of the expression of the CD8 α-chain molecule.

In humans, DCs under study are normally derived from the peripheral blood. Markers used to define subsets within the myeloid lineage include expression of the FcγRIII (CD16) and the blood DC antigens (BDCA) BDCA1 and BDCA3. No subpopulation of human DCs expressing CD8 has yet been identified. However, DC subsets expressing low and high levels of the integrin CD11b appear to correspond functionally to mouse CD8+ and CD8-DCs respectively.

Three other types of DC that may be of relevance to parasitic infection include CD103+DCs, Langerhans cells and plasmacytoid DCs. CD103+ expressing DCs are concentrated in the mucosal areas of the body, such as the respiratory tract and the intestine. CD103+DCs have been shown to produce the immunoregulatory cytokine transforming growth factor (TGF)-β, and they have the capability of expanding T regulatory (Treg) cells; therefore, they are sometimes referred to as 'regulatory DCs'.

Langerhans cells are specialised DCs that reside in the skin, and are defined by the expression of the C-type lectin Langerin (CD207). They are an important DC subset involved in processing and presenting antigen from the epidermis.

Plasmacytoid DCs are an important source of type I interferons (IFN-α and IFN-β) in viral infection. Morphologically, they look similar to plasma cells (antibody secreting B cells), but their capacity to present antigen led to their designation as a DC subset. Plasmacytoid DCs may contribute to the initiation of adaptive immune responses in some protozoan infections.

When DCs are tissue resident, they take up antigen from the extracellular environment via the processes of macropinocytosis and phagocytosis. Prior to activation, immature DCs express few MHC or co-stimulatory molecules on their surface, and correspondingly they have poor capacity to stimulate T cells. However, upon recognition of antigen via PRRs or receptors recognising opsonic molecules (e.g. antibody/Fc receptors or complement/complement receptors), DCs become activated (or 'mature'). Mature DCs are often found in lymphoid tissue; they have a low capacity for antigen uptake, but express high levels of peptide-loaded MHC and co-stimulatory molecules, and they have a high capacity for stimulating T cells.

The ability of different DC subsets to activate and polarise T cells is not identical. In mice, CD8+DCs and CD8–DCs have differing roles in T cell activation. CD8+DCs and CD103+DCs (but not CD8–DCs) can cross-present antigen (see below). Furthermore, some studies have observed differences in the type of CD4+ T cells expanded by different types of DC. While CD103+ 'regulatory' DCs have a tendency to expand Tregs, Th1 cells appear to be preferentially expanded by CD8+DCs; CD4+ T cells activated by CD8-DCs are more polarised towards a Th2 phenotype. In part, this could be due to the propensity of CD8+ (but not CD8–) DCs to secrete high levels of IL-12p70 upon activation, a critical cytokine involved in polarisation of CD4+ T cells to a Th1 phenotype.

1.6.4 Natural killer (NK) cells

Natural killer cells can be found in both lymphoid and non-lymphoid tissues throughout the body. As suggested by the name, NK cells are cytotoxic and are able to mediate lysis of infected cells by the targeted release of granules containing perforin and granzymes. In addition to a direct cytotoxic role, NK cells have a high immunomodulatory capacity, and they secrete cytokines to skew the immune response when activated. In some protozoan infections, they are considered to be an important innate source of IFN-γ facilitating up-regulation of the IL-12 receptor on naïve CD4+ T cells during priming, in turn permitting responsiveness to APC-secreted IL-12 and the expansion of Th1 cells.

In addition to the secretion of cytokines, activated NK cells also secrete chemokines such as macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4) and Regulated upon Activation, Normal T cell Expressed (RANTES/CCL5). This attracts other immune cells to the infected tissue and helps to focus immune defence mechanisms at the site of infection.

NK cells can be activated in response to ligation of receptors for the macrophage-derived cytokines IL-12 and IL-18. However, degranulation of NK cells is not indiscriminate, as this would undoubtedly result in extensive tissue damage. The identification and targeted lysis of infected cells by activated NK

cells is complicated by the fact that NK cells do not express a clonotypic receptor. Instead, infected target cells are recognised by NK cells via a cumulation of signals derived from multiple inhibitory and activatory receptors on the NK cell surface.

In general, NK cells become activated when activatory signals (ligation of receptors bearing immunoreceptor tyrosine-based activation motif (ITAM)) outweigh inhibitory signals (ligation of receptors bearing immunoreceptor tyrosine-based inhibitory motif (ITIM)). Antibody opsonisation of pathogens can facilitate the activation of NK cells via the ligation of FcγRIII, an activatory receptor on the NK cell surface (Figure 1.8 A). This leads to antibody-dependent cytotoxicity (ADCC) and lysis of the opsonised cell or parasite. In a similar way, ligation of other activatory receptors expressed on NK cells during infection may activate NK cells, although ligands recognised in parasitic infections have not been fully elucidated.

NK cells can also become activated by the lack of 'self' molecules, such as MHC-I, expressed on the surface of infected cells. In some infections with

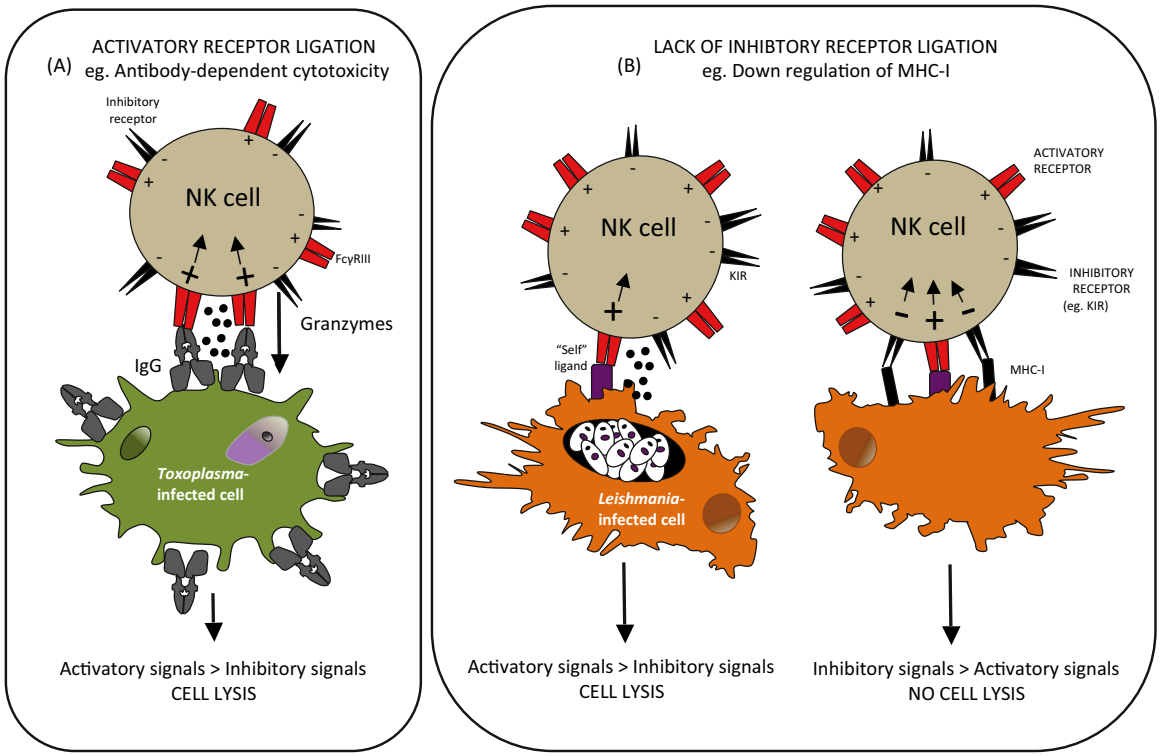


Figure 1.8 Activation of NK cells. Activation of NK cells occurs when activatory signals outweigh inhibitory signals. This can occur by the specific ligation of activatory receptors not normally activated, such as ligation of FcγRIII by IgG on an opsonised pathogen (A) or when inhibitory receptors, such as those normally ligated by MHC-I expressed on most nucleated cells, do not receive signals (B). This latter can occur in situations such as infection with intracellular pathogens that down-regulate MHC-I expression to avoid immune detection.
Abbreviations: Ig, immunoglobulin; KIR, natural killer cell immunoglobulin-like receptor; NK, natural killer; MHC, major histocompatibility complex.

intracellular pathogens, the expression of MHC-I is down-regulated on the surface of infected cells (in parasitic infection, one example is *Leishmania* infection in macrophages). Some of the inhibitory receptors expressed on NK cells (for example some of the natural killer cell immunoglobulin-like receptors (KIRs)) ligate with MHC-I molecules, delivering inhibitory signals that prevent activation of NK cells. The failure to ligate these inhibitory molecules delivers insufficient inhibitory signals to the NK cell, and the NK cells become activated as a result, degranulating to cause lysis of infected cells (Figure 1.8B).

The efficiency of NK cells in the immune response can be enhanced by interactions with phagocytic cells such as DCs and macrophages. These interactions are stabilised by the ligation of integrins such as CD11a/18 (leukocyte functioning antigen, LFA-1), CD11b/CD18 and CD11c/18 expressed on the surface of the NK cell, which can bind to cellular adhesion molecules (ICAMs) expressed on DCs and macrophages.

1.7 Communication in the immune system

Immune cells secrete and respond to a network of proteins known as cytokines. Some of the main cytokines involved in parasitic infection are shown in Table 1.3. The ability to respond to any particular cytokine is determined by the expression of cytokine receptors that can initiate signalling pathways to activate gene transcription in the nucleus. The Janus kinase (Jak)-signal transducer and activator of transcription (STAT) signalling system is a common pathway used to transmit signals from cytokine receptors to the nucleus. This system consists of several autophosphorylating Jak molecules that can phosphorylate the cytoplasmic tails of cytokine receptors. This, in turn, allows the binding of different combinations of STATs, which dimerise and translocate into the nucleus, where they switch on gene transcription.

Many cytokine receptors are dimeric, and the chains making up some of the cytokine receptors are promiscuous. For example, the common γ chain (CD132) is shared by a number of cytokine receptors (notably the receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21), and the IL-4R chain (IL-4Rα) pairs with IL-α13R to convey signals in response to IL-13.

1.8 Adaptive immunity

The adaptive immune response differs from the innate immune response because it has specificity and the ability to form immunological memory. Specificity in the immune system is mediated by antigen-specific antibodies and clonotypic receptors: the T cell receptor (TCR) on T cells and the B cell receptor (surface-bound antibody) on B cells. 'Adaptive immunity' is therefore a term used to describe immune responses carried out by T cells (both CD4+ T helper cells and CD8+ cytotoxic T cells) and B cells.

Antibodies/BCRs and TCRs have variable regions which dictate the differences in binding to specific antigen sequences, known as epitopes. Antibodies and BCRs can recognise epitopes on proteins from tertiary or linear structures of

Table 1.3 Some of the main cytokines involved in parasitic infections.

Cytokine	Main sources	Main functions
IL-1	Macrophages; Epithelial cells	Pro-inflammatory; macrophage activation; acute phase response; induction of fever.
IL-2	T cells	T cell proliferation; clonal expansion of T cells.
IL-3	T cells	Differentiation of basophils from progenitor cells.
IL-4	Basophils; Mast cells; Th2 cells; B cells	Polarisation of Th2 cells; proliferation factor for B cells; isotype switching to IgE; development of M2 macrophages.
IL-5	Th2 cells; Mast cells	Differentiation and expansion of eosinophils; isotype switching in B cells to IgA.
IL-6	Macrophages; Endothelial cells; Th2 cells	Acute phase response; induction of fever; growth and differentiation of adaptive immune cells.
IL-8	Macrophages	Chemoattractant for neutrophils.
IL-9	Th2 cells Th9 cells	Expansion and recruitment of mast cells.
IL-10	Macrophages Dendritic cells T cells B cells	Immunoregulatory cytokine that dampens most immune responses.
IL-12	Dendritic cells; Macrophages	Bioactive IL-12p70 composed of a p35 and a p40 subunit is pro-inflammatory; monomeric or dimeric p40 subunits inhibitory to IL-12R signalling; IL-12p70 is one of the main inducers of Th1 cells; activation of NK cells.
IL-13	Th2 cells	Mucous production; promotion of tissue fibrosis; development of M2 macrophages; antagonism of Th1 responses; promotion of eosinophil reactivity and airway hyper-responsiveness in allergic asthma; isotype switching to IgE in human B cells.
IL-15	Macrophages	Proliferation of NK cells; promotion of CD8+ memory T cell survival.
IL-17	Th17 cells $\gamma\delta$ T cells	Promotion of neutrophilic immune responses.
IL-18	Macrophages	Activation of NK cells; production of IFN-γ by NK cells; promotion of Th1 responses.
IL-21	Th2 cells Th17 cells NKT cells	Promotion CD8+ cytotoxic T cell responses; suppression of IgE production; down-regulation of IgE-mediated allergic responses; isotype switching of human B cells to IgG1 and IgG3.
IL-22	Activated NK cells; Human Th22 cells	Protects tissues from damage; enhances the innate immune responsiveness and regeneration of non-haematopoeitic cells such as epithelial cells and keratinocytes.
IL-23	Dendritic cells	Composed of the p40 subunit of IL-12 and a p19 subunit; polarisation of CD4+ T cells to Th17.

Table 1.3 (*Continued*)

Cytokine	Main sources	Main functions
IL-25	Th2 cells; Mast cells; Epithelial cells	Induces the production of Th2-associated cytokines; stimulates the expansion of eosinophils via Th2 cell induction.
IL-27	Dendritic cells; Macrophages	Heterodimer composed of two subunits: Epstein-Barr induced gene 3 (EBI3) and IL-27 p28; synergises with IL-12 to promote expansion of Th1 cells; suppresses the development of Th17 cells.
IL-33	Epithelial cells Endothelial cells Mast cells	Promotes Th2 cytokine release from granulocytes and Th2 cells.
IFN-γ	NK cells Th1 cells; CD8+ T cells	Increase expression of vascular adhesion molecules for cell trafficking; increase the efficiency of phagocytes; promotion of NK cell activity; isotype switching of mouse B cells to IgG2a.
TNF	Dendritic cells; Macrophages; NK cells Th1 cells	Formally TNF-α (TNF-β now lymphotoxin); induced as part of the acute phase response; pro-inflammatory cytokine that can induce cachexia and temperature dysregulation; increase expression of vascular adhesion molecules for cell trafficking.
TGF-β	Dendritic cells; Macrophages, Treg cells	Immunoregulatory cytokine that can dampen most immune responses; isotype switching of B cells to IgA.
TSLP	Fibroblasts; Epithelial cells Stromal cells	Activation of Langerhans cells; maturation of CD11c+ myeloid DCs.

Abbreviations: IFN, interferon; Ig, immunoglobulin; IL, interleukin; NK, natural killer; TGF, transforming growth factor; TNF, tumour necrosis factor; TSLP, thymic stromal lymphopoietin.

proteins, and the repertoire of sequences that can be detected by antibodies/BCRs is so vast that most sequences can be recognised. TCRs only recognise linear epitopes in the context of cell surface-bound Major Histocompatibility complexes (MHC)(see below) on APCs. Therefore, linear T cell receptor epitopes are restricted by MHC haplotype. As such, in any individual, the variability of the MHC molecules in the genome determines the epitope sequences that can be detected by that individual's T cells.

Once activated, antigen-specific adaptive immune cells undergo clonal expansion, resulting in a population of cells with identical antigen receptors and antigen specificities. Once the immune response has cleared the pathogen from the body, the antigen-specific cell population contracts in number via programmed cell death (apoptosis). However, some antigen-specific T and B cells remain in the body as long-lived memory cells which are capable of responding to a second infection of the same pathogen more efficiently than their naïve counterparts. Maximising the numbers and efficiency of these memory cells ('immunological memory') is the target of vaccination against parasitic infection (Chapter 25)

1.9 The role of the MHC in the immune response

The MHC is a polygenic family of glycoproteins that present peptides from digested pathogen proteins to TCRs on T cells. The MHC genes were originally defined in the context of their role in the compatibility and rejection of transplanted tissues (hence the name 'histo-compatibility'). In addition to the presence of several MHC genes in the human genome, each MHC gene is polymorphic, with multiple variants in the human species, and expression is co-dominant between the alleles inherited from each parent. The particular combination of MHC variant genes in a human is known as the MHC haplotype.

The MHC can be classified into MHC-I and MHC-II. MHC-I is expressed on most nucleated cells, whereas MHC-II is generally restricted to haematopoietic cells – in particular, APCs such as DCs, macrophages and granulocytes. APCs play a crucial role in the initiation of T cell responses because of their ability to present antigen on MHC-II molecules on the cell surface. MHC-II is also expressed on B cells to enable the acquisition of help from CD4+ T helper cells (see below).

The two classes of MHC differ in composition: MHC-I is composed of a polymorphic α chain with three domains, the structure of which is stabilised by dimerisation with a second non-polymorphic molecule called β2-microglobulin. MHC-II molecules are composed of two chains – the α- and the β- chain – each with a constant and variable domain.

The variable domains of each class of MHC molecule form the peptide-binding groove, a structure which varies in amino acid sequence. The peptide-binding groove binds to a peptide epitope using a combination of hydrogen bonding and ionic interactions which anchor the peptide into the groove. The sequence of the peptide-binding groove influences the size and sequence of the peptide that can be loaded and presented. The size of the peptide that can be presented by MHC molecules differs by class: MHC-I-associated peptides are generally 8–10 amino acids long, whereas MHC-II-associated peptides are slightly longer at 15–24 amino acids.

The variability among MHC molecules affects the peptide sequence or 'sequence motif' that can be presented to T cells. It is the combination of peptide sequence and the sequence of the MHC residues surrounding the peptide-binding groove that is recognised by each clonotypic TCR. Thus, the recognition of a linear T cell epitope presented in the context of the MHC is partially determined by the residues in the MHC molecule and is 'MHC-restricted'; individual T cell receptors react with peptides complexed with some variants of MHC molecules but not others.

MHC-I molecules present peptides that are intracellularly derived (for example, viral particles in virally-infected cells, tumour antigens or peptides derived from intracellular bacteria or protozoan parasites), whereas MHC-II presents antigens derived from the extracellular environment. This makes sense when viewed in the context of the types of T cells that become activated by the different classes of MHC molecule: the CD8 molecule on cytotoxic T cells

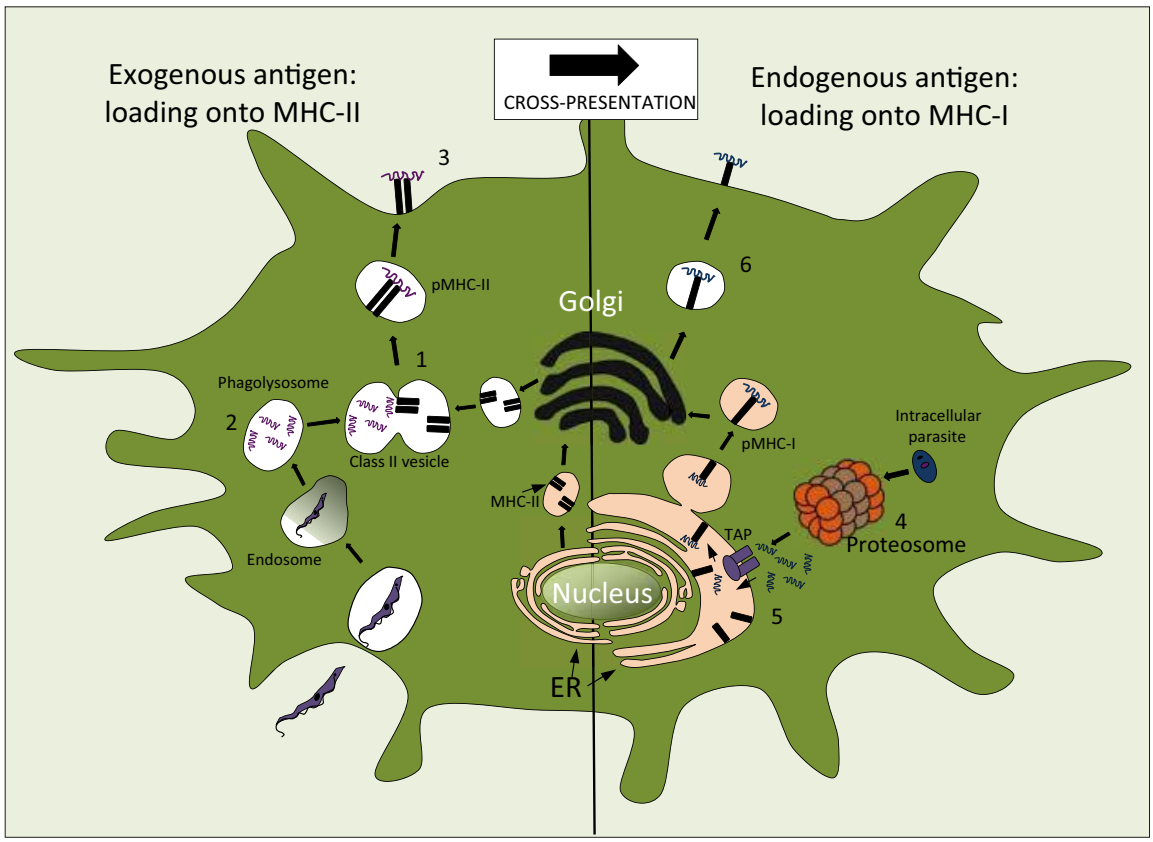


Figure 1.9 MHC processing pathways. MHC-II is loaded in specialised vesicles (Class II vesicles) (1) that fuse with phagolysosomes containing endocytosed digested pathogen particles (2) before peptide-loaded MHC-II traffics to the cell surface for display to T cells (3). Peptides from intracellular pathogens are generated by a multi-subunit structure known as the proteosome (4), and are pumped into the ER of the cell by TAP molecules (5), where MHC-I is loaded before trafficking to the cell surface via the Golgi (6). Cross-presentation, whereby endocytosed material 'crosses over' to the MHC-I loading pathway, occurs by mechanisms that are not fully understood.
Abbreviations: ER, endoplasmic reticulum; MHC, major histocompatibility complex; TAP, transporters associated with antigen processing.

can bind to MHC-I molecules, facilitating activation by pMHC-I, whereas the CD4 molecule on T helper cells can bind to the MHC-II molecules, facilitating activation by pMHC-II. Correspondingly, the lytic properties of CD8+ T cells can be implemented for lysis of infected cells expressing intracellularly-derived peptides, whereas the orchestration of multiple types of immune cells reacting against extracellular pathogens can be achieved by activation of CD4+ T cells.

Epitopes are loaded onto MHC molecules inside APCs, and different MHC-loading pathways have been determined according to the source of the antigen (Figure 1.9). However, this schema is simplified; the process of cross-presentation (see below), allowing CD8+ T cells to become 'primed' by DCs, demonstrates that the extracellularly-derived pathogen proteins can be endocytosed and 'cross over' to the MHC-I loading pathway in the endoplasmic reticulum.

1.10 T cell activation and cellular-mediated immunity

The main subsets of T cells are defined by their expression of CD4 (T helper cells) or CD8 (cytotoxic T cells). T helper cells provide 'help' to other cells of the immune system, such as the amplification of macrophage functions, the isotype switching of B cells or the amplification of CD8+ cytotoxic T cell functions. Cytotoxic T cells lyse cells infected with intracellular pathogens.

1.10.1 Three signals are required for CD4+ T cell activation

The activation of CD4+ T cells initially requires binding between T cells and APCs. This interaction is stabilised by the adhesion molecule leukocyte function-associated antigen (LFA)-1 on the T cell surface, and cellular adhesion molecules such as intercellular adhesion molecule (ICAM)-1 on the APC. Naïve CD4+ T cells become fully activated to become effector CD4+ T cells in response to three main signals received from the APC (Figure 1.10). The first is the ligation of the TCR with pMHC-II. The TCR is made up of two chains – α and β – neither of which contain intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). Therefore, signals are transmitted from the TCR via a complex of ITAM-containing CD3 molecules associated with the α and β chains making up the TCR.

The second signal received from the APC is the ligation of CD28 by co-stimulatory molecules of the B7 family (B7.1/CD80 and B7.2/CD86). Although

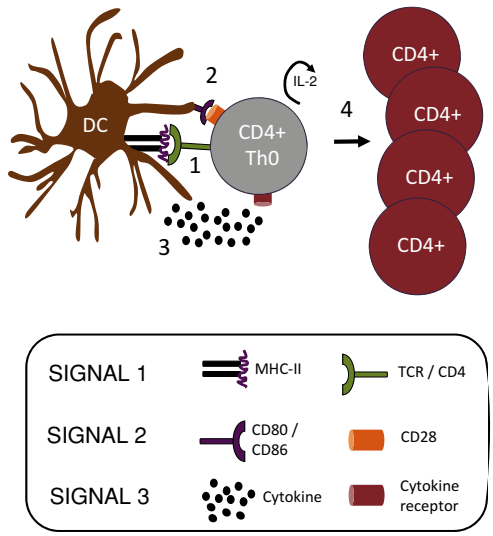


Figure 1.10 Molecular interactions leading to CD4+ T cell activation. CD4+ T cells require three main signals from APCs for activation. The first is received from ligation of the TCR by pMHC-II (1). The second signal is received by ligation of the co-stimulation molecule CD28 with CD80 or CD86 expressed on the APC (2), and this leads to the production of IL-2, allowing the T cell to proliferate. The third signal is received by cytokine receptors and determines the polarisation of the CD4+ T cell into a specific subtype (3). Upon receipt of all three of these signals, the CD4+ T cell expands and differentiates (4).
Abbreviations: APC, antigen presenting cell; pMHC-II, peptide loaded major histocompatibility-II molecules; TCR, T cell receptor.

Table 1.4 Cytokines required for polarisation of CD4+ T cell subsets.

T cell subset	Polarising cytokine
Th1	IL-12p70
Th2	IL-4
Th9	TGF-β and IL-4
Th17	TGF-β, IL-6, IL-21, IL-23
Th22	IL-6, TNF-α
iTreg	TGF-β

Abbreviations: IL, interleukin; iTreg, inducible T regulatory cell; TGF, transforming growth factor; Th, T helper; TNF, tumour necrosis factor.

this is regarded as one of the main co-stimulatory signals, many other molecules can contribute to the co-stimulatory signals required for full T cell activation upon ligation. Co-stimulation is critical to induce the expression of IL-2 and to up-regulate expression of the α-chain of the IL-2 receptor. Since T cell expansion is critically dependent on IL-2, this endows the T cell with the ability to proliferate. Activation of CD4+ T cells in the absence of co-stimulation leads to T cell anergy, whereby the T cells cannot proliferate.

Once CD4+ T cells have become activated, the proliferation of T cells gives rise to a critical mass of antigen-specific T cells to effectuate an immune response. Since all T cells arising from the original activated T cell are clonal, expansion is referred to as 'clonal expansion'.

T cell polarisation is facilitated by a third signal received from the APC – the ligation of cytokine receptors by APC-derived cytokines (Table 1.4). Signals received through cytokine receptors result in Jak-STAT signalling and the activation of transcription factors that promote the secretion of specific cytokines associated with the various polarised CD4+ T cell subsets (described in more detail below).

1.10.2 Cross-presentation and cross-priming of CD8+ T cells

Primed CD8+ T cells can traffic to areas of the body to lyse cells infected with intracellular pathogens. These are normally viruses, but also can be intracellular bacteria or parasites such as *Toxoplasma gondii* (Chapter 4) or *Trypanosoma cruzi* (Chapter 9). At the site of infection, they recognise infected cells via the expression of pMHC-I presenting peptides derived from the intracellular pathogen. However, naïve CD8+ T cells must be primed before they can function efficiently, in part because they also require co-stimulation to become fully activated. CD8+ T cells become primed by DCs in the secondary lymphoid organs.

DCs are associated with the endocytosis of foreign antigen from extracellular pathogens via phagocytosis. They also primarily present extracellularly-derived antigen complexed with MHC-II molecules for presentation to CD4+ T cells. This begs the question: how can DCs possibly prime CD8+ T cells that require presentation of peptide epitopes from intracellularly-derived antigen complexed with MHC-I? The process by which DCs present peptide derived

from intracellular pathogens with which they are not directly infected is called 'cross-presentation'. The priming of CD8+ T cells by DCs is essential, not just for defence against intracellular pathogens, but also to prime and generate memory CD8+ T cells induced by vaccination.

Cross-presentation involves the crossing of endocytosed extracellularly-derived peptides from the endocytic pathway for loading onto MHC-II molecules to the proteosome-derived pathway for peptide loading onto MHC-I molecules in the endoplasmic reticulum (Figure 1.9). The molecular events mediating cross-presentation are described in the References for Further Reading. However, the method of antigen uptake by the DC can influence this process; receptor-mediated phagocytosis by Fc receptors (see below) and some of the C-type lectins (Figure 1.3) can feed endocytosed peptides into the MHC-I loading pathway. Although several types of phagocytes can cross-present antigen, only certain subsets of DC can cross-present antigen to prime of CD8+ T cells. In mice, cross-presentation and priming is thought to be restricted to CD8+DCs and CD103+DCs.

Priming of CD8+ T cells also requires 'help' from CD4+ T helper cells in addition to ligation of the TCR with pMHC-I on DCs. The exact nature of CD4+ T cell help is still unclear, but it is thought to involve recruitment of naïve CD8+ T cells to the pMHC-I DC via secretion of chemotactic factors, up-regulation of appropriate co-stimulatory molecules to deliver signal 2 to the CD8+ T cells, and IL-2 production to assist in clonal expansion of the primed CD8+ T cells (Figure 1.11). The ligation of CD40L on the CD4+ T cell by CD40 on the DC is known to be critical in the provision of CD4+ T cell help during cross-priming of CD8+ T cells. Thus, DCs can simultaneously prime CD4+ T helper and CD8+ cytotoxic T cells and act as a bridge, allowing CD4+ T cells to provide help in the priming of CD8+ T cells.

1.10.3 CD4+ T cell phenotypes

During priming of CD4+ T cells, the cytokines secreted by APCs can polarise the CD4+ T cells into one of several different phenotypes. Originally, the field of CD4+ T cell polarisation centred around a Th1/Th2 paradigm, whereby CD4+ T cells were thought to differentiate into either Th1 (pro-inflammatory IFN-γ secreting CD4+ T cells) or Th2 (anti-inflammatory CD4+ T cells secreting several cytokines, of which IL-4 was the main protagonist). The cross-regulatory nature of IFN-γ and IL-4 was considered a factor in the observed dominance of either response, Th1 responses during infection with microorganisms such as viruses, bacteria and parasites, or Th2 responses during allergic reactions and infection with macroparasites such as helminths. Since the original description of the Th1/Th2 paradigm in the 1980s, the polarisation of CD4+ T cells is now known to be more complex than the Th1/Th2 paradigm, with the identification of several other distinct types of CD4+ T cells, including Tregs and, more recently, Th17, Th9 and Th22 cells.

Each subset of CD4+ T cells secretes a distinct profile of cytokines (Figure 1.12) and provides a different form of 'help' to amplify specific effector mechanisms of the immune system. Thus, the effector mechanisms mediated by IFN-γ secreted by Th1 cells are effective at clearing protozoan parasites, whereas IL-4

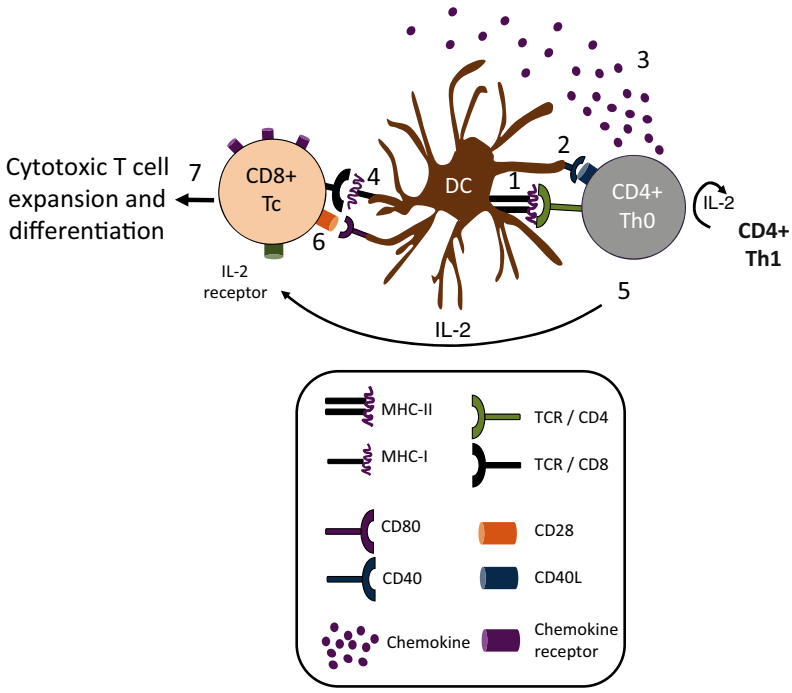


Figure 1.11 CD4+ T cell help is required for cross-presentation and priming of CD8+ cytotoxic T cells. CD8+ or CD103+DCs can display endocytosed antigen on pMHC-II molecules(1) for recognition by CD4+ T cells. The ligation of CD4+ T cell-expressed CD40L with CD40 expressed on the DC (2) permits CD4+ T cell help in cross-priming of CD8+ T cells. CD4+ T cells secrete chemokines to attract CD8+ T cells to the DC (3), where the TCR of the CD8+ T cell recognises endocytosed antigen that has 'crossed over' to the MHC-I loading pathway, presented as pMHC-I on the DC surface (4). CD4+ T cells induce the up-regulation of co-stimulatory molecules, which allows CD4+ T cell production of IL-2 to support CD8+ T cell proliferation (5), as well as co-stimulation of the CD8+ T cell (6). These events lead to the expansion of CD8+ T cells to exogenously-derived antigen (7).
Abbreviations: DC, dendritic cell; IL, interleukin; MHC, major histocompatibility complex; TCR, T cell receptor.

secreted by Th2 cells activates effector mechanisms that can provide protection against helminths. Furthermore, all immune responses must be controlled because, when excessive, they can cause immunopathology. Regulation of immune responses is carried out by the immunoregulatory cytokines IL-10 and TGF-β, both of which can be produced by the Treg subset of CD4+ T cells.

1.10.3.1 Th1

Th1 cells are a feature of protozoan infections, and the signature cytokine of Th1 cells is INF-γ. T-box, expressed in T cells (T-bet), is a key transcription factor for the IFN-γ gene, and CD4+ T cells expressing T-bet are generally considered to be of a Th1 phenotype. APC-derived IL-12 is a key driver of Th1 polarisation. However, naïve CD4+ T cells (Th0) do not express receptors for IL-12 but up-regulate expression of the IL-12 receptor in response to stimulation from IFN-γ. Since IFN-γ is not produced in significant quantities by APCs, often the source of IFN-γ comes from other cells of the innate immune system. NK cells

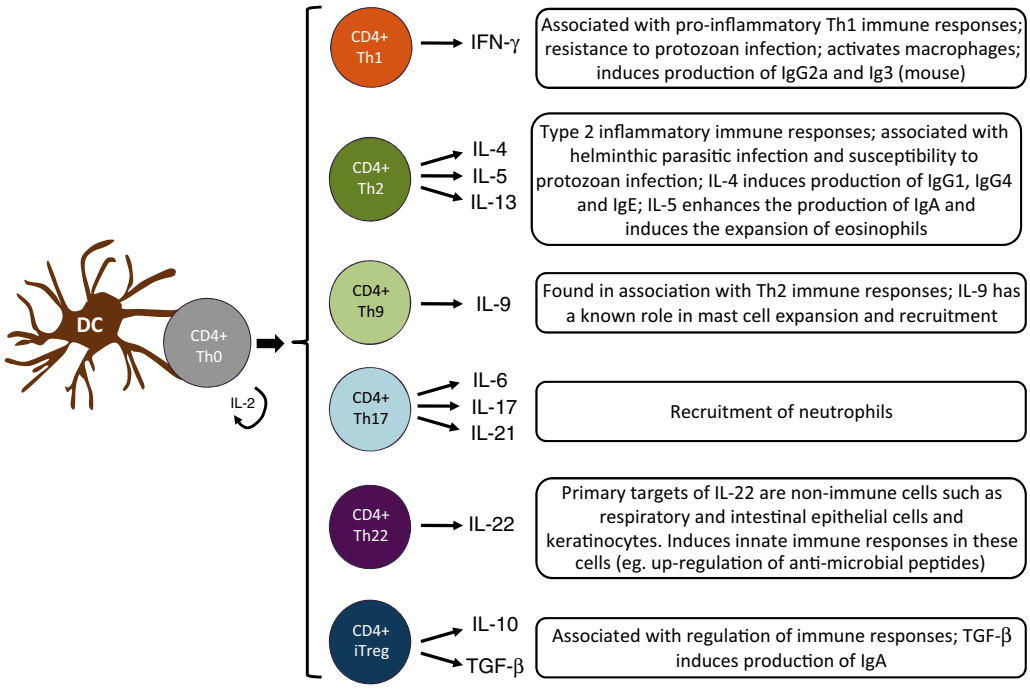


Figure 1.12 Functions of different CD4+ T cell phenotypes.

and γδ T cells (see below) are important sources of innate IFN-γ, and their activation can therefore play a key role in the development of pro-inflammatory immune responses via the expansion of Th1 cells.

Signalling through the IFN-γ receptor results in the transcription of IFN-inducible genes, the products of which enhance the microbicidal activity of a number of immune cells. In macrophages, reaponding to IFN-γ promotes phagocytosis and induces the production of the enzymes, mediating respiratory burst. The expression of MHC molecules is up-regulated in APCs, leading to enhanced antigen presentation. Furthermore, in mice IFN-γ induces B cells to isotype-switch to the IgG2a, a cytophilic antibody isotype effective at opsonising protozoan parasites.

1.10.3.2 Th2

Th2 cells are induced in helminth infections and in allergic responses. Although it is undoubtedly true that Th2 cells and Th1 cells are counter-regulatory, Th2 cells field of parasitology (in particular helminthology) has played a major role in determining that Th2 cells do not only arise when Th1 responses are absent; they can be actively induced, and one of the strongest inducers of Th2 cells can be found in the eggs of Schistosomes (Chapter 16). Counter-regulation of Th1 responses by Th2 associated cytokines is also not always absolute, with instances where some IL-4 can be necessary for the induction of a Th1 response (for example in *Cryptosporidium* infection – see Chapter 5).

The signature cytokine of Th2 cells is IL-4, although Th2 cells also produce significant quantities of IL-5 and IL-13 (Figure 1.12). Previously, Th2 cells were thought to be the major producers of IL-9 and T cell-derived IL-10, but this is no longer the case; IL-9 producing T helper cells have recently been designated as a separate subset, and IL-10 has now been observed to be produced in large quantities from most CD4+ T cell subsets, including Th1 cells under certain conditions.

Naïve CD4+ T cells polarise towards a Th2 phenotype in response to IL-4. DCs do not produce significant quantities of IL-4, and the early source of IL-4 from the innate immune system has remained an area of investigation. Recent studies suggest that basophils may play an important role in this regard (see Chapter 12).

The IL-4 receptor is composed of two chains (IL-4Rα and the common γ chain). IL-4R is also a component of the IL-13R. The IL-13R is responsive to IL-13 when paired with the IL-13R1 chain and both the IL-4R and the IL-13R signal through STAT6 activation. Pairing of the IL-13R1 chain with IL-13R2 rather than the IL-4Rα results in the formation of a soluble, high affinity binding protein that inhibits IL-13 signalling. Indeed understanding how positive signaling via IL-4R/IL-13R1 and inhibition by the IL-13R2 is regulated is not currently known but will be of great importance for many Th2-associated diseases including asthma, ulcerative colitis and schistosomiasis.

Th2-effector mechanisms centre around the effects of IL-4 on B cells. IL-4 is an important growth factor for B cells, and it induces isotype switching to IgE. Since IgE is a central molecule mediating degranulation of eosinophils, mast cells and basophils, Th2-induced isotype switching of B cells to IgE production is critical in supporting some of the main granulocyte-based effector mechanisms against helminth parasites. Th2 cytokines also polarise macrophages towards an M2 phenotype, possibly contributing to the repair of tissue damaged by helminth infection, but also leading to susceptibility of macrophages to infection with some protozoan parasites.

1.10.3.3 Th17

Th17 cells were first described as producers of IL-17, but more recently they have also been shown to also produce IL-21. The polarisation of Th17 cells was originally thought to depend on the production of IL-23, a pro-inflammatory cytokine related to IL-12 (see Table 3), by the activating APC. However, Th17 cells are now known to differentiate in response to IL-6 and TGF-β. The development of Th17 cells can be suppressed by both IFN-γ and IL-4. The main function of Th17 cells in infectious diseases is the recruitment of neutrophils to control infection, although they may also have other, as yet undiscovered, roles.

1.10.3.4 Th9 and Th22

IL-9 was historically considered to be a product of Th2 cells, but recently a subset of T helper cells that produces IL-9 alone has been identified and designated as a separate Th9 subset. Information regarding the contribution of Th9 cells and, indeed, the role of IL-9 in immune responses during parasitic

infection, is currently sparse. IL-9 is known to be involved in the expansion and recruitment of mast cells, in turn influencing the recruitment and activation of eosinophils via mast cell-derived release of eosinophil chemotactic factor. IL-9 is known to have a protective effect against *Trichuris* infection (Chapter 14) and promote mucous production and mast cell activation in schistosome infection (Chapter 16), but it is currently unclear whether the source of IL-9 in these infections is from Th2 or Th9 cells.

IL-22 is secreted by activated CD4+ T cells (now termed Th22 cells) that are found in immune responses in humans, but not in mice. The function of IL-22 (and Th22 cells) is still being investigated, but on a molecular level IL-22 is related to the immunoregulatory cytokine IL-10. It is known that the functional receptor for IL-22 is predominantly expressed on epithelial cells in the mucosal tract and keratinocytes in the skin. However, no expression of the IL-22 receptor has been detected on immune cells. Cells responsive to IL-22 can produce innate immune molecules, such as anti-microbial peptides and type 1 interferons, in response to ligation of the IL-22 receptor. Therefore, IL-22 may play a role in infection immunity via the induction of innate immune responses. Although the evidence suggests that IL-22 may contribute to the body's defence against bacterial infection, a role for IL-22, and for the Th22 cells that produce this cytokine, awaits elucidation in parasitic infection.

1.10.3.5 Tregs

Natural regulatory CD4+ T cells (nTreg) maintain immunological homeostasis, preventing activation of auto-reactive cells, while inducible Treg (iTreg) cells appear to control the magnitude of immune responses to exogenous antigenic challenge, including that posed by invading pathogens. Both nTregs and iTregs provide essential immunological control to prevent immune-mediated pathology. The discovery of Forkhead box protein 3 (Foxp3) as a definitive transcription factor for Treg cells has allowed investigators to identify, isolate and study the role of these cells in many immunological systems.

Markers such as the α-chain of the IL-2 receptor (CD25), inhibitory co-receptor cytotoxic T lymphocyte antigen-4 (CTLA-4), and the glucocorticoid-inducible tumour necrosis factor receptor (GITR) provide clues to the function and mechanisms used by Tregs, but these markers are shared with activated effector T cells. CD103, an αEβ7 T cell integrin also expressed on regulatory DCs, is required for cell-cell contact and is highly expressed on activated Treg cells. Neuropilin-1 (also called BCDA-4) is a receptor involved in axon guidance, angiogenesis and the activation of T cells, and it has also been identified on nTreg cells during *S. mansoni* infection. Originally identified through global gene expression studies, high expression of surface neuropilin-1 correlates with Foxp3 expression and is rapidly down-regulated following TCR-ligation.

iTregs are a more heterogeneous population of CD4+ T regulatory cells than nTregs. They are often distinguished by the panel of cytokines they secrete upon activation. Subsets of iTregs include 'Th3' cells that secrete IL-4 in addition to TGF-β and IL-10, and 'Tr1' cells that secrete TGF-β and IL-10 but not IL-4. Regulatory CD4+CD25+Foxp3− cells that can secrete IL-10 extend the repertoire of immunoregulatory CD4+ T cells.

Collectively, iTreg and nTreg cells represent a population of professional suppressor cells, with the unique and primary function to suppress immune responses and prevent immune-driven pathology. Tregs are able to suppress both Th1 and Th2 responses. In addition to the Treg-mediated suppression of the cell function and proliferation of other CD4+ T cell subsets, Tregs can also influence the function of macrophages, CD8+ cells, B cells and granulocytes.

1.10.4 Other T cells of the innate immune system

Other TCR-expressing T cells that may play a role in parasitic infections in addition to CD4+ T helper cells and CD8+ cytotoxic T cells are γδ T cells and NKT cells.

1.10.4.1 γδ T cells

γδ T cells are defined by the expression of TCRs that are composed of γ and δ chains rather than α and β chains. γδ T cells recognise monophosphate and diphosphate esters, and do not require presentation of these antigens by APCs to become activated. As such, they are capable of producing cytokines before T cells expressing TCRs composed of α and β chains (αβ T cells). In some protozoan infections, γδ T cells may be an important early source of IFN-γ for the up-regulation of IL-12 receptors on pre-Th1 cells. The repertoire of cytokines secreted by γδ T cells is not confined to IFN-γ, and these cells have been shown to also secrete IL-4, IL-10 and IL-17, depending on their environment.

1.10.4.2 NKT cells

NKT cells express a range of markers associated with NK cells, such as the NK cell receptor NK1.1, as well as αβ-TCRs, and they are therefore defined as both NK cells and T cells. In mice, NKT cells have a TCR that disproportionately uses the variable regions Vα14Vβ8. NKT cells can recognise lipids and glycolipids – in particular α-galactosylceramide (α-GalCer) – in the context of MHC-I-like molecule CD1d. Upon activation, NKT cells are flexible with regards to cytokine production and can produce both IL-4 and IFN-γ, depending on their environmental stimuli.

1.11 B cells and the humoral response

The predominant function of B cells is to produce antibodies, the main component of the humoral immune response. Upon activation, naïve B cells differentiate to become antibody-secreting cells called plasma cells. Some B cells differentiate to become long-lasting memory B cells that participate in memory responses upon challenge infections with the same pathogen.

B cells are a heterogeneous population of cells that can respond to antigen in a T-dependent or T-independent manner, the former requiring 'help' from CD4+ T cells. B cells can be divided into B1 and B2 cells B1- and B2-B cells. B1-B cells largely produce antibody to T-independent antigens, and they secrete IgM that can interact with multiple epitopes with a low affinity. B1-B cells are activated

by B cell mitogens that can directly initiate the division of B cells. They respond to polysaccharides found on pathogens such as the TLR-4 agonist LPS, a cell wall constituent of gram-negative bacteria. B1 cells also spontaneously secrete antibody and are the main B cell subset contributing to natural serum antibodies present in the bloodstream.

B2-B cells make up the majority of B cells in the body and require T cell help from CD4+ T cells to produce high-affinity, isotype-switched antibody. The acquisition of help from CD4+ T cells dictates that B2-B cells have a capacity for antigen presentation and, indeed, they can act as APCs. It is the B2 subset of B cells that form long-lasting memory B cells, a capability not shared with B1-B cells. The antibody secreted by B2-B cells recognises particular epitopes in pathogen-derived molecules with high specificity.

1.11.1 B cell activation against T-dependent antigens

The activation of B cells to produce antibody against T-dependent antigens occurs via the BCR, essentially surface-bound antibody. Naïve B cells express predominantly IgM and IgD on their surface, and the antigen-binding fragment of the BCRs on any one B cell are identical. Upon binding to multivalent antigen, cross-linking of the BCRs on the B cell surface induces internalisation of the antigen which becomes degraded and loaded onto MHC-II molecules for presentation to CD4+ T cells at the cell surface (Figure 1.13). This process is

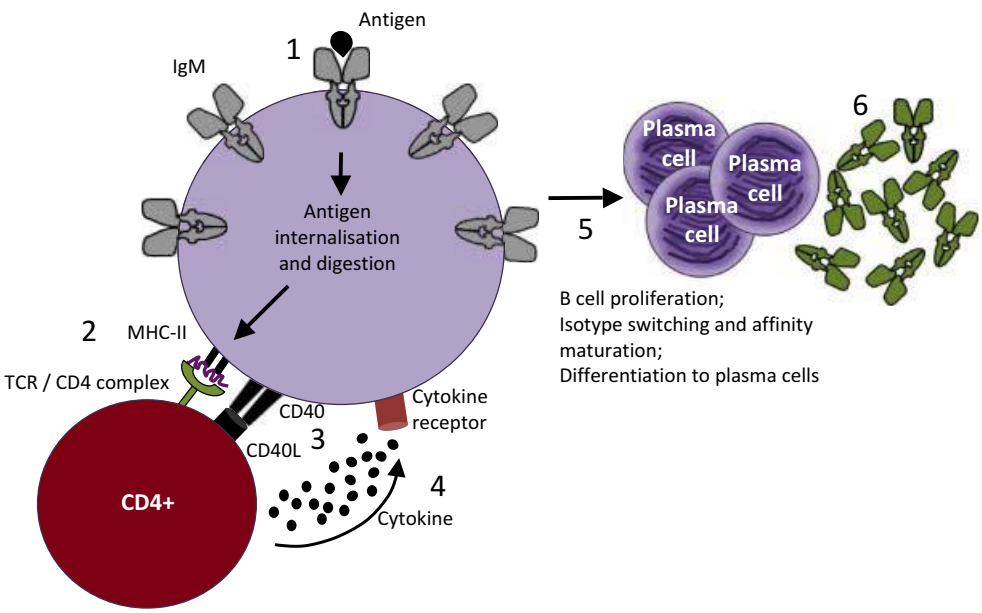


Figure 1.13 Activation of B cells towards T-dependent antigen. Ligation of the BCR by antigen (1) leads to internalisation and digestion of the antigen. Digested peptides are loaded onto MHC-II molecules, and peptide loaded MHC-II is displayed on the B cell surface for recognition by cognate CD4+ T cells (2). The ligation of CD40 by CD4+ T cell expressed CD40L (3) and the production of cytokines by the CD4+ T helper cell (4) leads to B cell activation and the eventual formation of plasma cells (5) that secrete isotype-switched, high-affinity antibodies (6).
Abbreviations: BCR, B cell receptor; MHC, major histocompatibility.

amplified by the presence of the B cell co-receptor (not shown), a complex of molecules consisting of CD19, CD21 and CD81. Indeed, expression of CD19 is often used to distinguish B cells from other immune cells, particularly in studies involving mice.

The recruitment of a cognate CD4+ T cell (i.e. one that can recognise the same antigen as the B cell) is essential for the B cell to receive signals derived from the T cell to initiate B cell proliferation, isotype switching and somatic hypermutation (a process known as "affinity maturation" which increases the affinity of the antibody for the antigen). The ligation of CD40 on the B cell surface with CD40 ligand (CD154) on the CD4+ T cell is essential to induce B cell proliferation and this interaction induces the up-regulation of co-stimulatory molecules on the B cell surface (Figure 1.13). This, in turn, increases the ability of the B cell to co-stimulate the CD4+ T cell, stimulating the production of T cell-derived cytokines such as IL-2, IL-4 and IL-5 that drive proliferation and isotype switching.

Isotype switching exchanges the constant regions of the heavy chain of the antibody molecules (Figure 1.14), leading to the production of specific types of antibody, each with different functions in the immune system (see below). The constant region of each isotype can react with specific Fc receptors, differentially expressed on the cells of the immune system. Affinity maturation alters the sequence of the variable region of the antibody, increasing the affinity of the antibody for the antigen.

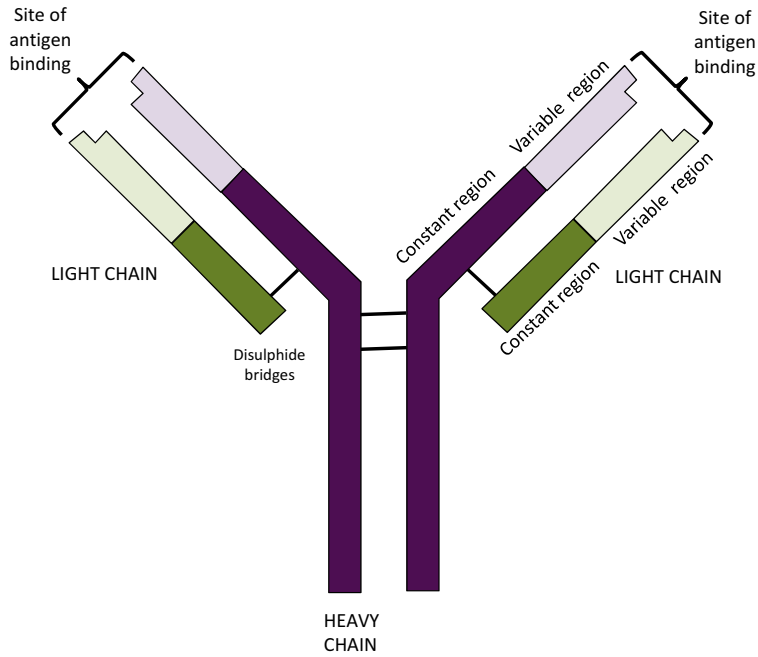


Figure 1.14 Generic structure of an antibody. Antibodies are composed of two heavy chains and two light chains, connected by disulphide bridges. Each heavy and light chain is composed of a constant region and a variable region, and the sites of antigen binding are composed of the variable regions of the heavy and light chains. The constant region of the heavy chains determines the isotype of the antibody and is the portion of the antibody that binds to Fc receptors.

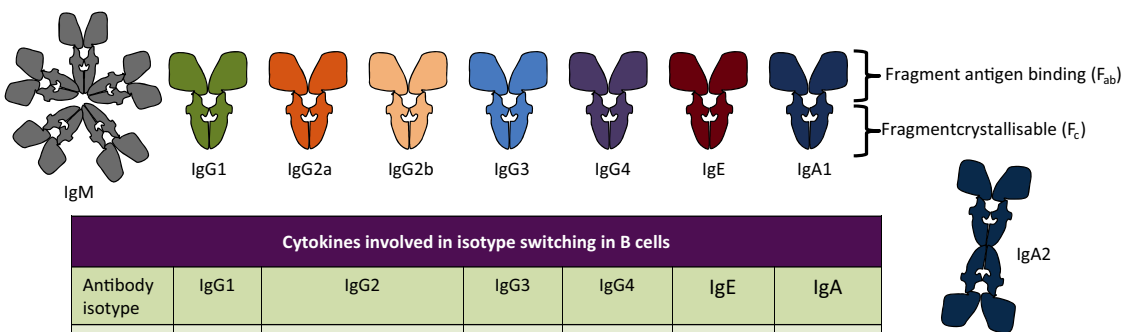
Antibody-secreting B cells (plasma cells) can secrete up to 2,000 antibodies per cell per second for 1–2 weeks, and this intensive output of protein is the reason that plasma cells have extensive rough endoplasmic reticulum and Golgi apparatus. Antibodies have a half-life and do not live forever in the circulation. However, plasma cells continuously secrete antibody once activated, and they maintain antibody in the circulation. Long-lived plasma cells (a type of B memory cell) can secrete antibodies for many years while resident in the bone marrow.

The production of antibody is not the only function of B2-B cells. They also play an important role as APCs in the activation of CD4+ T cells, and are also producers of cytokines. In particular, B cells have also become appreciated as important immunoregulatory cells, capable of producing the immunoregulatory cytokines IL-10 and TGF-β in certain situations.

1.11.2 Antibody isotypes

Antibodies are of distinct isotypes (or classes), designated according to the constant regions of the heavy chains from which they are composed (Figure 1.14). There are five classes of antibody isotypes: IgM, IgD, IgG, IgE and IgA. The IgG class is further divided into four subclasses. Isotype switching is determined by the CD4+ T cell-derived cytokines received during T cell help (Figure 1.15), and this is influenced by the subclass of CD4+ T cell that provides help for the B cell. Different isotypes of antibody can be concentrated in different locations of the body (eg. IgA is the major isotype at the mucosal surfaces).

Antibodies perform numerous effector functions in the immune system. Some of these functions are carried out by particular isotypes of antibody. The main



Cytokines involved in isotype switching in B cells							
Antibody isotype	IgG1	IgG2		IgG3	IgG4	IgE	IgA
Human	IL-4 IL-21	Not established		IL-21	IL-4 IL-10	IL-4 IL-13	TGF-β IL-5 IL-21
Mouse	IL-4 IL-5 IL-13	IgG2a: IFN-γ	IgG2b: TGF-β	IL-5	_____	IL-4	TGF-β IL-5
Abbreviations: IFN, interferon; Ig, immunoglobulin; IL, interleukin; TGF, transforming growth factor.							

Figure 1.15 Cytokines involved in isotype switching. Monomeric antibodies can be split into two fragments of antigen binding (Fab) and a crystallisable fragment (Fc) when digested by the protease papain. All antibodies shown are monomeric, with the exception of IgM (pentamer) and IgA2(dimeric).

Table 1.5 Functions of antibody isotypes.

Function	Isotypes	Effects
Neutralisation	IgA IgG1, IgG2, IgG3, IgG4	Neutralisation of pathogen toxins
Opsonisation	IgG1 and IgG3 (IgG2a in mice)	Prevention of pathogen attachment and invasion Targeting of pathogens for phagocytosis Targeting of pathogens for antibody-dependent cell-mediated cytotoxicity (NK cells)
Activation	IgE IgD IgM IgG1 and IgG3	Mast cell degranulation IL-4 induction from basophils Complement activation

roles of antibodies can be categorised into neutralisation of pathogen toxins, opsonisation of pathogens and activation of immune effector mechanisms (Table 1.5). Some isotypes of antibody – in particular some subclasses of IgG – can perform more than one function effectively.

1.11.2.1 IgM

IgM is one of the first antibody isotypes secreted in a primary immune response. It is found on the surface of naïve B cells and is the major isotype secreted by B1-B cells in response to T-independent antigens. IgM normally does not undergo affinity maturation, so it is generally capable of binding to several different antigens with weak affinity. Soluble IgM forms pentameric structures, and it is the majority isotype in natural antibody found in the serum. This isotype is effective at activating the complement cascade.

1.11.2.2 IgD

IgD is found on the surface of naïve B cells prior to class switching. This isotype can also can be found in extremely low concentrations in the serum. The function of IgD has been debated for some time, but it was recently shown to bind to an unknown receptor on basophils. Complexes of IgD can lead to the production of IL-4 from basophils, and therefore this antibody isotype may be an important player in the production of innate-derived IL-4.

1.11.2.3 IgG

IgG is one of the main antibody isotypes in the body. Humans have four subclasses of IgG antibody (IgG1, IgG2, IgG3 and IgG4), of which IgG1 and IgG3 are cytophilic ('affinity for cells') and are particularly good opsonins. Mice also have four subclasses of IgG, but have two forms of IgG2 (IgG2a and IgG2b) and do not produce IgG4. C57BL/6, C57BL/10 and non-obese diabetic (NOD) mice express IgG2c instead of IgG2a, although commercial reagents to measure IgG2a often cross-react with the IgG2c isotype.

IgG is a multifunctional isotype, and the cytophilic nature of the IgG1 and IgG3 subclasses make the generation of these antibodies desirable for efficient pathogen clearance. The subclass of IgG produced is heavily influenced by the cytokine environment at the time of B cell priming (Figure 1.15). Of particular relevance for parasitic infections in mice, IgG1 is often used as an indicator of a Th2/IL-4 environment, whereas IgG2a is used as an indicator of a Th1/IFN-γ environment.

1.11.2.4 IgE

IgE is induced when B cells receive help from IL-4 producing Th2 cells (in both humans and mice). IgE is the main antibody isotype that induces degranulation of mast cells, eosinophils and basophils. Therefore, the production of IgE is the main isotype responsible for allergic hypersensitivity reactions. It is also a key isotype that facilitates anti-helminthic effector mechanisms mediated by the degranulation of granulocytes.

1.11.2.5 IgA

IgA is an important component of breast milk and is the major type of antibody found in the mucous secretions of the body, particularly in the gastrointestinal tract and in the respiratory tract. IgA plasma cells normally reside in the lamina propria of the gut. There they secrete dimeric IgA which is able to trancytose across the epithelium of the gut and into the lumen by complexing with the polymeric immunoglobulin receptor (pIgR). IgA1 is less abundant than IgA2 and can be found circulating in the serum. IgA performs an important role in defence against gastrointestinal parasites such as *Giardia* (Chapter 6).

1.11.3 Fc receptor recognition via Fc receptors

One of the main functions of antibody is to opsonise pathogens. Opsonised pathogens can be recognised by immune cells expressing Fc receptors that bind onto the Fc portion of antibodies. Cross-linking of Fc receptors by antibodies complexed by a multivalent antigen (or on the surface of a pathogen) activates intracellular signalling cascades. In the case of Fcγ receptors this occurs via an ITAM present on the cytoplasmic portion of the Fc receptor chain (FcγRII-A), or by association of the Fc receptor chain with an ITAM-containing Fcγ adaptor protein (Figure 1.16). Cross-linking of Fc receptors activates effector pathways for destruction of the opsonised pathogen. Immune cells expressing Fc receptors include APCs such as macrophages (endocytosis and digestion of the pathogen), NK cells (lysis by ADCC) or granulocytes (damage by exposure to the granule contents after induced granule release). The FcγRII-B receptors (both B1 and B2) are the exception, because the cytoplasmic tail of these receptors contain an ITIM that inhibits the signaling cascade.

Fc receptors are designated by Greek symbols that match the relevant antibody isotype recognised: IgG antibodies ligate Fcγ receptors, IgE antibodies ligate Fcε receptors, and IgA antibodies ligate Fcα receptors (Figure 1.16). However,

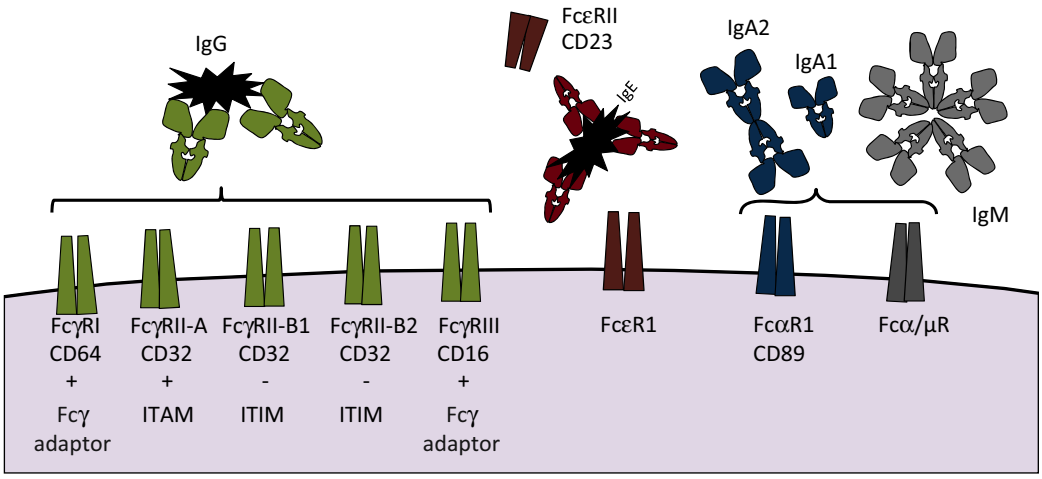


Figure 1.16 Soluble antibodies bind to Fc receptors expressed on the surface of effector cells. IgG is recognised by Fcγ, IgE is recognised by Fcε receptors, one of which is cleaved to form a soluble Fc receptor, and IgA is recognised by Fcα receptors. IgM is recognised by the Fcα/μR.
Abbreviations: Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif.

Fcα/μR can bind both IgA and IgM. While most Fc receptors are cell-surface bound, one exception is FcεRII, an Fc receptor that binds IgE with low affinity. FcεRII is a C-type lectin and it can be cleaved into soluble FcεRII (also called CD23). The binding of IgE/antigen complexes with FcεRII is thought to promote further IgE production from B cells by promoting uptake of the antigen by APCs and presentation of the antigen to CD4+ T cells to provide B cell help.

1.12 Cell trafficking around the body

Parasitic infection can occur in different areas of the human body. As such, tissue-resident sentinel APCs, such as macrophages and DCs, must detect antigen in the breached area and traffic to the local lymph nodes to present antigen to adaptive immune cells for initiation of an adaptive immune response. For effective containment of the infection, effector-immune cells must then traffic towards the area of infection. The ability of immune cells to travel around the body in a controlled and specific manner is mediated by a group of molecules distinct from cytokines called chemokines.

Chemokines are produced by activated immune cells and tissue resident APCs. Secreted chemokines attract additional immune cells to the infected tissue. Immune cells move towards gradients of chemokines which are detected by G-protein-coupled chemokine receptors expressed on the cell surface. In this way immune responses can be targeted to particular areas of the body, as required.

Chemokines are classified into four different groups, according to the sequence of cysteine motifs found in the amino terminal of the molecule. Chemokines

containing CC and CXC motifs are the most common chemokines. The CC class of chemokines includes RANTES (CCL5) and eotaxin (CCL11) which are both important in the chemotaxis of T cells and eosinophils. CXC chemokines include IL-8 (CXCL8), secreted by macrophages to recruit neutrophils to the site of infection, and IP-10 (CXCL-10), secreted by endothelial cells and monocytes to attract T cells and NK cells. Lymphotactin (CXL1) and Fractalkine (CX_3CL1) belong the C and CX_3C classes of chemokines respectively, attracting DCs, NK cells (CXL1) and T cells (CX_3CL1).

Once immune cells are recruited to the site of infection, they must attach to the endothelium and leave the bloodstream to mediate effector functions in the infected tissue. The up-regulation of integrins on immune cells activated in the secondary lymphoid organs, and adhesion molecules on the endothelium, is normally induced by inflammatory cytokines and facilitates the accumulation of immune cells at sites of inflammation.

Integrins such as leukocyte functioning antigen (LFA)-1, CD11b:CD18 and CD11c:CD18, and adhesion molecules such as members of the selectin family (e.g. E-selectin or P-selectin) or cellular adhesion molecules (e.g. ICAM-1), allow attachment and traversal of the endothelium. Interactions between integrins and selectins initiate a rolling adhesion before cellular adhesion molecules support a tighter binding of the immune cell to the endothelium. Leukocyte extravasation, that is traversal between endothelial cells and into the tissue in a process known as diapedesis, completes the journey of the activated immune cells to the site of infection.

1.13 Cellular immune effector mechanisms

1.13.1 Phagocytosis and pathogen digestion

Phagocytosis ('cell-eating') is carried out by macrophages, granulocytes (in particular neutrophils) and DCs. Macrophages and neutrophils clear pathogens from the body by phagocytosis, while DCs phagocytose pathogens to enable digested pathogen fragments to be displayed on the cell surface, complexed with MHC molecules to activate T cells. Unlike phagocytosis of pathogens and their products, which activate macrophages to produce inflammatory molecules to recruit immune cells to the area of infection, the clearance of senescent or lysed (see below) cells undergoing programmed cell death does not lead to the generation of an inflammatory immune response.

Phagocytes generally engulf pathogens or pathogen products using extensions known as pseudopodia. The initial compartment formed is known as a phagosome (Figure 1.6). Acidification of the phagosomes to form phagolysosomes, occurs after fusion of lysosomes, Golgi-derived compartments full of acidic hydrolases. Conditions in phagolysosomes are optimal for the digestion and destruction of the engulfed pathogen. Peptides derived from digested pathogen proteins are loaded onto MHC-II molecules in Class II vesicles (Figure 1.9). These peptide loaded MHC II molecules are trafficked to the surface of the cell for presentation to CD4+ T cells.

1.13.2 Cellular-mediated lysis of pathogens

Lytic responses can be mediated by activation of complement (Figure 1.5). However cellular-mediated lysis of pathogenic organisms or infected cells is carried out by CD8+ cytotoxic T cells and NK cells. Although the molecules identifying target cells for lysis are different for each of these cell types (pMHC-I for CD8+ T cells or the lack of MHC-I or opsonic IgG antibodies/reduced levels of MHC-I on the infected cell surface for NK cells), the mechanisms used for lysis are similar in that both cell types release cytotoxic granules (which are modified lysosomes) stored in the cytoplasm.

CD8+ T cells are cross-primed by DCs in the secondary lymph nodes, and then traffic to the site of infected tissue by up-regulating the expression of chemokine receptors to detect chemokines produced in the area of infection. The TCR of primed CD8+ T cells is able to recognise infected cells within the tissue by their expression of pMHC-I molecules displaying peptide from the intracellular pathogen.

Upon recognition, the release of granzymes and perforins (serine proteases) from the cytotoxic granules of the CD8+ T cell creates pores in the target cell, resulting in apoptotic lysis. CD8+ T cells do not randomly release granule contents, but do so in a controlled manner. The enzymes within the granules induce programmed cell death (apoptosis) via activation of caspases rather than necrotic cell death, because the latter would induce inflammation via recognition of DAMPs by PRRs in the surrounding tissue. Apoptotic infected cells are then ingested by phagocytic cells recruited to the area of infection by chemokines.

NK cells similarly contain cytotoxic granules of pre-formed perforin and granzymes. NK cells act in a quicker time frame than CD8+ T cells because they are activated by invariant receptors; they do not require priming. The lytic activity of NK cells is amplified by exposure to pro-inflammatory cytokines such as IL-12.

1.13.3 Granuloma formation as a method of containment

Immune cells traffic to the site of infection attracted by chemokines that are produced as a result of the inflammatory immune response generated by cells already in the infected tissue. When microbes are not effectively destroyed by this action, immune cells can develop a structure known as a tissue granuloma. Granulomas can form in the environment of both type 1 and type 2 inflammation, although the type of immune response under way influences the composition of the immune cell types that form the granuloma. Macrophages (M1 or M2) are integral to granuloma formation and in type 1 granulomas they can fuse to form multinucleated giant cells. Both T and B cells can be found in a granuloma, and type 2 granulomas have a greater proportion of granulocytes.

Granulomas essentially act as cellular cages, containing the pathogen and, in some cases, causing death or inactivation of the pathogen by starvation due to restricted access to the components necessary for growth. Granulomas in

Table 1.6 Summary of immune effector mechanisms against parasitic infection.

Soluble			Cellular		
Component	Function	Innate or adaptive	Cell	Process	Innate or adaptive
Complement	Opsonin lysis	Innate and adaptive	Macrophages, dendritic cells Neutrophils Eosinophils	Phagocytosis	Innate
Acute phase proteins	Opsonin complement activation	Innate	CD8+ T cells	Cell lysis	Adaptive
Anti-microbial peptides	Opsonin lysis	Innate	NK cells	Cell lysis	Innate
Antibody	Opsonin complement activation	Adaptive	Granulocytes	Granuloma formation	Innate

parasitic infection are most commonly associated with schistosome infection, in particular the formation of type 2 granulomas around schistosome eggs trapped in the liver (see Chapter 16). In this case, the prolonged induction of Th2 inflammation, and the involvement of M2 macrophages, can lead to fibrosis in the liver. Granulomas can also form around some parasitic nematodes, although it has not been established whether this is a cause or a consequence of, nematode death. Type 1 granulomas are most commonly associated with tuberculosis as a method of containment of *Mycobacterium tuberculosis* bacteria in the lung. They also occur in Crohn's disease as an over-reaction to the commensal gut flora.

Some of the different immune effector mechanisms against parasitic infections are summarised in Table 1.6.

1.14 Hypersensitivity reactions

Hypersensitivity reactions are of relevance to this book, due to the powerful effects that parasitic infections, in particular helminth infections, can have on the development of allergies and allergic asthma. This is discussed in section 5 under the 'hygiene hypothesis' (Chapter 23), which attempts to explain why the incidence of allergies and asthma have increased in developed countries when compared with their developing counterparts. Hypersensitivity reactions are classified into four different types, with types 1, 2 and 3 occurring immediately and type 4 taking some time to develop fully (delayed hypersensitivity reactions).

1.14.1 Type 1 hypersensitivity reactions (immediate)

Type 1 reactions are mediated by cross-linking of FcεRI on the surface of mast cells by complexes of innocuous multivalent antigens ('allergens') with IgE (Figure 1.17 A). This leads to the release of vasoactive mediators, such as histamine, and symptoms of allergic reactions such as hay fever.

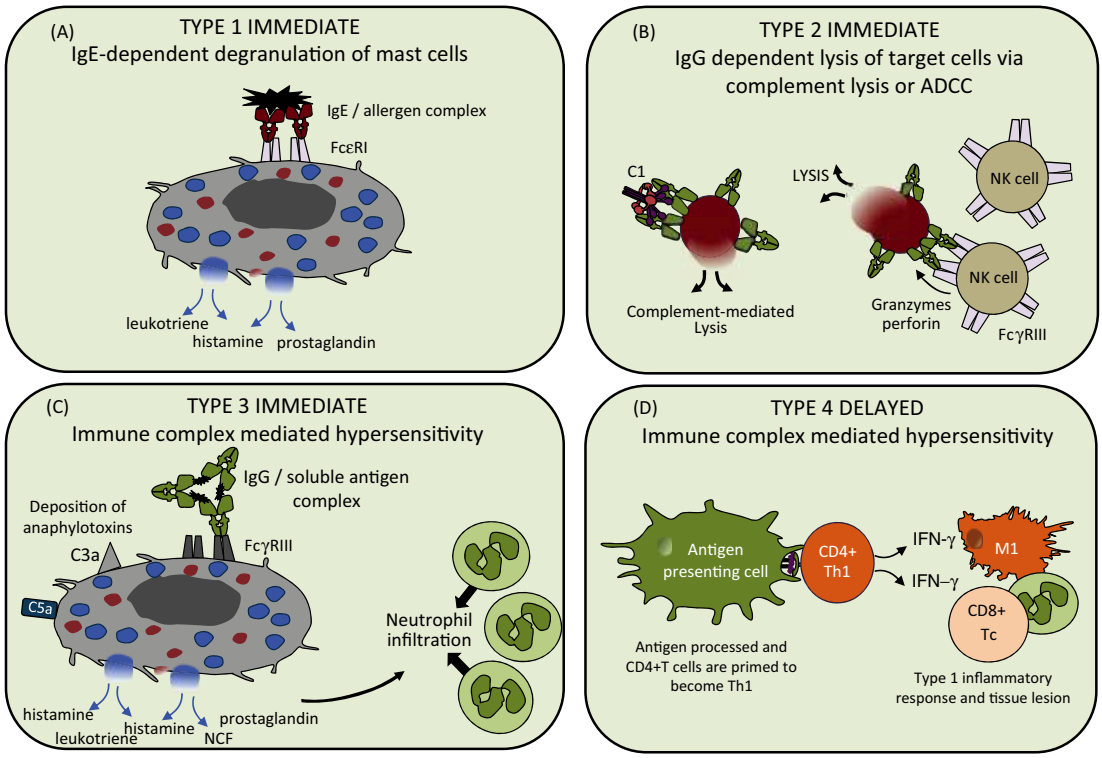


Figure 1.17 Hypersensitivity reactions. Hypersensitivity reactions can be classified into four types. Type 1 hypersensitivity reactions (A) arise from the degranulation of mast cells by IgE complexed with multivalent antigen. Type 2 hypersensitivity reactions lead to ADCC-mediated lysis of red blood cells and platelets opsonised by IgG or IgM (IgM not shown) (B). The deposition of immune complexes and neutrophil mediated inflammation cause type 3 hypersensitivity reactions (C). Cell-mediated delayed type hypersensitivity reactions are the result of activation of Th1-mediated inflammation in the tissue (D) Abbreviations: ADCC, antibody-dependent cytotoxicity; Ig, immunoglobulin; NCF, neutrophil chemotactic factor; NK, natural killer.

1.14.2 Type 2 hypersensitivity reaction (immediate)

Type 2 reactions result from the opsonisation of red blood cells and platelets by antibodies of the IgG or IgM isotype. These antibodies are made in response to drugs such as the antibiotic penicillin. The drugs bind onto the surface of red blood cells and platelets, serving as a target for opsonising antibodies resulting in ADCC from ligation of FcγRIII on NK cells by IgG or complement lysis from IgM opsonisation. Clearance of the opsonised cells leads to haemolytic anaemia (opsonised red blood cells) or thrombocytopaenia (opsonised platelets).

1.14.3 Type 3 hypersensitivity reactions (immediate)

Type 3 reactions are mediated by immune (IgG/ antigen) complexes that form in response to soluble antigen. The deposition of these complexes in various tissues causes an inflammatory response, initially mediated by mast cell degranulation in response to anaphylotoxins from the complement pathway

and cross-linking of FcγRIII, which leads to the degranulation of mast cells. This response is subsequently amplified by the infiltration of neutrophils into the tissue. Serum sickness from the administration of therapeutic antibodies, or anti-venom against snake bites and glomerulonephritis (deposition of immune complexes in the kidneys), are some examples of type 3 hypersensitivity reactions.

1.14.4 Type 4 hypersensitivity reaction (delayed)

Type 4 reactions are delayed type hypersensitivity reactions (DTH) that occur when inflammatory Th1 cytokines activate macrophages and cytotoxic CD8+ T cells, causing direct cellular damage. DTH is delayed due to the time required to activate the cellular component of the reaction. Examples of DTH reactions include rejection of transplanted tissue, and the delayed reaction to the tuberculin test normally used to determine whether an individual has been previously infected with *M. tuberculosis*.

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Section 2

Introduction to Protozoan Infections 2

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2.1 The protozoa

Protozoa, Protoctists, and Protoctista are all generic terms used to encompass unicellular microorganisms with a nucleus (eukaryotes). The organisms that have historically been included in these catch-alls are morphologically, trophically, ecologically, and phylogenetically diverse and occupy every niche possible. These include some of the most hostile environments found on Earth, such as in the human body. With over 40,000 different species described, it is widely accepted that many thousands of additional species await discovery.

While our understanding of the evolutionary history of the protozoa is rapidly changing, analyses of large-scale genomic and phylogenetic studies suggest that they can be partitioned into six supergroups (Figure 2.1): the Hacrobia (or CCTH: cryptomonads, centrohelids, telonemids, and haptophytes), the Harosa (or SAR: stramenopiles, alveolates and *Rhizaria*), Archaeplastida (*Plantae* and algae), Excavata, Amoebozoa and Opisthokonta (*Metazoa* and fungi). With the exception of the Hacrobia, parasitic species have been identified within all of these groups.

The complex life cycles and reproductive strategies that many protozoan parasites display often involve colonisation of multiple host species and belie their apparent morphological simplicity. Many are exquisitely adapted to life within particular host(s), while others are generalists and able to colonise diverse groups of hosts. Only a handful of these parasitic species are human pathogens (Table 2.1). However, a few of these organisms have been historically, and continue to prove to be, tremendous burdens to public health, killing millions of people annually (e.g. *Apicomplexa*) or rendering agricultural practices impossible in certain parts of the world (e.g. *Kinetoplastida*). Some of these parasites have applied such powerful selection pressures on their human hosts that they

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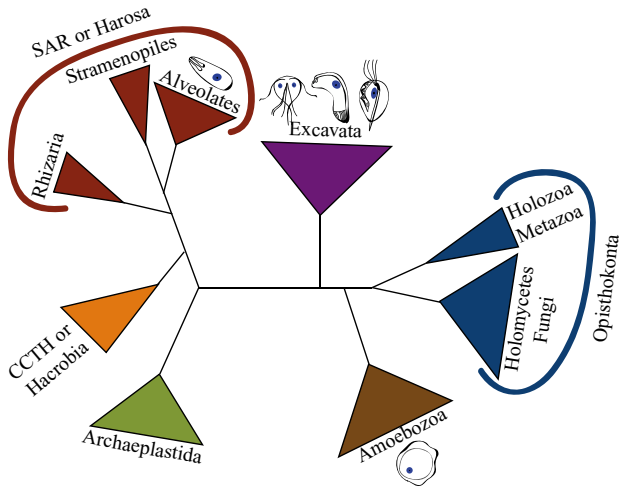

Figure 2.1 The phylogeny of the protozoa. This illustration (adapted from Walker, G *et al*. (2011). Eukaryotic systematics: a user's guide for cell biologists and parasitologists. *Parasitology* 138(13), 1638–1663) is based on the analysis of several protein-encoding gene families and shows the phylogenetic relationship of the different protozoan supergroups containing parasitic species. Within these supergroups, the positions of the major species infecting humans is also shown with a pathogen cartoon.

have helped to fix mutations that can results in serious medical conditions, such as sickle cell anaemia or thalassemia, into endemic populations.

This chapter serves as an introduction to the world of protozoa. We have confined our discussion to the three protozoan supergroups that contain major human pathogens – the Amoebozoa, Excavata and Harosa. The general anatomy and life cycles of these pathogens will be summarised, as will the use of more recent innovations in genomic analysis that are reshaping our understanding of their biology and evolution.

2.2 Amoebozoa

This supergroup consists of thirteen clades of amoebae and amoeboid flagellates. The Amoebozoa contains free-living, commensal, and parasitic species. Free-living species inhabit both terrestrial and aquatic environments. Most move via directed cytoplasmic flow and extension pseudopodia, although a few groups utilise flagella during particular points in their life cycle. Organisms within this group also show a great deal of diversity in their organelle complement. Some species have branching mitochondria with irregular cristae, degenerate or no mitochondria, or harbour a variety of endosymbionts. Several members of this group are parasites. of which *Entamoeba histolytica* is the most important to human health.

2.2.1 *Entamoebidae*

This family is comprised of species that are commensals or parasites of the digestive systems of arthropods or vertebrates. Genera and species have

Table 2.1 There are 30 species of protozoa that commonly infect humans. Many of these species are commensals or opportunistic pathogens that only develop into medically important parasites in immunocompromised individuals. These species belong to five supergroups which are listed in underlined text, along with their mode of transmission, tissue tropism and global distribution. Species which were formally considered protozoa but are now known to be fungi are not listed.

		Transmission	Tissue tropism	Distribution
Amoebozoa				
Archamoebae				
Entamoeba	*histolytica*	environmental cysts or direct transmission	small intestine	worldwide
	coli	environmental cysts or direct transmission	small intestine non-pathogenic	worldwide
	dispar	environmental cysts or direct transmission	small intestine non-pathogenic	worldwide
Endolimax	*nana*	environmental cysts	cecum non-pathogenic	worldwide
Iodamoeba	*bütschlii*	environmental cysts	large intestine non-pathogenic	worldwide
Centramoebida				
Acanthamoeba	*castellanii and other spp.*	environmental cysts and trophozoites	respiratory and CNS	worldwide
Balamuthia	*mandrillaris*	environmental cysts and trophozoites	respiratory and CNS	North America
Flabellinea				
Sappinia	*diploidea*	environmental cysts and trophozoites	respiratory and CNS	North America
Excavata				
Metamonad				
Diplomonadida				
Giardia	*lamblia*	environmental cysts or direct transmission	small intestine	worldwide
Retortamonadida				
Chilomastix	*mesnili*	environmental cysts	cecum and colon non-pathogenic	worldwide
Trichomonadida				
Trichomonas	*vaginalis*	direct transmission	vagina, prostate, urethra and seminal vesicles	worldwide
	tenax	direct transmission	oral cavity non-pathogenic	worldwide
Pentatrichomonas	*hominis*		cecum and large intestine non-pathogenic	worldwide
Dientamoeba	*fragilis*	unknown	large intestine	worldwide

(*Continued*)

Table 2.1 (Continued)

		Transmission	Tissue tropism	Distribution
Discoba				
Kinetoplastida				
Leishmania	*braziliensis*	Sandfly	causes mucocutaneous disease	Brazil
	donovani	sandfly	causes visceral disease	India and China
	infantum	sandfly	causes infantile visceral Leishmaniasis	old world
	major	sandfly	causes cutaneous disease	old world
	mexicana	sandfly	causes cutaneous disease	new world
	tropica	sandfly	causes cutaneous disease	Middle East and India
Trypansoma	*cruzi*	reduviid bug	systemic	Central and South America
	rangeli	reduvid bug or faecal	systemic non-pathogenic	Central and South America
Trypanosoma	*brucei brucei*		systemic non-pathogenic	Africa
	b. gambiense		systemic chronic disease	Africa
	b. rhodesiense		systemic acute disease	Africa
Heterolobosea				
Naegleria	*fowleri*	environmental cysts and trophozoites	respiratory and CNS	worldwide
Harosa				
Aveolata				
Apicomplexa				
Babesia	*microti and other spp.*	tick	systemic generally non-pathogenic	USA
Cystoisospora	*belli*	environmental oocytes	small intestine	worldwide
Cryptospordium	*parvum*	environmental oocysts	small intestine	worldwide
	homis	environmental oocysts	small intestine	worldwide
Plasmodium	*falciparum*	mosquito	systemic	worldwide
	knowlesi	mosquito	systemic	South East Asia
	malariae	mosquito	systemic	worldwide
	ovale	mosquito	systemic	old world
	vivax	mosquito	systemic	worldwide
Toxoplasma	*gondii*	ingestion of environmental oocysts or tissue bradyzoites	systemic	worldwide

Table 2.1 (Continued)

		Transmission	Tissue tropism	Distribution
Ciliophora				
Balantidium	*coli*	environmental cyst	large intestine	Common in the Philippines, worldwide
Stramenophiles				
Blastocystis	*sp.*	environmental cyst	intestine	worldwide
Opisthokont				
Ichthyosporea				
Rhinosporidium	*seeberi*	environmental	nasal mucosa	Asia
Archeaplastida				
Chlorellales				
Prototheca	*wickerhamii*	environmental	dermis	worldwide

traditionally been differentiated based on nuclear morphology. The most important species, *Entamoeba histolytica*, is the causative agent of amoebic dysentery and infects millions of people annually, causing at least 100,000 deaths per year. While it is primarily transmitted via contaminated water or food, sexual transmission is also common in certain communities.

2.2.2 *Entamaoba histolytica*

The life cycle of this parasite is relatively simple and comprised of five stages: trophozoites, precysts; cyst; metacyst; metacystic trophozoites. Trophozoites inhabit the crypts of the large intestine and are highly motile, feeding on luminal content as well as mucus and material scavenged from the destruction of epithelial cells. While vacuoles containing digesting material are readily apparent in *E. histolytica* trophozoites, they have a number of unusual ultrastructural characteristics (Figure 2.2A and 2.2B). The cell is generally divided into two sections: a granular central mass called the endoplasm, that contains the nucleus; and a clear outer zone called the ectoplasm, that surrounds the endoplasm. The ectoplasm trails behind endoplasm as the protozoa moves. The nuclei of these organisms have a prominent endosome at the centre, with achromatic fibrils radiating to the inner surface of the nuclear membrane.

Typical endoplasmic reticulum and Golgi apparatus are not readily apparent in these organisms, although recent studies indicate that they have membrane compartments that perform similar functions. They also lack conventional mitochondria, instead having structures called mitosomes or cryptons. The function(s) of the mitosome is still unclear. Like conventional mitochondria, they possess a double membrane, and nuclear-encoded proteins are targeted to them with mitochondrial-like signal sequences. They do not have an organellular DNA, nor do they perform metabolic functions seen in aerobic organisms such as oxidative phosphorylation. Currently, they are believed to be

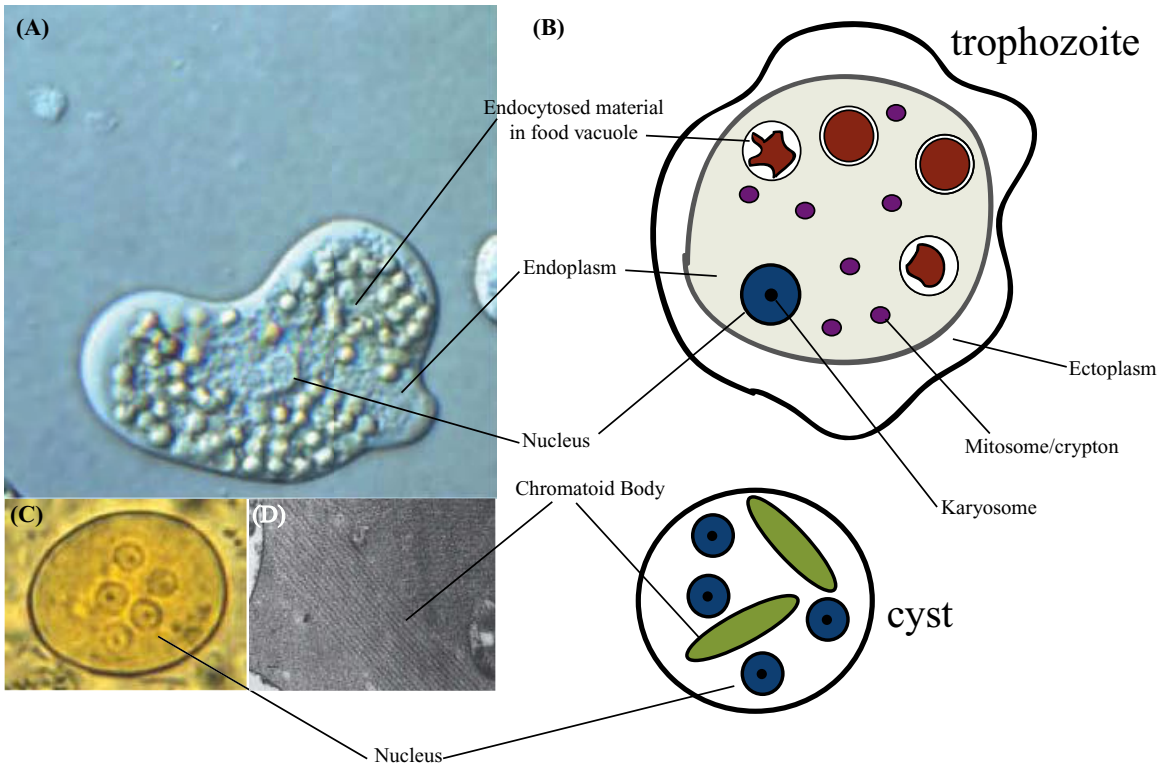

Figure 2.2 ***Entamoeba histolytica* basic anatomy.** (A) A phase contrast image of an *E. histolytica* trophozoite. (B) The illustration shows an idealised image of a trophozoite and a cyst. The major organelles and other subcellular features are labelled. (C) A phase contrast image of an *E. coli* cyst showing the characteristic multinucleate structure. (D) A transmission electron microscopy (TEM) image of the chromatoid body within the *E. histolytica* cyst.

degenerate mitochondria which play a role in Fe-S cluster assembly. However, additional metabolic functions will undoubtedly be identified.

Trophozoites multiply by binary fission. Under certain conditions, trophozoites invade the submucosa, causing inflammation and ulceration that spreads to the underlying muscularis mucosae and serosa. These ulcers can develop into serious lesions, via a combination of tissue destruction by the invading parasites and immune reactions to amoebas and luminal bacteria carried into the wound. In the most extreme cases, there is perforation of the colon and dissemination of parasites to other organs, leading to life-threatening conditions.

In normal, asymptomatic infections trophozoites pass in stool formations to the lower sections of the colon. Dehydration of the stool stimulates them to condense into a precyst sphere that secretes a hyaline wall around itself. The cyst initially has a single nucleus, but these rapidly divide into four nuclei as the cyst matures (Figure 2.2B and 2.2C). Chromatoid bodies containing large amounts of RNA are prominent during the development of the cyst (Figure 2.2B and 2.2C). This quadrinucleate cyst (or metacyst) is then passed into the external environment, where it can then infect a new host. After ingestion and

excysting in the small intestine, the cytoplasm and nuclei divide, forming eight small metacystic trophozoites.

2.3 Excavata

The Excavata are a group of largely heterotrophic flagellates which are found in oxygen-poor environments, although there are a number of prominent exceptions to this generalisation. The term 'excavate' is derived from the distinctive longitudinal feeding groove present in many lineages. Food particles are collected in these grooves after being swept there by currents generated from the beating of posteriorly directed flagella. Excavates can be divided into two major divisions: the amitochondriate metamonads and the predominately mitochondriate Discoba.

The lack of mitochondria in metamonads, other morphological features and early molecular phylogenetic analyses have lead to a consensus that this group contained the most 'basal' extant eukaryotes. However, advances in genomics and more recent molecular phylogenetic studies are reshaping our understanding of the evolution of these organisms, and it is now apparent that adaptation to their chosen habits has driven the evolution of many of the characteristics that have previously been labelled as being 'primitive'.

2.3.1 *Metamonad,* Diplomonadida

Many members of this class of small amitochondriate protozoa have the typical excavate feeding groove but also display an unusual double cell structure containing two nuclei with a complex of associated kinetosomes (Figure 2.3A and 2.3B). They often inhabit of the gastrointestinal (GI) tracts of animals living as commensals or parasites, although a number of free-living groups can be found in aquatic habitats. Like *E. histolytica*, they also possess atypical degenerate mitochondria (mitosomes) that have severely reduced or altered metabolic functions. Typical stack-like Golgi structures are also not apparent at an ultrastructural level although, again, like *E. histolytica,* equivalent functions are carried out in other membrane compartments. Several members of this group are parasites of humans, the most important of which is *Giardia lamblia* (see Chapter 6), a member of the *Hexamitidae*.

2.3.1.1 *Giardia*

This was probably the first parasitic protozoa ever described; Antony van Leeuwenhoek gave a clear description of *G. lamblia* in 1681 after examining his own diarrhoeic stool. Also sometimes referred to as *G. intestinalis* and *G. duodenalis*, this species is thought to be the most common flagellate of the human digestive tract. Nomenclature and taxon/species identification remain somewhat controversial, with 11 species being proposed by some authors. Assignments are complicated by high levels of genetic diversity within the *G. duodenalis/ lamblia/intestinalis* assemblage and the broad host range displayed by these parasites.

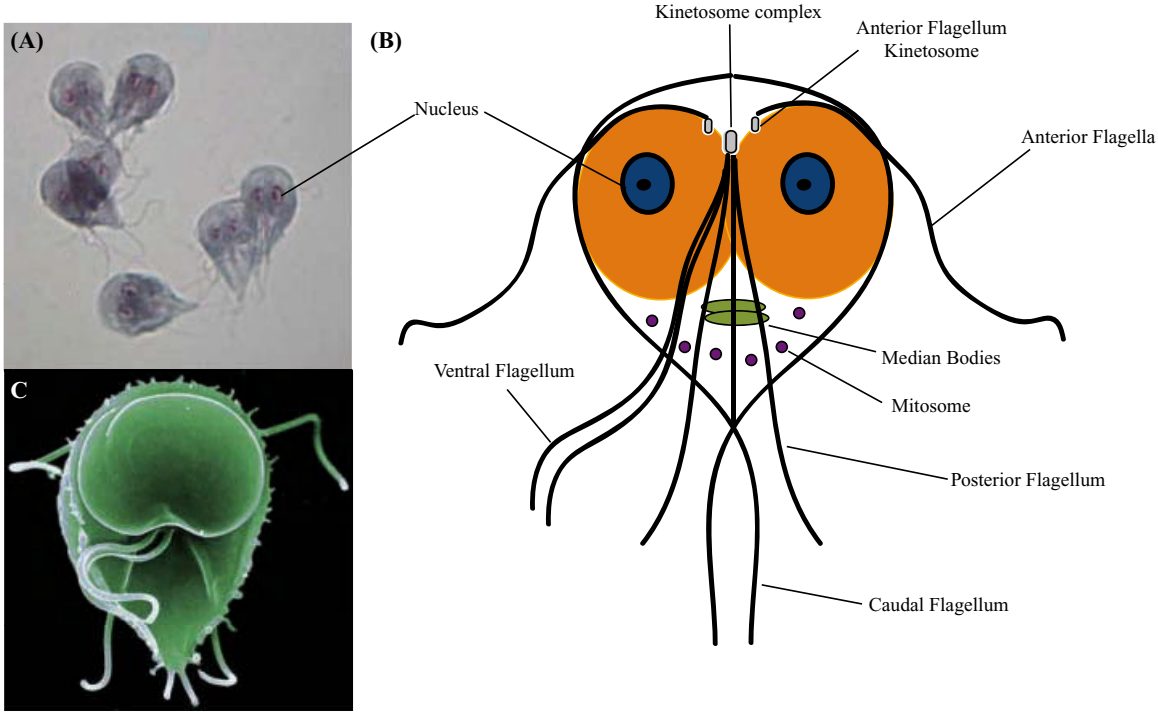

Figure 2.3 ***Giardia lamblia* basic anatomy.** (A) A light microscopy image of Giemsa-stained *G. lamblia* trophozoites. (B) The illustration shows an idealised image of a *G. lamblia* trophozoite. The major organelles and other subcellular features are labelled. (C) A false coloured scanning electron microscopy (SEM) image of *G. lamblia* trophozoite showing the adhesive disc on the surface the parasite.

Like *Entamoeba,* infection is initiated by the consumption (or sexual transmission) of environmentally resistant cysts. These cysts are ovoid and contain four nuclei. Once inside the small intestine, the parasite excysts, divides into trophozoites and attaches to the surface of an epithelial cell with an adhesive disc and ventral flagella (Figure 2.3A, B and C). Trophozoite populations expand rapidly by asexual binary fission, and some estimates of parasite numbers have been upwards of 1.4×10^{10} trophozoites and 3×10^{8} infectious cysts in a single diarrhoeic stool.

Trophozoites are highly motile, swimming with the aid of their eight flagella. Ultra-structurally, these flagella originate from a complex kinetosome structure located at the anterior end of the parasites two nuclei (Figure 2.3A and 2.3B). Below the nuclei and adhesive disc, there is an organelle made of bundled microtubules, called the median body. This organelle is specific to the genus and is often used to discriminate between species. Its function is unknown, although it is speculated that it might be involved in the formation or maintenance of the adhesive disc.

These parasites are aerotolerant anaerobes that will consume oxygen when it is present but they lack cytochrome-mediated oxidative phosphorylation, instead relying on fermentative metabolism. Trophozoites do not directly damage the intestinal epithelium, but they do strip mucus from the surface of cells, which

can precipitate other complications. High parasite loads can lead to malnutrition by interfering with nutrient absorption. Immune reactions to the parasites induce diarrhoea, which is the discomforting hallmark of this disease, although many individuals remain completely asymptomatic.

Like other parasitic protozoa, such as *Plasmodia* (see Chapter 3) and *Trypanosoma* (Chapter 8), *Giardia* exhibits large amounts of antigenic variation, which presumably deflects humoral responses that normally help control infections. Dehydration of stool stimulates trophozoites to develop into cysts, which are then passed out into the external environment.

2.3.2 *Metamonad, Parabasalia*

Parabasalids are heterotrophes found strictly in association with the mucosal surfaces of animals. Most are gut commensals, with a few species adopting parasitic lifestyles. This phylum is characterised by the presence of one or more specialised organelles called parabasal bodies. These organelles are a highly modified Golgi complex and associate with the kinetosomes that are basal to the cells flagella (Figure 2.4A and 2.4B). While the number of flagella present

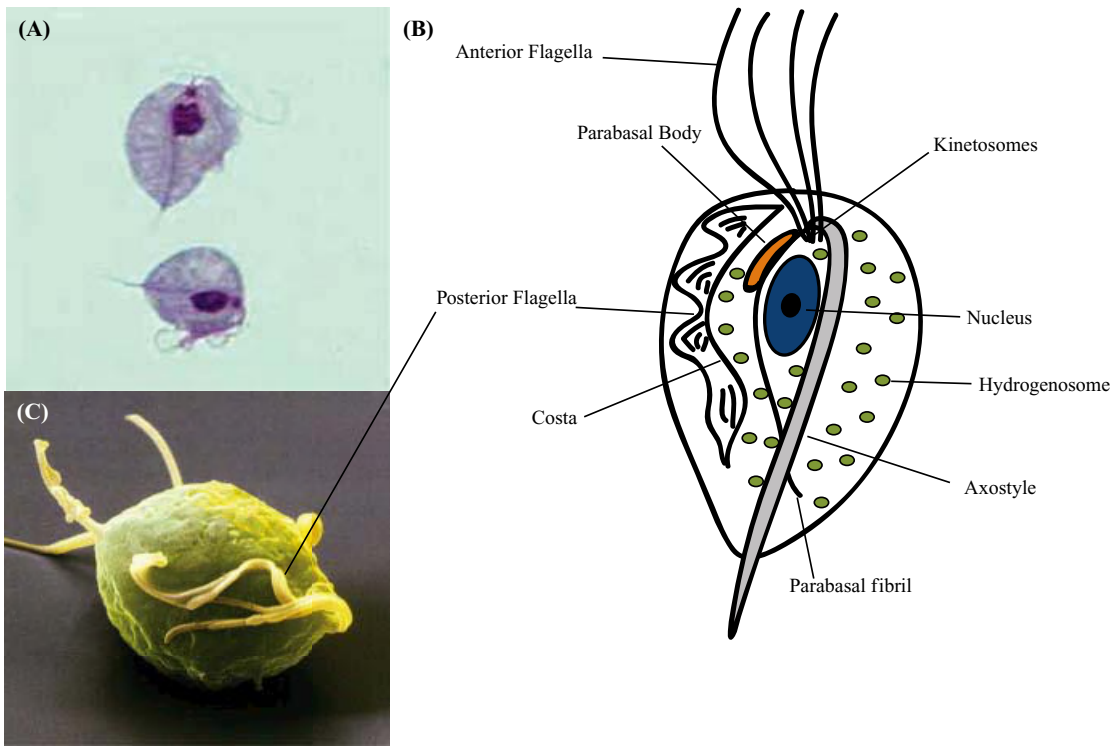

Figure 2.4 ***Trichomonas vaginalis* basic anatomy.** (A) A light microscopy image of Giemsa-stained *T. vaginalis* trophozoites. (B) The illustration shows an idealised image of a *T. vaginalis* trophozoite. The major organelles and other subcellular features are labelled. (C) A false coloured scanning electron microscopy (SEM) image of *T. vaginalis* trophozoite, showing the flagella and posterior flagellar membrane on the surface of the parasite.

can vary dramatically, most parabasalids are mononucleate, although some species can be multinucleate.

Like *Giardia* and *Entamoeba*, these organisms lack conventional mitochondria, instead having mitochondria-like structures called hydrogenosomes (Figure 2.4B). These organelles generate hydrogen as a by-product of pyruvate metabolism to acetate and ATP. Like the mitosomes of other parasitic protozoa, these organelles lack an endogenous genome, but appear to import nuclear encoded proteins in a manner similar to conventional mitochondria. Analogous H_2-producing structures have been found in other protozoa and fungi that still harbour a genome, so whether these structures are representative of a highly divergent mitochondria or a separate endosymbiotic association is still unclear. Several species of parabasalids can colonise humans, with the most important belong to the *Trichomonadidae*.

2.3.2.1 Trichomonadidae

Three different species of *Trichomonadidae* are found in association with humans: *Trichomonas tenax*, *T. vaginalis* and *Pentatrichomonas hominis*. *T. vaginalis* is the only species of great medical importance. These commensals sometimes 'moonlight' as venereal pathogens, colonising the vagina and urethra of women or the prostate, seminal vesicles and urethra of men. They have only one trophic stage of development and are morphologically oblong, with four free anterior flagella associated with the nuclei (Figure 2.4B and 2.4C). A fifth flagellum is fixed to a short undulating membrane that ends in the middle of the cell body. A structure called the costa runs underneath the undulating membrane. An axostyle originating near the flagellar kinetosomes runs the length of the cell and protrudes from the posterior end of the cell. The parabasal body is found associated with kinetosomes, and the parabasal filament runs most of the length of the cell (Figure 2.4B).

Transmission of *T. vaginalis* is via sexual contact, but most strains show low pathogenicity, with infected individuals being asymptomatic. At the mucosal surfaces, these protozoa voraciously consume material that they can engulf, including commensal bacteria, leukocytes and cell debris. They reproduce by longitudinal fission. Dysbiosis or immune reactions to pathogenic strains can lead to intense inflammation and leukocyte infiltration. These parasites do not form environmentally resistant structures such as cysts.

2.3.3 *Discoba,* Euglenozoa

This diverse group of mitochondriate flagellates includes the euglenids, kinetoplastids (trypanosomes and bodonids), diplonemids and symbiontids. The *Euglenozoa* are united by the presence of two flagella, which are inserted parallel to one another in an apical or subapical pocket. These are usually associated with a unique feeding apparatus called a cytostome, which is used to ingest smaller prey or other material. Most members of this group are heterotrophic. One lineage (the euglenids) has acquired a plastid derived from an endosymbiotic association with a photosynthetic alga. Taxa from this group occupy almost every trophic niche, with species identified having free-living, commensal,

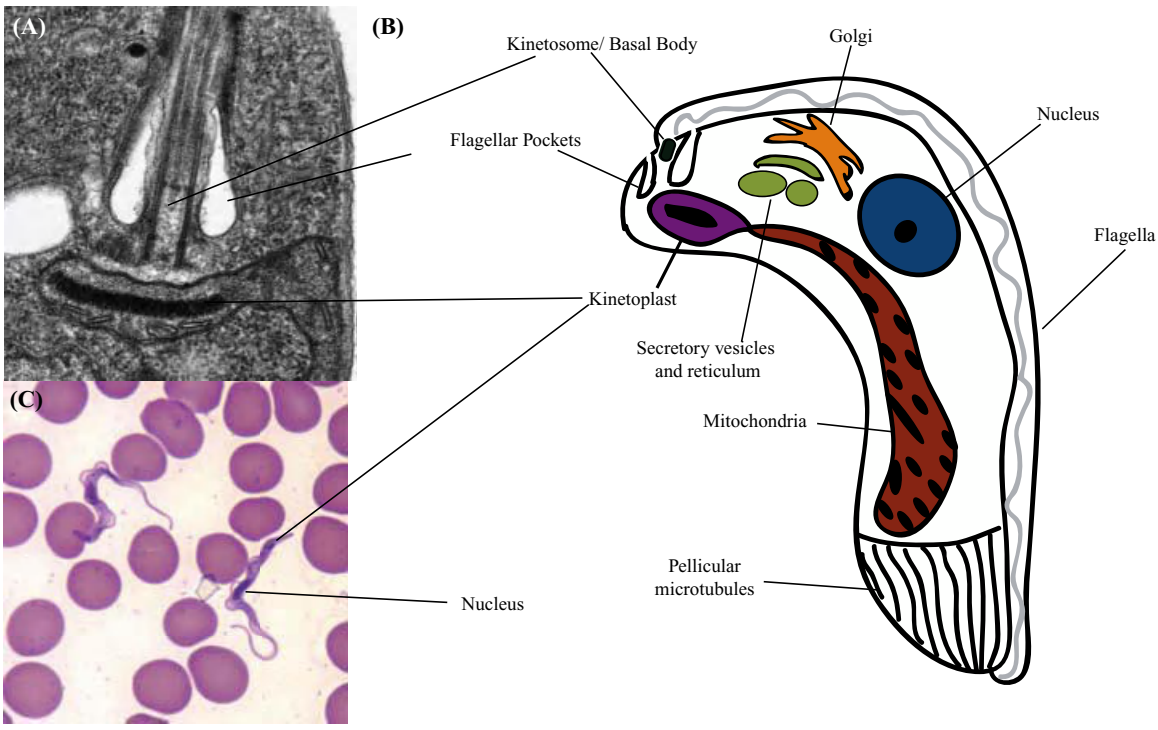

Figure 2.5 Kinetoplastida basic anatomy. (A) A TEM image of a *Leishmania* promastigote kinetoplast and kDNA complex at the base of the kinetosome and flagella. (B) The illustration shows an idealised image of a *T. brucei* stumpy trypomastigotes. The major organelles and other subcellular features are labelled. (C) A light microscopy image of Wright Giemsa-stained thin blood smear containing *T. brucei* trypomastigotes.

endosymbiotic, and parasitic lifestyles. The *Kinetoplastida* contain the species that are of medical importance to humans.

2.3.3.1 *Kinetoplastida*

The kinetoplastids are an extremely diverse order, containing both free-living and parasitic members. As well as having the defining characteristics of the Euglenozoa, this order possess a single large mitochondrion containing a massed collection of mitochondrial DNA that forms a complex structure called the kinetoplast (kDNA). The kinetoplast is associated with the two kinetosomes at the base of the flagella (Figure 2.5A and 2.5B).

The *Kinetoplastida* contains two suborders: the predominately free-living *Bodonina* and the parasitic *Trypanosomatidae*. The *Trypanosomatidae* are one of the most diverse groups of parasites known; they are unusual because they have members which not only infect animals and plants (*Phytomonas sp.*), but can do so in both terrestrial and aquatic environments using different groups of intermediate hosts.

Their life cycles can range from simple to complex and, while it is believed that the group may have evolved from parasites of insect digestive tracts, many

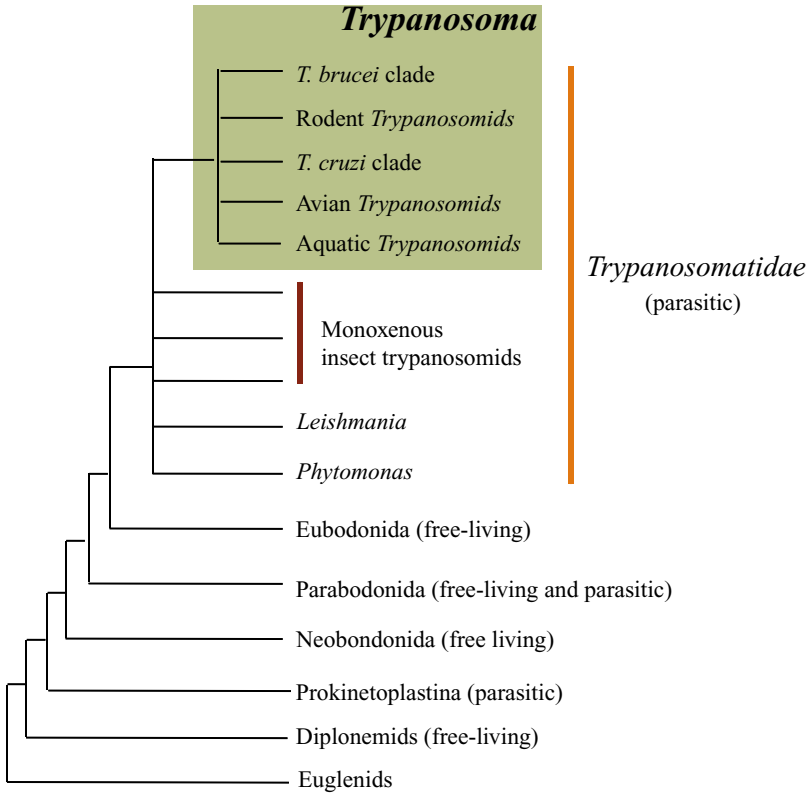

Figure 2.6 The phylogeny of the Kinetoplastida. The cladogram shows the phylogenetic relationship of the different free living or parasitic kinetoplastid groups. This illustration is based the analysis of the small subunit (SSU) ribosomal RNA and several protein encoding gene families. Adapted from Simpson, AG *et al.* (2006). The evolution and diversity of kinetoplastid flagellates. *Trends in Parasitology* 22(4), 168–174.

vertebrate-infecting species are transmitted via an invertebrate host. Understanding how parasitism evolved in this group and their placement with in the Kinetoplastids, has been an area of great interest. Figure 2.6 shows a phylogenetic tree that summarises our current knowledge of the evolutionary relationships of the members of this order.

The data suggests several important points. Importantly, the previous belief that the closest free-living ancestors of the Kinetoplastida are photosynthetic Euglenids is not supported by these analyses. Instead, they indicate the Kinetoplastida may have evolved from an obscure group of surface-associated heterotrophes called the Diplonemids. Within the kinetoplastids, parasitism has evolved at least three different times. However, while there is support for the monophyly of the Trypanosomatids and the major groupings within this clade, the details of how many of these organisms are related to each other remain unresolved.

The two genera containing important humans pathogens are *Trypanosoma* and *Leishmania*. These genera undergo replicative cycles in both their insect and

vertebrate hosts and, while it is poorly described, there is evidence that these parasites also undergo periodic sexual cycles in their insect hosts. Besides the kinetoplast a second unusual organelle called the glycosome is found in the *Trypanosoma* and *Leishmania*. A peroxisome derivative, this specialised vesicle is bound by a single membrane and is the primary site of glycolysis and glycolytic regulation in the parasite.

Trypanosoma brucei

This is a collection of three subspecies: *T. brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense* (see Chapter 8). *T. brucei brucei* is a pathogen of ruminants causing a disease called nagana. As well as infecting native game, *T. b. gambiense*, and *T. b. rhodesiense* are the etiological agents of African sleeping sickness, causing chronic and acute forms of the disease respectively. Infection is initiated by the bite of an infected tsetse flies (*Glossina sp.*), which can inoculate a host with thousands of metacyclic trypomastigotes. Once in the vertebrate host, the protozoa transform into slender trypomastigotes and begin replication in the blood and lymph. The parasites remain extracellular, although there are reports of recovery of amastigote forms from certain tissues during experimental infection of animals. In chronic infections, the parasites eventually invade the CNS and brain of the host. While in the vertebrate, the parasites are pleomorphic, with slender (containing sparse short tubular cristae in their mitochondria), stumpy (containing many tubular cristae in their mitochondria) and intermediate trypomastigotes, forms easily detected in circulation.

Studies of isolated varieties of these pleomorphic forms have shown that morphological differences are linked to the forms of metabolism that the parasites are currently using. Slender forms are highly replicative and have repressed mitochondrial function, metabolising glucose as far as pyruvate. They do not use the TCA cycle or oxidative phosphorylation for further ATP generation. Plentiful glucose and oxygen supplies in the blood apparently favour this form of respiration, and the glycosomes serve as the centre of this respiratory process.

In contrast, the stumpy forms that are infectious to the tsetse fly do not divide and have active mitochondria, fully oxidising glucose. Changes in mitochondrial respiration are linked with changes in kDNA localisation, but it is unclear whether the localisation of the kDNA regulates this process. Once the stumpy forms are taken up in a blood meal by a tsetse fly, the parasites migrate to the posterior midgut, where they replicate. After about ten days, slender form trypomastigotes migrate to the foregut and then the salivary glands, via the oesophagus and pharynx. In the salivary glands, they develop into epimastigotes, undergo further replication and transform into infectious metacyclic trypomastigotes.

The major mechanism of immune evasion, variation of surface antigen repertoires, is one of the best-studied aspects of *T. brucei* biology. One of the most interesting features of its genome is the organisation of more than 1,000 different variant-specific surface glycoprotein genes (VSG) in sub-telomeric arrays, and the mechanisms employed to restrict expression to a single VSG.

Trypanosoma cruzi

Restricted to the new world, the causative agent of Chagas disease, *T. cruzi* (see Chapter 9), has a radically different life cycle to its cousin *T. brucei*, opting instead for an intracellular lifestyle within the vertebrate host. A number of genetically distinct *T. cruzi* populations have been identified in South America, and phylogenetic analyses estimate that some of these groups may have diverged at least ten million years ago.

Infection is initiated by the feeding of a reduviid bug and its subsequent defecation on the vertebrate host. Infectious metacyclic stages are passed onto the host in the faeces of the insect. These either invade the host directly, via the feeding wound, or by mechanical transfer to mucosal surfaces. Once in the blood, trypomastigotes of *T. cruzi* do not replicate, but instead invade host cells. While a wide variety of cell types can be invaded, the most common are reticuloendothelial and muscle cells. The mechanisms underlying the invasion process are still being elucidated, but they involve the release of Ca^{+} by the host cell Golgi and recruitment of lysosomes to the contact point with the parasite. *T. cruzi* then enters the cell via modification of these lysosomes into a parasitophorous vacuole (PV). As the lysosome re-acidifies, a porin-like toxin secreted by the parasite ruptures the PV membrane, releasing the parasite into the cell cytoplasm where they lose their flagella and develop into amastigotes. The amastigotes replicate quickly, transform back to trypomastigotes and eventually rupture the infected cell. In certain larger cells, such as muscle cells, cyst-like pockets of parasites can form pseudocysts.

Released trypomastigotes can invade new cells or enter the blood, where they can continue their life cycle if they are ingested by feeding reduviid bugs. Once in the midgut of the insect, they transform into short epimastigotes, which replicate by longitudinal fission into a longer, slender form. Short, infectious metacyclic trypomastigotes appear in the rectum of the insect eight to ten days after it is infected.

Leishmania sp.

The genus *Leishmania* (see Chapter 7) contains over a dozen recognised species and several species complexes which have been defined by biochemical or molecular criteria. Distributed throughout both the old and the new world, two sub-genuses are recognised, *Leishmania* and *Viannia*, both of which contain pathogens relevant to human health. The five most important species from a medical perspective are *L. tropica*, *L. infantum*, *L. major*, *L. donovani*, *L. mexicana*, and *L. brasiliensis*, and these can cause visceral or cutaneous disease. Infection in a vertebrate is initiated when promastigotes are transmitted into the skin by the bite of female phlebotomine sandfly. Promastigotes are quickly phagocytosed by macrophages that reside in or underneath the dermis. Instead of being dispatched by the macrophages, the parasites reside in a modified lysosome, which matures into a PV. Once in the PV, the parasites transform into replicative amastigotes, which eventually rupture out of the infected cell and can infect new macrophages or other phagocytic cells.

Morphologically, the different amastigote species look fairly similar and are among the smallest known eukaryotic cells (2.5–5 μm wide). In stained

microscopic preparations, the nucleus and kinetoplast are the most prominent features, and the small cytoplasmic space appears vacuolated. Successive cycles of infection and intracellular replication ensue with different species displaying tropisms for specific organ sites. These tropisms eventually lead to the distinct pathologies that each *Leishmania* species causes.

A new sandfly is infected when *Leishmania*-containing cells in skin and blood are ingested during a meal. When infected cells are digested, amastigotes are released and migrate to the mid or hindgut, where they transform into promastigotes, attach to the fly's gut wall and multiply by binary fission. By the fourth or fifth day after infection, the promastigotes migrate to the oesophagus and pharynx, which they eventually clog. The fly clears the obstructing parasites by pumping the contents of the oesophagus in and out, and this action inadvertently inoculates the promastigotes onto the skin of a new vertebrate host.

2.4 Harosa

This supergroup (also called the SAR clade) was proposed in 2009 by Thomas Cavalier-Smith, and it is still undergoing revision based on new analyses of available and forthcoming genome datasets. Each of the three subgroups of which it is comprised, stramenophiles, alveolates, and *Rhizaria*, contain photosynthetic, mixotrophic and heterotrophic members. The stramenopiles and alveolates were initially grouped in the clade Chromalveolata, but this assemblage was subsequently revised, with rhizerians being added and some taxa being moved to the Hacrobia clade. The Harosa also contain a number of prominent parasitic members of metazoans and plants. The most important human pathogens belonging to the Harosa are found in the phylum *Apicomplexa*, which are members of the *Alveolata*.

2.4.1 Aveolata

This group of organisms are united on the basis of two shared ultra-structural features:

1. The presence of membrane-bound sacs beneath the plasma membrane (alveoli)
2. A plastid-like organelle which is believed to have been acquired by secondary endosymbiosis of a red algae in a Chromalveolate ancestor.

This grouping has been supported by subsequent molecular analyses, although exactly how the *Alveolata* are related to other Harosa members remains controversial.

2.4.1.1 Apicomplexa

Two major groups of human pathogens found within the Apicomplexa are the opportunistic Coccidian species such as *Toxoplasma gondii* and *Cryptosporidium sp.*, and the Haemosporidian parasites of the genus *Plasmodium*.

Historically named the *Sporozoa*, this phylum is comprised of a diverse group of mitochondria bearing parasitic protozoa with life cycle stages that include an infective motile 'zoite' form. The apical end of the zoite is comprised of a specialised secretory organelle complex – the 'apical complex' – and this is the basis for the name of the phylum and a requisite for invasion and colonisation of host cells.

Generally, the life cycles of the *Apicomplexa* are comprised of three stages. The first is a growth stage after infection of a host (or host cell) by the zoite. In many species, this is accompanied by mitotic reproduction. The second is a sexual cycle via the production of gametes, followed by fertilisation and the formation of zygotes within a thick-walled structure called an oocyst. Finally, sporogenesis occurs, during which there are successive divisions of the sporoplasm within the oocysts to form new infective zoites (now termed 'sporozoites'). In those *Apicomplexa* that infect new hosts via transit through the external environment, highly resistant spore structures (sporocysts), which shelter the developing sporozoites, form in the oocyst.

The *Apicomplexa* is a large phylum made of over 2,500 described species. It can be divided up into three major groups:

1. *Gregarina*, which infect invertebrates such as arthropods and prochordates.
2. *Coccidia*, which infect a wide variety of animals.
3. *Aconoidasida*, which is composed of the Piroplasmids and Haemosporidians, whose heteroxenous life cycles alternate between a vertebrate and an arthropod vector.

Parasites within all three of these classes can either be generalists, which infect a wide range of hosts or related species, or can have extremely narrow host specificities.

Ultra-structural analysis of the apical complex of the invasive zoites has shown they are composed of a cap formed by two conoidal rings (in some species), a conoid which is composed of spirally arranged microtubules, and a polar ring which acts as an organising centre connecting the conoid to microtubules that extend backwards underneath the plasma membrane (Figure 2.7A and 2.7B). This unique microtubule structure gives the complex its characteristic shape. Within the complex, there are generally two pedunculate secretory organelles called rhoptries, and a variety of other organelles such as the micronemes and dense bodies that are believed to traffic material such as secreted proteins from the Golgi to the apical complex.

In the *Apicomplexa*, the plastid organelle (or apicoplast) is surrounded by four membranes but has lost any photosynthetic capacity. Despite not being photosynthetic, the inner membrane of the apicoplast in some species such as *Plasmodium* still retains tubular 'whorls' which resembles thylakoids found in photosynthetic plastids. The function of these membrane structures remains unknown. The apicoplast still contains its own organellular DNA and is believed to perform a variety of important biosynthetic functions in lipid, heme

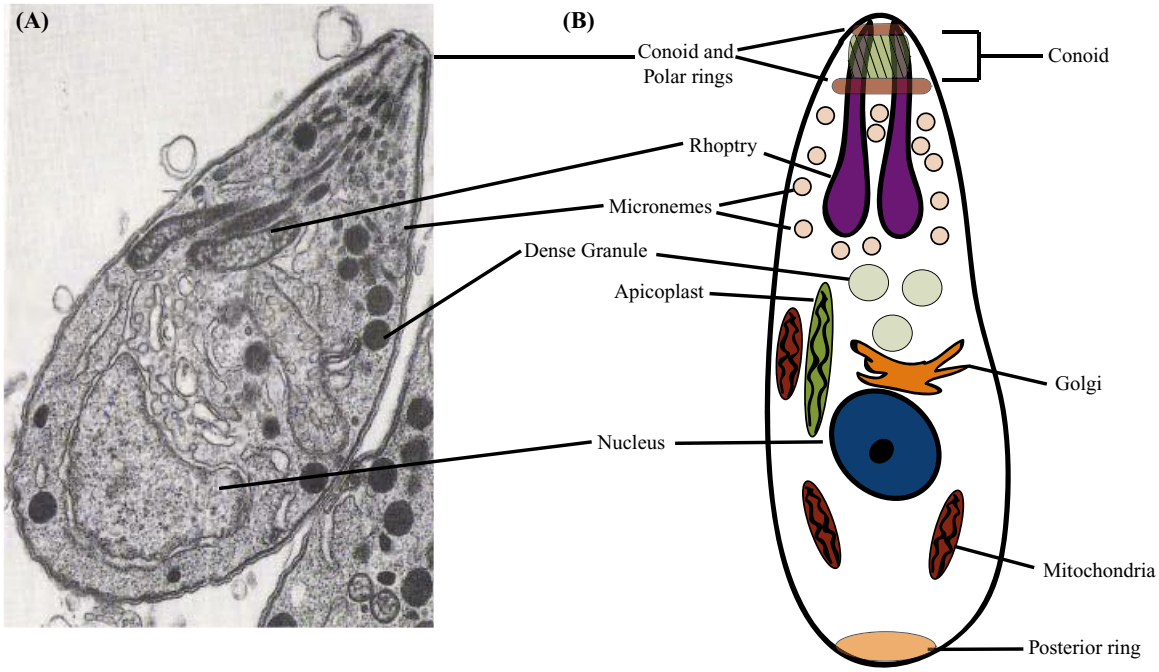

Figure 2.7 Apicomplexa basic anatomy. (A) A transmission electron microscopy (TEM) image of a *Toxoplasma gondii* tachyzoite. (B) The illustration shows an idealised image of a *Toxoplasma gondii* tachyzoite. The major organelles and other subcellular features are labelled.

and amino acid metabolism. The unique metabolic pathways used in the apicoplast are being developed as novel anti-apicomplexan drug targets.

Understanding the evolution of this important group of parasites, and the *Apicomplexa* in general, has offered a number of challenges. Traditional morphology or ultra-structure-based taxonomies are often conflicting and have not offered robust resolution within the genus *Plasmodium* or the phylum *Apicomplexa*. Phylogenies based on molecular data have also offered conflicting results, as genes which have served as workhorses for these types of analyses in other groups, such as the small subunit (SSU) ribosomal DNA (rDNA) gene, appear to have heterogeneous evolutionary rates or atypical composition biases in some *Apicomplexa*.

Figure 2.8A depicts a simplified phylogenetic tree, with only the major apicomplexan groups and representative species listed. Surprisingly, in these and other analyses, the *Cryptosporidia* are consistently placed outside of the clade containing other Coccidea, such as *Eimeria* and *Toxoplasma*. If these are truly unrelated groups, this may serve as a starting-point for elucidating why traditional anti-coccidial drugs are not effective in treating *Cryptosporidia* infections. Recent phylogenetic analyses using mitochondrial genes has also helped resolve relationships with the Haemosporidians (Figure 2.8B) and has lent support to the choice of some rodent and primate *Plasmodium* species as models for human malarial biology and vaccinology. Mammalian *Plasmodium sp.*

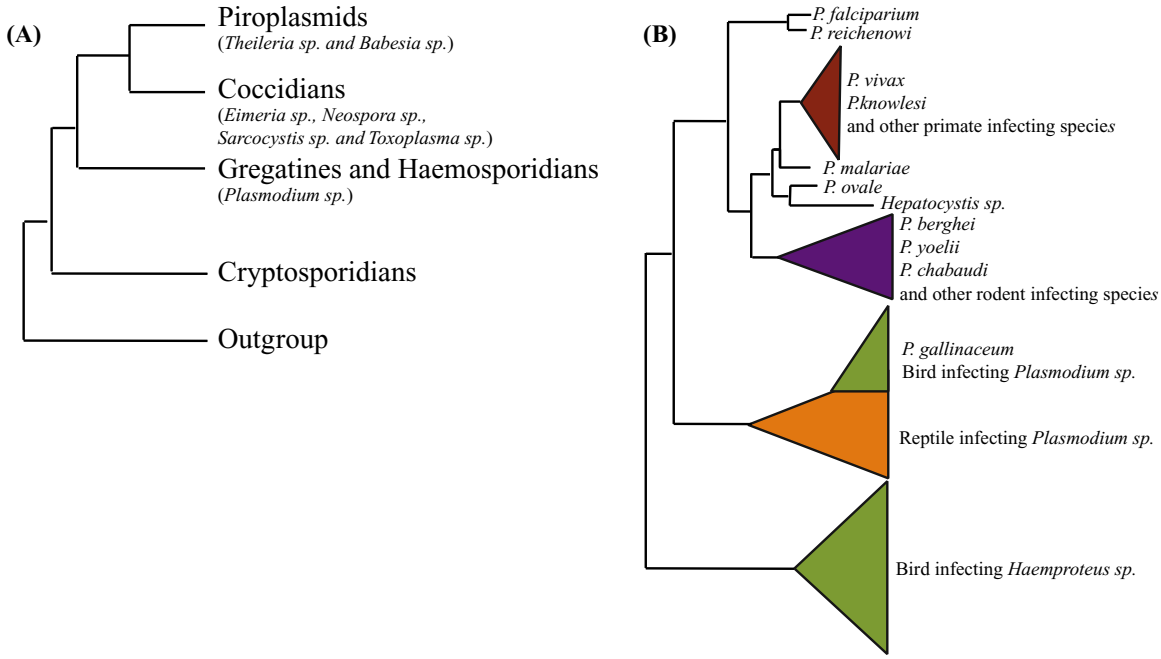

Figure 2.8 The phylogeny of the *Apicomplexa*. (A) The cladogram shows the phylogenetic relationship of the major *Apicomplexa* groups. This illustration, based the analysis of the SSU, has been adapted from Morrison, DA (2009). Evolution of the Apicomplexa: where are we now? *Trends in Parasitology* 25(8), 375–382. (B) This illustration is based the analysis of the mitochondrial cytochrome b gene and shows the phylogenetic relationships of the major Haemosporidian groups and *Plasmodium* species of medical importance (adapted from Perkins, SL and Schall, JJ (2002). A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. *Journal of Parasitology* 88(5), 972–978)

are now believed to have evolved from an ancestral species infecting reptiles or birds, and species capable of infecting humans appear to have evolved several times potentially as species jumped back and forth between primate and human hosts.

Coccidia, Cryptosporidium sp.

The Cryptosporidia are parasites of the brush boarders of a variety of mammals, birds, reptiles and fish, causing diarrhoea in human infections. *Cryptosporidium parvum* and *C. hominis* are the species that cause most human infections (see Chapter 5), although other species have been documented to cause disease. Infection begins after ingestion of a sporulated oocyst that contains four infectious sporozoites. Sporozoites parasitise epithelial cells in the digestive tract (or occasionally the respiratory epithelium). In these cells, parasites undergo alternating cycles of asexual division as type I meronts (schizogony) and sexual reproduction as type II meronts (gametogony). During gametogony, micro- and macrogamonts (gametocytes) develop. Microgamonts undergo asexual replication and then invade the adjacent tissue until they find and fertilise a macrogamont. The fertilised zygote then forms a thick- or thin-walled oocyte. The thick-walled oocytes exit the host in the faeces and

sporulate into infectious oocytes. The thin-walled oocytes sporulate within the host and sporozoites released initiate an autoinfectious cycle. *Cryptosporidium* infections are usually self-limiting, with only young and immunocompromised hosts developing serious complications.

Coccidia, Toxoplasma gondii

This cosmopolitan species has been found in almost every part of the world and in just about every warm-blooded mammal. In human populations, infection is widespread but is often asymptomatic outside of the very young or immunocompromised individuals. As an intracellular parasite, *T. gondii* can infect a wide variety of cells, including epithelia, muscle and neuronal cells. Sexual reproduction (the enteroepithelial phase) is confined to the definitive feline host, while asexual reproduction (the extra-intestinal phase) and the formation of long-lived cyst structures occurs in humans and other intermediate hosts.

Extra-intestinal stages begin upon ingestion of environmental oocysts or cyst-like structures contained in the tissue of an infected intermediate host called bradyzoites. Once ingested, sporozoites released from the oocysts or bradyzoites released from cysts rapidly penetrate the gut and invade a host cell, where they live within a PV. The parasites develop into rapidly replicating tachyzoite stages. These disseminate and invade muscle and neural tissue (including the brain). In most cases, after the acute phase, the onset of chronic infection is characterised by slower replication of the parasite and formation of bradyzoites. Cyst formation coincides with the onset of protective immune responses, but these long-lived structures occasionally rupture, releasing parasites that can re-initiate an acute phase of infection if the host becomes immunosuppressed.

If a feline definitive host ingests an oocyst or tissue-encysted bradyzoite in addition to the asexual systemic infection, a short phase of sexual reproduction occurs. Sporozoites or bradyzoites invade the intestinal epithelial, transform into replicating merozoites and produce microgametocytes and macrogametocytes. Microgametocytes divide and break out of the infected cell, and invade adjacent cells until they find and fuse with a macrogametocyte, where fertilisation occurs. The fertilised zygote forms an immature oocyte, which passes out of the feline in it's faeces.

Haemosporida, Plasmodium sp.

Composed of three major genera, *Haemoproteus*, *Leucocytozoon*, and *Plasmodium,* this group of organisms is one of the most successful parasitic assemblages known. In humans, all major pathogens belong to the genus *Plasmodium* (see Chapter 3), with the five important species currently recognised being *P. vivax*, *P. falciparum*, *P. ovale*, *P. malariae and P. knowlesei*. All the *Plasmodium* species have very similar life cycles, requiring both an invertebrate host (mosquito) and a vertebrate host.

The mosquito acts as the relatively brief definitive host, where sexual reproduction occurs, in contrast to several phases of asexual reproduction that occur within the vertebrate intermediate host. Infection in the vertebrate host takes place after deposition of saliva-containing sporozoites during mosquito feeding on the host blood. These sporozoites are highly motile and they rapidly migrate to the liver, where they specifically invade hepatocytes. Specific recognition of hepatocytes by receptors on the surface of the sporozoite confers the cell tropism observed by this stage of the parasite. Invasion of hepatocytes is facilitated by secretion of proteins from the secretory organelles of the apical complex. Entry into the hepatocyte initiates a stage of asexual reproduction called the pre-erythrocytic cycle, where the parasite transforms into a trophozoite, feeds on intracellular material and begins a process of asexual reproduction called schizogony. However, in some *Plasmodium* species (*P. vivax* and *P. ovale* in humans), some of the parasites develop into a state of dormancy and become hypnozoites, which can reactivate many years after the initial infection.

The schizont (also known as a cryptozoite) initially undergoes a series of nuclear divisions without cytokinesis, forming a large polynucleate cell. Once nuclear division is finished, other organelles also undergo division and, eventually, individual merozoites are formed. When the merozoites leave the hepatocytes, they initiate the erythrocytic cycle by invading a blood erythrocyte. Within the erythrocyte, they again transform into a trophozoite and feed, forming a large food vacuole (ring stage), where haemoglobin digestion occurs. They undergo schizogony, and finally rupture the host erythrocyte, releasing a new generation of merozoites. After an indeterminate number of asexual generations, a proportion of merozoites enter erythrocytes and develop into macro- or micro-gametocytes. These are the sexual stages of the parasite and the only transmissible stages of *Plasmodium* that are infective to mosquitoes.

After ingestion by a suitable mosquito host, gamonts are released from the gametocytes. The microgametocytes undergo a series of cell divisions, and daughter cells seek a suitably mature macrogamete to penetrate and fertilise. The resulting diploid zygote transforms into a motile ookinete, which penetrates the gut wall of the mosquito to form an oocyst on the hemocoel side of the gut. The oocyst undergoes a complex cycle of DNA replication and segregation that culminates in the formation of thousands of new individual sporozoites, which break out of the oocyst. The free sporozoites then migrate and enter the salivary glands, where they are in a prime position to be deposited in a new host when the insect feeds again.

One particularly interesting molecule produced by the parasite is a heme polymer called hemozoin. Free heme released during the digestion of erythrocyte haemoglobin would build to toxic levels if left uncompartmentalised. *Plasmodium*'s solution to this problem is to sequester the heme in the form of a polymer, which is then released when the merozoites escape the erythrocyte. Hemozoin's activity on the immune cells is complicated and has been shown both to activate (via Toll-like receptor 9) and to suppress innate immune cell function. Formation of hemozoin is an important metabolic process for the parasite, and several drugs currently used to treat malaria infection target

various aspects of heme polymerisation or food vacuole function (chloroquine and artemisinin).

2.5 Protozoa that are now fungi

Historically, two taxonomically enigmatic groups of organisms – Pneumocystids and Microsporidians – have been classified as protozoa. The Pneumocystids' initial grouping with the *Apicomplexa* was suggested, despite the presence of chitin in the organism's cell wall and other fungal characteristics. This assignment was made on the basis of resistance of *Pneumocystis carinii* (now *Pneumocystis jiroveci*) to standard anti-fungal drugs such as amphotericin B, the ability to form amoeboid pseudopodia and sensitivity to drugs used to treat protozoal infections.

In the case of *Microsporidia*, taxonomists initially grouped them with the *Apicomplexa* and then with the *Myxozoa* in the now-defunct class *Cnidosporidea*. The fact that these organisms lacked mitochondria, instead possessing mitosome-like structures similar to those seen in anaerobic protozoa like *Giardia*, *Entamoeba*, and *Trichomonas,* suggested to many taxonomists that they were 'primitive' protists that should be placed in what was then called the *Archezoa* (now the *Excavata*). However, molecular phylogenetic analysis based on rDNA and various protein-encoding genes have now established a firm placement for both of these groups within the Fungi.

Pneumocystis jiroveci is the major species of Pneumocystids that infects humans. It lives as an extracellular organism in the interstitial tissues in the lung. *P. jiroveci* has three life cycle stages – the trophozoite, precyst, and cyst stages – and engages in both asexual and sexual reproduction. In immunocompromised hosts, *P. jiroveci* can become a lethal respiratory pathogen, causing pneumonia with lung alveoli filling with a foamy exudate full of parasites. There are high carriage rates of this organism in normal, healthy people, suggesting that they act as a reservoir that can infect immunocompromised individuals via aerosol droplets. Molecular studies indicate that while *Pneumocystis spp.* are widespread, they exhibit fairly narrow ranges with each species, infecting a small number of closely related hosts.

There are at least fourteen microsporidian species that can infect humans, and all are obligate intracellular pathogens. *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* are two of the most common species. Infection is initiated by ingestion or inhalation of an infectious spore, which extrudes a polar tube or filament that pierces a target cell and injects sporoplasm into it. The sporoplasm transforms into replicative intracellular trophozoites. Development can occur either within the cytoplasm of the host cell (*Enterocytozoon bieneusi*) or within a PV (*Encephalitozoon intestinalis*).

During cell division, some trophozoites undergo sporogony, where a thick wall develops around them, forming a new environmentally resistant spore. These eventually burst out of the host cell and can infect a new cell or pass into the external environment. While *Microsporidia* are rarely pathogenic in normal, healthy individuals, they can develop into life-threatening illness in AIDs

patents or other immunocompromised individuals, where they can cause serious diarrhoeal disease or respiratory infection.

2.6 Taxonomy and the evolution of the parasitic protozoa

Taxonomic classification of single-celled eukaryotes or other microbiota has been, and remains, one of the most controversial areas of research in microbiology. While often seeming a very Victorian exercise to modern scientists, the building of robust taxonomic classification systems remains an important tool in helping us to understand the biology and evolution of species or groups. From the perspective of those interested in anti-parasite drug design or the immunology of parasitic infection, having a robust understanding of how a pathogen of interest is related to other members of its group is invaluable for the selection of appropriate model systems. Movement away from taxonomies based purely on morphological features has been slow, and parasitologists have been especially guilty of clinging to historical taxonomic systems, even in the light of genomes' worth of molecular data indicating these groupings are incorrect.

Historically (i.e. prior to the 1970s), all protozoan taxonomies were based on gross morphological features observable under a light microscope, mechanisms of motility and shared life cycle traits. Five major groupings (the Mastigophora, Sarcodina, Ciliophora, Sporozoa, and Microsporidia) were recognised, and many parasitology textbooks still organise their discussions of protozoa based on these outdated taxonomic groupings. With the introduction of glutaraldehyde as a fixation technique in the 1960s, data from ultrastructural studies using transmission electron microscopy began to call into question the validity of the five groups. Many taxa found themselves orphaned, because their ultra-structure indicated that they did not fit well with other members of a group into which they previously had been included.

Things did not progress further until the early 1990s, when a series of publications by Sogin and colleagues used SSU rDNA sequences to construct molecular phylogenies. These techniques precipitated a revolution in our understanding of how different protozoa were related to each other, and they also highlighted species that might represent the most basal lineages relative to the evolution of eukaryotes. These included a number of amitochondriate organisms such as *Giardia*, *Trichomonas* and *Encephalitozoon*. As well as lacking mitochondria, these species also lacked a number of other features present in 'higher' eukaryotes, such as introns, Golgi apparatus and peroxisomes, which again supported the hypothesis that they may have diverged at an early point in eukaryote evolution. This group of protozoal 'living fossils' was dubbed the Archezoa, and they sat at the base of a phylogenetic tree where the three 'higher' kingdoms comprised of animals, plants and fungi sat at the top, forming the 'crown group'. The rest of protozoan life sat somewhere in the middle, and this positioning was always rather tentative.

Unfortunately, the crown/Archezoa hypothesis proved incorrect. By the late 1990s, additional molecular studies of basal members of the tree identified

mitochondria-like genes in their genomes that were targeted to double-membrane bound organelles reminiscent of mitochondria, such as hydrogenosomes and mitosomes. In addition, sampling of SSU rDNA and protein gene sequences from additional species within each protozoan group established that some groups were evolving more rapidly (molecularly) than others. These rapidly evolving sequences would always cluster together, or with the bacterial sequences that were used as comparators to the eukaryotic sequences for rooting of the phylogenetic tree.

Once this phenomena – called 'long-branch attraction' – was accounted for, a number of rearrangements in the tree occurred, including the movement of several of the basal taxa into higher order groups (i.e. Microsporidia into the Fungi), with no one group(s) appearing to branch away earlier than the others. In the aftermath of the demise of the Archezoa, single-gene phylogenies were abandoned in favour of those combining multiple characteristics, such as two or more genes, and ultra-structural data to form a consensus view.

A new 'holistic' taxonomy of protozoa has recently been outlined, and this proposes six major taxonomic groupings: the Rhizeria, Chromalveolates, Archaeplastida, Excavata, Amoebozoa and Opisthokonta. This taxonomy has been revised several times, with whole or partial genome sequences becoming available for numerous additional species and groups. The tree presented in Figure 2.1 shows a snapshot of the current global view of protozoan taxonomy and evolution. This tree will undoubtedly change as more taxa, genes and characteristics are added, but it is the most robust framework that has ever been available.

2.7 Genomic and post genomic exploration of protozoan biology

In 1996, the genome of *Saccharomyces cerevisiae* was published in *Science*, heralding the arrival of whole genome sequencing for eukaryotes. It was six years before the first parasitic protozoan genome was completed (*P. falciparum*) but, within three years, seven more parasite genomes had joined *P. falciparum* in GenBank. Additional projects rapidly joined the queues at the sequencing centres and, to date, there are over 20 completed genomes from parasitic protozoa (Table 2.2), as well as numerous genome sequences of free-living species.

Many of these projects proved extremely challenging because of genome composition (i.e. the high AT content of *Plasmodium sp.*) or content (more than 50 per cent of the *T. cruzi* genome is repetitive elements). However, the effort to acquire the genome sequences has borne numerous fruits, increasing our knowledge of the biology of these organisms, as well as providing numerous novel drug or vaccine targets to the research community. In addition, it is now common for several pathogens within a single group to have fully sequenced genomes, and this has kick-started the era of comparative genomic analysis, facilitating the exploration of phylum and genus-specific biology.

Table 2.2 A selection of protozoa genome projects that have been completed (C) or are nearing completion (P). These have been listed along with the research institution(s) contributing to the project and associated references. There have been a large number of protozoa gene discovery and transcriptome-based projects that have also been published but are too numerous to be listed here.

	Model	Status	Sequencing Institute	References
Amoebozoa				
Entamoeba histolytica	human pathogen	C	TIGR, WSTI	Loftus, B *et al.* (2005). The genome of the protist parasite *Entamoeba histolytica*. *Nature* 433(7028), 865–868.
E. dispar	Commensal and model *Entamoeba* species	P	JCVI	
E. invadens	Reptile pathogen and model *Entamoeba* species	P	JCVI	
Acanthamoeba castellanii	human pathogen	P	HGSC	
Excavata				
Giardia lamblia (assemblage A)	human pathogen	C	KI	Morrison, HG *et al.* (2007). Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. *Science* 317(5846), 1921–1926.
Giardia intestinalis (assemblage B)	human pathogen	C	WSTI	Franzen, O *et al.* (2009). Draft genome sequencing of giardia intestinalis assemblage B isolate GS: is human giardiasis caused by two different species? *PLoS Pathog* 5(8), pe1000560.
Giardia intestinalis (assemblage E)	human pathogen	C	KI	Jerlstrom-Hultqvist, J *et al.* (2010). Genome analysis and comparative genomics of a *Giardia intestinalis* assemblage E isolate. *BMC Genomics* 11, 543.
Trichomonas vaginalis	human pathogen	C	TIGR/JCVI	Carlton, JM *et al.* (2007). Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. *Science* 315(5809), 207–212.
Naegleria gruberi	Model *Naegleria sp.*	C	CIG	Fritz-Laylin, LK *et al.* (2010). The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell* 140(5), 631–642.
Leishmania braziliensis	human pathogen	C	WSTI	Peacock, CS *et al.* (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nature Genetics* 39(7), 839–847.
L. donovani	human pathogen	P	WSTI	

Table 2.2 (*Continued*)

	Model	Status	Sequencing Institute	References
L. infantum	human pathogen	C	WSTI	Peacock, CS *et al.* (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nature Genetics* 39(7), 839–847.
L. major	human pathogen	C	WSTI	Ivens, AC *et al.* (2005). The genome of the kinetoplastid parasite, *Leishmania major*. *Science* 309(5733), 436–442.
Trypanosoma brucei	human pathogen	C	WSTI/TIGR	Berriman, M *et al.* (2005). The genome of the African trypanosome *Trypanosoma brucei. Science* 309(5733), 416–422.
T. cruzi	human pathogen	C	TIGR	El-Sayed, NM *et al.* (2005). The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* 309(5733), 409-415.
Harosa				
Babesia bovis	Major cattle pathogen and model of *B. microti*	C	WSU-CVM	Brayton, KA *et al.* (2007). Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. *PLoS Pathogens* 3(10), 1401–1413.
Cryptospordium parvum	Opportunistic human pathogen	C	BMGC	Abrahamsen, MS *et al.* (2004). Complete genome sequence of the apicomplexan, *Cryptosporidium parvum. Science* 304(5669), 441–445.
C. homis	Opportunistic human pathogen	C	BMGC	Xu, P *et al.* (2004). The genome of *Cryptosporidium hominis. Nature* 431(7012), 1107–1112.
C. muris	Rodent model of *Cryptosporidium* infection	P	TIGR/JCVI	Heiges, M *et al.* (2006). CryptoDB: a *Cryptosporidium* bioinformatics resource update. *Nucleic Acids Research* 34(Database issue), D419-422.
Plasmodium berghei	*Rodent model of malaria infection*	C	WTSI	Hall, N *et al.* (2005). A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science* 307(5706), 82–86.
P. chabaudi	*Rodent model of malaria infection*	C	WTSI	Hall, N *et al.* (2005). A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science* 307(5706), 82–86.
P. falciparum	*human pathogen*	C	SGTC, TIGR and WTSI	Summarised in Gardner, MJ *et al.* (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum. Nature* 419(6906), 498–511.

(*Continued*)

Table 2.2 (*Continued*)

	Model	Status	Sequencing Institute	References
P. knowlesi	*Primate model of malaria infection. Can also infect humans.*	C	WTSI	Pain, A *et al.* (2008). The genome of the simian and human malaria parasite *Plasmodium knowlesi*. *Nature* 455(7214), 799–803.
P. ovale		P	WTSI	
P.vivax	*human pathogen*	C	TIGR	Carlton, JM *et al.* (2008). Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 455(7214), 757–763.
P. yoelii yoelii	*Rodent model of malaria infection*	C	TIGR	Carlton, JM *et al.* (2002). Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii. Nature* 419(6906), 512–519.
Toxoplasma	*gondii*	C	TIGR/JCVI	Unpublished but summarised in Gajria, B *et al.* (2008). ToxoDB: an integrated *Toxoplasma gondii* database resource. *Nucleic Acids Research* 36(Database issue), D553–556.
Blastocystis	*hominis*	C	GSP	Denoeud, F *et al*. (2011). Genome sequence of the stramenopile *Blastocystis*, a human anaerobic parasite. *Genome Biology* 12(3), R29.

Abbreviations: CIG, Center for Integrative Genomics, University of California, Berkley, CA USA; BMGC, Biomedical Genomics Center, University of Minnesota, St. Paul, MN USA; GSP: Genoscope (CEA) and CNRS UMR 8030, Université d'Evry, Evry, France; HGSC, Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX USA; JCVI, J. Craig Venter Institute Rockville, MD. USA; KI: Karolinska Institute, Stockholm, Sweden; SGTC, Stanford Genome Technology Center, Stanford School of Medicine, Palo Alto, CA USA; TIGR, The Institute for Genome Research (now J. Craig Venter Institute) Rockville, MD. USA; WSU-CVM, Washington State University, College of Veterinary Medicine, Pullman, WA USA; WTSI, Wellcome Trust Sanger Institute, Cambridge UK.

From these studies, there have been several global observations that are relevant to those interested in the immunology of these organisms.

First, many of these organisms have large gene families, encoding species-specific surface antigens. Many of these gene families are novel and were identified during the genome annotation. It is unclear what their functions are, or what proportion of these gene families are utilised by the parasites at any given time. However, this data suggests that, like *T. brucei*, many protozoan parasites may frequently vary their antigenic determinants as an immune-avoidance mechanism. Surprisingly, many of these organisms have opted to organise these gene families in large sub-telomeric arrays. In *T. brucei*, expression of a clone-specific VSG is controlled by its position within the genome. Whether other parasites have independently developed analogous mechanisms to regulate the expression of polymorphic surface antigens is being explored.

Second, for some species, genomes of a number of different strains or isolates have been (or are being) sequenced, or genomic data has facilitated large-scale

genotyping efforts. Assessments from these projects have hinted at high levels of genetic diversity within some protozoan populations. These two observations have important implications for the development of vaccines, which will need to target either highly conserved antigens or contain polyvalent components that are effective against a broad spectrum of the parasite population.

Raw genome data can be a tremendous resource, and has stimulated many new avenues of enquiry in the protozoan research community; indeed, studies building on this platform hold the prospect of a number of important advances. For instance, transcriptomic and proteomic analyses have become important tools, allowing parasite gene expression and/or protein content to be assayed at any life cycle stage or from limited clinical samples. Subcellular proteomics now allow the biology of important organelles like the apical complex, hydrogenosomes or apicoplasts to be explored at a level of detail not previously possible.

Most importantly, methods for genetically manipulating many of the major protozoan parasites are now routinely used. This contrasts with the helminths, where, despite many years of research, these techniques are still being developed. In a few species, such as *T. brucei*, transgenesis and RNA interference (RNAi) are efficient techniques for over-expressing or down-regulating gene expression, allowing the function of potential pathogenicity factors and immune evasion genes to be directly interrogated. This, in combination with the ease of genetic manipulation of relevant model hosts such as mice, gives immunologists an unprecedented opportunity to explore the details of how specific parasite or host genes shape the course of infections and offer potential intervention points.

2.8 Summary

Protozoa are one of the most diverse groups of pathogens known. Their success as parasites, and the difficulties encountered by vaccine development efforts, is a testament to how exquisitely evolved their association with their hosts is. The last twenty years have seen an explosion in our knowledge of the biology and immunology of these organisms. As new molecular data and analytical techniques have been developed, our understanding of protozoan biology, taxonomy and evolution has undergone a series of major revisions. The availability of parasite genome datasets and the advent of methods for genetic manipulation of protozoa has changed the scope of what it is possible to explore as a parasite immunologist.

However, a number of important goals have not yet been realised. To date, the development of an effective vaccine against any of the parasitic protozoa discussed in this book remains years, or perhaps decades, away. Continued exploration of the biology of these exquisitely adapted organisms holds the tantalising prospect of the improvement of human health and also offers an unprecedented opportunity to expand our basic understanding of the immune system and how it functions.

2.9 General information on protozoa

For those wishing to obtain a more detailed view of the biology of the protozoa an excellent starting-point is offered by the following textbooks:

- Schmidt, GD & Roberts, LS (1996). *Foundations in Parasitology*. Wm. C. Brown, USA.
- Smyth, JD (1994). *Introduction to Animal Parasitology*. Cambridge University Press, Cambridge, UK.
- Margulis, L (1990). *Handbook of Protoctista*. Jones and Bartlett, Boston, MA.

However, please note that some of these texts do not hold current accounts of the taxonomy and evolution of these organisms which has undergone a number of dramatic shifts in the last decade. The websites maintained by the USA CDC's Division of Parasitic Diseases (http://dpd.cdc.gov/dpdx/Default.htm) also offers in-depth account of all groups and species discussed. Information presented in the text and tables above is derived from these sources, as well as those listed below.

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Apicomplexa: Malaria 3

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3.1 Malaria

Over half of the world's population is at risk from catching malaria, a disease that results from infection with protozoan parasites of the genus *Plasmodium*. At the turn of the 20th century, Sir Ronald Ross discovered that malaria resulted from bites of infected *Anopheline* mosquitoes, but before that it was believed that malaria was the result of unhygienic conditions, or 'mal-air' (bad air). There are five species of *Plasmodium* that infect humans:

- *Plasmodium falciparum* and *Plasmodium vivax* account for the majority of morbidity and mortality associated with malaria.
- The distribution and prevalence of *Plasmodium ovale* and *Plasmodium malariae* infections are both much lower.
- *Plasmodium knowlesi* (formerly known as a primate malaria) is now recognised as a significant pathogen of humans in South-east Asia.

Malaria is currently endemic in 109 countries in four continents and, of the 500 million cases of malaria estimated to occur annually, approximately one million result in death. Most of the fatalities are in children under the age of five years old and pregnant women.

The degree to which populations become exposed to malaria can vary, depending on the transmission rate. In areas where malaria is holoendemic (with most of the population infected to some degree), transmission can be high, but a non-sterile immunity that accommodates asymptomatic infection builds with age. In other areas, where rain patterns dictate fluctuations in the mosquito population, malaria can be both seasonal and unstable.

3.1.1 The life cycle of malaria

The life cycle of *Plasmodium* consists of several different developmental stages (both intracellular and extracellular), controlled by a genome consisting of over

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5,000 genes. The parasite largely replicates as a haploid organism, with the only diploid stages occurring in the mosquito (ookinete/oocyst stage).

3.1.1.1 Sporozoites in the skin

Malaria infection is initiated upon deposition of sporozoites into the avascular tissue of the skin from the salivary glands of a female mosquito as it probes for a blood meal (Figure 3.1). Within one minute, the sporozoites become highly motile, traverse the capillary wall and enter the blood stream. However, some sporozoites can remain motile in the skin for several hours, while others enter the lymphatic system and can be found in the draining lymph nodes, where the host mounts an immune response.

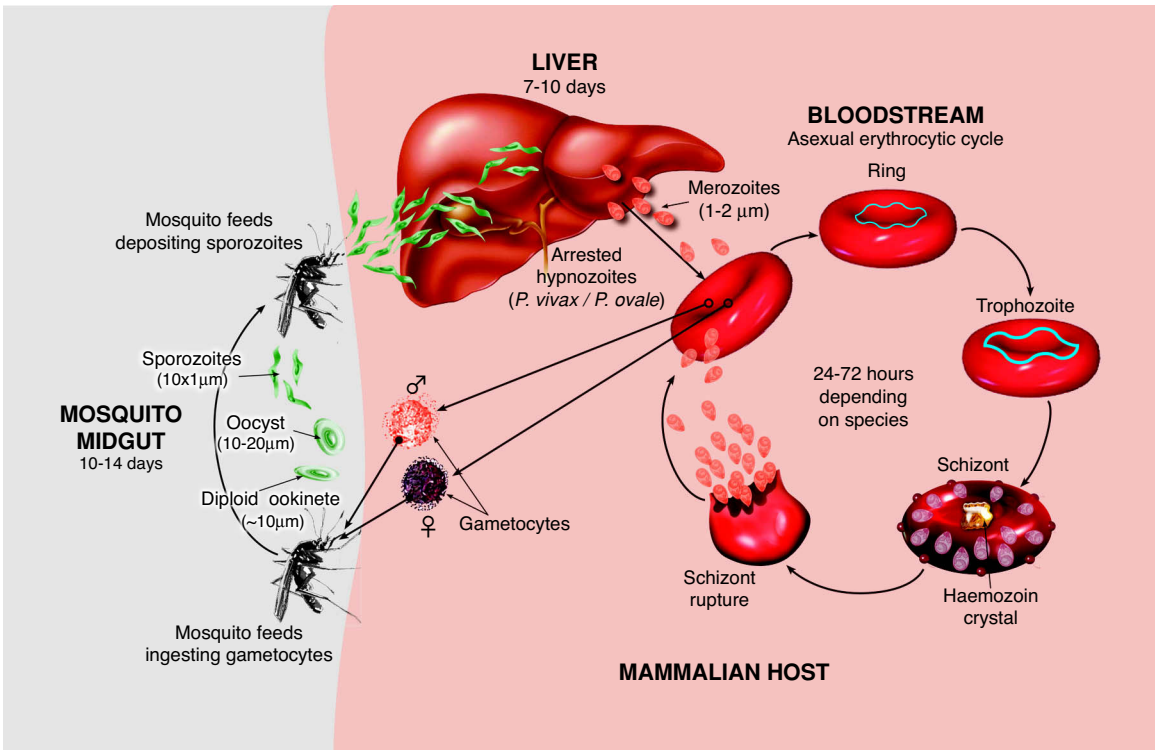

Figure 3.1 The life cycle of malaria. Malaria sporozoites are deposited in the vascular beds of the skin by a mosquito bite; these then actively traverse the endothelium, migrating to the liver via the bloodstream. They traverse several hepatocytes before developing in to a large exoerythrocytic form (LEF). In *P. vivax* and *P. ovale* infections, some sporozoites invade hepatocytes but undergo arrested development to form hypnozoites, which are largely resistant to drug treatment and are responsible for relapsing malaria infections. Merozoites develop inside the LEFs and burst out of the hepatocyte to invade erythrocytes. During maturation in erythrocytes, the malaria parasites export proteins to the surface of the erythrocyte, remodelling it and enabling removal from the circulation via sequestration to the endothelium of a number of organs in the body. Some erythrocytes invaded by merozoites do not continue cycling, instead developing to become transmissible stages known as gametocytes. When taken up by mosquitoes, male and female gametocytes mate in the midgut of the mosquito and undergo several stages of development before becoming sporozoites, which migrate to the salivary glands and are deposited upon the next feed.

3.1.1.2 Liver-stage malaria

Once in the liver, sporozoites glide along the sinusoidal epithelium traversing several Kupffer cells (resident liver macrophages) before invading a final hepatocyte, in which a parasitophorous vacuole (PV) forms. The infected hepatocyte then grows into a large exoerythrocytic form (LEF), which eventually gives rise to between 10,000 and 20,000 merozoites over a 7–10 day time period.

The liver-stage of the life cycle is not associated with notable disease in malaria infection, but allows the parasite to multiply. Relapsing malaria infections caused by *P. vivax* and *P. ovale* arise from arrested liver-stage parasites known as hypnozoites, which are generally resistant to anti-malarial drugs.

3.1.1.3 Asexual erythrocytic cycle

Once merozoites burst from hepatocytes they invade red blood cells (RBC) and enter into the asexual erythrocytic cycle. This stage of malaria is associated with most of the pathology in malaria infection, and the length of each replication cycle differs depending on the species of malaria parasite (Figure 3.1).

Invasion of new RBCs by merozoites involves secretion of proteases from structures found at the apical end of the merozoites called micronemes, rhoptries and dense granules. One of the major surface proteins of merozoites is a 200 kDa protein called merozoite surface protein (MSP)-1. This protein is essential for asexual cycling in RBC stages; it is proteolytically processed in a number of stages after it reaches the merozoite surface, a process necessary for invasion. *P. vivax* requires the presence of a glycoprotein on the RBC surface (known as Duffy antigen) to attach. This species is not a major contributor to the malaria burden across Sub-Saharan Africa, where most of the population are of the Duffy-negative blood type, making their RBCs refractory to invasion by *P. vivax*.

Once inside the RBC, the parasite becomes once again encased by a PV membrane but it is able to remodel the surface of the RBC. Parasite proteins are synthesised, secreted from the parasite and exported over the PV membrane to the RBC surface. The passage of the proteins across the PVM and into the RBC cytosol is mediated by a complex of proteins forming a translocation channel (or translocon) known as *Plasmodium* translocon of exported proteins (PTEX). Proteins that traffic to the surface of the infected RBC (e.g. *P. falciparum* erythrocyte membrane protein (*Pf*EMP)-1) tend to contain motifs known as *Plasmodium* export elements (PEXEL motifs) to facilitate this process, although some exceptions exist.

RBCs do not have nuclei and are essentially metabolically inactive cells. Replicating parasites obtain the amino acids they require by digesting haemoglobin. Additionally, RBC modification by the parasite renders the RBC more permeable to essential anions, sugars, amino acids and organic cations from the blood plasma, in a process termed the 'new permeation pathway'. At schizogony, once infected RBCs burst, between 10–32 merozoites are released, and these invade fresh RBCs to being a new erythrocytic cycle.

3.1.1.4 Transmission back to mosquitoes

A small proportion of iRBC differentiate in to transmissible male and female gametocytes, but the exact molecular cues leading to the development of male and female gametocytes are unknown. Once inside the mosquito midgut, the temperature shift and pH change induces gametogenesis and fertilisation leading, to the formation of motile diploid ookinetes that leave the blood meal bolus and traverse the midgut epithelium to become sessile oocysts. Over 10–14 days, sporozoites develop within the oocyst via mitosis, and these escape via an enzymatic process into the mosquito body cavity. The sporozoites circulate via the haemolymph and attach onto the basal lamina of the mosquito salivary glands, ready for introduction into the next host.

3.1.2 Mouse models of malaria

Much of the current knowledge on immune responses to malaria infection have been derived from a combination of observations in human malaria infections, *in vitro* modelling of immune responses to malaria, and mechanistic investigations using animal models of malaria. The mouse has a well-characterised immune system that is easy to manipulate and, although no one mouse model replicates all of the symptoms of human malaria infections, there are many similarities in the immune responses and pathology observed.

The phenotype of infection that results in mouse models of malaria depends on the parasite species, as well as the strain of mouse being infected. Cerebral malaria caused by *P. falciparum* is often modelled by *Plasmodium berghei* infections of mice (on a susceptible C57BL/6 background) or *Plasmodium yoelii* infections, whereas malarial anaemia and memory immune responses are often studied using non-lethal infections such as *Plasmodium chabaudi*.

3.2 Recognition of malaria parasites

Several studies have demonstrated the presence of pathogen-associated molecular patterns (PAMPs) in malaria antigens and/or various parasite products, which are conserved across malaria parasite species and strains. Such antigens can be recognised by cells of the innate immune system via pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) and scavenger receptors.

Knowledge of all malaria parasite-derived molecules and their respective PRRs involved in the innate immune recognition of malaria is incomplete and, in some cases, controversial. The infected RBC has been the focus of most work to date, and possible molecules containing pathogen-associated molecular patterns (PAMPs) are outlined in Table 3.1. Many of the molecules proposed to interact with PRRs are released as by-products from infected RBCs at schizogony. Indeed, there is some support for the idea that lysed, rather than intact, infected RBCs are required for activation of antigen presenting cells. The molecules and receptors involved in recognition of intact infected RBCs by phagocytic cells are less clear, although there are likely roles for opsonins such as natural

Table 3.1 Innate recognition of malaria-derived molecules and infected red blood cells.

Receptor	Cellular location of the receptor	Parasite molecule
TLR2	Extracellular	GPI-anchors – attached to most malarial proteins
TLR9	Intracellular: (endosomal compartments)	Parasite DNA – complexed to hemozoin or other parasite proteins
TLR11	Intracellular: (endoplasmic reticulum)	Apicomplexan profilin-like molecule (see Chapter 4)
Nod-like receptors	Intracellular (cytosolic)	Uric acid derived from degradation of hypoxanthine and xanthine from infected RBC
Fc receptors	Extracellular	Opsonising antibodies (natural, then acquired)
Complement receptors	Extracellular	Opsonising complement
CD36	Extracellular	GPI anchors (in association with TLR2) Molecules on adhered platelets; *Pf*EMP-1 (possibly other exported parasite proteins)
Unknown	————————	RBC-derived micro particles

Abbreviations: GPI- glycosylphosphatidylinositol anchors; RBC – red blood cell; TLR – Toll-like receptor.

antibody (Fc receptors), complement (complement receptors) or adhered platelets (CD36) expressed on innate immune cells (Table 3.1).

3.3 Innate effector mechanisms

3.3.1 Pre-erythrocytic stages

The exact mechanisms governing immune responses targeted against the hepatic stages of malaria are still unclear. Mouse models of malaria have elucidated some of the immune mechanisms that may be important and, in general, the innate immune response to liver-stage malaria is known to involve the secretion of interferon (IFN)-γ from natural killer (NK) cells, NKT cells and γδ T cells (Figure 3.2). IFNγ promotes the production of interleukin (IL)-12 and IL-18 by localised phagocytes to boost NK cell activation. Effective immune responses against hepatic stages rely on the involvement of cells with a lytic capacity, such as NK cells, to lyse infected hepatocytes, thereby facilitating destruction of the developing LEFs.

3.3.2 Asexual erythrocytic cycle

Many of the symptoms of malaria can be attributed to the inflammatory responses generated from cells of the innate immune system in response to the erythrocytic cycle of malaria. Inflammatory cytokines, such as tumour necrosis factor (TFN), IL-12 and IFN-γ, can be measured in serum around the time that merozoites first burst from hepatocytes before the adaptive immune

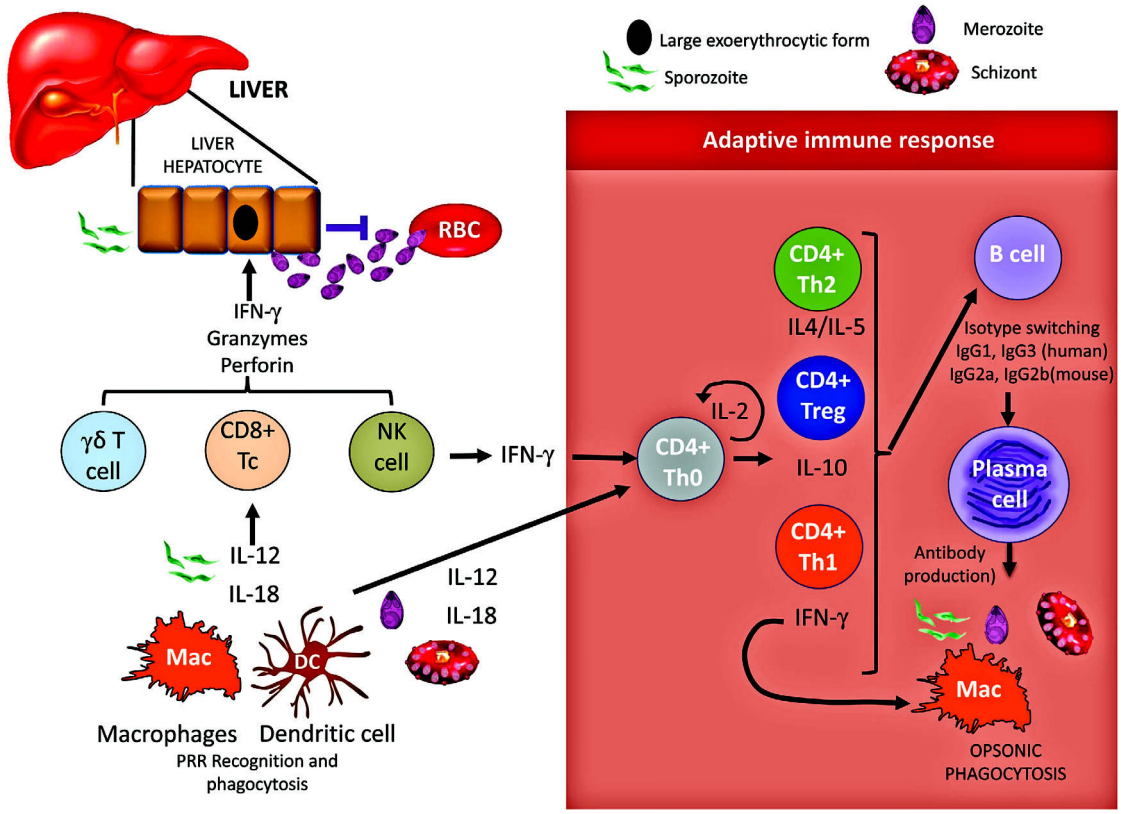

Figure 3.2 Features of the immune response mounted against malaria. Recognition of parasite stages by phagocytic cells such as macrophages and dendritic cells induces the production of pro-inflammatory cytokines, which potentiate cytolytic cells such as NK cells, γδ T cells and CD8+ cytotoxic T cells to produce IFN-γ and target infected hepatocytes with large exoerythrocytic forms. Lysis of infected hepatocytes can prevent the development of merozoites and, in turn, cycling of asexual forms of the parasite in erythrocytes. Effective immune responses against sporozoites and infected hepatocytes is known to be potentiated by phagocytic cells in the draining lymph nodes of the biting site, rather than in the liver *per se*. Adaptive immune responses leading to antibodies specific for sporozoites are able to neutralise further challenge infections from mosquito bites, providing some protection against reinfection. Immune response against erythrocytic stages is known to occur in the spleen, the main organ filtering the blood stream. This leads to the activation of CD4+ T helper cells, which develop into different phenotypes of CD4+ T cells, depending on the immunological environment at the time of challenge. CD4+ T cells 'help' B cells to become activated, proliferate and differentiate in to plasma cells that produce antibodies of different isotypes, including cytophilic antibodies that are highly effective at opsonisation and neutralisation of different parasite stages. The production of IFN-γ from Th1 cells augments the phagocytic capacity of macrophages to clear opsonised parasites from the body.

system becomes activated. Innate immune responses against malaria can help limit the density of the parasite population, but complete elimination of parasitaemia requires more parasite-specific adaptive immune effector mechanisms that take longer to develop.

3.3.2.1 Macrophages/monocytes

Both the number and activation of macrophages is increased during malaria infection. Macrophages play a significant role in the clearance of malaria-infected RBCs – particularly splenic macrophages, due to their location in the main lymphoid organ filtering the bloodstream. The expression of a number of PRRs,

CD36, as well as receptors recognising opsonins, allows macrophages to recognise RBC stages of malaria and associated malarial products (Table 3.1). Typical macrophage/monocyte responses to infected RBCs involve the production of pro-inflammatory cytokines such as IL-12 or IL-18 that, in turn, are important in the activation of other innate immune cell types such as NK cells (see below). Acute phase response cytokines are also produced by macrophages in malaria infection. TNF is secreted in conjunction with IL-1 and IL-6 in response to GPI anchors released at schizogony, causing the cyclical fevers associated with malaria infection.

Macrophages are able to phagocytose infected RBCs in both an antibody-dependent and antibody-independent manner. Antibody-dependent cellular cytotoxicity (ADCC) occurs when macrophages recognise antibody-opsonised infected RBCs or free parasite stages via surface Fc receptors. Antibody-independent phagocytosis by macrophages is thought to be mediated by the scavenger receptor CD36 via recognition of the *Pf*EMP-1 variant antigen on *P. falciparum* and/or platelets adhered on infected RBCs.

3.3.2.2 Granulocytes

The role of granulocytes in malaria infection is not well studied. Molecules released from neutrophil granules, such as myeloperoxidase, can be measured in the serum of patients infected with *P. falciparum*, demonstrating neutrophil activation in malaria infection. *P. falciparum* merozoites opsonised by antibody can induce respiratory burst in neutrophils, providing some protection against parasite growth. However, intact infected RBCS are not thought to activate neutrophils.

3.3.2.3 NK cells

The serum of non-immune humans experimentally infected with *P. falciparum* has been found to contain granzyme A and IFN-γ before the onset of clinical symptoms or detectable circulating parasitaemia, and before activation of CD8+ T cells. This suggests that innate NK cells are activated early after infection. NK cell numbers increase in the circulation of infected children and have a greater capacity for lysis.

Infected RBCs can be recognised by NK cells; incubation of a human NK cell line with *P. falciparum*-infected RBCs results in rosetting around the NK cells, an interaction that has putatively been linked to the interaction of *Pf*EMP1 with CD36 expressed on the NK cell surface. Once NK cells have made physical contact with infected RBCs, exposure to the pro-inflammatory cytokines IL-12 and IL-18 from macrophages is required for full activation. Thus, there is cooperation between macrophages/monocytes and NK cells in NK recognition of malaria-infected RBCs.

NK cells are thought to play an important immune-modulatory role in anti-malarial immune responses early after infection, via the secretion of IFN-γ. These cells are the main source of IFN-γ production when PBMCs from humans are incubated with infected *P. falciparum* RBCs. Mouse malaria infections suggest that, in the absence of NK cells, IFN-γ levels are decreased in infected animals, and parasites are detectable earlier after infection.

3.3.2.4 γδ T cells

Infection with either *P. falciparum* or *P. vivax* results in an expansion of γδ T cells that are thought to recognise malaria-derived non-peptidic phosphoantigens. Activation of γδ T cells in malaria infection requires exogenous cytokine stimulation from other cells of the immune system. However, when activated, these cells produce IFN-γ and perform cytotoxic actions on infected RBCs.

3.3.2.5 Dendritic cells

Myeloid (but not plasmacytoid) DCs are generally believed to play an important role in priming T cells in malaria infection, in turn activating adaptive immune responses against malaria. Uptake of infected RBCs, as in macrophages, can occur via opsonic or non-opsonic routes. Again, non-opsonic uptake of *P. falciparum* infected RBC is thought to occur via *Pf*EMP-1 interactions with CD36. This interaction has been shown to suppress the response of DCs to secondary TLR stimulation, as evidenced by a diminished up-regulation of MHC-II or co-stimulatory molecules CD40, CD80, CD86 in response to lipopolysaccharide (LPS).

In the *P. yoelii* mouse model of malaria, DCs phagocytose infected RBCs and digest them in acidified mature phagosomes. However, this process does not always appear to result in activation of DCs, which may be dependent on recognition of parasite products released at schizogony. Lysed *P. yoelii* infected RBCs activate DCs via activation of the MyD88, an adaptor protein used by PRRs to activate the transcription of pro-inflammatory cytokines. Some of the products of lysed infected RBCs responsible for this activation are listed in Table 3.1.

In contrast to the *P. yoelii* model of mouse malaria, intact *P. chabaudi*-infected RBC can activate mouse DCs, inducing up-regulation of MHC-II and co-stimulatory molecules CD80/CD86. One of the primary splenic DC subsets in the mouse to prime CD4+ T cells towards a Th1 phenotype in *P. chabaudi* infection are CD8+ DCs. Activation of CD8+ T cells via cross-presented antigens on MHC-I in *P. berghei* infection has also been shown to occur via the CD8+ DC subset. The activation of regulatory DC subsets, such as the $CD11c^{low}CD45RB^{high}$ DCs in the spleen during *P. yoelii* infection, may play a role in immunoregulation of malaria infection via the priming and expansion of IL-10 expressing CD4+ T cells, a T cell subset that is able to reduce immunopathology in malaria infection.

3.4 Adaptive immunity

3.4.1 Immunity to pre-erythrocytic stages

3.4.1.1 Anti-sporozoite antibodies

At high titres, pre-existing anti-sporozoite antibodies can immobilise the extracelluar sporozoites deposited in the skin by mosquitoes, thereby preventing invasion of hepatocytes. Sporozoite antigens known to be recognised by antibodies from malaria-infected individuals include circumsporozoite protein (CSP).

Antibody-opsonised sporozoites are susceptible to destruction by complement mediated-lysis, in addition to Fc receptor-mediated lysis by NK or NKT cells and phagocytosis by macrophages.

3.4.1.2 T cell immunity to intra-hepatic stages

Sporozoites that escape antibody-based mechanisms are able to infect hepatocytes. Developing LEFs are susceptible to immune responses mediated by both CD4+ and CD8+ T cells. In the *P. yoelii* mouse model, immunisation of mice with linear peptides derived from the intra-hepatic stage *P. yoelii* proteins, surface sporozoite protein (*Py*SSP2), or hepatic and erythrocytic stage protein of 17kDa (*Py*HEP17), generates a CD4+ T cell response that confers solid protective immunity against challenge infection in an IFN-γ-dependent manner.

CD8+ T cells and IFNγ are indispensable for effective immune responses to the intra-hepatic stages. *In vivo* depletion of CD8+ T cells completely abrogates protection afforded by injection with sporozoites that are attenuated (and unable to develop) due either to exposure to radiation or to genetic modification. Mice that are engineered to be deficient in MHC-I expression (and are therefore unable to present parasite-derived peptides on infected hepatocytes for targeted destruction by CD8+ T cells) are also not protected from a challenge infection by immunisation of attenuated sporozoites.

The anti-parasitic effect of CD8+ T cells is dependent on IFN-γ, since *in vivo* depletion of IFN-γ leads to abrogation of protective anti-sporozoite immunity. CD8+ T cells could kill infected hepatocytes directly via targeted release of cytotoxic pore-forming molecules such as perforin and granzymes, leading to necrotic destruction. Alternatively, apoptosis of infected hepatocytes could be induced via Fas-FasL signalling. In both cases, entry of the parasites to the asexual erythrocytic cycle is prevented.

Although infected hepatocytes and/or resident liver antigen-presenting cells can present parasite antigens complexed to MHC-I molecules to prime CD8+ T cells, evidence suggests that dendritic cells (DCs) play an essential role in the priming of T cells against pre-erythrocytic stages. It is not clear where DCs prime CD8+ T cells capable of recognising infected hepatocytes, but the antigenic specificity of T cells reactive against sporozoites and against infected hepatocytes has some similarity (for example thrombospondin-related anonymous protein (TRAP) and CSP are antigens common to both stages). Therefore, it is thought that DCs that become primed in the skin, while sporozoites reside in the avascular space (or in the lymph nodes draining the site of sporozoite deposition), may have an important role in activating CD8+ T cells that can subsequently lyse infected hepatocytes containing LEFs.

3.4.2 Immunity to the asexual erythrocytic cycle

3.4.2.1 CD4 T cells

Infected RBCs express parasite-exported antigens on the surface, facilitating recognition, uptake and processing by antigen presenting cells followed by subsequent activation of CD4+ T cells. The activated CD4+ T cells secrete a variety

of cytokines and/or parasiticidal molecules that will have either a direct or an indirect effect on parasite killing.

During the acute phase of a malaria infection, the responding CD4+ T cells are predominantly of the Th1 phenotype and are thought to induce cell-mediated parasiticidal mechanisms by phagocytic cells. Subsequently, CD4+ T cells with a Th2 phenotype arise, reflecting the importance of CD4+ T cell help for antibody production by B cells that eventually limits parasite density. Consistent with a role for CD4+ T cell help for protective antibody production by B cells, mice that are deficient in CD4+ T cells cannot make a sufficient antibody response to control malaria parasitaemia in rodent models of malaria infection.

3.4.2.2 B cells and antibodies

Antibodies were shown to be a critical component of naturally acquired immunity to the blood-stages of malaria when purified IgG was passively transferred from 'malaria-immune' adults to patients with clinical malaria, resulting in resolution of symptoms as well as significant reductions in parasitaemia. Protective antibody against erythrocytic stage parasites can be targeted to the free merozoite surface, the parasite infected erythrocyte, or to gametocytes, as described in Table 3.2.

Antibodies can function to control parasite density by opsonising infected RBC or free merozoites, thereby facilitating their removal by macrophages and

Table 3.2 Protective mechanisms of antibodies against the erythrocytic stages of malaria.

Antibody target	Example of target antigens	Mechanism of neutralisation
Free merozoites	MSP-1 MSP-2 MSP3 AMA-1	1. Blockage of parasite attachment onto uninfected red blood cells (invasion) 2. Interference with proteolytic processing of proteins critical for invasion 3. Prevention of shedding of invasion ligands 4. Opsonisation to facilitate removal by phagocytic cells
Infected RBCs, gametocytes	*Pf*EMP-1	1. Prevention of sequestration via adherence to ICAM-1 or VCAM-1, by blocking the adherence domains on *Pf*EMP-1 2. Prevention of gametocyte maturation 3. Complement mediated lysis of infected RBCs 4. Opsonisation to facilitate removal by phagocytic cells
'Malaria toxins' released at schizogony	GPI anchors	Neutralisation, thereby prevention ligation of TLR2 and subsequent pathogenic inflammation

Abbreviations: AMA-1- apical membrane antigen-1; ICAM-1- intercellular adhesion molecule; MSP-1- merozoite surface protein-1; *Pf*EMP-1- *Plasmodium falciparum* erythrocyte membrane protein-1; RBC- red blood cell; TLR-2-Toll-like receptor 2; VCAM-1- vascular cellular adhesion molecule.

preventing the reinvasion of merozoites into RBCs. Antibodies can also help to ameliorate pathogenic processes in malaria infection by helping to prevent sequestration of infected RBC or release of 'malaria toxins' at schizogony.

3.5 Memory responses

A protective immunity that limits parasite load and prevents disease symptoms develops in people living in areas endemic for malaria, but only after several years of exposure to parasites. Naturally acquired immunity to malaria is not sterile, and there is anecdotal evidence suggesting that it may be lost in the absence of continued exposure.

Most of the studies investigating memory responses in malaria infection have focused on serology, because of the technical challenges of identifying and quantifying antigen-specific memory lymphocytes. In young children, antibody responses to defined antigens are often extremely short-lived, suggesting defects in the establishment of long-lived plasma cells. However, rapid boosting of antibody responses to various antigens has been reported in some (but not all) studies, suggesting that children can generate memory B cells to malaria, in agreement with observations that the malaria antigen-specific memory B cell pool increases with repeated infections.

Attenuated sporozoite immunisation of TCR-transgenic mice, in which all CD8+ T cells specifically recognise a peptide of CSP, indicates that the generation of central and effector memory CD8+ T cells against liver-stages requires priming by DCs in the lymph nodes. These cells are then disseminated to the liver, where they mediate the killing mechanisms that eliminate LEFs in challenge infections. Central memory CD8+ T cells generated in human sporozoite immunisation models can last for up to six months.

Although erythrocytic stages of malaria are intracellular, the absence of MHC molecule expression on the infected RBC surface means that infected RBCs will be recognised as extracellular organisms, and CD4+ T helper cell-associated responses will be necessary for clearance. There is extensive evidence for CD4+ T cell recall responses to selected erythrocytic stage malaria peptides in people exposed to malaria. However, both the frequencies of responding cells and the prevalence of responders within exposed populations are low. The association between the loss of CD4 T cells and reduced antibody responses to malaria antigens in malaria-infected HIV-positive individuals, who are particularly susceptible to malaria (see Chapter 19), strengthens the evidence for a role for CD4+ T helper cells in the production of protective antibodies.

3.6 Immune evasion

Many vector-borne parasites establish long-lasting chronic infections to maximise opportunities for transmission. Malaria is no exception, and a variety of immune evasion mechanisms have evolved in malaria parasites that allow evasion of the host immune system, at both the individual and local population levels.

3.6.1 Subversion of T cell responses by liver-stage parasites

As sporozoites traverse hepatocytes, they shed vesicles with CSP and TRAP on their surface. It is unknown how these vesicles are recognised, but they may leave a trail of activated antigen-presenting cells in the liver that present these antigens in the context of MHC molecules (in particular, stationary antigen-presenting cells such as Kupffer cells, Stellate cells and liver sinusoidal epithelial cells). On the other hand, infected hepatocytes are known to have defective synthesis of MHC molecules. These mechanisms may act as decoys to the immune response in order to avoid destruction of developing LEFs.

3.6.2 Immune evasion in the erythrocytic cycle

3.6.2.1 Modulation of T cell responses

A number of observations suggest that malaria parasites subvert T cell responses that would otherwise mediate parasite clearance mechanisms. Erythrocytic stages of malaria may inhibit the ability of human DCs to respond to infected RBCs, in turn reducing the capability for T cell stimulation. Acute malaria infections of children have been associated with diminished expression of HLA-DR on DCs in the circulation. Migration of DCs towards infected RBCs may also be subverted by parasite production of a homologue of human MIF macrophage-migration inhibitory factor (MIF) in *P. falciparum* infections. Again, this would reduce the capacity for the activation of T cells in malaria infection.

Human malaria infection is associated with increased frequencies of T regulatory cells and an increased level of transforming growth factor (TGF)-β in the circulation. It has been hypothesised that malaria parasites could activate the T regulatory cells in order to evade adaptive immune responses. This hypothesis is in agreement with observations from West Africa, where the Fulani tribe, who are more resistant to malaria compared with their sympatric Mossi neighbours, were found to have lower numbers of circulating T regulatory cells and an associated decrease in TGF-β in their blood. Consistent with this hypothesis, the Fulani also accumulate stronger T cell responses to *P. falciparum* antigens than do the Mossi.

3.6.2.2 Modulation of antigens: antigenic variation

Infected RBCs express several different parasite-encoded variant surface antigens on their surface, the best characterised of which is PfEMP-1. There are 60 variant (*var*) gene copies in each parasite genome, each of which encodes a different version of the *Pf*EMP-1 molecule. Each infected RBC expresses only one of these variants at a time, but several variants can be expressed within the population of parasites in an individual host. Within a single infection, the clearance of each variant follows an antibody response specific for that variant.

Upon reinfection of the new RBCs, the daughter infected RBCs switch to express (and export) a different *Pf*EMP-1 variant to that expressed on their

parental infected RBC, and are not cleared by the antibody response. Thus, infected RBCs expressing the new variant survive and multiply. This sequence is repeated many times, resulting in successive peaks of parasite density over time. This enables the malaria parasites to avoid the host immune response, allowing the establishment of a chronic infection.

*Pf*EMP-1 also functions to facilitate sequestration of infected RBC to the endothelium, a survival strategy effective by removal of infected RBCs from the circulation, in turn preventing clearance by the spleen and liver. The phenomenon of rosetting, whereby infected RBCs become surrounded by uninfected RBCs, facilitating a close and protected environment for newly released merozoites upon schizogony, has also been attributed to the *Pf*EMP-1 molecule.

3.7 Immunopathology

Malaria is a complex multisystem disorder, with many similarities to bacterial sepsis. Disease can manifest as a heterogeneous set of symptoms, varying from person to person but broadly defined as mild and severe malaria. The vast majority of cases of malaria are mild in nature, but severe life-threatening malaria occurs mostly in children under five years of age infected with *P. falciparum*. Excessive inflammatory immune responses against the erythrocytic asexual cycle stages of malaria results in pathogenesis of malaria infection but this can be counteracted by the induction of the immunoregulatory cytokines IL-10 and transforming growth factor (TGF)-β, which down-regulate production of IFN-γ in malaria infection.

3.7.1 Thermoregulation

Malaria is associated with fevers that coincide with rupture of infected RBCs upon schizont rupture (Figure 3.1), with the concomitant release of inflammatory parasite products such as GPI anchors, uric acid and parasite DNA (Table 3.1). Stimulation of macrophages, and other antigen presenting cells harbouring the relevant PRRs, results in an acute phase reaction and the release of cytokines such as TNF-α. Fever is caused by the interaction of acute phase response cytokines with the hypothalamus in the brain. Neutralisation of TNF-α via monoclonal antibody treatment can reduce fevers in *P. falciparum* infection.

3.7.2 Severe malarial anaemia

Severe malarial anaemia (SMA) is often associated with chronic and repeated infections of malaria, and it can lead to a drop in haemoglobin in the blood to <5 g/dl (normal values are between 10–15 g/dl for humans). Anaemia in malaria infection can be due to loss of RBCs during parasite replication, as well as removal of infected RBCs as part of immune-mediated clearance mechanisms. In addition, increased phagocytic mechanisms in the spleen lead to

premature clearance of uninfected RBCs; around ten times more uninfected RBCs are removed from the circulation that infected RBCs.

RBC loss is normally compensated for by the development and release of new RBCs from progenitor cells in a process known as erythropoiesis. Parasite products such as haemozoin, and anti-malarial immune responses to these products, can depress normal haematopoietic mechanisms in the bone marrow and spleen.

The amount of TNF in children infected with malaria is positively correlated with the severity of malarial anaemia, in particular when levels of the immunoregulatory cytokine IL-10 are low. In addition, the TNF-238A promoter allele, controlling expression of TNF, is correlated with the development of SMA. The production of haematopoietic stimulant proteins (such as erythropoietin produced from the kidneys) is also depressed in malaria infection.

3.7.3 Metabolic acidosis and respiratory distress

The development of metabolic acidosis, whereby the pH of the blood lowers due to increased production of hydrogen in the body or defective removal of bicarbonate from the body by the kidneys, is often accompanied by respiratory distress and is strongly correlated with fatal malaria infection. Metabolic acidosis is exacerbated by the lack of circulating RBCs in patients with SMA, due to a reduction in the amount of oxygen delivered to the tissues and anaerobic metabolism. Hypovolaemia, whereby the volume of circulating blood decreases (presumably volume loss is partially due to lost RBC mass), is associated with severe anaemia, and this also exacerbates metabolic acidosis.

3.7.4 Cerebral malaria

This has a high case fatality and is a pathological condition resulting from infection with *P. falciparum*. A number of hypotheses have been proposed to explain the phenomenon of cerebral malaria, but in general it is thought to stem from immune responses against sequestered infected RBCs. Without the adhesive properties of *P. falciparum*, cerebral malaria does not generally occur.

Sections of brain tissue from fatal *P. falciparum* infections reveal microvascular obstruction in the brain due to the accumulation of sequestered infected RBCs, autoagglutinates (whereby infected RBC adhere to each other) and rosettes of infected RBCs, as well as infiltrates of lymphocytes. Brain-resident macrophages, or macrophage/monocyte populations that migrate to the brain tissue as a result of inflammatory immune responses against sequestered infected RBCs, directly contribute to the pathogenesis of cerebral malaria.

Secretion of chemokines such as macrophage inflammatory protein (MIP)-1α and MIP-1β by macrophages augments the migration of lymphocytes to the brain, in turn magnifying the pro-inflammatory response already under way. The release of pore-forming molecules such as perforin from CD8+ T cells and NK cells that have migrated to the brain tissue contributes to disruption of the

blood-brain barrier (BBB). Release of inflammatory molecules such as TNF-α up-regulates the expression of adhesion molecules such as ICAM-1 and VCAM-1 on the endothelium of the brain tissue, in turn amplifying the sequestration of infected RBC via *Pf*EMP-1.

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Apicomplexa: *Toxoplasma gondii* 4

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4.1 Introduction

Toxoplasma gondii is an obligate intracellular protozoan and – it could be argued – the most successful parasite on the planet. Prevalence rates range from 15–30 per cent in parts of Europe and the United States, to up to 80 per cent in France and Brazil, and no mammal or bird population has yet been found that does not harbour *Toxoplasma*. This success is likely due to a number of adaptations that distinguish *T. gondii* from its other apicomplexan cousins, including its ability to invade any nucleated cell and to survive outside the mammalian host.

Toxoplasma consists of one species – *Toxoplasma gondii* – containing clonal populations with three lineages: type I, type II and type III. These range in virulence, with fewer than ten parasites of a type I strain able to kill a mouse, and type II and III being avirulent, leading to chronic infections. In comparison to *Plasmodium* or the more complex multicellular helminths, this parasite is simple to maintain *in vitro*, it can easily be genetically manipulated and the mouse is a natural host. As a result, it is an ideal model system for parasite immunologists and is the 'gold standard' for the study of type 1 immune responses.

4.2 Life cycle and pathogenesis

As with all Apicomplexans *Toxoplasma* can undergo both asexual (shizogony) and sexual (gametogony) reproduction. Thus, *Toxoplasma* exists in one of three states:

1. Sexually, as sporozoites within a dormant highly resistant oocyst.
2. Asexually, as bradyzoites within a latent tissue cyst.
3. As fast-replicating tachyzoites (Figure 4.1).

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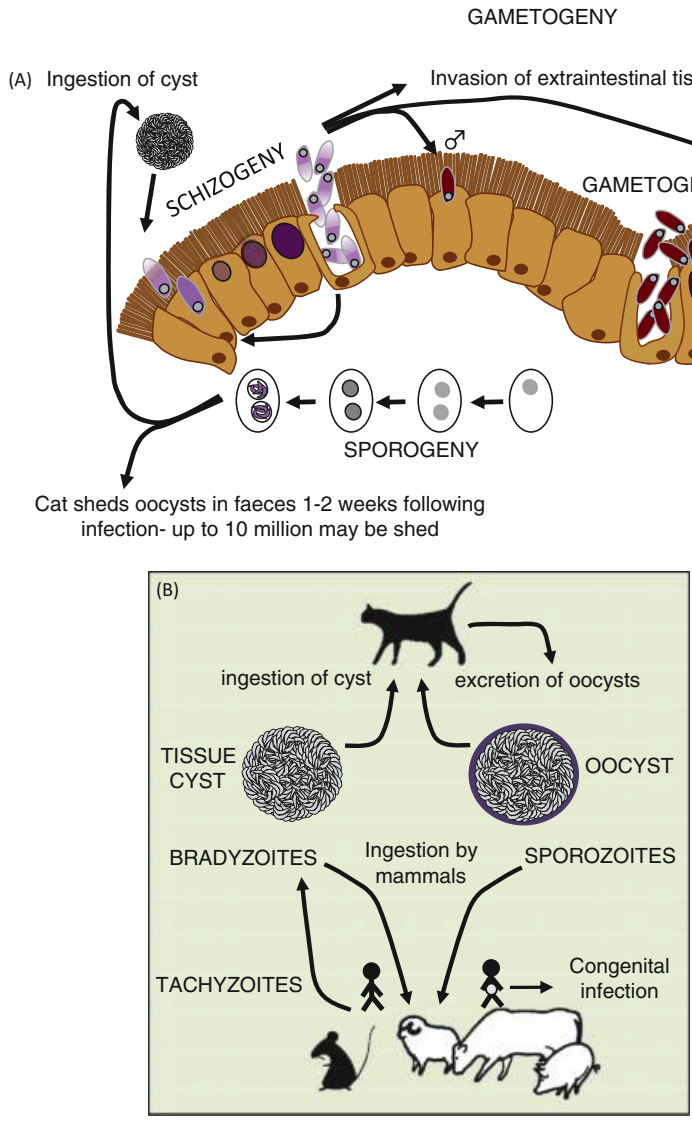

Figure 4.1 *Toxoplasma gondii* life cycle. *Toxoplasma* exists in one of three states: 1) sexually as sporozoites within a dormant highly resistant oocyst; or asexually as 2) bradyzoites within a latent tissue cyst; or 3) as fast replicating tachyzoites. A. Sexual reproduction can only occur in feline intestinal epithelial cells. B. Excretion of oocysts leads to their ingestion by mammalian intermediate hosts, asexual reproduction by tachyzoites and their dissemination throughout host tissues. Tachyzoites then convert to cysts, forming bradyzoites.

The definitive host is the feline, and thus sexual reproduction of sporozoites can only occur in gut epithelium of the cat. Although it is not unusual for a parasite to have such restrictions, the reasons for this specificity are unknown.

Following ingestion, parasites are released and invade the intestinal epithelium, where they undergo gametogony and oocyst formation. Oocysts are incredibly resilient structures known to resist desiccation and concentrations

of bleach. The cat sheds up to ten million oocysts in the faeces for up to two weeks, and this leads to contamination of the environment. Studies that have evaluated the prevalence of *T. gondii* in the cat population suggest that up to 80 per cent of cats are infected in the USA, and the estimate of contaminated cat faeces in the environment is in the tonnage! The ability of *Toxoplasma* to survive outside the mammalian host in such a resistant form is a likely contributor to its success as a parasite. The oocyst can now cause infection via ingestion by grazing animals, or in humans through the consumption of unwashed fruit and vegetables.

Ingestion of *T. gondii* by any mammal other than a cat leads to the development of tissue cysts. These cysts are formed in muscle, heart, liver and brain, and they remain in the tissue for the lifetime of the host. Therefore, a second route of infection is the consumption of tissue cysts in under-cooked meat.

Following infection of an intermediate host, cysts rupture and sporozoites from oocysts or bradyzoites from tissue cysts will be released into the lumen. At this point, parasites convert to fast-replicating tachyzoites and invade cells of the small intestine. Unlike *Plasmodium* spp., which spends most of its time in anucleated red blood cells, and where distinct life cycles and species require different host cells, *T. gondii* can replicate in any cell that has a nucleus. As it invades, it invaginates the host cell membrane, forming a protective parasitophorous vacuole (PV) isolating it from the inner workings of the cell – a process that takes less than 20 seconds. Tachyzoites proceed to divide within the PV approximately every eight hours. Continued replication results in cell lysis, and therefore it is the proliferation of tachyzoites that is the primary cause of pathology associated with infection.

Generally, one week post-infection is the height of parasitaemia and the immune response, after which a contraction phase occurs (Figure 4.2). Tachyzoites convert back to cyst-forming bradyzoites, a process that can be stimulated by stress conditions *in vitro* (e.g. low pH). As it does not occur in immune-deficient animals, it is likely to be stimulated in part by the immune response *in vivo*.

By three weeks post-infection, parasites are largely confined to the brain in cyst form. Here, continued reactivation of bradyzoites into tachyzoites necessitates a lifelong immune response in the central nervous system (CNS) to prevent Toxoplasmic encephalitis (TE). Although there are drugs that can inhibit the fast-replicating tachyzoites, none are yet available to remove the cyst form of the parasite. The cycle is completed following the ingestion of tissue containing cysts by a feline, so in most cases (except on safari?), *Toxoplasma* infection in humans represents a dead end for the parasite.

4.2.1 Clinical manifestations

One of the anomalies of *T. gondii* is that disease is rare, despite the high prevalence rates. Infection is generally asymptomatic in immune-competent individuals and may, at most, manifest as a non-specific swelling of the

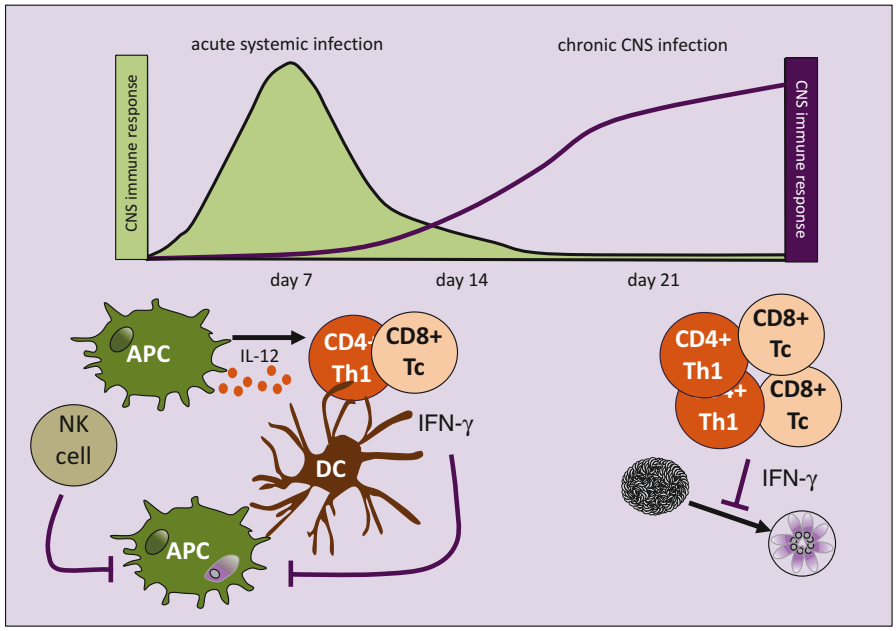

Figure 4.2 General immune response to *Toxoplasma gondii*. Production of IL-12 by neutrophils, DCs and macrophages, and innate production of IFN-γ early following infection, controls parasite replication and initiates Th1 immune responses. During chronic infection, T cells and IFN-γ production are required to prevent parasite replication.
Abbreviations: APC, antigen presenting cell; DC, dendritic cell; IFN, interferon; IL, interleukin; NK, natural killer; Tc, cytotoxic T cell; Th, T helper cell.

cervical lymph nodes and flu-like symptoms. However, when disease does occur, it can be severe. Retinal toxoplasmosis is the uncontrolled replication of tachyzoites in the retina, and is one of the lead causes of blindness. Congenitally acquired *Toxoplasma* infection remains one of the largest causes of foetal abnormalities, including hydrocephalus, intracranial calcification and mental retardation.

Historically, there was an increased focus of research on *Toxoplasma* following the advent of HIV and AIDS, which resulted in large numbers of patients with severe neurological conditions due to the reactivation of latent *T. gondii* cysts in the brain, causing TE. This patient population highlighted the requirements of the immune response in maintaining *Toxoplasma* latency. It is now recognised that all genetic or acquired immune-compromised patients are susceptible, and Toxoplasmosis is reported following organ transplant or chemotherapy.

The generalised immune response to *T. gondii* is the production of interleukin (IL)-12, driving interferon (IFN)-γ production by Th1 cells and, subsequently, type 1 effector responses (Figure 4.2). In mouse models, the absence of IL-12, IFN-γ or T cells leads to a failure to control parasite replication, and mice succumb to infection. In addition to the requirement for controlling and killing the parasite, susceptibility to infection is also apparent in the absence of immune molecules responsible for controlling the pro-inflammatory response.

4.3 Innate immune responses

Innate immune responses to *T. gondii* are required for initial recognition of the parasite, early control via effector mechanisms and the recruitment of the adaptive immune response. The systemic nature of *Toxoplasma* infection and the parasite's ability to invade any nucleated cell brings into play potentially every cell in the body. Here we will focus on a few key players and pathways that are essential during infection.

4.3.1 Innate recognition

As with the majority of pathogens, there is a requirement for initial detection of infection through Pattern Recognition Receptors (PRR) present on all innate immune cells and many tissue-resident cells. These receptors recognise Pathogen Associated Molecular Patterns (PAMPS) and are an evolutionarily conserved defensive mechanism against infectious agents.

Toll-like receptors (TLRs) are the most well-known group of PRRs, and they play an essential role in the generation of protective innate immune responses to *T. gondii* through the activation of myeloid cells and the production of IL-12. Thus, mice that do not have the adapter protein encoded by the myeloid differentiation primary response gene (88) (MyD88), required for nearly all TLR signalling, or the associated molecule UNC93B1, succumb early to infection with a complete lack of IL-12 production.

Investigations into the parasite-derived molecule that activates this pathway revealed several molecules that bind to distinct TLRs. A well-characterised molecule is *Toxoplasma* profilin, which is required for parasite invasion of host cells. Profilin binds to TLR11, inducing IL-12 production and TLR11-/- mice are susceptible to infection. A requirement for this specific TLR in recognition and protection to *Toxoplasma* is complicated by the absence of TLR11 in humans. As we are quite able to mount protective immune responses, it seems that TLR11 plays another role in *Toxoplasma* infection in mice.

Toxoplasma glycosylphosphatidyl inositol (GPI)-anchored proteins, present on the parasite surface and required for survival, bind to both TLR2 and TLR4. However, IL-12 production in response to *T. gondii* infection is TLR2 and TLR4 independent. The contrast between the absolute lack of IL-12 in MyD88-/- mice following infection, and the lack of susceptibility of individual TLR deficient mice, suggests that other ligands or pathways may signal through MyD88 for protective immune responses. The PRR family includes many other non-TLR receptors and molecules. Particularly relevant is the family of NOD proteins that form intracellular signalling structures called inflammasomes. These have been implicated in immunity to *Toxoplasma* and can also signal through MyD88.

4.3.2 Neutrophils

Neutrophils, circulate in the bloodstream and are rapid responders, congregating at the site of infection within hours. They are an early source of IL-12

production, as this cytokine is pre-formed within the cell. This population is also designed to control intracellular pathogens with the secretion of effector molecules, including nitric oxide (NO) and reactive oxygen intermediates (ROI). The other important role for neutrophils early on, following infection, is the production of chemokines that recruits further effector cell populations, including monocytes and T cells.

4.3.3 Monocytes and macrophages

The initial identification of neutrophils entailed using the antibody Gr-1, which binds to the surface of neutrophils. Gr-1 depletion during *Toxoplasma* infection leads to a susceptible phenotype, pointing to an absolute requirement for neutrophils in protection and clearance of the parasite.

However, it has been recently recognised that the antibody Gr-1 recognises two distinct molecules: Ly6C and Ly6G. The latter, Ly6G, is expressed only by neutrophils, whereas inflammatory monocytes express Ly6C. Inflammatory monocytes are recruited from the bone marrow in a manner that is dependent on the chemokine receptor CCR2. This again distinguishes them from neutrophils, and CCR2-/- mice fail to recruit inflammatory monocytes while the neutrophil population remains intact. In addition, CCR2-/- mice produce equivalent Th1-associated cytokines, IL-12 and IFN-γ; however, they are extremely susceptible to infection. This demonstrates that the primary population of cells responsible for the initial control of *Toxoplasma* are the inflammatory monocytes, not neutrophils.

Macrophages are, as their name predicts, essential phagocytes of the immune response. In the context of *Toxoplasma* infection, they are a source of IL-12 and are able to phagocytose and kill the parasite. This is mediated following the activation of macrophages by IFN-γ, and it leads to the production of NO. In addition, macrophages up-regulate interferon-inducible p47 GTPases (IRGs) that bind to the PV and ultimately lead to its degradation and parasite killing within the cell. This process of autophagy can also occur independently of IFN-γ, mediated by CD40. CD40 ligation is known to play an important role in IL-12 production but, in addition, it leads to the recruitment of IRG molecules that allow the disintegration of the intracellular PV, thereby exposing the parasite to lysosome degradation. The importance of this mechanism for killing the parasite is paramount, as *Toxoplasma* has employed methods to inhibit this pathway (as discussed later).

4.3.4 NK cells

Prior to the recruitment of the adaptive immune response, NK cells play a critical role in the early production of IFN-γ, the activation of surrounding innate cells and the polarisation of the immune response. During *T. gondii* infection, the activation of NK cells is through the production of IL-1 and IL-18, and they are rapidly recruited to sites of infection within hours, a process that is dependent on CCR5. In addition to the production of IFN-γ, they are cytolytic, producing granzymes and perforin to kill infected cells. Interestingly, there may be a role for NK cells in the early production of IL-10. This cytokine is a

potent and necessary anti-inflammatory molecule that inhibits the production of IL-12 and, thereby, the downstream Th1 immune response, suggesting that NK cells are also an early control mechanism.

4.3.5 Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs). They are the link between the innate and adaptive immune response, because they are activated in the periphery by pathogens or inflammatory stimuli and they migrate to the lymph nodes to present to naïve T cells. They express a wide expanse of PRRs and, during *Toxoplasma* infection, this expression and the downstream production of IL-12 is critical to generating a protective immune response.

One of the questions in studying *Toxoplasma* is how the parasite rapidly disseminates in the host. Several studies have pointed to a role for DCs in this process, coining the concept of a 'Trojan Horse', where the parasite utilises the host's own immune cells to carry it around the body. Thus, DCs infected with tachyzoites are highly migratory compared with uninfected cells and, following transfer, they can increase parasite dissemination. Therefore, it is DCs that have been implicated in carrying *Toxoplasma* from the intestine, across the blood brain barrier and into the CNS.

However, the primary and distinct role for DCs is in the presentation of antigen to T cells. The importance of this population in generating protective immune responses to *Toxoplasma* is observed by loading DCs *in vitro* with *Toxoplasma* antigen. Transfer of these loaded cells into mice confers protection against a subsequent *T. gondii* challenge. Therefore, antigen-presenting DCs alone are sufficient for vaccination against *Toxoplasma*. Furthermore, depletion of DCs prevents the formation of a parasite-specific T cell response and, during infection with avirulent type II parasites, robust DC activation occurs where large populations of parasite specific T cells are generated. In contrast, infection with a virulent strain of *T. gondii* leads to poor activation of DCs and a significantly diminished protective T cell response.

DCs first encounter Toxoplasma in the gut, where they probably directly phagocytose parasites or indirectly pick up antigen from dying endothelial cells. Following their migration to the lymph node, they encounter naïve T cells. Both of these cell populations migrate through collagen conduits that are coated with the chemokine CCL21. DCs and T cells express the CCR7 receptor that binds to CCL21, facilitating the chances of an antigen-loaded DC encountering a naïve T cell specific for its peptide. Long-lived interactions between DCs and T cells can be observed within six hours following *Toxoplasma* infection, and are primarily with DCs cross-presenting antigens rather than infected DCs.

4.4 Evasion strategies

Toxoplasma lives directly inside host cells, and part of its success can be attributed to multiple mechanisms of immune evasion (Figure 4.3). One of the

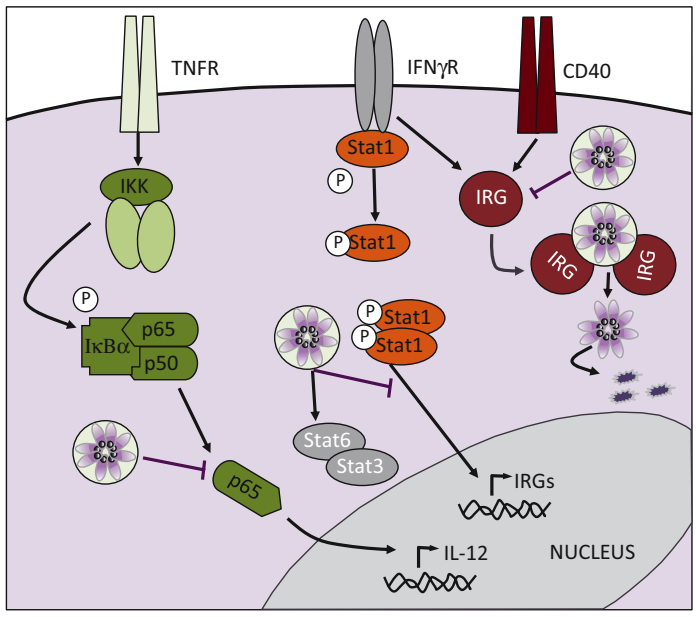

Figure 4.3 Inhibition of cell signalling by *Toxoplasma gondii*. Targeting the NF-κB pathway: *T. gondii* prevents nuclear localisation of NF-κBp65 and therefore inhibits pro-inflammatory immune responses. Inhibition of Stat1 signalling and activation of Stat3 and Stat6: *T. gondii* phosphorylates IRG proteins, preventing their activity and binding to the parasitophorous vacuole.
Abbreviations: NF-κB, nuclear factor-κB; Stat, signal transduction and activator of transcription; IRG, interferon inducible p47 GTPases.

first descriptions of manipulation by *T. gondii* was the direct inhibition of NF-κB. This transcription factor is necessary for downstream pro-inflammatory immune responses, including IL-12 production as well as anti-apoptotic signalling. There are multiple family members within the NF-κB family, and many of them are required for immunity to *Toxoplasma*. However, the parasite is able transiently to inhibit NF-κB activation within infected cells, leading to inhibition of IL-12 and TNF-α production. This inhibition is associated with the virulence of the parasite.

Recent data shows that GRA15, a dense granule protein secreted by *Toxoplasma* upon host cell invasion, is responsible for the inhibition of NF-κB-avirulent type II strains that do not possess GRA15 activate NF-κB. *Toxoplasma* can also target STAT signalling pathways, including inhibition of STAT1 and induction of STAT6 and STAT3, the net result being inhibition of Th1 immune responses.

As discussed previously, one of the primary mechanisms of killing *T. gondii* is the disruption of the PV by IRG proteins. Therefore, perhaps not surprisingly, our parasite has evolved ways to inhibit this pathway by phosphorylating IRG proteins, thereby preventing their activation (Figure 4.3). Avirulent parasites expressing different rhoptry (ROP) proteins are unable to harness this pathway and are, therefore, more susceptible to IRG-mediated lysis.

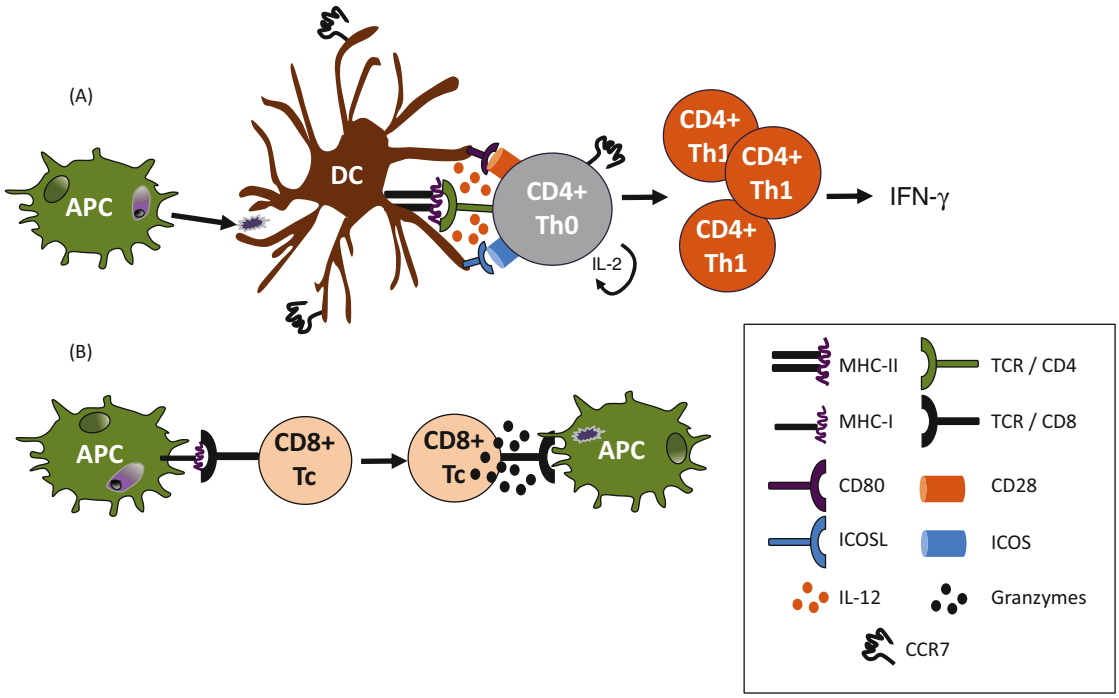

Figure 4.4 T cell activation pathways important during *Toxoplasma gondii* infection. A. DCs pick up parasite antigens from infected cells and increase surface expression of co-stimulatory molecules. DCs migrate to the lymph node, where they encounter naïve T cells in a CCR7-dependent manner. Activation of T cells occurs by antigen presentation via MHC-II and co-stimulatory molecules CD28 and ICOS. IL-2 production induces T cell proliferation, and IL-12 production polarises to Th1 cells, leading to the production of IFN-γ. B. Presentation of parasite antigens by infected cells activates CD8+ CTLs to initiate granzyme and perforin production that is released directly onto the infected cell, killing parasites and cells. Abbreviations: APC, antigen presenting cell; CCR7, chemokine receptor of CC-motif containing chemokines 7; DC, dendritic cell; ICOS, inducible T cell co-stimulator; IFN, interferon; IL-interleukin; MHC, major histocompatibility complex; Tc, cytotoxic lymphocytes; TCR, T cell receptor; Th, T helper cell.

4.5 Adaptive immune responses

The reactivation of *Toxoplasma* seen in AIDS patients is mimicked in mice without T cells. In addition, depletion of CD4+ or CD8+ T cells leads to parasite reactivation and TE. This demonstrates not just the requirement for adaptive immunity but that T cells are needed continuously for the lifetime of the host to prevent cyst reactivation.

4.5.1 *T. gondii*-specific T cells

T cell activation is threefold: signal 1 from antigen presentation, signal 2 from co-stimulation and signal 3 from polarising cytokines. Using transgenic parasites, we know that both professional and non-professional APCs can present parasite antigens to CD8+ T cells. In addition, secretion of antigen into the PV enables antigen presentation to MHC class II restricted CD4+ T cells more effectively than if it remains within the cytosol of the parasite. Investigations into endogenous peptides responsible for the T cell repertoire used advanced

tetramer-based technology to show that the dominant population of T cells changes over the course of infection. During acute infection, T cells specific for proteins secreted from the dense granules of *T. gondii* (GRA4 and GRA6) dominate, while during chronic infection, the T cell population is specific for the rhoptry-secreted protein ROP7. All of these molecules play an important role in the invasion of cells by the parasite, and they are strong indicators of virulence.

4.5.2 Costimulation

There have been numerous costimulatory molecules implicated in the optimal protective immune response to *Toxoplasma*. CD28 is the primary activating molecule on T cells that binds to CD80 and CD86 present on APCs. Its ligation leads to IL-2 production by T cells and T cell proliferation. However, in the absence of CD28, mice are still able to generate protective (though diminished) T cell responses. This likely reflects a degree of redundancy, and other molecules, including CD40L and ICOS, also play an important role. Indeed, blockade of ICOS in CD28-/- mice resulted in increased susceptibility due to poor T cell activation and low production of IFN-γ.

4.5.3 T cell polarisation

Examples of the importance of the polarisation of CD4+ T cells for protective immune responses to parasites are evidenced throughout this book. Indeed, some of the first demonstrations of Th1 versus Th2 cells were conducted using parasite infections. During *T. gondii* infection, IL-12 binding to the IL-12 receptor signals through STAT-4, tumour progression locus-2 (Tpl2) and the transcription factor T-bet, induces IFN-γ production and the generation of a polarised Th1 response.

The differentiation of CD4+ T cells was limited to two subsets for over two decades. Now, in addition to Th1 and Th2, there are T regulatory cells, Th17 cells, Th9 and Th22 cells! At present, there is no evidence that Th9 or Th22 cells play a role in immunity to *Toxoplasma*. However, Th17 cells, primed by the cytokines IL-23, IL-6 and TGF-β, and inhibited by the cytokine IL-27, develop following infection. Thus, IL-17-/- mice are more susceptible and have higher cyst burdens in the brain, and IL-27 receptor -/- mice have uncontrolled inflammation that leads to acute mortality, associated with increased levels of IL-2, IL-17 and IFN-γ.

In addition to the need to polarise CD4+ T cells, the activation of CD8+ T cells plays an important role in the adaptive immune response against *T. gondii*. As well as IFN-γ production that provides indirect killing of the parasite by activating infected cells, CD8+ T cells, once activated, can directly kill infected cells via the production of granzymes and perforin. This is through the presentation of parasite antigens via MHC class I, and although it plays a limited role during acute infection, it may be important in cyst containment during chronic infection in the brain.

Possibly due to the high antigen burden during such a prolific infection and the profound production of pro-inflammatory cytokines needed to control the

parasite, it is essential that inflammation is rapidly and continuously controlled. The two key cytokines responsible for this are IL-27 and IL-10. Although IL-10 is a cytokine associated with T regulatory cells, a role for Tregs during *T. gondii* infection is not as clear, and IL-10 is produced by non-Treg CD4+, CD8+ T cells and macrophages during infection.

IL-10 does not work by directly inhibiting T cells, and instead inhibits the production of IL-12 from accessory cells such as DCs. In the absence of IL-10, mice succumb to infection within ten days, due to massive inflammation and production of IFN-γ. IL-10 is required throughout the chronic infection, continuing to inhibit the CD4+ T cell response, and recent evidence points to the existence of a negative feedback loop with CD4+ T cells, producing both IFN-γ and IL-10, so that the cells responsible for killing the parasite are also regulating subsequent inflammation.

4.5.4 T cell memory

It is clear that strong memory responses are generated to *Toxoplasma* infection. Mice can be infected with mutant strains that do not replicate *in vivo*. These mice can then be challenged with a virulent strain and survive infection. The development of such immunity is dependent on CD8+ T cells, with help from CD4+ T cells. In contrast to effector cell generation, there is evidence that IL-12 actually inhibits memory T cell generation, and instead the cytokines IL-7 and IL-15 are the key mediators of CD8+ T cell memory survival.

4.6 CNS infection

Throughout infection in the brain, there is infiltration of peripheral immune cells. This includes the presence of CD4+ and CD8+ T cells, DCs and macrophages. Inflammation of the CNS is normally associated with profound pathologies such as multiple sclerosis (MS) or meningitis. Although, in some mouse models, profound inflammation can cause pathology, the majority of infections do not result in overt mortality. Furthermore, the prevalence of *T. gondii* in the human population throughout the world suggests that a large proportion is infected, and yet it is only the severely immune-compromised who exhibit neurological problems. The mechanisms that control the immune response in the brain so that it is robust enough to inhibit *T. gondii* replication without causing pathology have only just begun to be analysed.

Endothelial cells at the blood brain barrier (BBB) up-regulate adhesion molecules, enabling early migration of cells into the brain that are likely to be carrying *T. gondii* parasites with them. Significant T cell populations can be seen in the brain after two weeks of infection, and CD8+ T cell entry is controlled by the availability of antigen. Thus, only antigen-specific CD8+ T cells enter the brain and they undergo a vast array of behaviours associated with parasite burden and their activation status. CD4+ T cell entry seems to be additionally controlled by the presence of CCL21 and matrix metalloproteinases implicated in facilitating migration into the parenchyma.

In the brain, CNS resident cells include astrocytes, microglia and neurons, all of which can harbour *T. gondii*. As these cells are intrinsic to brain function, it is likely that all of these cell types play a role during infection, including the production of cytokines and chemokines and direct killing of the parasite. In addition, it is thought that cysts are maintained intracellularly in neurons.

This brings up the intriguing question of what affect this could have on neuronal signalling in the brain. A surprising study demonstrated the potential for *Toxoplasma* to induce profound changes in mouse behaviour. In contrast to an uninfected mouse, one that has been infected with *Toxoplasma* runs towards cat urine defying countless rounds of natural selection and implying that the parasite manipulates its host to increase the chances of returning to the definitive host. This may be facilitated by the localisation of the parasite to particular areas associated with behaviour, namely the frontal cortex and the amygdale. What behaviour could be triggered by infection in humans is a much more difficult analysis. However, this subject is now gathering attention.

4.7 Conclusions

Toxoplasma gondii is a highly successful parasite and is an excellent model for studying host-pathogen interactions. Indeed, this parasite has elicited a profound expanse of knowledge on the immune system – more than there is room to discuss here. The parasite's ability to inhibit the host immune response is vast, yet parasite-specific immune responses remain exquisitely balanced. *Toxoplasma* will likely lead to many more advances in our understanding of immunity in the future.

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5 Apicomplexa: *Cryptosporidium*

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Note: The findings and conclusions in this presentation are those of the authors and do not necessarily represent those of the Centers for Disease Control and Prevention.

Protozoa of the genus *Cryptosporidium* are recognised as one of the most ubiquitous parasites of animal gastrointestinal (GI) and respiratory tracts. While respiratory and disseminated cryptosporidiosis in humans is relatively uncommon, intestinal disease is commonplace and widespread. Despite the identification of *Cryptosporidium* in mice over 100 years ago, it was not until 1976 that human infections were reported, and another six years would pass before the significance of cryptosporidial diarrhoea in humans was emphasised by the recognition of severe diarrhoea in *Cryptosporidium*-infected immunocompromised patients with acquired immune deficiency syndrome (AIDS).

The majority of human infections appear to be caused by two species: *Cryptosporidium parvum* and *Cryptosporidium hominis*. Microscopical morphology techniques applied to clinical isolates provide little discrimination between cryptosporidial species infecting humans (oocyst size and shape are very similar – approximately 4.5–5 μm in diameter). Molecular biological techniques, coupled with limited cross-transmission studies, have led to the naming of many new species of *Cryptosporidium* (currently 12 are recognised and validated). As epidemiological and molecular taxonomical studies progress, the frequency and distribution of species infective for humans will no doubt expand. Nevertheless, most experimental studies relevant to humans have focused on *C. parvum* and, to a lesser extent, *C. hominis* – primarily due to limitations related to experimental laboratory animal hosts. A lack of effective cryopreservation techniques further limits experimental studies, requiring isolates to be maintained by serial passage in permissive hosts.

Immunity to Parasitic Infection, First Edition. Edited by Tracey J. Lamb.

5.1 Life cycle

Fundamentally, *Cryptosporidium* species are eukaryotic apicomplexan parasites with typical apical complex structures (e.g. micronemes, rhoptries, apical rings), and are homoxenous (entire life cycle completed in a single host). Like other Apicomplexan parasites, including *Plasmodium* and *Toxoplasma*, *Cryptosporidium* has a complex life cycle, producing asexual and sexual stages. Development proceeds following oocyst excystation through merogony (asexual growth), gametogony (production of sexual stages), fertilisation, and sporogony (the production of oocysts containing four naked sporozoites, i.e. no sporocysts enclose the sporozoites within the oocyst) (Figure 5.1).

Permissive hosts ingest exogenous sporulated, infectious, thick-walled oocysts which excyst in the gut. Sporozoites released from the oocyst invade the brush border of epithelial cells and occupy a location described as 'intracellular but

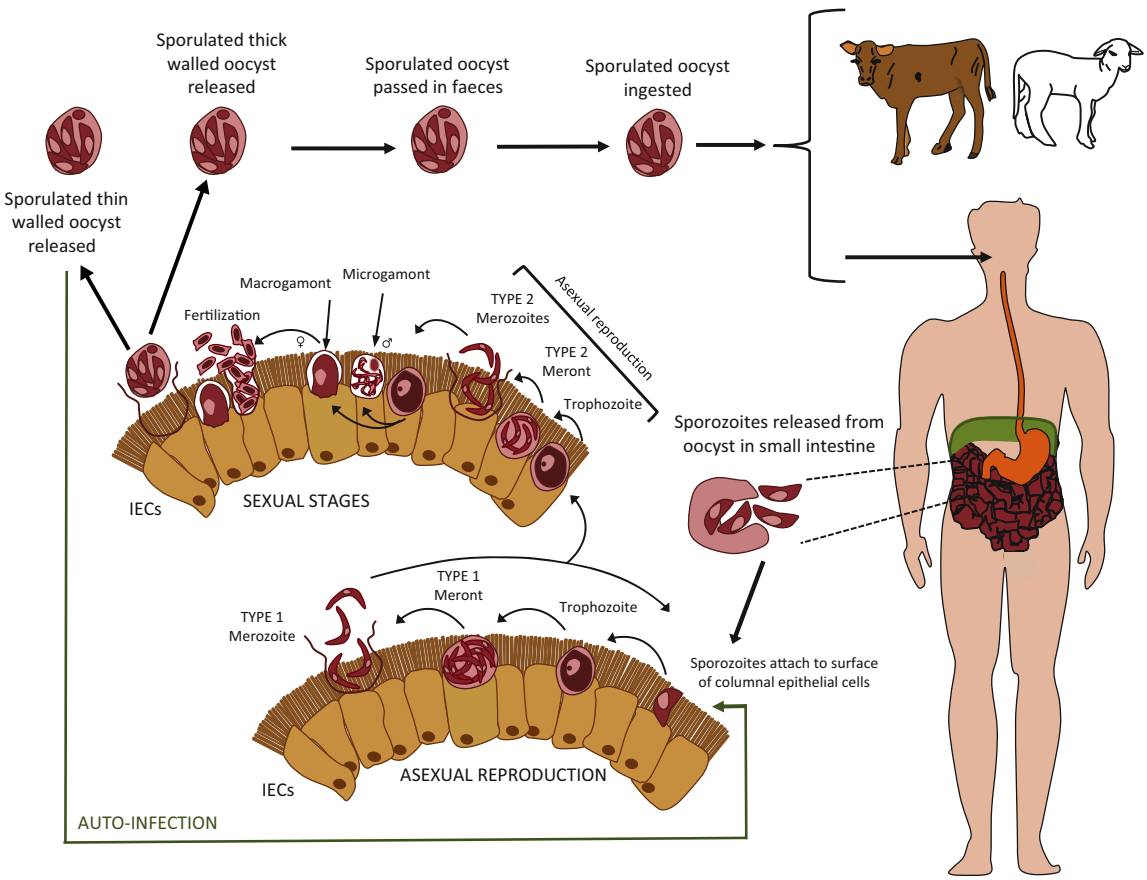

Figure 5.1 Diagrammatic life cycle of *Cryptosporidium parvum*. Trophozoites (growing stage) differentiate following sporozoite invasion and develop into Type I meronts. Type I merozoites are released from Type I meronts, and these invade epithelial cells and produce trophozoites in a cyclical fashion. Type II meronts are derived from invasion of IECs by Type I merozoites. Type II merozoites are released from Type II meronts. Invasion of epithelial cells by Type II merozoites produce trophozoites, which will differentiate into male or female gamonts. Microgametocytes (male) are released from a mature microgamont, and a zygote is derived from a macrogametocyte fertilised by a microgametocyte. Thick-walled sporulated oocysts are excreted in the faeces of the host. IECs, intestinal epithelial cells.

extracytoplasmic', in that the parasite is enclosed in a parasitophorous vacuole of both parasite and host cell origin. The base of the parasitophorous vacuole is adjacent to the host cell cytoplasm and a complex 'feeder organelle' is located at the interface, presumably to facilitate transport into and out of the host cell cytoplasm. The parasitophorous vacuole, nestled in the epithelial cell brush border, has an apical surface facing the gastrointestinal (GI) lumen. The newly invaded sporozoite differentiates into a trophozoite (growing stage) and further differentiates into a Type I meront, which upon maturation produces eight merozoites.

These Type I merozoites are released from the mature meront, invade epithelial cells, produce trophozoites and differentiate into additional Type I meronts or Type II meronts. The latter produce four merozoites, which invade epithelial cells, differentiate into trophozoites and, ultimately, produce gamonts (sexual stages). It is thought that Type II merozoites are committed to producing gamonts, while Type I merozoites produce both Type I and Type II meronts (as observed in the GI tract; however, the 'recycling' of Type I meronts *in vitro* is rare or nonexistent).

The gamonts derived from Type II merozoites differentiate into macrogamonts (female) and microgamonts (male). Microgamonts differentiate and produce 12 microgametocytes, which are released and fertilise mature macrogametocytes, which differentiate from the macrogamonts. Zygotes derived from fertilised macrogametocytes differentiate into oocysts. Two forms have been observed in the GI tract:

- Thin-walled oocysts (≈20 per cent of total) are thought to excyst in the gut and release sporozoites capable of infecting the same host (autoinfective cycle).
- Thick-walled oocysts (≈80 per cent of total) are shed in the faeces and are immediately infectious.

Production and purification of cryptosporidia for laboratory studies is generally accomplished using experimentally infected animals, especially rodents and livestock. Although other life cycle stages have been isolated in modest numbers, it is the oocyst stage that is available in abundance. Oocyst production is labour-intensive, but commercial sources are available.

5.2 Clinical presentation

The clinical signs associated with cryptosporidiosis are not pathognomonic, i.e. they are not sufficiently unique to facilitate a diagnosis in the absence of parasite detection. Parasitological confirmation may involve directly identifying life cycle stages, e.g. oocysts in stool, or developing stages in epithelial cells in stained biopsy material, or indirectly by detecting antigens or parasite DNA in clinical specimens.

Clinical signs vary considerably, depending on the immunologic status of the host. Onset of symptoms following exposure is typically 2–10 days, with a mean of seven days. Symptoms may persist for approximately 1–2 weeks, but oocyst shedding in stool may continue for an additional 1–2 weeks after clinical

signs abate. Immunocompetent individuals may experience watery diarrhoea, abdominal pain (cramping), nausea, vomiting, anorexia, mild fever (<39°C), neuralgia (headache) and fatigue. Immunocompromised individuals often experience the same clinical signs, but they are more severe, protracted, and possibly involve extraintestinal sites of infection, including the gall bladder, hepatobiliary ducts, pancreatic ducts and respiratory tract. While immunocompetent individuals typically recover fully from infection by mounting specific immune responses, immunocompromised individuals may develop chronic infections. Individuals with cryptosporidiosis related to immunosuppressive therapy may recover from infection by temporarily suspending immunosuppressive therapy.

Efficacious chemotherapy of cryptosporidiosis is not yet fully realised. The drugs used for treatment may provide some relief from symptoms, but they do not generally accomplish parasitological cure, or may not do so in a desirable time frame, and they are generally not effective in immunocompromised patients. Certainly, compared to very effective treatments available for related parasites (e.g. trimethoprim sulfamethoxazole for *Cyclospora* and *Cystoisospora*), the current therapies for *Cryptosporidium* are disappointing.

5.3 General immune responses in cryptosporidiosis

Intestinal epithelial cells (IECs) and mucosa in the gut form a physical protective barrier against many pathogens and also serve as an interface between the lumen and underlying immune system. Upon encountering microorganisms, intestinal epithelial cells are able to detect pathogens through innate receptors, and they respond by generating cytokines and up-regulating chemokines that attract and activate other immune cells. Injury to these barriers due to infection and inflammation can alter tight junctions between the epithelial cells, resulting in increases in the uptake of solutes and microbial antigens.

Cryptosporidium infections cause both increased permeability of the epithelial barrier and induction of pro-inflammatory responses. The main features of the anti-cryptosporidial immune response are summarised in Table 5.1.

Table 5.1 Generalised features of the anti-cryptosporidial immune response.

Cell type	Molecules involved
Intestinal epithelial cells	Activation by TLRs Up-regulation of MHC-I and MHC-II Induction of IFN-α Production of NO via induction of iNOS Production of anti-microbial peptides
CD4+ T cells	γδ CD4+ T cells contribute to resistance via undefined mechanisms αβ CD4+ T cells secrete IFN-γ
NK cells B cells	Secretion of IFN-γ Secretion of IgG and IgA antibodies

Abbreviations: IFN, interferon; Ig, immunoglobulin; iNOS, inducible nitric oxide synthase; NK, natural killer; NO, nitric oxide; TLR, Toll-like receptor.

Up-regulation of chemokines, histocompatibility complex (MHC) class I and class II molecules, and activation of Toll-like receptor (TLR) molecules have been reported in response to cryptosporidial infection. Nitric oxide (NO), produced through the induction of nitric oxide synthase (iNOS) of epithelial cells, is significantly increased in *C. parvum* infection. In both human and animal studies, immune effector mechanisms that are dependent on interferon (IFN)-γ are important for a protective responses.

5.4 Innate effector mechanisms

5.4.1 Toll-like receptors

Toll-like receptors (TLRs) are constitutively expressed on IECs and are important activators of the innate immune system, targeting intestinal bacterial and protists such as *Toxoplasma*. However, their significance in cryptosporidial infections is not clear. It has been shown that mice lacking MyD88, an adaptor protein in TLR signalling, are more susceptible to infection compared with wild-type mice, and more so if the mice are made immunodeficient by IFN-γ neutralisation. Additionally, *in vitro* infections lead to up-regulation of TLR2 and TRL4, and infections are increased when siRNA is used to knock down the expression of MyD88.

TLR11 plays a major role in Th1 responses in regulating the production of IL-12 in *T. gondii*-infected mice, and TLR11 is stimulated through TgPRF, a profilin-like ligand that is indispensable for gliding motility and host cell invasion (see Chapter 4). *Cryptosporidium parvum* has a similar profilin-like ligand that can activate TLR11-dependent signalling, but to a lesser extent than TgPRF. This may be due to sequence differences in the acidic loop. TLR9 may also play a role in immunity, since neonatal mice treated with an unmethylated CpG oligonucleotide, a ligand for TLR9, show resistance to infection.

5.4.2 Chemokines and chemokine receptors

Chemokines play key roles in attracting immune cells to sites of infections. Increased expression of CXCL-8, IL-8 and growth regulated oncogene (GRO-α) occurred in *C. parvum*-infection intestinal epithelial cells, mainly on the basal side of the cell. In neonatal mice, C, C–C, and C–X–C class chemokines have been shown to be up-regulated in response to *C. parvum* infection. The lack of CCR5, an important receptor of epithelial cells, may increase the susceptibility of *C. parvum* infections in neonatal mice, but not in adults, and it appears to be unnecessary for subsequent parasite elimination. In AIDS patients with cryptosporidiosis, the level of CXCL10 (secreted in response to IFN-γ) is significantly increased, compared to the levels in AIDS patients without cryptosporidiosis or in normal volunteers.

5.4.3 Mannose-binding lectin

Mannose-binding lectin (MBL) is a conserved protein that functions in innate immunity by binding to microbial surfaces and promoting phagocytosis. MBL

has been shown to bind to *Cryptosporidium* sporozoites, and earlier work has suggested that the protective role of MBL may be most important in childhood. In five per cent of the world's population, polymorphisms in the *MBL2* gene create low MBL-producing *MBL2* genotypes, which may lead to an increased susceptibility to particular diseases, including *Cryptosporidium*. *C. parvum*, can activate both the classical and lectin pathways, leading to the deposition of C3b on the parasite. *Cryptosporidium* parasites can develop in MBL-deficient – but not C1q-deficient – adult mice, demonstrating that the lectin pathway (but not the alternative pathway of complement) plays an important role against infection.

5.4.4 Type 1 interferons

The secretion of type I IFNs is mainly thought of as an important innate immune mechanism in viral infections. However, *in vitro* assays have demonstrated that IFN-αβ is expressed by infected epithelial monolayers and by bone marrow-derived dendritic cells (DCs) exposed to live parasites, and that pretreatment with recombinant IFN-αβ inhibited parasite development. Additionally, *in vivo* treatment with antibodies to remove IFN-αβ in neonatal SCID mice can increase oocyst production.

5.4.5 Anti-microbial peptides

Natural and synthetic lytic peptides can be active against cryptosporidial sporozoites *in vitro*. In mammals, there are two large families of anti-microbial peptides: defensins and cathelicidins. β-defensins are widely expressed in epithelial cells, and infection of the human colonic HT29 cell line with *Cryptosporidium* induced the expression of human β-defensin-1 (hBD-1). In another study, four mammalian anti-microbial peptides belonging to the cathelicidin peptide family were tested for anti-cryptosporidial activity and found to exert a strong cytotoxic effect on sporozoites.

Interestingly, a recombinant antibody-peptide antibody-biocide fusion protein, composed of the human anti-microbial peptide LL37 and an antibody directed against the circumsporozoite-like antigen (CSL) expressed on sporozoites, reduced infection by up to 81 per cent when administered orally to neonatal mice in a prophylactic model of cryptosporidiosis.

5.4.6 NK cells

In humans, NK cells may play more of a role, as treatment of PBMCs with IL-15 was observed to increase expression of the NK marker, NKG2D, and enhance lysis of *Cryptosporidium*-infected epithelial cells. Human volunteers infected with *Cryptosporidium* expressed higher levels of IL-15 in the jejunum and shed fewer oocysts than did seronegative volunteers not expressing this cytokine.

Mice deficient in NK cells, such as neonates and NIH III mice with the beige mutation, are more susceptible to *Cryptosporidium* infection than are control mice. Conversely, SCID mice, which lack adaptive immunity but have an intact innate immune system, are initially relatively resistant to *C. parvum* infection. Their resistance to *C. parvum* infection has been shown to be IFN-γ-dependent, and NK cells may be the initial source of innate IFN-γ in cryptosporidial infection.

Although NK cell activation is associated with protection against cryptosporidial infection in mice, it is not an essential protective mechanism, because neither depletion of NK-cells with monoclonal antibody treatment or stimulation of NK-cells with administration of interleukin (IL)-2 alters infection levels in these mice.

5.4.7 Macrophages and dendritic cells

Macrophages and dendritic cells (DCs) play an essential role in activation and regulation of T cells, but to date have garnered little attention in *C. parvum* research. It is known that CD40-positive bone marrow-derived cells (which include both macrophages and DCs) are needed to clear parasites in infected mice. Cytokines such as IFN-γ are important in the up-regulation of DC-attracting chemokines, because decreased DC recruitment occurs in neonatal C57BL/6 IFN-γ deficient mice infected with *Cryptosporidium*.

It is likely that DCs are involved in the degradation and transport of antigens to lymph nodes. DCs also release chemokines in response to infection with *C. parvum*, which will attract additional immune cells, such as macrophages or neutrophils, to clear *Cryptosporidium* parasites. Macrophages and neutrophils are thought to play a role in host resistance against acute *C. parvum* infection.

In a study comparing SCIDbgMN mice (severe combined immunodeficiency (SCID) mice that lacked macrophages/neutrophils) and SCIDbg mice (SCID mice that lack NK cells), a higher mortality rate resulted from acute *C. parvum* infection in SCIDbgMN than in SCIDbg mice.

5.5 Adaptive immunity

As important as innate immunity is in the initial stages of infection, adaptive immunity is required to clear the parasites completely. Experimentally, infections in nude and severe combined immunodeficiency (SCID) mice cannot be cleared by the immune system and are chronic. T cells are particularly important in providing protection against cryptosporidial infection in mice. Innate T cells such γδ T cells are found in abundance in the gut mucosa. In cryptosporidiosis, they contribute to resistance to infection; neonates deficient in these cells are more susceptible than control mice. However, it is the adaptive αβ T cells that are necessary for control of infection, as shown by the fact that adult mice lacking αβ T cells develop chronic infections.

5.5.1 T cells: CD4+ cells

CD4+ cells proliferate and produce IFN-γ in response to infection. CD4+ lymphocytes are necessary for the resolution of infection. In general, patients with CD4+ counts greater than 200 cells/mm^3 tend to have less severe disease than those with less than 50 cells/mm^3. Individuals with a depleted CD4+ T cell number due to HIV infection (see Chapter 19) have more severe and potentially life-threatening disease, demonstrating the importance of T cells in resistance and recovery from infection. Mice rendered deficient in CD4+ T cells by monoclonal antibody treatment have markedly decreased immunity, while adoptive transfer of CD4+ T cells to T cell-deficient mice can greatly reduce infection levels.

Intestinal epithelial lymphocytes (IELs) are nonconventional lymphocytes located among the epithelial cells in the lumen. These are important effector cells in *C. parvum* infections, and a proportion of them are CD4+ cells. Adoptive transfer of CD4+ IELs cells in SCID mice have been shown to reduce parasite load and give better protection against *Cryptosporidium* infections than adoptive transfer of CD8+ IELs, despite the fact that the majority (≈85 per cent) of IELs in mice are CD8+. This is consistent with infections of mice that are deficient in MHC molecules; MHC-II deficient mice that lack functional CD4+ are more susceptible than are MHC-I deficient mice that lack CD8+ T cells.

5.5.2 T cells: CD8+ cells

Although not as protective as CD4+ T cells, CD8+ T cells also play an important role in response to infection. Increased numbers are observed in animals that have recovered from challenge infection, and adoptively transferred CD8+ cell populations decrease infection, albeit not as markedly as CD4+ cells. The mechanism by which CD8+ T cells confer protection is not entirely clear, but it may be via the production IFN-γ early in infection. CD8+ T cells have been shown to produce IFN-γ when stimulated with the cryptosporidial-specific antigen gp15 *ex vivo*. Additionally, they may act through cytotoxicity, as antigen-sensitised CD8+ T cells can reduce the parasite load in infected intestinal epithelial cell cultures by lysing infected intestinal epithelial cells.

5.5.3 Cytokines

5.5.3.1 IFN-γ

In humans, increased amounts of IFN-γ are generated in response to cryptosporidial specific antigen after prior exposure. Treatment with an antibody to remove IFN-γ enhances infection in mice and SCID mice have shown that resistance to *C. parvum* infection is IFN-γ-dependent. This evidence suggests that IFN-γ plays an important role in innate, as well as adaptive, immunity. More severe infection in neonatal BALB/c mice developed upon

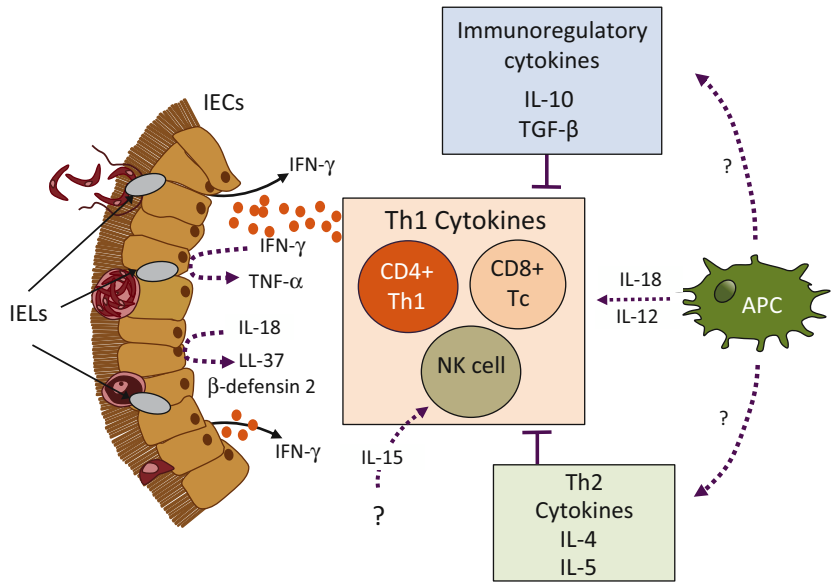

Figure 5.2 The putative role of cytokines in *Cryptosporidium* infection. The intestinal immune response to Cryptosporidium is largely Th1 in nature, driven by the cytokine IFN-γ (orange dots). The production of immunoregulatory or Th2 cytokines are hypothesised to dampen Th1 responses in the intestine. The purple dashed lines depict hypothetical or unknown sources or roles of cytokines.
Abbreviations: IEC, intestinal epithelial cell; IEL, intestinal epithelial lymphocyte; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor.

administration of monoclonal antibodies to remove CD4+ T cells and IFN-γ, when compared to antibody treatment-mediated removal of CD4+ T cells or CD8+ T cells, strongly suggesting that IFN-γ might be produced in *Cryptosporidium*-infected mice by non-T cells such as NK cells.

Several different mechanisms of resistance mediated by the cytokine have been proposed. A common mechanism of action is through the induction of iNOS synthesis of NO in infection (Chapter 1). However, IFN-γ does not seem to mediate expression of iNOS by the intestinal epithelium or synthesis of NO in response to *C. parvum* infection *in vivo*. In *Cryptosporidium*-infected cells exposed to exogenous IFN-γ, depletion of intracellular iron may be a possible mechanism of action responsible for inhibition of *C. parvum* growth. Alternatively, it is possible that IFN-γ activation of TNF-α expression via up-regulation of its transcription factor NF-κβ could impact on *Cryptosporidium* infection (Figure 5.2).

5.5.3.2 TNF-α

While TNF-α is increased during *C. parvum* infection, and may enhance other immune responses, the lack of this cytokine does not appear to impact significantly on infection or enteric symptoms. Although treatment with recombinant TNF-α can reduce parasite numbers in infected epithelial cells, neutralisation

of TNF-α by antibody treatment in mice has no effect on infection, and TNF-α deficient mice are no more susceptible to infection than immunologically intact mice.

5.5.3.3 IL-12

IL-12 is a key inducer of IFN-γ, critical in resistance and protection of other Apicomplexan parasites. In *cryptosporidiosis*, treatment of both immunocompetent and immunodeficient mice with IL-12 before infection prevented or greatly reduced the severity of infection, and was attributed to a decrease in IFN-γ reduction. IL-12 deficient mice are susceptible to infection, but they generate some IFN-γ and are able to recover from infection. Infected calves produced IL-12 in response to infection, but treatment with recombinant IL-12 does not provide protection from challenge inoculation with *C. parvum* oocysts. IL-12 may have other roles besides induction of IFN-γ, as IL-12p40 gene knockout mice treated with IFN-γ-neutralising antibody excreted over eightfold higher oocyst numbers than IFN-γ knockout mice.

5.5.3.4 IL-18

IL-18 is a pluripotent cytokine involved in innate and adaptive immune mechanisms, and it is produced by epithelial cells and a number of different immune cells. It is up-regulated in response to *C. parvum* infection *in vitro* and in mice. Recombinant IL-18 can inhibit intracellular development of the parasite in HCT-8 and HT-29 cell lines, possibly by increasing expression of bactericidal antibiotic peptides LL-37 and α-defensin 2 (see Figure 5.2). IL-18 deficient mice are susceptible to infection, supporting a protective role for this cytokine in cryptosporidial infection.

Although IL-18 is well known to act in conjunction with IL-12 as an IFN-γ inducer, treatment of IL-12 deficient mice with recombinant IL-18 decreased *C. parvum* load markedly demonstrating a protective role for this cytokine alone. Additionally, treatment of IFN-γ deficient mice with neutralising anti-IL-18 antibodies resulted in an increased parasite excretion, suggesting an additional role for IL-18 besides induction of IFN-γ. Potentially, this role could be through the induction of Th2 cytokines, since increased gene expressions of IL-4 and IL-13 were observed in splenocytes of anti-IL-18-treated mice deficient in the production of IL-12 and IFN-γ.

5.5.3.5 Th2 cytokines

Th2 cytokines such as IL-4 and IL-5 have been detected following infection. These cytokines probably play a role in effective control of *C. parvum* infection by suppressing the production of Th1 cytokines (see Figure 5.2) although, paradoxically, it is also possible that IL-4 may enhance the Th1 response by stimulating IFN-γ production. Although one study has demonstrated that IL-4 deficient mice shed oocysts for longer than immunologically intact mice, the majority of studies have failed to demonstrate a critical role for IL-4 in cryptosporidial infection. In human volunteers, IL-4 expression was associated with prior sensitisation to *Cryptosporidium* infection, but it did not correlate with symptoms or oocyst production.

5.5.3.6 Immunoregulatory cytokines

IL-10 and TGF-β are important immunoregulators in the intestinal tract, and they inhibit synthesis of pro-inflammatory cytokines. IL-10 increases in both HIV-infected and malnourished individuals infected with *Cryptosporidium*, as well as in infected calves and in some mouse models of cryptosporidial infection. TGF-β also has been shown to increase in both animals and humans after infection. This cytokine is important in the repair of epithelial cells and can down-regulate Th1 cytokines such as IFN-γ activity *in vitro*, as well as inhibit *C. parvum* development.

5.5.4 Antibody response

In the general population, the seropositivity rate in humans is high and reported to be anywhere from 25 to >60 per cent, depending on the location and population being surveyed. While antibody responses (specifically immunoglobulin (IgG and IgA)) are mounted against parasite antigens following primary infection, the role of these antibodies during recovery appears limited. There is some evidence that humoral responses play a role, albeit modest, in protection from reinfection and, indeed, individuals with antibody deficiencies such as X-linked hyper-IgM and IgA deficiencies are more susceptible to *Cryptosporidium* infection. On the contrary, high titres of parasite IgG and IgA can be found in HIV-infected individuals, who remain susceptible to infection with *Cryptosporidium*.

Antibodies are directed against several immunodominant antigens and these can block invasion *in vitro* and reduce parasite loads in mice. In calves, oocyst shedding and parasite load was reduced by passive transfer of colostrum from cows immunised with P23 antigen of *C. parvum*, suggesting that partial protection may be achieved if local antibody concentrations are generated and maintained. However, antibodies do not seem to be essential, at least in mice; B cell deficient mice are no more susceptible to infection than immunologically intact control mice.

5.6 Memory responses

Secondary infections with *C. parvum* result in decreased oocyst shedding and reduced parasite colonisation, compared with primary infections. In mice, immunity is reduced upon depletion of CD8+ T cells, and completely abrogated by depletion of CD4+ cells. This suggests that both CD4+ and CD8+ cells are involved in resistance to secondary infection. In particular, IEL cells are important memory effector cells, because adoptive transfer of both CD4+ and CD8+ IELs into susceptible SCID mice can reduce parasite load and protect against *Cryptosporidium* infection.

It not known how long immunity persists after resolution of cryptosporidial infection. Experiments in human volunteer studies show that individuals challenged 1 year after experimental infection have reduced infection levels and disease but are not completely resistant to reinfection. It may be that there is

a gradual decline of memory T cell responses, like that observed in malaria (see Chapter 3) or that protective memory cell responses are not sufficiently generated. T cell clones isolated from the blood of patients previously infected with *Cryptosporidium* and then stimulated with cryptosporidial antigen fractions or recombinant peptides *ex-vivo* are predominantly α/βTCR+CD4+ CD45RO+, markers of memory cells. Challenge infections can lead to an increase in the antibody response in mice, indicating the presence of memory B cells, and a pre-existing antibody response was associated with a reduction in oocyst shedding in human volunteers challenged with a secondary infection of *Cryptosporidium*.

5.7 Antigens eliciting the immune response

Several antigens of *Cryptosporidium* have been identified as immunodominant, some of which are surface and/or apical complex proteins that may mediate attachment and invasion. Sera from infected animals or humans have been shown to recognise a number of immunodominant sporozoite antigens, including polypeptides of approximately 11, 15, 23, 44, 100, 180 and >200 kDa. These include the surface antigens CSL, Cp900, Cp23/27, Cp40/45, Cp15/17, Muc4 and Muc5. Many of these proteins provide or are associated with protection (see Table 5.2). In addition, passively administered monoclonal and polyclonal antibody preparations to native proteins are partially protective in animal models of cryptosporidiosis.

The longevity of the antibody response has not been thoroughly investigated, although it is known that the 15–17 kDa and the 23 kDa/27 kDa antigens can elicit IgA and IgG antibody reactivity 4–6 weeks post infection. The gp60 protein is expressed as a precursor protein that is proteolytically cleaved into two mature glycopeptides, the gp15–17 kDa and 40 kDa peptides. These proteins are secreted and co-localise to the surface membrane of sporozoites and merozoites. Ribosomal proteins P0, P1 and P2 have been described as prominent antigens in other parasitic diseases, and a cryptosporidial 60S acidic ribosomal protein P2 (CpP2) in the 15–17 kDa size range has also been identified as an immunodominant antigen.

Some of the immunodominant antigens in *Cryptosporidium* infection are partially or heavily glycosylated. The *C. parvum* 17-kDa antigen GPI anchor is composed of a very basic Manα1,2-Manα1,6-Manα1,4-glucosamine glycan core, but there are also minor GPI phospholipids that are recognised by serum antibodies in infected humans. The microneme Cp900 antigen, known to participate in the invasion process, is a heavily glycosylated protein that displays mucin-type O-linked and N-acetylgalactosamine (GalNAcα1-Ser/Thr) glycotopes.

5.8 Immune evasion

Little is known about the parasite's ability to evade or alter the immune response. It has been suggested that the unique location of *Cryptosporidium* parasites (intracellular, but surrounded by host cell membrane) in the host cell may limit exposure to parasitic antigen. Other Apicomplexans have utilised

Table 5.2 Antigens recognised by the immune system in cryptosporidial infection.

Antigen	Observation	Reference
Cp23/27	In experimentally infected humans, those that had pre-existing serum IgG to the Cp23/27 antigen excreted fewer oocysts compared to those that did not.	Moss, DM *et al*. (1998). The antibody response to 27-, 17-, and 15-kDa *Cryptosporidium* antigens following experimental infection in humans. *Journal of Infectious Diseases* 178(3), 827–833.
	Antibody and cell-mediated responses to the Cp23 antigen are of a higher magnitude from patients with prior exposure to cryptosporidiosis when compared with unexposed individuals. In natural infections, IgG levels to Cp23/27 antigen are higher in older children.	Smith, LM *et al*. (2001). Human T and B cell immunoreactivity to a recombinant 23-kDa *Cryptosporidium parvum* antigen. *Journal of Parasitology* 87(3), 704–707. Priest, JW *et al*. (2006). Longitudinal analysis of *Cryptosporidium* species-specific immunoglobulin G antibody responses in Peruvian children. *Clinical and Vaccine Immunology* 13(1), 123–131.
	In natural infections, IgG levels to Cp23/27 antigen are higher in HIV-infected patients that asymptomatic patients.	Frost, FJ *et al*. (2005). Protective immunity associated with a strong serological response to a *Cryptosporidium*-specific antigen group, in HIV-infected individuals. *Journal of Infectious Diseases* 192(4), 618–621.
	Children with a shorter duration of diarrhoea tend to have higher antibody levels to the Cp23/27.	Moss, DM *et al*. (1998). Enzyme-linked immunoelectrotransfer blot analysis of a cryptosporidiosis outbreak on a United States Coast Guard cutter. *American Journal of Tropical Medicine and Hygiene* 58(1), 110–118.
Cp15/17	In natural infections, IgG levels to Cp15/17 antigen are higher in older children.	Priest, JW *et al*. (2006). Longitudinal analysis of *Cryptosporidium* species-specific immunoglobulin G antibody responses in Peruvian children. *Clinical and Vaccine Immunology* 13(1), 123–131.
	In natural infections, IgG levels to Cp15/17 antigen are higher in HIV-infected patients that are asymptomatic.	Frost, FJ *et al*. (2005). Protective immunity associated with a strong serological response to a *Cryptosporidium*-specific antigen group, in HIV-infected individuals. *Journal of Infectious Diseases* 192(4), 618–621.
Gp40	Antibody to gp40 can inhibit parasite binding to the epithelial cells.	Cevallos, AM *et al*. (2000). Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. *Infection and Immunity* 68(7), 4108–4116.
Gp900	Purified native gp900 has been shown to bind to intestinal epithelial cells and competitively inhibit *C. parvum* infection *in vitro.*	Strong, WB *et al*. (2000). Cloning and sequence analysis of a highly polymorphic Cryptosporidium parvum gene encoding a 60-kilodalton glycoprotein and characterisation of its 15- and 45-kilodalton zoite surface antigen products. *Infection and immunity* 68(7), 4117–4134.
CpP2	Prominent anti-CpP2 IgG antibody responses are generally found only among residents of countries in the developing world where *Cryptosporidium* infection occurs early and often. This may be a proxy for the intensity of infection and/or acquired immunity.	Barnes, DA *et al*. (1998). A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. *Molecular and Biochemical Parasitology* 96(1–2), 93–110.

strategies to modulate and interfere with programmed cell death or apoptosis (for example *Toxoplasma gondii*; see Chapter 4). *C. parvum* can also inhibit apoptosis early in infection, but pro-apoptotic gene expression has been shown to occur preferentially at late stages. B7-H1, a co-stimulatory molecule for T cell stimulation, is induced in *C. parvum*-infected cholangiocytes. Although B7-H1 can facilitate the activation of naive T cells to trigger cellular immune responses, this molecule may also have a negative effect on T cells by inducing apoptosis, as enhanced apoptotic cell death has been identified in activated human T cells after co-culture with *C. parvum*-infected epithelial cells.

Another potential mechanism of immune evasion involves inhibiting signal transduction pathways in the host cell; bacterial pathogens of the gut are able to block or down-regulate signal transduction pathways that are involved in cytokine production. As an example, *Escherichia coli* can inhibit STAT-1-mediated signal transduction from the IFN-γ receptor in epithelial cells, in turn reducing IFN-γ production. A similar mechanism appears to be in operation in *Cryptosporidium* infection, whereby signalling pathways emanating from the IFN-γ receptor are disrupted in epithelial cells that are infected with *C. parvum*.

5.9 Immunopathology in the gut and intestinal tract

Cryptosporidium parvum is a minimally invasive pathogen in immunocompetent hosts, but does result in villus atrophy and reduced epithelial barrier function. In immunodeficient hosts, where infections can be more severe and chronic, greater influx in the number of inflammatory cells (including macrophages, dendritic cells, and lymphocytes) has been observed.

The parasite has been reported to cause flare-ups in patients with inflammatory bowel disease or to cause severe infections in individuals with Crohn's disease. However, the immune cells responsible for this are currently undetermined. Hypersensitivity with mast cell accumulation in the gut wall after infection has been noted with certain isolates of *C. parvum* in an un-weaned rat model. On the other hand, neutrophils in human and animal cryptosporidiosis may play a role in enhancing the epithelial cell barrier function, rather than in mediating the pathological sequelae of *C. parvum* infection. They do not appear to contribute to the pathology of disease during infection, as they do not contribute to peroxynitrite formation or have any impact on the severity of epithelial infection, villus atrophy or diarrhoea in a pig model of cryptosporidiosis.

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Diplomonadida: *Giardia* 6

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6.1 The life cycle and pathogenesis of *Giardia* infection

Giardia intestinalis is a binucleated protozoan that replicates in the lumen of many species of mammalian hosts. The nomenclature of *Giardia spp.* is rather complicated. There are three separate names currently used to refer to the same species: *G. lamblia, G. duodenalis* and *G. intestinalis*. Moreover, recent molecular studies have identified eight different genetic groups (referred to as assemblages A-H) within this species. The assemblages clearly not only differ in their DNA sequence, but also in the host species from which they have been isolated. Because of the molecular and biological differences among them, it is likely that these assemblages will be designated as distinct species in the near future.

Importantly, only parasites from assemblage A and B have been isolated from human hosts. Parasites from assemblages A and B, however, have also been recovered from numerous species of animals, including mice, beavers and livestock, suggesting that both human-to-human and animal-to-human modes of transmission exist.

6.1.1 Life cycle

Infection with *Giardia* occurs through ingestion of contaminated food and water (Figure 6.1). Parasite cysts are shed in the faeces of infected humans and animals, and these cysts can remain viable in aqueous environments for several months. As few as ten cysts have been shown to be capable of initiating an infection. After ingestion and passage through the stomach, two trophozoites – the replicative form of the parasite – emerge from each cyst in the small intestine. Within the small intestine, the parasites attach to the mucosal surface through a mechanical process thought to involve unique cytoskeletal structures underlying the parasite plasma membrane. Trophozoites divide by binary fission while in the small intestine, and they can reproduce

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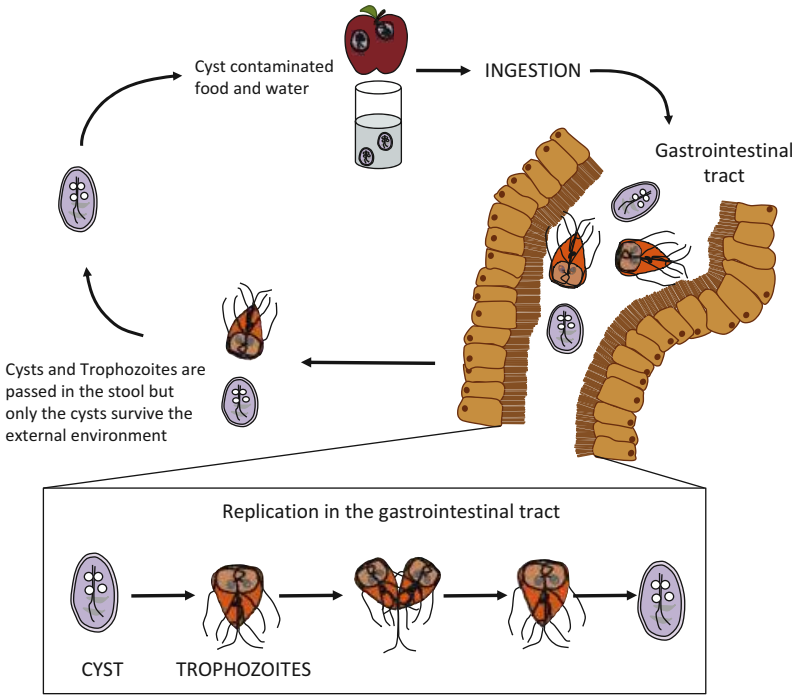

Figure 6.1 Life cycle of *Giardia*.

to millions of trophozoites per infected individual. As trophozoites migrate down the digestive tract, changes in the environment, particularly reductions in the amount of cholesterol and other lipids, trigger differentiation into the environmentally stable cyst.

6.1.2 Epidemiology and treatment

The incidence of *Giardia* infection is quite high in most countries. Prevalence of cyst shedding of ≈20 per cent has been found in numerous studies from many geographic areas, and anti-*Giardia* antibodies in sera are found in ≈99 per cent of children in endemic areas by the age of three. Estimates for infection rates in the USA are close to two million annually, with a peak incidence in the summer months, when recreational use of water sources such as pools, rivers and lakes increases.

Metronidazole (Flagyl™) is the primary treatment for giardiasis. A five-day course is recommended and usually results in cessation of cyst shedding, as well as amelioration of symptoms. Resistance to treatment, reinfection and recrudescence, however, have all been reported. Recently, tinidazole has been approved by the FDA for treatment of *Giardia* and numerous studies have shown albendazole to be equally effective. Given the frequency of side-effects with Flagyl (e.g. metallic taste and gastrointestinal upset), patients often fail to complete the five-day course of treatment.

6.1.3 Pathogenesis

Giardia infection can result in a variety of clinical presentations, ranging from sub-clinical disease characterised only by nutrient malabsorption, to severe diarrhoea accompanied by cramps, fever and vomiting. Numerous studies have tried to associate severe disease with the presence of a particular genetic assemblage. Thus, in one study, assemblage B parasites were found in symptomatic patients and assemblage A parasites were more frequent in patients with sub-clinical disease. However, in other studies, the opposite association has been found. At present, no specific virulence factors which can induce symptoms of infection have been identified. It is still likely that different parasite isolates will differ in their ability to induce clinical symptoms, but it may not be as simple as differences between the assemblages.

The normal pattern of infection is a pre-patent period of 5–7 days. This is followed by 1–2 weeks of acute symptoms and then elimination of the parasite. As noted above, acute symptoms like diarrhoea and cramps are often not present during this 'acute' phase. In most individuals, the parasite is eliminated within 3–4 weeks, but chronic infections can also occur. Chronic infections are common in individuals with certain immunodeficiencies, in particular common variable immune deficiency (CVID) and X-linked immunodeficiency (XID). Both of these syndromes are characterised by reduced production of IgG, suggesting that antibody responses may play a pivotal role in preventing chronic infection (see below).

6.2 Recognition of *Giardia* by the immune system

The initial recognition of *Giardia* by the host occurs through the innate immune system. Parasites can be killed by complement after activation with specific antibodies, i.e. the classical pathway of complement activation.

6.2.1 Mannose binding lectin

Biochemical and genetic studies of *Giardia* have shown that the parasite is able to glycosylate numerous proteins with the addition of N-Acetyl-Glucosamine (GlcNAc). GlcNAc is a known ligand for the mannose binding lectin (MBL), and trophozoite lysis by naïve complement-containing sera *in vitro* can occur following MBL binding. The cyst walls of *Giardia* also contain a large amount of carbohydrate polymer-containing GlcNAc. The ability of this cyst wall material to stimulate any type of immune response, however, has not been investigated.

6.2.2 Epithelial cell recognition

The cells of the innate immune response are also capable of recognising *Giardia* antigens. The main cell type with which the trophozoites have contact are the epithelial cells lining the wall of the intestine. *In vitro* studies using cell lines have demonstrated that *Giardia* can induce cytoskeletal rearrangements

in the intestinal epithelial cells (IECs), and that some parasite strains can induce apoptosis of these cells.

Two groups have examined the ability of IECs to produce cytokines and chemokines in response to *Giardia*. The first group reported that they could not detect the chemokine CCL2 or the cytokines IL-1 or TNF-α in supernatants following exposure of the human colorectal epithelial cell line Caco2 to live parasites. A second group used a microarray analysis to examine changes in chemokine RNA levels, and they found increased expression of CCL2, CCL20, CXCL1, CXCL2 and CXCL3. They also found CCL2 protein in the supernatants of these cell cultures.

6.2.3 Dendritic cells

Dendritic cells (DCs) are also able to recognise and respond to *Giardia* trophozoites. However, the receptors involved and parasite ligands have not been investigated. Exposure of mouse bone marrow-derived DCs to *Giardia* results in up-regulation of the surface co-stimulatory molecules CD40, B7–1 and B7–2, although up-regulation of the B7 molecules is relatively weak. Similarly, DCs produce small amounts of cytokine, including IL-6 and TNF-α, in response to *Giardia*.

Interestingly, mice lacking either IL-6 or TNF-α exhibit delayed clearance of infections with *G. intestinalis*. However, it is unclear if DC production of these cytokines is responsible for this effect, or if cytokine production by other cells is also important. Interestingly, addition of *Giardia* extract to DCs stimulated with various Toll-like receptor (TLR) agonists, including lipopolysaccharide, CpG-containing DNA and PAM-3-Cys, results in the inhibition of the responses (particularly IL-12 production) to these canonical pathogen-associated molecules. Inhibition can be significantly reversed by treatment with the phosphoinositide-3-kinase (PI3K) small molecule inhibitor wortmannin, suggesting that inhibition may be mediated through a PI3K dependent pathway.

Addition of *Giardia* extracts to these TLR agonists also increases IL-10 production by the DCs. Thus, *Giardia* infection is likely to promote an anti-inflammatory environment within the intestinal tract.

6.3 Innate effector mechanisms against *Giardia*

Several innate effector mechanisms have been shown to have anti-*Giardia* activities. For example, components of normal breast milk and normal serum can kill parasites *in vitro*. As noted above, complement activation through the MBL can also lead to parasite lysis *in vitro*.

6.3.1 Defensins

The small intestine is also protected by a unique set of anti-microbial peptides (α-defensins) produced by Paneth cells in the crypts of Lieberkuhn. Human

Paneth cells secrete two peptides, known as HD5 and HD6, while mouse Paneth cells secrete six related peptides (Cryptdins 1-6). *In vitro* studies have again shown that *Giardia* trophozoites are susceptible to killing by mouse cryptdins, although some peptides are much more efficient than others.

An *in vivo* study used mice lacking the enzyme matrix metalloprotease-7 (MMP-7, also known as matrilysin) to determine the role of cryptdins during infection. Cryptdins are synthesised as inactive precursor peptides, which are activated upon cleavage by MMP-7. Thus, mice lacking MMP-7 cannot produce active defensins. These mice actually exhibited lower levels of *G. muris* at one week following infection than did wild-type counterparts, suggesting that cryptdins may not be a major factor in controlling *Giardia* infection. The authors of this study speculated that changes in microbial flora due to a lack of cryptdins might have resulted in the decreased parasite burden.

6.3.2 The contribution of the microbial flora

Resistance to *Giardia* infection can, indeed, be due to the presence of beneficial microbes in the intestinal tract. In the adult mouse infection model, the susceptibility of commercial mice differs, based on where the mice are bred. Housing susceptible mice with resistant mice in the same cage for a few weeks can make them resistant to infection. Conversely, treatment of resistant mice with antibiotics can make them susceptible. These data suggest that commensal bacteria influences the ability of *Giardia* to colonise mice.

In vitro studies have shown that supernatants of certain strains of *Lactobacilli* can kill *Giardia* trophozoites, and *in vivo* studies in mice and gerbils have shown an inhibition of infection by *Lactobacilli* as well.

6.3.3 Nitric oxide

A discrepancy between *in vitro* and *in vivo* effects was also found in studies of the effect of nitric oxide (NO) on *Giardia*. Initial *in vitro* studies showed that NO could inhibit trophozoite replication as well as differentiation into cysts. *In vivo* studies were then conducted using mice lacking the enzyme inducible NO synthase (NOS2), which showed no deficit in control of the infection. Interestingly, additional studies using pharmacologic inhibitors of NO synthase showed that NO production by the neuronal isoform of NO synthase (NOS1), but not by NOS2, was actually important for normal elimination of this infection.

6.4 Adaptive immunity against *Giardia*

6.4.1 B cells and antibodies

Infection with *Giardia* typically results in a strong antibody response against the parasite. Incubation of trophozoites with specific antibody can lead to

inhibition of parasite replication as well as parasite death, depending on the particular antibodies and the presence of complement. While IgG is made in significant amounts, IgA is believed to be more important in parasite control. IgA is transported across epithelial surfaces by the poly-Immunoglobulin receptor (pIgR) in a process called transcytosis. Thus, IgA is the most abundant isotype in intestinal secretions, and it is also the dominant isotype in mother's milk. Studies have shown that passive transfer of anti-*Giardia* antibodies in milk can help protect newborn humans, as well as neonatal mice. IgA in adults has also been shown to be protective in studies using mice lacking the pIgR.

As noted earlier, humans with CVID or XID are prone to developing chronic giardiasis, suggesting that antibodies may be important for preventing chronic infection. Interestingly, infection of mice unable to produce any antibodies with the GS strain of *G. intestinalis* resulted in parasite elimination with kinetics identical to wild-type control mice. This experiment shows that antibody-independent mechanisms can contribute to parasite elimination, but it does not mean that antibodies are unimportant in normal infections.

6.4.2 T cells

The other major aspect of adaptive immune responses is the T cell response. Several studies have shown that T cells are essential for proper control of *Giardia* infections. Given that *Giardia* is an extracellular pathogen, it is not surprising that CD4+ helper T cells are primarily responsible for this protective effect.

One role of helper T cells is to promote antibody production and isotype switching. Other roles include cytokine production to help recruit other effector cells of the immune response. For example, mice lacking IL-9 have a reduced ability to control infection, and this is accompanied by a reduced ability to recruit mast cells to the small intestine. Indeed, several studies have shown that mast cell recruitment is an important aspect of immunity to *Giardia* (Figure 6.2). Interestingly, mast cell responses are usually thought of in context of helminth infections, but they may be more a reflection of the site of the infection, the intestinal mucosa.

T cells can produce several distinct patterns of cytokines following infection. Studies on cytokine responses to *Giardia* are currently rudimentary. Mesenteric lymph node cells from mice infected with *G. muris* produce a mix of the Th2 cytokine IL-5 and the Th1 cytokine IFN-γ after stimulation with the mitogen Concanavalin A.

The role of IFN-γ in the clearance of parasites in *Giardia* infection is unclear. Treatment with monoclonal antibodies to mop up IFN-γ delays parasite clearance but, in contrast, IFN-γ deficient mice eliminate *G. intestinalis* infections with kinetics similar to wild-type mice. Mice lacking IL-4 or components of the IL-4 signalling pathway also exhibited rapid elimination of *G. intestinalis*. Studies in humans have shown that infection is associated with elevated levels of a variety of cytokines in serum, including IL-2, IL-4, IL-5, IL-6, IFN-γ, TNF-α and the soluble IL-2 receptor (sIL-2R).

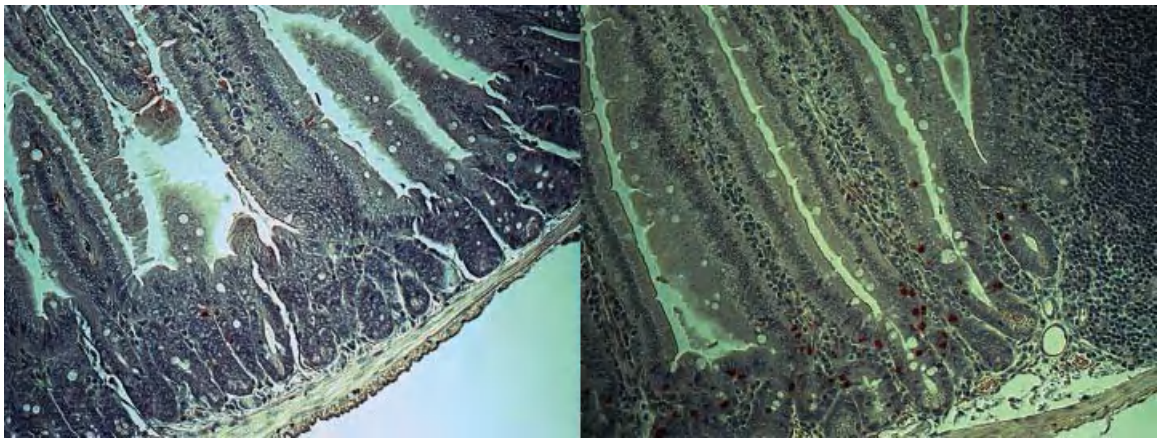

Figure 6.2 Mast cell accumulation following *Giardia* infection. Mice were infected with one million trophozoites of the GS strain of *G. intestinalis*. Five days (left panel) or 14 days (right panel) later, segments of the small intestine were fixed, sectioned and stained for chloroacetate esterase activity, turning the mast cells fuchsia. Parasites can be seen in the intestinal lumen on day 5. Original magnification = 200×. Source: E. Li and S. Singer (unpublished data).

6.4.3 Activation of mast cells by the adaptive immune response

An important aspect of adaptive immune responses to *Giardia* infection is their effect on intestinal motility. Infections in wild-type mice, but not in severe combined immunodeficiency (SCID) mice which have very few functional T or B cells, lead to an increased rate of transit in the small intestine. *In vitro* data suggest that mast cell activation contributes to this hypermotility, through a pathway involving increased intestinal smooth muscle contractions, mediated by the hormone cholecystokinin (CCK). Interestingly, one of the normal functions of CCK is to trigger smooth muscle contractions in the gall bladder and release of bile into the small intestine following a meal. *Giardia* is unable to synthesise lipids and requires bile for growth. Finally, blocking the increased transit using the μ-opioid agonist loperamide resulted in delayed parasite elimination, indicating that this hypermotility response contributes to successful parasite elimination.

6.5 Memory responses

6.5.1 Evidence from outbreaks of Giardiasis

Most of the innate and adaptive responses described above examined primary infections with *Giardia*. Vaccine development, however, requires an understanding of secondary responses. In humans in endemic regions of the world, recurrent episodes of giardiasis are common. However, it is unclear if these reflect recrudescence of an initial infection that was not completely eradicated, repeated infections with the same strain of parasite, or a new infection with a different strain.

The best data supporting the ability of memory responses to protect against a repeated infection comes from a series of *Giardia* outbreaks in British Columbia that occurred several years apart. Individuals who had been infected during the first outbreak were significantly less likely to have giardiasis during the second outbreak. This suggests that recovery from a prior infection can protect against subsequent infection, or at least against developing symptomatic disease during subsequent infections (stool surveys of individuals without diarrhoea were not performed).

A similar conclusion was found in a giardiasis outbreak in Colorado; individuals who had resided in the area for longer periods of time were much less likely to become ill during a giardiasis outbreak, suggesting that previous exposure might have generated a protective immune response.

6.5.2 Giardiavax™

Studies in animals have also suggested that memory responses can help prevent infection. A commercially available vaccine, Giardiavax™, has been shown to generate antibodies against the parasite and to help prevent infections in dogs and cats. Vaccine administration to chronically infected dogs also promotes parasite elimination. It is unclear from these studies whether the antibodies that are observed are responsible for the protective effects of the vaccine.

6.5.3 Anti-cyst vaccines

Additional studies of memory responses in mice have targeted the cyst stage of the parasite. Mice immunised with recombinant cyst wall protein (CWP)-2, then challenged with *G. muris*, exhibited reduced cyst shedding. Vaccinated mice were shown to produce antibodies against the CWP2, and increased cytokine mRNA levels were detected in spleen and mesenteric lymph nodes.

An anti-cyst vaccine might function to reduce transmission of the parasite without affording direct benefit to those who have received the vaccine – a so-called 'altruistic vaccine'. It is possible that antibodies against the cyst might serve to block infection, although this effect has not been observed in animal studies.

6.6 Antigens eliciting the immune response

The most prominent antigens recognised by the adaptive immune response against *Giardia* are the variant-specific surface proteins (VSPs – see below). Other invariant proteins have been shown to stimulate robust immune responses in humans and animals. One study using sera collected from patients during an outbreak in Sweden identified 16 proteins commonly recognised following infection. Another study used mice unable to secrete IgA into the intestinal lumen (due to a deletion in the pIgR), resulting in high levels of serum antibodies which were used to identify additional potential antigens. Importantly, passive transfer of these serum antibodies to naïve recipient mice that could

transport IgA to the intestinal lumen indicated that these antibodies could provide protection against a challenge infection.

Activators of the innate immune response are not well defined. One group has identified a set of metabolic enzymes that are released by trophozoites after exposure to epithelial cells. Moreover, they have shown that some of these secreted molecules are sufficient to inhibit production of NO by epithelial cells, possibly promoting colonisation of the small intestine. Another approach has been to define the proteins which are modified by the addition of N-Acetylglucosamine (Glc-NAc). This is the only sugar found to decorate trophozoite proteins, and it is a ligand for the host MBL. These modified proteins are, therefore, possible activators of complement early during infection.

In addition to trophozoite antigens, cyst wall proteins and carbohydrates can also be targets of host immune responses. As noted above, CWP2 has been used as a vaccine target in order to reduce cyst shedding following infections. Antibodies to CWPs are also the basis for an antibody-based diagnostic test for *Giardia* that is typically more sensitive (albeit more expensive) than microscopy.

6.7 Immune evasion

Variant-specific surface proteins (VSPs) are cysteine-rich and often contain zinc-finger domains that are thought to promote protein-protein interactions, in turn helping to form a dense coat on the surface of the trophozoite. Thus, VSPs may contribute to pathogenesis in ways that are independent of the host antibody response, perhaps by providing resistance to host proteases, or innate immune factors that contribute to parasite killing and host specificity. However, the major role of VSPs in pathogenesis is undoubtedly to contribute to immune evasion through a process of antigenic variation.

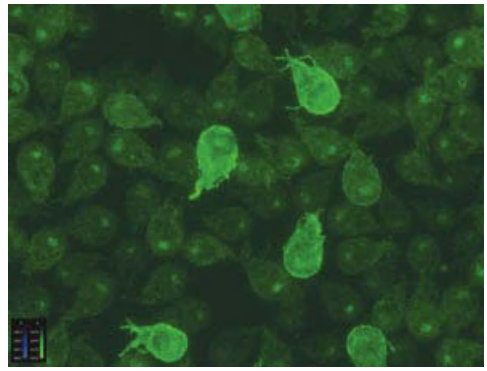

Figure 6.3 Intestinal IgA responses detect variant *Giardia* antigens. Intestinal fluid was collected from mice infected for 12 days and used to stain parasites that had been grown *in vitro* in the absence of antigenic selection. FITC labelled anti-mouse IgA reveals strong staining of a subset of the trophozoites. All parasites exhibit faint labelling throughout, as well as a strong reaction in the centre of the parasite, possibly the microtubule organising centre or a component of the ventral disc. Original magnification = 400×. Source: E. Li and S. Singer (unpublished data).

Antigenic variation has been shown to occur following *G. intestinalis* infections in both humans and in animal models. Experimental infections using cloned *Giardia* lines expressing a single VSP on most of the parasites typically initially results in a homogenous population of parasites and an antibody response against that VSP. In mice able to mount an antibody response, the appearance of anti-VSP antibodies typically results in selection for parasites which have spontaneously switched expression to a different VSP. There does not appear to be a preference for switching to any particular VSP and, indeed, expression of many different VSPs can be observed following depletion of the initial VSP.

Each individual parasite contains approximately 150 different VSP genes, but each trophozoite expresses only one VSP at a time (Figure 6.3). Moreover, the different strains of *Giardia* appear to have predominantly non-overlapping repertoires of VSP genes. Switching among VSP genes

appears to occur via a stochastic process that does not involve gene rearrangements, although the mechanism of switching remains unknown.

Recent work has shown that genes involved in double-stranded RNA mediated gene silencing appear to be important for maintaining the expression of only one VSP per parasite. Infection in gerbils using *G. intestinalis* that express multiple (perhaps all) VSPs at the same time has been shown to provide protection against a second infection, suggesting a possible novel route of vaccination against *Giardia*.

IL-6 deficient mice exhibit prolonged infections, despite the ability to produce anti-*Giardia* IgA. Thus, IL-6 deficient mice provide a useful vehicle to investigate the antibody-specificity of IgA in chronic infection. While IgA in intestinal fluid of IL-6 deficient (and wild-type) mice two weeks post-infection reacts with a restricted subset of parasite surface antigens, intestinal fluid eight weeks post-infection contains IgA that reacts with all parasites in a population containing a mixture of parasites expressing different VSPs. This result suggests that, during the course of *Giardia* infections, the IgA response initially targets variable epitopes on VSPs, then later matures to recognise either conserved epitopes on VSPs, invariant antigens or both.

IgA produced early in a *G. intestinalis* infection has also been found to react with different epitopes from those targeted later in infections, and it is these later specificities which may be important in controlling the development of chronic infections. Antibodies against variant-specific epitopes are likely only to select for antigenic variants, rather than to lead to parasite elimination.

6.8 Immunopathology

Giardia infection can result in a spectrum of clinical presentations, ranging from severe diarrhoea with cramps, nausea and fever to a sub-clinical syndrome marked only by nutrient malabsorption and moderate reductions in lactase activity. The mechanisms involved in producing these symptoms are still controversial, although recent work has highlighted the potential role for immune responses in producing some of these effects. Parasite virulence factors may also contribute to the development of symptoms, although no molecular determinant of pathogenicity have so far been defined.

Many intestinal pathogens cause diarrhoea by activation of a generalised inflammatory response. Interestingly, inflammation during *Giardia* infection does not correlate well with the development of severe symptoms. As noted above, *Giardia* appears to be able to modulate the innate response so as to diminish production of IL-12, a major pro-inflammatory cytokine, and to augment production of IL-10, an anti-inflammatory cytokine.

Perhaps the best evidence for the role of the immune response in contributing to symptomatic giardiasis comes from the studies of intestinal motility mentioned previously. *Giardia* infection in immunocompetent (but not SCID) mice leads to increased rates of intestinal transit and hyper-contractility of intestinal smooth muscle. Specifically, the force of spontaneous muscle contractions in

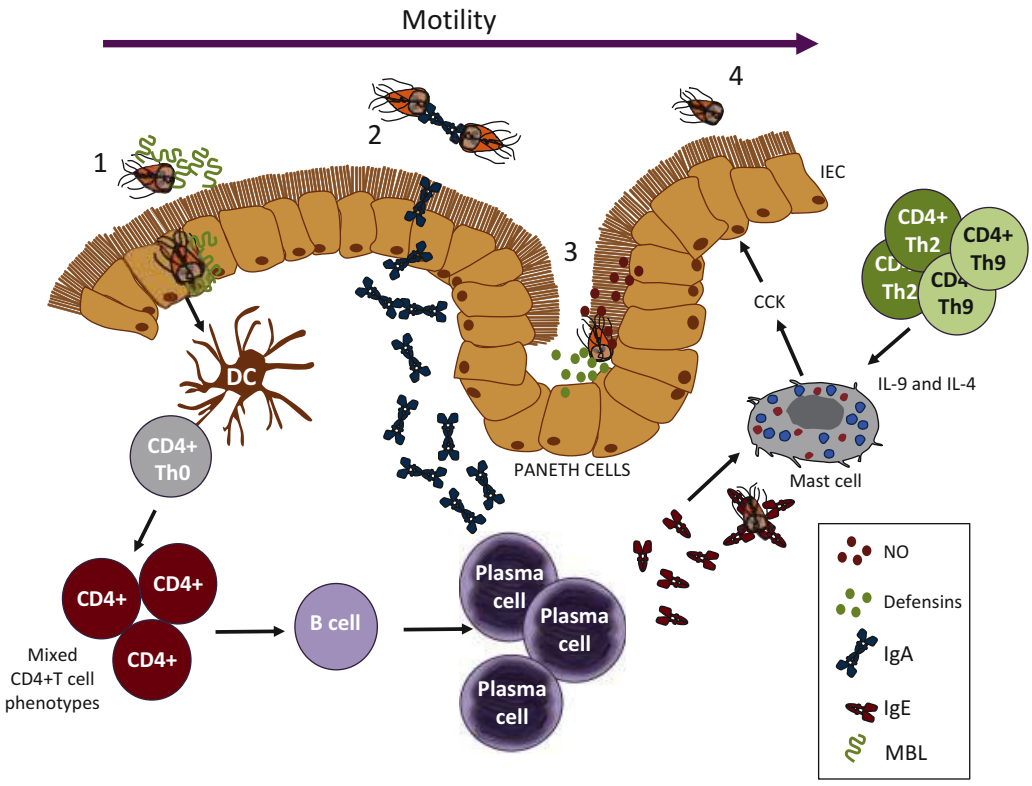

Figure 6.4 Model of immune mechanisms controlling infection. Parasites stimulate innate responses (1), leading to activation of adaptive responses (IgA and T cells) (2). Enhanced production of defensins by Paneth cells and NO by IECs kill parasites attached to the epithelium (3), while mast cell activation leads to increased motility in a CCK-dependent manner (4). These increased propulsive forces help to detach parasites and to flush non-attached parasites down the gastrointestinal tract. Abbreviations: CCK, cholecystokinin; DC, denritic cells; IEC, intestinal epithelial cell; Ig, immunoglobulin; IL, interleukin; MBL, mannose binding lectin; NO, nitric oxide; Th, T helper cell.

infected mice is roughly double that found in non-infected mice. These muscular contractions are analogous to the severe cramps seen in human infections. While intestinal transit was shown to depend on the presence of adaptive immune responses, muscle hyper-contractility *in vitro* was shown to depend on mast cell responses and the hormone CCK. It is likely that these are connected, as both B cell production of IgE and T cell production of cytokines (e.g. IL-4 and IL-9) can contribute to recruitment and activation of mast cells (Figure 6.4).

Interestingly, anti-*Giardia* IgE production has been correlated with symptomatic human infections in some studies supporting a role for IgE-mediated cross-linking of FcεR in the activation of mast cells in *Giardia* infection. Furthermore, urticaria (skin rash commonly caused by activation of skin-resident mast cells) is a common sequella of *Giardia* infections.

Other mechanisms that contribute to diarrhoea and nutrient malabsorption in giardiasis include loss of epithelial barrier integrity, increased ion secretion and reduced levels of digestive enzymes such as sucrose and lactase. Changes in epithelial barrier integrity are thought to be due to alterations in the levels

and localisation of tight junction proteins, and to increased rates of epithelial cell apoptosis. These changes have been seen *in vitro*, using epithelial cell lines and parasites, suggesting that they are not dependent on the host immune response.

The reduced levels of disaccharidases such as sucrase and lactase are thought to be due to reductions in the surface area of microvilli. Reduced levels of sucrase and lactase activity are seen even in infections where more overt symptoms like cramps and diarrhoea are absent. These epithelial changes have also been observed in animal models of infection, and have been attributed to CD8+ T cell responses. Infections in wild-type (but not T cell-deficient nude) mice resulted in reduced microvillous surface area and reduced sucrose and lactase activities. Moreover, adoptive transfer of CD8+ (but not CD4+) T cells into naïve recipient mice induced a similar change in the intestinal epithelium. It is unclear how CD8+ T cells are activated following *Giardia* infection, although cross-presentation of extracellular antigens in the MHC-I pathway is now a well-described phenomenon (see Chapter 1).

6.9 Summary

Immunity to *Giardia* is complex. Multiple pathways are involved in elimination of the parasite from the GI tract and in prevention of chronic and recurrent infection. These pathways likely exhibit functional redundancy, such that elimination of one pathway still allows the immune system to combat the parasite (Figure 6.4). Conversely, antigenic variation within individual parasite strains, as well as genetic variation among parasite strains, can allow *Giardia* to circumvent immunological memory responses. Finally, mechanisms involved in parasite elimination may also contribute to symptoms of the disease. Thus, vaccines that promote immune responses might lead to an increase in symptomatic disease, along with a reduced incidence of infection.

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Kinetoplastids: *Leishmania* 7

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The leishmaniases, a group of vector-borne parasitic diseases, represent a major public health problem worldwide. The diseases affect an estimated 12 million people in 88 countries, and approximately 350 million people are at risk. The leishmaniases belong to the most neglected tropical diseases, affecting the poorest populations, for whom access to diagnosis and effective treatment are most difficult. Much effort has been put into the discovery of new drugs for the treatment of these diseases, but still the most widely used drugs remain the pentavalent antimonials which were introduced over 50 years ago.

Patients presenting with *Leishmania* infection display a wide range of symptoms, from the self-healing cutaneous form to the visceral form, the most severe manifestation of leishmaniasis, in which the mortality rate approaches 100 per cent without treatment. The drugs used to treat the leishmaniases have many limitations, such as the long course of treatment, severe side-effects and development of resistance. No efficient vaccine is available to date.

7.1 The pathogenesis of *Leishmania* infection

The protozoan parasite, *Leishmania*, is responsible for the disease leishmaniasis, and there are several different species of *Leishmania* parasites that can cause the different forms of the disease (Table 7.1). Cutaneous leishmaniasis (CL) manifests as localised cutaneous, mucosal or mucocutaneous and diffuse cutaneous disease:

- Localised CL is characterised by single or multiple localised lesions on exposed areas of skin that often heal spontaneously and leave a scar.
- Mucosal lesions are caused by *L. infantum*, *L. major*, *L. tropica* and *L. aethiopica* in the Old World and may develop in the mouth, the nose or on the genital mucosa.

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Table 7.1 ***Leishmania*** **species causing disease in humans.**

Disease	Cutaneous leishmaniasis		Mucocutaneous leishmaniasis	Visceral leishmaniasis
Subgenus	*L. (Leishmania)*	*L. (Viannia)*	*L. (Viannia)*	*L. (Leishmania)*
New World	*L. infantum* *L. mexicana* *L. pifanoi* *L. venezeluensis* *L. amazonensis*	*L. braziliensis* *L. guyanensis* *L. panamensis*	*L. braziliensis* *L. panamensis*	*L. infantum*
Old World	*L. major* *L. tropica* *L. aethiopica* *L. infantum*			*L. donovani* *L. infantum*

- Mucocutaneous leishmaniasis is a metastatic complication of the mucosal tissue and upper respiratory tract, caused by *L. braziliensis* and *L. panamensis* infection. It occurs in the New World.
- Diffuse CL is characterised by numerous non-ulcerating nodules, with an abundant parasite load.

Visceral leishmaniasis (VL) is caused by parasites of the *L. donovani-L. infantum* complex in the Old World and by *L. infantum* in the New World (Table 7.1), and there are an estimated 30–100 subclinical infections for every one symptomatic case. Visceral leishmaniasis is clinically comparable in both the New and the Old World and is characterised by fever, malaise, weight loss and splenomegaly.

The leishmaniases are mainly zoonoses that are transmitted from animals to humans by the bite of infected sandflies. Domestic and wild animals can be reservoir hosts; wild animals carry the parasite but do not develop disease whereas dogs can develop symptoms of leishmaniasis. The leishmaniases can also be transmitted anthroponotically, with human populations being the main reservoir for infection. Anthroponotic transmission of visceral and cutaneous leishmaniasis is significant in the Indian subcontinent, in East Africa and in urban regions.

7.2 Life cycle

Leishmania parasites are transmitted between hosts by the bite of infected female phlebotomine sandflies. The vectors of leishmaniases are species and subspecies of *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Table 7.2). *Leishmania* have a dimorphic life cycle: the extracellular promastigotes (the flagellated form of the parasites) develop within the digestive tract of sandflies, while the intracellular amastigotes (the non-flagellated form) reside and multiply within phagolysosomal vacuoles of mammalian phagocytes.

Table 7.2 The distribution of sandfly species susceptible to *Leishmania*.

Sandfly species	Geographical distribution	Species of *Leishmania*
Phlebotomus papatasi; *Phlebotomus dubosqui*; *Phlebotomus salehi*	Central and West Asia, North Africa, Sahel of Africa, Central and West Africa	*Leishmania (Leishmania) major*
Phlebotomus sergenti *Phlebotomus longipes*; *Phlebotomus pedifer*	Central and West Asia, North Africa Ethiopia, Kenya	*Leishmania (Leishmania) tropica* *Leishmania (Leishmania) aethiopica*
Phlebotomus argentipes; *Phlebotomus orientalis*; *Phlebotomus martini*	Indian subcontinent, East Africa	*Leishmania (Leishmania) donovani*
Phlebotomus ariasi; *Phlebotomus perniciosus*	Mediterranean basin, Central and West Africa	*Leishmania (Leishmania) infantum*
Lutzomyia longipalpis	Central and South America	*L. (L.) infantum (syn. chagasi)*
Lutzomyia olmeca olmeca	Central America	*Leishmania (Leishmania) mexicana*
Lutzomyia flavis cutellata	South America	*Leishmania (Leishmania) amazonensis*
Lutzomyia wellcomei; *Lutzomyia complexus*; *Lutzomyia carrerai*	Central and South America	*Leishmania (Viannia) braziliensis*
Lutzomyia peruensis; *Lutzomyia verrucarum*	Peru	*Leishmania (Viannia) peruviana*
Lutzomyia umbratilis	South America	*Leishmania (Viannia) guyanensis*
Lutzomyia trapidoi	Central America	*Leishmania (Viannia) panamensis*

Parasites are delivered to a host as promastigotes in the saliva of the sandfly and a mucin-rich gel produced by the parasites in the sandfly midgut (Figure 7.1, 1). *Leishmania* parasites are then taken up by neutrophils and subsequently establish residence inside macrophages; they are obligate intracellular parasites of macrophages in their mammalian hosts. Inside the macrophages, the promastigotes transform into, and multiply as, amastigotes within phagolysosomal vacuoles (Figures 7.1, 2 and 3). The infected phagocytic cells rupture and release amastigotes that are taken up by neighbouring phagocytic cells (Figures 7.1, 4).

When a sandfly takes a blood meal from an infected host, it ingests infected macrophages (Figure 7.1, 5). In the midgut of sandflies, the amastigotes are rapidly released from the macrophages and transform into promastigotes completing the life cycle (Figure 7.1, 6 and 7).

7.3 Parasite transmission and avoidance of immune responses

All natural infections with *Leishmania* parasites are initiated by the bite of infected female sandflies that can initiate infection in humans as well as

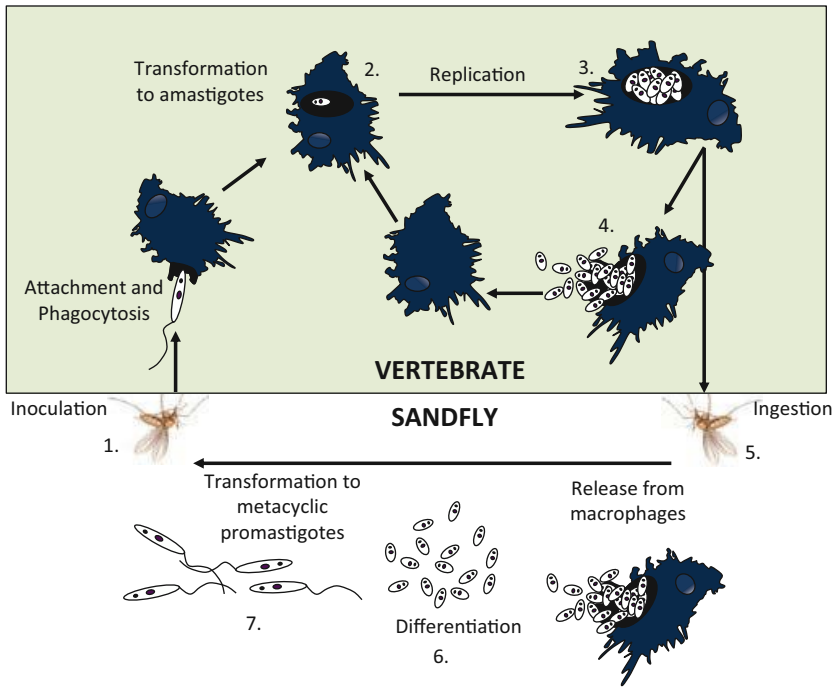

Figure 7.1 The life cycle of *Leishmania* parasites.

other warm-blooded reservoir hosts. The parasites are deposited as infective metacyclic promastigotes into the dermis of the skin. Sandflies belong to a family of pool-feeding arthropod vectors that obtain blood by lacerating surface capillaries of the upper dermis with their barbed mouthparts, and they salivate into the wound to prevent blood clotting. The tissue injury caused by the sandfly bite initiates not only a wound-healing response, but also an inflammatory response at the bite site. Since the skin is an immunologically active organ, the transmitted parasites must resist destruction by innate cells present in (or rapidly recruited to) the skin in order to survive and establish residence in the host.

The infected sandfly delivers live promastigotes as well as apoptotic parasites, its own salivary components and also a product of parasite origin, a mucin-rich gel called the promastigote secretory gel (PSG). The active ingredient of PSG is filamentous proteophosphoglycan secreted by multiplying promastigotes during their development in the insect vector. High concentrations of secreted PSG obstructs the anterior midgut of the sandfly, and the presence of this plug reduces the size of the blood meal, in turn enhancing the frequency of bites by the sandfly and thus increasing the chances that the parasites will be transmitted. This blockage needs to be cleared for effective blood feeding, and this induces flies to regurgitate both infective promastigotes and PSG during feeding.

Both vector- and parasite-derived components present in the inoculum play an active role in modulating the immune response of the host to *Leishmania*

parasites. The regurgitated PSG exacerbates cutaneous leishmaniasis and two mechanisms are known to be involved in this exacerbation:

1. PSG recruits macrophages (the main host cells of *Leishmania*) to the site of parasite inoculation.
2. PSG ensures increased synthesis of nutrients for the inoculated parasites by enhancing the expression of arginase, an enzyme which hydrolyses arginine into ornithine (the first building block for the synthesis of polyamines).

Polyamines are small cationic molecules required for the growth of eukaryotic cells. The infecting parasite takes advantage of the increased pool of polyamines present in the macrophages for their early nutrition and growth. Thus, *Leishmania* has evolved to exploit the wound-healing response to the bite of the sandfly for initial survival in the vertebrate host.

7.4 Innate effector mechanisms: the role of neutrophils in *Leishmania* infection

Parasites deposited into the skin are initially confronted with the highly conserved host immune response to the tissue injury caused by the fly bite. Neutrophils (also known as polymorphonuclear (PMN) cells) are the first cells to arrive within minutes, and they play a decisive role at the onset of infection. Neutrophils can drive macrophage differentiation into pro- or anti-inflammatory states and can recognise, phagocytose and destroy pathogens via the generation of oxygen metabolites and the release of lytic molecules from their cytoplasmic granules (see Chapter 1).

The precise role of neutrophils in the pathogenesis of leishmaniasis is not fully understood. Initiation of infection by needle injection in mice has generated contradictory results: some studies suggest that they are protective to the host, while others show that neutrophils exacerbate infection. Recently, elegant studies visualised the dynamics of neutrophil recruitment and neutrophil-parasite interactions in sandfly transmitted infections. Using intravital microscopy and flow cytometry, it has been shown that neutrophils rapidly infiltrate the local site of a sandfly bite and efficiently engulf *L. major* promastigotes. These studies demonstrated unequivocally the importance of neutrophils in the establishment and exacerbation of leishmaniasis. *Leishmania* parasites release neutrophil-attracting factors and trigger chemokine/cytokine secretion by neutrophils, which in turn recruits and activates other cell types.

Neutrophils release lower levels of cytokines and chemokines than other cell types. However, since they are the dominant cell type in the early phase of infection, the factors released by the masses of recruited neutrophils are likely to shape the type and magnitude of the *Leishmania*-specific immune response. Neutrophils not only infiltrate in the initial phase of infection, but they are also prominent in lesions and therefore may play different roles in different stages of leishmaniasis.

Since the primary function of neutrophils is to destroy invading pathogens, *Leishmania* parasites have evolved several mechanisms to survive phagocytosis by neutrophils. Ultra-structural examination shows that *Leishmania* parasites regulate granule fusion of neutrophils in a selective way to ensure their survival. Inside neutrophils *Leishmania* parasites survive in phagosomes by preferentially mobilising and fusing azurophilic granules to the parasite-harbouring vacuole, while avoiding fusion with specific and tertiary granules, which contain the machinery for microbicidal effector mechanisms (such as the generation of reactive oxygen species and acidification). *Leishmania* parasites are able to prolong their stay in the neutrophil by delaying programmed cell death (apoptosis), but infected neutrophils eventually undergo apoptosis and are subsequently phagocytosed by macrophages (the main host cell of *Leishmania* parasites).

Imaging studies were the first to show the release of *Leishmania* parasites by neutrophils *in vivo* and their subsequent uptake by other phagocytic cells. This is beneficial for the parasite, because recognition of apoptotic neutrophils is known to modulate macrophage function, allowing the silent entry of *Leishmania* into macrophages. This is thought to be one mechanism of immune evasion that also contributes to the early silent phase of disease when protective immune responses are not generated. Thus, neutrophils provide a transient and safe shelter for *Leishmania*, and the parasites use them as intermediate passenger cells for delivery to the macrophages in which they will replicate. The cross-talk between neutrophils and cells of the innate and adaptive immune system needs to be further dissected to identify the precise mechanisms by which these cells influence the manifestation of leishmaniasis.

The strategies employed by *Leishmania* parasites to evade destruction by neutrophils are not completely effective, and consequently some studies have been able to show a protective role for neutrophils in *Leishmania* infection. After appropriate activation *in vitro*, neutrophils can release DNA, histones and granule proteins that form fibrous structures known as neutrophil extracellular traps (NETs); these structures can kill bacteria and fungi. Several species of *Leishmania* parasites have been shown to be able to induce NET formation *in vitro* in a dose-dependent way. *L. amazonensis* is destroyed in NETs, whereas other species have been shown to be retained in the traps but remain alive. Interestingly, meshes of DNA and elastase (highly suggestive of NET formation) were found in skin biopsies of patients with cutaneous leishmaniasis.

7.5 Adaptive immunity: lessons from *L. major* infections of mice

In all forms of leishmaniasis, both immunity and pathology are mediated predominantly by T lymphocytes. Experimental studies in inbred strains of mice with *L. major* have contributed significantly to our understanding of host/parasite interactions, as well as establishing basic immunological principles such as the involvement of the Th1/Th2 paradigm of T helper (Th) cell subsets in infectious diseases. Therefore, this chapter will now focus on immune responses to *L. major* infections in mice.

Inbred strains of mice are usually infected by needle inoculation with *L. major* promastigotes (doses of 10^4–10^7). This inoculation can be performed at different sites, but the most commonly used are the hind footpads, the rump, or the base of the tail. Of note, there are different ways to perform infection of mice with *Leishmania* parasites, and this can result in different outcomes of infection. Factors which impact on the immune response generated include injection of lower numbers of parasites, different developmental stages of parasites and the route and site of injection in the mouse.

7.5.1 CD4+ T cells

In the classical model of infection of mice with *L. major*, control of infection and healing have been associated with a polarised Th1 response, whereas non-healing has been ascribed to an interleukin (IL)-4-dominated polarised Th2 response (Figure 7.2).

The majority of inbred strains of mice, such as CBA or C57BL/6, develop small lesions that will spontaneously heal within a few weeks, leaving the mice immune to reinfection. This ability to control infection is associated with the preferential expansion of antigen-specific CD4+ Th1 cells, characterised by the production of interferon (IFN)-γ. Indeed, mice from a genetically resistant background lacking the IFN-γ receptor cannot control *L. major* infection.

Th1 cells efficiently contribute to the control of parasites by promoting the ability of macrophages to kill the intracellular *Leishmania* parasites via the induction of nitric oxide (NO), a key anti-microbial effector molecule (Figure 7.2).

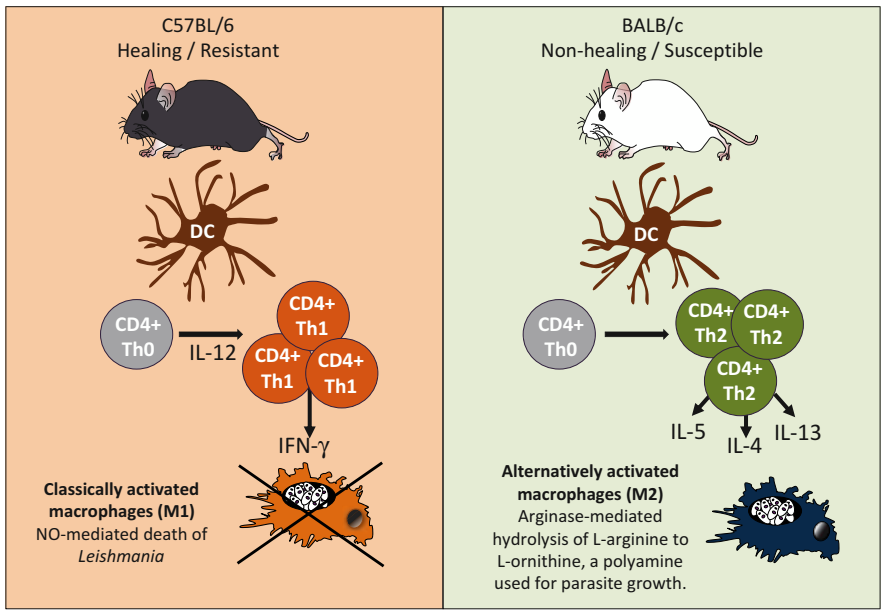

Figure 7.2 Resistance and susceptibility to *Leishmania* in mouse models: the Th1/Th2 paradigm.

NO is generated from L-arginine by the enzyme inducible NO synthase (iNOS), and iNOS-deficient mice are unable to control *L. major* infections, even though their capacity to mount a strong Th1 response is not impaired. Polarisation of Th1 cells is mainly directed by the production of IL-12 by dendritic cells (DC).

Early studies have shown that infection of resistant mice with *L. major* induces IL-12 production *in vivo*, and that IL-12 is essential for the development of protective Th1 response. Further, uptake of *L. major* by dendritic cells leads to the production of IL-12 and the subsequent priming of Th1 cells. The selective loss of IL-12 signalling by antigen-specific CD4+ T cells has been proposed to contribute to the susceptibility of BALB/c mice to *L. major* infection.

In contrast to the healing phenotype, a few strains of mice, such as BALB/c, develop non-healing disease following infection with *L. major* parasites. This non-healing phenotype is attributed to the preferential expansion of Th2 cells, characterised by the production of IL-4, IL-5 and IL-13. The development of a Th2 response in BALB/c mice infected by *L. major* has been attributed to the early production of IL-4 by Vβ4Vα8 CD4+ T cells. These cells are known to recognise the *Leishmania* antigen LACK (*Leishmania* homolog of receptors for activated C kinase), and they can be detected as early as 16 hours post infection. LACK-specific CD4+ T cells are known to be involved in Th2-mediated susceptibility to *L. major* infection, because mice made tolerant to LACK display a decreased Th2 response and control *L. major* infection. In addition, Vβ4-deficient BALB/c mice display a stronger Th1 response than genetically intact BALB/c mice and are able to control parasite replication.

IL-4 is important in the development of Th2 responses and the associated non-healing phenotype of BALB/c mice following infection with *L. major* parasites, and treatment of BALB/c mice with anti-IL-4 monoclonal antibodies (mAb) results in control of parasite replication. However, despite these results, there is now mounting evidence that IL-4 is not always sufficient, or even necessary, for susceptibility. Even in the face of a protective Th1 response, non-healing lesions can develop in a genetically resistant strain of mice infected with a virulent strain of *L. major* in the absence of IL-4. Furthermore, IL-4-deficient BALB/c mice infected with *L. major* sub-strains IR173 or LV39 do not generate a protective Th1 response and are only slightly less susceptible, or as susceptible, as wild-type mice, indicating that IL-4 is not essential for non-healing.

The reason why IL-4 is not necessary for susceptibility is because, in mice, a non-healing phenotype is multi-factorial. The IL-4 receptor α-chain (IL-4Rα) is a component of both the IL-4 receptor and the IL-13 receptor; IL-4Rα-deficient mice can control infection with *L. major* IR173, suggesting that IL-13 plays a role in susceptibility to *L. major* infection. Indeed, experiments performed in IL-13 deficient mice confirmed the importance of IL-13 as a susceptibility factor.

Susceptibility can also be mediated by the suppression of protective Th1 responses by the immunomodulatory cytokine IL-10. This cytokine can be produced by many cell types, including B cells, macrophages, DCs and some populations of T cells, such as regulatory T cells (Treg). *L. major*-infected BALB/c mice that are genetically deficient in IL-10 are more resistant to infection than

their wild-type counterparts, and blocking the IL-10 receptor with a mAb results in more efficient control of parasite replication.

Recently, a novel population of *Leishmania*-specific T cells that are CD4+CD25−Foxp3− produce both IL-10 and IFN-γ have been shown to be immunosuppressive and abrogate acquired immunity. This T cell population may be an important source of IL-10, and their development during *Leishmania* infection may play an important role in mediating a non-healing susceptible phenotype.

7.5.2 Regulatory T cells

Tregs are essential for the maintenance of immunological homeostasis. Several types of regulatory T cells have been described to date, some of which develop during selection in the thymus (natural Tregs), and some of which are induced in response to infectious challenge (induced Tregs). Natural Tregs play an important role in the regulation of the immune response to *Leishmania* parasites. These cells, constituting 5–10 per cent of peripheral CD4+ T cells (both in mice and humans), have been shown to control excessive immune responses. The hallmarks of natural Tregs are the surface expression of the IL-2Rα-chain (CD25) and the intracellular expression of the transcription factor FoxP3. However, the expression of molecules such as Cytotoxic T lymphocyte antigen (CTLA)-4 and glucocorticoid-induced TNFR-related protein (GITR) have also been associated with these cells.

When genetically resistant mouse strains heal, *Leishmania* parasites persist in the body (latency). Pioneer work with the low-dose intradermal challenge in a genetically resistant strain of mice has shown that natural Tregs accumulate at the site of inoculation, where they suppress the ability of CD4+ T effector cells to eliminate *Leishmania* parasites once pathogenesis has been resolved. Natural Tregs can use both IL-10-dependent and IL-10-independent mechanisms to contribute to parasite persistence.

Interestingly, in this study, when parasites were eliminated and sterile cure was achieved, the capacity of cured mice to control secondary infections was abrogated, demonstrating that equilibrium between Tregs and effector T cells is crucial for immunity to reinfection. This study also showed that natural Tregs proliferate in response to *L. major*-infected DCs, further supporting the concept that natural Treg can recognise microbial antigens.

7.5.3 Th17 cells

Recently a new subset of T helper cells, Th17, has been described; these cells produce the pro-inflammatory cytokine, IL-17. Macrophages, endothelial cells and fibroblasts produce inflammatory mediators such as TNF-α, IL-1 and chemokines in response to IL-17 which, in turn, results in the recruitment of neutrophils and leukocytes. Th17 cells have been shown to play an important role in inflammatory diseases, and a recent study has shown that IL-17 deficient mice on a susceptible background were able to control *Leishmania*

parasite load more efficiently than intact mice. IL-17 did not seem to play a role in the differentiation of Th1 and Th2 immune responses in this study. Rather, a reduction of neutrophil recruitment to the site of infection reduced the initial survival of *Leishmania* parasites (presumably by reducing the successful entry of parasites into macrophages).

7.5.4 CD8+ T cells

No protective role for CD8+ T cells has been shown so far in the control of primary *Leishmania* infections in the standard mouse model of cutaneous leishmaniasis, i.e. subcutaneous infection with high dose of parasites. Mice deficient in CD8+ T cells or in MHC-I (and therefore unable to present antigen to CD8+ T cells) remain resistant to *Leishmania* infection. In contrast, in low-dose models of intradermal infection with *L. major* parasites, CD8+ T cells participate in the pathogenesis and immunity to primary infection. Genetically resistant mice deficient in CD8+ T cells fail to control infection under these conditions.

CD8+ T cells undergo expansion and produce IFN-γ in genetically resistant immunologically intact mice injected intradermally with low doses of *L. major*. Primed CD8+ T cells from these mice can transfer protective immunity to recombination-activating gene (RAG)-deficient mice (these mice lack both T and B cells) infected with *L. major*. Therefore, the role of CD8+ T cells in immunity to *Leishmania* appears to depend on the dose of parasites administered, and this may be highly relevant in humans, whereby infection from the bite of a sandfly arises from a low dose of *Leishmania* parasites.

7.6 Arginase promotes *Leishmania* parasite growth

The metabolism of L-arginine by arginase during experimental leishmaniasis is emerging as a crucial mechanism for parasite survival. *Leishmania* are obligate intracellular parasites and, after transmission to the mammalian host, they invade macrophages that will either kill or host the intracellular parasites, depending on the balance between Th1 and Th2 cytokines. While Th1 cytokines induce classical activation of macrophages and the generation of inducible nitric oxide synthase (iNOS) to oxidise L-arginine into nitric oxide (NO), a metabolite that will kill the parasite, Th2 cytokines result in alternative activation of macrophages and the induction of arginase (see Chapter 1).

A novel role for arginase-1 in the pathogenesis of non-healing leishmaniasis has been recently unveiled. The activity of this enzyme has been shown to promote a non-healing phenotype and uncontrolled parasite growth *in vivo* by hydrolysing L-arginine into L-ornithine, the latter of which is the main intracellular source for the synthesis of the polyamines necessary for the growth of *Leishmania* parasites. As such, arginase regulates parasite growth directly, by affecting polyamine synthesis in macrophages (Figure 7.2).

In addition to a direct role in controlling *Leishmania* parasite multiplication, the metabolism of L-arginine by arginase is also emerging as a crucial mechanism for the regulation of immune responses. Arginase has been shown to

impair T cell responses by reducing the bioavailability of L-arginine. High arginase activity results in increased uptake of extracellular L-arginine into myeloid cells, causing a reduction of L-arginine levels in the microenvironment, in turn leading to T cell hypo-responsiveness.

In experimental leishmaniasis, high arginase activity has been shown to be a hallmark of non-healing disease, via the induction of local suppression of antigen-specific T cells responses and uncontrolled parasite replication. Competitive inhibition of arginase, as well as supplementation with L-arginine, restored T cell effector functions and reduced parasite growth. In addition, *Leishmania* parasites encode their own arginase, which has been shown to modulate both infectivity and disease pathogenesis.

7.7 Memory responses

The detailed understanding of immunological memory is crucial for the development of vaccines against leishmaniasis. Many experimental vaccines have been developed to protect against leishmaniasis (see Chapter 25.2) but, to date, there is still no efficient human vaccine. 'Leishmanisation' is the only immunisation strategy that has been shown to protect humans; it is a practice that has been used for centuries, in which live parasites from the lesions of infected people are transferred to non-infected individuals in order to confer protection against leishmaniasis. Although complications or infection can occur in 5–10 per cent of cases, leishmanisation demonstrates clearly that protective immunity can be induced in humans.

In the mouse model of infection with *L. major*, it is well established that genetically resistant mouse strains that are able to control parasite replication and heal primary infection become immune to reinfection. Following challenge, these mice will mount a delay-type hypersensitivity (DTH) reaction, mediated by IFN-γ-producing T cells that rapidly home to the site of challenge and control parasite replication. Similarly to primary infection, IL-12 produced from antigen-presenting cells plays an important role in the control of secondary infections; IL-12-deficient mice that have been chemotherapeutically induced to heal *L. major* infection will develop progressive non-healing disease upon reinfection.

Another crucial factor in the maintenance of immunity to reinfection is parasite persistence. Subclinical infections with low numbers of *L. major* parasites in susceptible BALB/c resulted in clearance of the parasite, but did not confer protection following challenge. Similarly, removal of the immune-regulatory cytokine IL-10 results in a sterile cure, but with a loss of effective protective memory responses.

Memory T cells are heterogeneous and can be separated into at least two categories: central memory (CM) T cells, which will migrate through lymph nodes and provide limited help; and effector memory (EM) T cells, which migrate to tissues and exert effector functions. Following activation and differentiation, CM T cells can differentiate to become effector cells, providing protection against reinfection.

It is still a matter of debate whether live *Leishmania* parasites are required for a protective memory response. Pioneering work has established that CM T cells isolated from lymph nodes from immune mice can mediate long-term immunity against *L. major* by differentiating into tissue-homing effector T cells after challenge. Importantly, this study also showed that the persistence of CM T cells does not depend on the presence of live *Leishmania* parasites. These findings have a great relevance for the development of vaccine against leishmaniasis, as reactivation of CM T cells could be a target for non-live vaccine to generate cell-mediated immunity.

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Kinetoplastids: Trypanosomes 8

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8.1 The African trypanosomes (*Trypanosoma brucei* ssp.)

The African trypanosomes cause serious disease in humans and livestock in sub-Saharan Africa, and they have had a major impact on the economic and agricultural development of that continent. These organisms have also rightly gained the reputation of being prime exponents of immune evasion by antigenic variation. However, as we will learn in this chapter, the interactions of African trypanosomes with the host-immune system are broad and complex, and define both the survival and the pathogenetic mechanisms of these parasites.

8.1.1 Life cycle of *Trypanosoma brucei*

A range of taxonomically related trypanosomes belonging to the genus *Trypanosoma* are transmitted to mammalian hosts in the saliva of tsetse files (*Glossina sp.*) in sub-Saharan Africa. They are referred to as salivarian trypanosomes to distinguish them from the more cosmopolitan stercorarian trypanosomes (such as *Trypanosoma cruzi*; see Chapter 9). All trypanosomes belong to the order Kinetoplastida, a grouping of protozoa distinguished by the possession of a unique complex mitochondrial DNA structure known as the kinetoplast (see Chapter 2). A simplified account of the species concerned is presented in Table 8.1.

All these species have similar life cycles, with the exception of *T. evansi*, and in some instances *T. vivax*, which are subject to mechanical transmission from host to host by other blood-feeding insects. This chapter will primarily refer to the infection immunology of *T. brucei*, the causative agent of human African trypanosomiasis (HAT or sleeping sickness), as this is most extensively studied. In general, however, the immunology and host-parasite interactions of the other species are similar. *T. brucei* causes disease in humans and domestic

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Table 8.1 African trypanosomes causing disease in humans and livestock.

Species	Veterinary and clinically significant hosts	Disease
T. brucei brucei	Cattle, goats, sheep, pigs, horses	Nagana
T. brucei rhodesiense *T. brucei gambiense*	As above plus humans As above plus humans	Human African trypansomiasis (sleeping sickness)
T. congolense *T. vivax*	Cattle, goats, sheep, pigs, horses, camels	Ngana*
T. evansi	Cattle, horses, camels, buffalo	Surra*

**T. vivax* and *T. evansi* may be mechanically transmitted and their distribution has spread outside the tsetse fly belts of sub-Saharan Africa to S. America and South/South-East Asia.

ungulates, and it also infects a range of other wild mammals in tsetse fly habitats, creating a large reservoir of infected hosts. Thus, control of the trypanosomiasis is complicated by the zoonotic nature of this parasitism.

African trypanosome life cycles are digenetic, involving a mammalian host and a tsetse fly vector (Figure 8.1). The mammalian infective stage, known as the metacyclic trypomastigote, is inoculated into the mammalian host in association with saliva when the tsetse fly probes its host prior to a blood meal. In some hosts, including humans, sheep and cattle, a localised skin reaction known as a chancre forms at the fly bite site. The chancre is characterised by a painful swelling, local erythema, and at the cellular level by sub-dermal

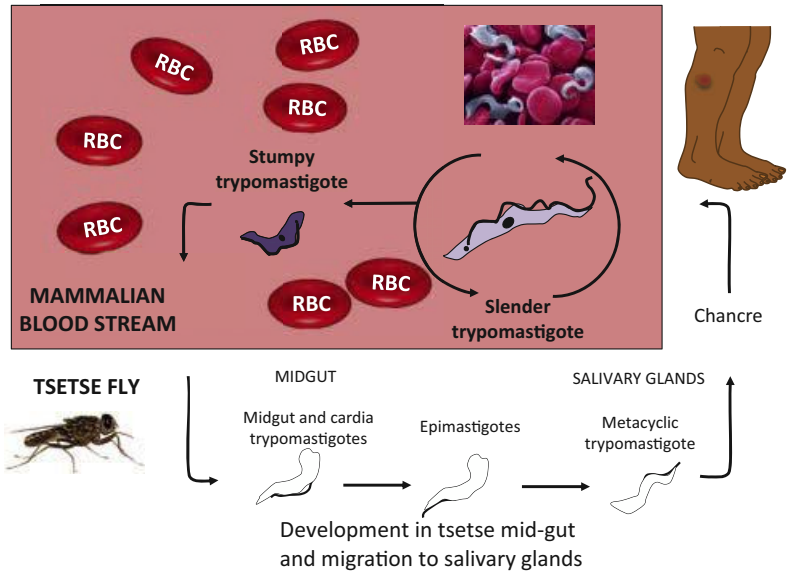

Figure 8.1 The life cycle of *Trypanosoma brucei*. The mammalian infective metacyclic stage of *T. brucei* develops in the salivary glands of the tsetse fly and is inoculated into the mammalian host when the fly takes a blood meal. Metacyclic trypanosomes develop into the bloodstream trypomastigote stages and migrate into the lymphatic and blood vascular systems. The stumpy stage is pre-adapted and preferentially established in a tsetse fly if taken up in a blood meal.

inflammatory leukocyte infiltration over a period of 5–7 days after an infective fly bite. The chancre typically resolves after a further 3–4 weeks. The metacyclic trypanosomes initially proliferate in the chancre and subsequently migrate to the systemic circulation via the local draining lymph node, while at the same time differentiating to the bloodstream trypomastigote stage.

The bloodstream stage has two morphotypes. The first to appear is the slender bloodstream stage that proliferates exponentially. This differentiates to a non-proliferative stumpy bloodstream stage that is pre-adapted to survival if ingested by a further tsetse fly. There is evidence that this differentiation is triggered by a parasite density-dependent factor, but this has not been identified to date. The mammalian host life cycle stages are completed when stumpy stage parasites are taken up by a tsetse fly in a blood meal; the developmental stages in the tsetse fly are outside the scope of this chapter.

8.2 Pathogenesis of sleeping sickness

In humans, the infection progresses through two clear stages that are characterised and distinguished by pathology and symptoms. In the haemolymphatic or early period of infection, the trypanosomes proliferate in the blood and lymphatic system. Clinical signs during the early stage of infection are largely associated with an inflammatory response to the parasitaemia, including fever, joint pain, headache, anaemia, splenomegaly and lymphadenopathy. Additionally, severe pruritis and anorexia are common.

The second stage of infection occurs when the parasites enter the central nervous system, crossing the blood-brain barrier from capillary vasculature in the brain. This is known as the meningoencephalitic or late stage of infection, and it is characterised by a range of neurological disturbances, including daytime somnolence (giving rise to the disease name ‘sleeping sickness’), ataxia, tremors, confusion, incontinence, neuropathy and, eventually, a deepening coma and death. These symptoms are associated with inflammatory changes in the brain, including an infiltration of mononuclear cells and an activation of astrocytes and microglia. Clinical diagnosis of the late stage depends on the detection of trypanosomes or elevated leukocyte counts in the cerebrospinal fluid.

The rate at which the disease develops and progresses from early to late stage shows considerable variation between individuals. In general, *T. b. rhodesiense* infections are acute, with progression to the late stage measured in weeks, while *T. b. gambiense* infections are chronic, with late stage taking months to develop. However, there are examples of chronic presentation of *T. b. rhodesiense* and acute presentation of *T. b. gambiense* and, at least for *T. b. gambiense* infection, there is evidence of chronic asymptomatic infections persisting for several years. However, once parasites have invaded the central nervous system (CNS), infection is invariably fatal without treatment.

The existing repertoire of drugs to treat this disease is limited and prone to serious side-effects. This is especially so for the treatment of late stage disease, where arsenical drugs are still the only effective frontline treatment.

8.3 Variant surface glycoprotein – the key to trypanosome-host interactions

One parasite molecule, the variant surface glycoprotein (VSG), dominates the interactions of trypanosomes with their mammalian hosts and defines the immunology of infection (Figure 8.2). About 5×10^6 glycosylphosphatidyl inositol (GPI)-anchored VSG molecules coat the surface of the cell, comprising more than 95 per cent of all surface membrane proteins and some 15 per cent

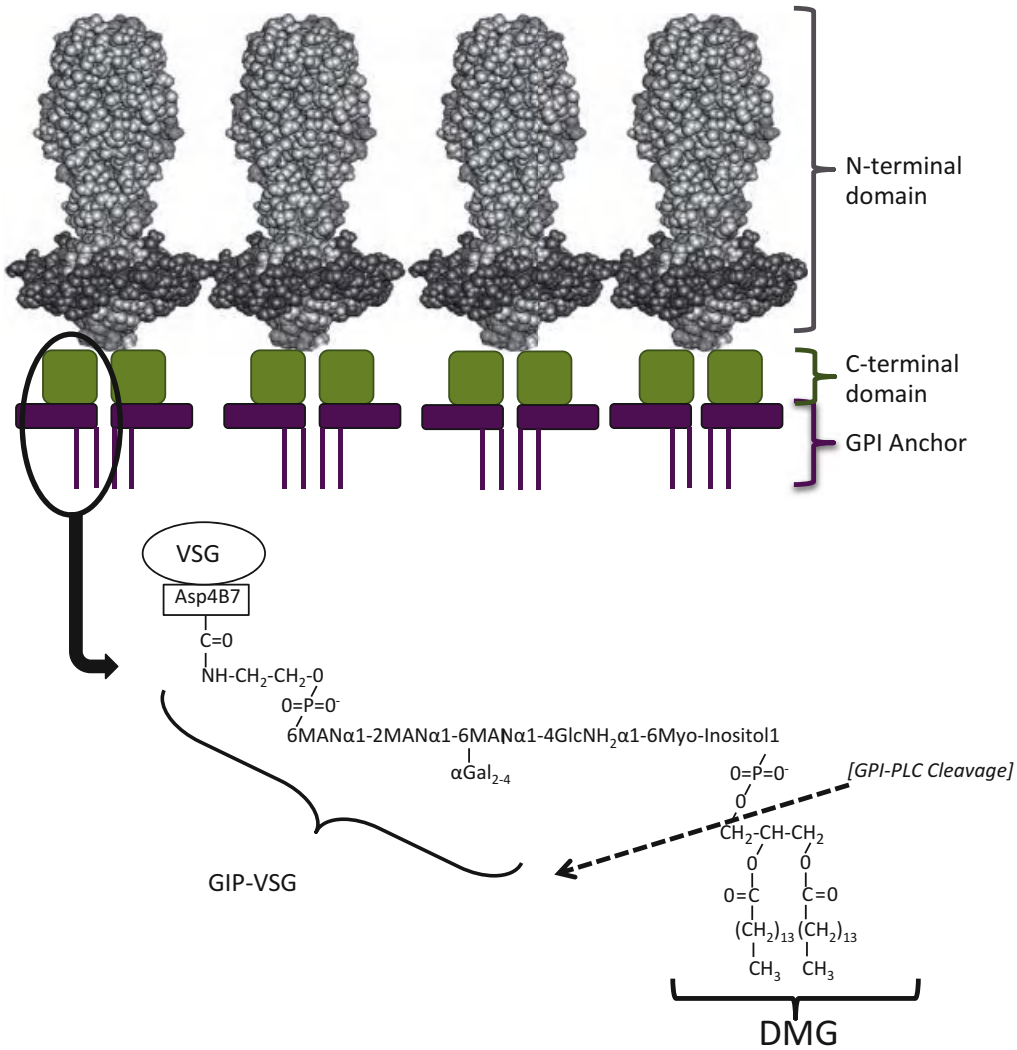

Figure 8.2 Variant surface glycoprotein and the GPI anchor. The upper panel represents VSG polypeptide dimers (shown as space filling models) and the GPI anchor, the acyl groups of which are inserted in the lipid bilayer membrane. The lower panel shows the chemical structure of the anchor and the GPI-PLC cleavage point Adapted from Paulnock, DM & Coller, SP (2001). Analysis of macrophage activation in African trypanosomiasis. *Journal of Leukocyte Biology* 69, 685–690; and Field, MC and Carrington, M (2004). Intracellular membrane transport systems in *Trypanosoma brucei*. *Traffic* 5, 905–913.

of total cellular protein – a clear indication of the biological importance of this molecule to the trypanosome. In order to enable antigenic variation, VSG genes represent 20 per cent of the protein-coding capacity of the trypanosome genome, and they are highly divergent in primary sequence, while retaining similar tertiary structure resulting from conserved cysteine residues. It is this sequence, and therefore epitopic diversity, that underpins the role of VSG in antigenic variation.

VSG molecules and their GPI anchor components also mediate immunopathology in trypanosomiasis, and a truncated VSG gene product (termed Serum Resistance Associated (SRA)) gene plays a critical role in defence against innate immunity. The VSG is a 400–500 amino acid protein linked via its carboxyl terminus to a GPI anchor. The N terminal domain, most of which is exposed externally, forms the majority of the protein, and this presents a uniform molecular coat surrounding all exposed areas of plasma membrane of the parasite. The VSG coat is a dynamic structure in which all VSG molecules are cycled through the active endocytic system of the trypanosomes every 12 minutes.

VSG is a highly immunogenic protein, eliciting a strong T cell independent IgM response that yields cross-linking and opsonising antibodies that are more than sufficient to eliminate the infection. However, the membrane cycling of VSG actively removes bound IgG and IgM and slows the accumulation of potentially trypanocidal levels of immunoglobulin on the cell surface.

As indicated in Figure 8.2, the GPI anchor of the VSG is cleaved by glycosylphosphatidylinositol-specific phospholipase-C (GPI-PLC) in African trypanosomes, releasing a soluble form of VSG carrying the glycosylinositolphosphate residue of the GPI anchor (GIP-VSG), with the dimyristoylglycerol (DMG) component remaining associated with the membrane. Current research suggests that this enzyme is located on the exterior of the flagellar membrane, but contact with the GPI anchors of VSG molecules must be tightly regulated, as GIP-VSG release from trypanosomes only takes place in stressed cells.

8.3.1 VSG and antigenic variation

In 1910, Ross and Thomson conducted the first study on the development of sleeping sickness in a human host, during the course of the unsuccessful treatment of a patient who had been infected with trypanosomes in modern-day Zambia. It was noted that the parasite numbers in the blood fluctuated, with waves of parasitaemia followed by periods when parasites were undetectable (Figure 8.3). Remarkably, given that the biological basis of antigenicity and the action of antibodies were unknown at that time, these early pioneers had the prescience to propose that the undulating course of parasitaemia resulted from antigenic variation. Later, once the biology of the immune system was understood, the role of antigenic variation in defining the course of trypanosome parasitaemia in the infected mammalian host was confirmed using serological and, subsequently, molecular biological methods.

VSG molecules are immunogenic and elicit high-titre lytic IgM responses which would be expected to effectively opsonise trypanosomes. However,

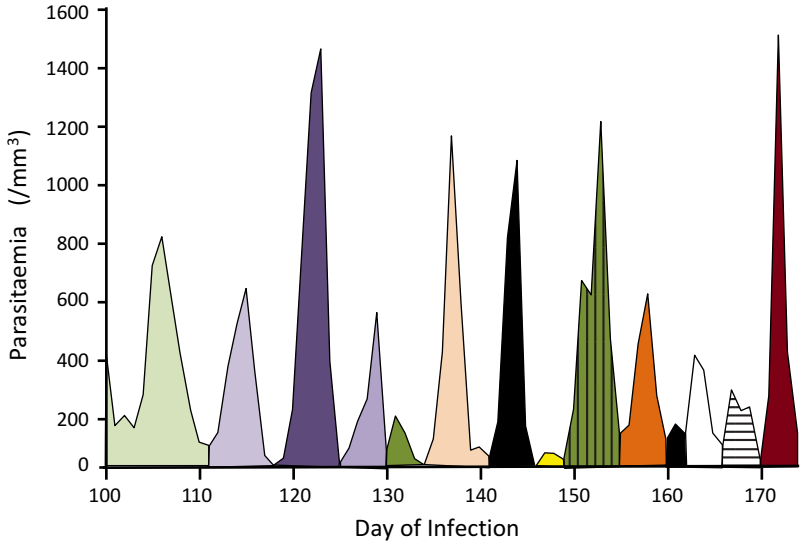

Figure 8.3 Typical parasitaemia profile in human African trypanosomiasis. Each wave of parasitaemia is the result of antigenic variation, with one or more novel VATs being expressed.

trypanosome infections persist because a small number of cells in the population undergo antigenic variation to express a new VSG coat that is not antigenically cross-reactive with its predecessors.

It is important to note that antigenic variation is a stochastic process, occurring independently of any immunological challenge to the parasite, at a rate of between 10^{-2} and 10^{-6} per cell division for any individual member of the trypanosome population in an infected host. Each immunologically distinct VSG (and its set of epitopes) is known as a Variant Antigen Type (VAT). The full complement of VSGs that may be expressed in a trypanosome clone, typically in excess of 1,600, is known as the VAT Repertoire. VAT repertoires are subject to rapid evolution, and trypanosome clones isolated from geographically close areas may have quite different repertoires.

8.3.1.1 The mechanism of antigenic variation

The mechanism of antigenic variation in African trypanosomes has been the subject of intense and detailed research, and our understanding of the process at the molecular level is summarised in Figure 8.4. Essentially, one gene is selected for expression from a repertoire of non-expressed 'basic copy' genes (that act as a 'library' of VATs). A critical feature of this system is that VSG expression is mono-allelic, so only a single VAT may be expressed at any one time; it is thought that mixed VAT expression would disrupt the surface coat structure.

The basic copy genes are distributed with up to 1,500 copies in large tandem arrays of VSG genes on large chromosomes and a further 200 copies at sub-telomeric sites on minichromosomes (Figure 8.4a). Of the basic copy genes in arrays in large chromosomes, a substantial proportion do not code for

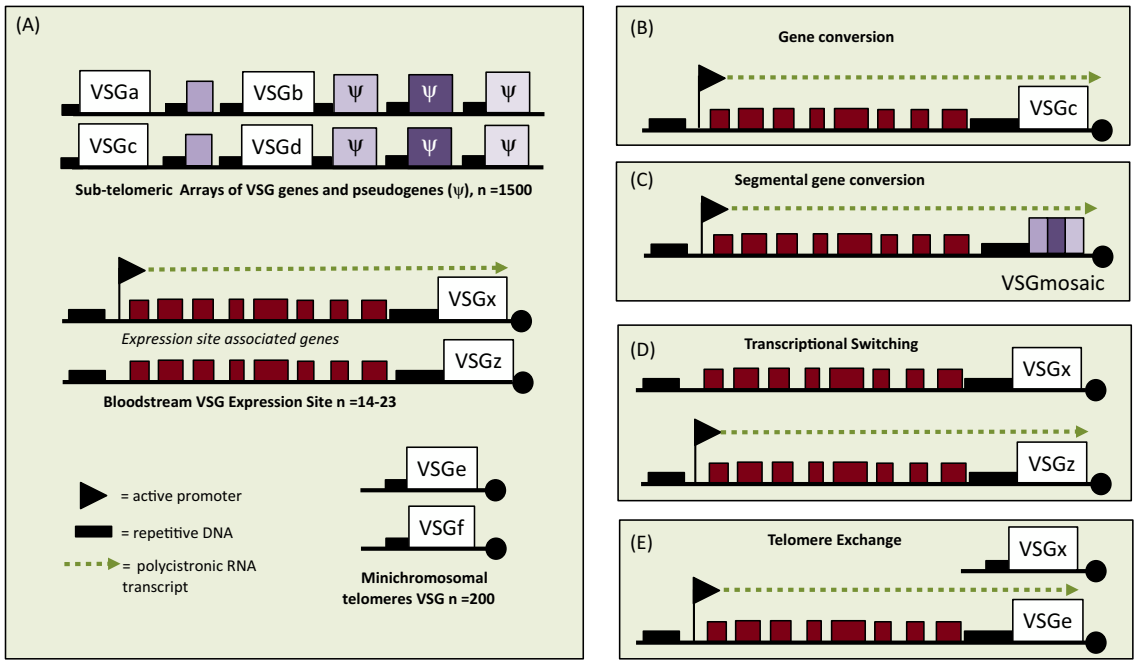

Figure 8.4 Antigenic variation mechanisms in *T. brucei*. a: Genetic organisation of variant surface glycoprotein (VSG) genes in trypanosome expressing hypothetical VSGx in active expression site. Basic copy genes are in arrays (both coding sequences, pseudogene and gene fragments) on large chromosomes, at inactive expression sites and at the telomeres of minichromosomes. b and c: Gene conversion leads to expression of intact or segmental basic copy genes. d: Transcriptional switching of expression sites. e: Reciprocal recombination, leading to exchange of telomeric VSG.

functional VSGs but, rather, exist as gene fragments or pseudogenes that require segmental gene conversion to enable expression.

Although one might expect that the requirement for mono-allelic VSG expression could be satisfied with a single expression site, there are in fact are least 14 bloodstream-stage VSG expression sites and up to a further 25 metacyclic-stage VSG expression sites per diploid genome. These expression sites are under tight control, with only one actively expressed at any one time and, unusually, VSG gene transcription is carried out by RNA polymerase I. The control of which VSG gene expression site is active is at the level of transcription elongation, and it also appears to be connected to a specialised region of chromatin in the trypanosome nucleus, known as the expression site body.

Antigenic variation thus involves either the activation of alternative VSG expression sites (Figure 8.4d), or the switching of a new basic copy gene into an expression site. The latter process primarily involves a duplicative transposition (or gene conversion), although reciprocal telomere recombination may also take place (Figure 8.4e). Duplicative transposition may involve intact basic copy VSG genes (Figure 8.4b) or may involve the reassembly of VSG gene and VSG pseudogene fragments to produce novel chimaeric VSGs (Figure 8.4c); this is thought to be important late in chronic infections as the basic copy repertoire becomes exhausted.

8.3.1.2 Expression site associated genes

The VSG expression sites of bloodstream form trypanosomes are not simply vehicles for VSG gene expression but, rather, are part of a much larger transcription unit with the promoter far (45-50kB) upstream from the VSG coding sequence. Within this large transcription unit are a series of genes and pseudogenes presumed to be derived from ancestral VSG gene duplication events. These genes are known as Expression Site Associated genes (ESAGS), and they contribute to a polycistronic transcription unit that includes the cognate VSG gene. ESAGs include the heterodimeric transferrin receptor and the Serum Resistance Associated (SRA) gene specifically found in *T. b. rhodesiense* and discussed further below.

The transferrin receptor, as with other eukaryotic and prokaryotic pathogens, scavenges iron-transferrin complexes from the host plasma and may provide a clue to the reason why trypanosomes utilise multiple expression sites. It has been suggested that ESAG gene polymorphisms between different expression sites allow trypanosomes to 'select' transferrin receptors optimal for capture of the transferrin variants in each of their natural hosts, and this may be an adaptive explanation for the existence of multiple VSG expression sites in trypanosomes.

8.4 The humoral response to African trypanosomes

The humoral response to African trypanosomes is dominated by anti-VSG antibodies. These are predominantly IgM antibodies directed to exposed VSG epitopes, and they arise as a result of T cell independent B cell activation. At low titres, these antibodies appear to be ineffectual, probably as a result of membrane recycling and endocytosis of VSG. However, at high titres, this IgM response effectively opsonises the trypanosomes, potentially leading to either phagocytosis by Kupffer cells or complement mediated lysis. This is the main mechanism by which parasitaemic waves are eliminated but, as described above, antigenic variation allows sub-populations of parasites to escape.

T cell dependent IgG antibodies appear to play a minimal role in trypanosome clearance. In part, this is due to the fact that IgG responses are slower to develop and appear to be largely elicited by cryptic antigens that presumably have been exposed during antigen processing. Various invariant surface antigens have been described that elicit IgG and IgM responses in experimental hosts, but it is likely that these antigens are sterically unavailable to antibodies in viable trypanosomes, being hidden under the VSG coat.

In addition to VSG specific antibodies, as infection progresses, IgM and IgG auto-antibodies are detected in the mammalian host. These arise from polyclonal B cell activation and target a wide range of host antigens.

Finally, immunosuppression of B cell responses develops as infection progresses, and this is related to the anatomical destruction of both marginal zone and follicular B cell centres in the spleen. This also effectively prevents the development of memory B cells, thus rendering the possibility of a vaccine against

African trypanosomes – either against invariant antigens or 'cocktails' of VSG molecules – extremely unlikely. Furthermore, it is highly likely that this destruction of the memory compartment may also destroy existing B cell memory. This has been demonstrated experimentally with bystander vaccine antigens, and it may well impact on both human and veterinary vaccination programmes in trypansomiasis endemic areas.

8.5 T cell responses in African trypanosome infections

While T cell-dependent antibody responses play a minimal role in the direct control of *T. brucei* parasitaemia, T cell cytokine mediated-effector mechanisms are involved in the control of infections, especially during early parasitaemic waves. VSG-specific Th1 cell responses appear – at least in animal model infections – to play an important role in the control of early parasitaemia in infection through the secretion of IFN-γ. This is linked to early classical (M1) activation of macrophages, the products of which, such as nitric oxide (NO) and reactive nitrogen intermediates (ROI), are trypanocidal in tissues, as described below. However, similar to B cells, as infection progresses, specific and non-specific T cell responses become severely suppressed in a macrophage-dependent manner.

8.6 Innate defence mechanisms: trypanosome lytic factor

While antigenic variation renders adaptive humoral responses to African trypanosomes ineffective, there is a humoral component of innate immunity that is remarkably effective at controlling African trypanosomes. This was discovered by researchers investigating why it was that although *T. b. rhodesiense* and *T. b. gambiense* infected humans, the third subspecies, *T. b. brucei*, was unable to do so. This is due to the presence of a lytic factor in human serum. The factor, known as Trypanosome lytic factor (TLF) comprises two fractions of High Density Lipoprotein (HDL), and the active component of each of these is Apolipoprotein L1 (Apo-L1).

Apo-L1 contains a Bcl2-like membrane pore-forming domain that is the basis of its trypanolytic activity. TLF1 is taken up by the trypanosome haptoglobin-haemoglobin receptor. TLF2 may also use this route of uptake, but it also appears to have an as yet undefined independent pathway. Uptake of TLF1 and/or TLF2 leads to the release of Apo-L1 inside the lysosome, leading to the formation of membrane pores that, in turn, lead to osmotic lysis of the parasite.

So, given the anti-trypanosomal activity of TLF, why is it that humans are infected with African trypanosomes? In the case of *T. b. rhodesiense*, the reason lies in the serum resistance associated (SRA) gene product mentioned earlier. The SRA gene is an ESAG within the bloodstream VSG expression site transcription unit in *T. b. rhodesiense* and is a derived from a truncated VSG gene. The SRA protein is able to stoichiometrically bind Apo-L1 in the lysosome and disrupt its pore-forming ability. So here we have an exquisite example of a parasite-host arms race, with the evolution of a potent innate immune factor being overcome by the evolution of a resistant population of parasites that we

now describe as *T. b. rhodesiense*. *T. b. gambiense* also escapes lysis by TLF but, at the time of writing, the identity of the resistance factor that neutralises Apo-L1 is not known.

While human Apo-L1 is neutralised by SRA, other primates, such as baboons, have Apo-L1 variants that are not bound by SRA. These species are resistant to infection with *T. b. rhodesiense*, and it has been suggested that purified recombinant Apo-L1 from these species may be used as a novel therapeutic agent, or even that transgenesis of livestock to express baboon Apo-L1 would provide trypanosome-resistant animals that could be farmed in tsetse-infested areas.

Natural polymorphisms in human Apo-L1 have been shown to have dramatic effects on resistance to trypanosomiasis in humans. For example, a human Apo-L1 variant allele, G2, that SRA does not bind to, has been identified. This confers resistance to *T. b. rhodesiense* in carriers and has been found to be selected for in certain West African populations, but at the cost of predisposing the carrier to increased susceptibility to kidney disease. On the other side of the coin, individuals who have rare Apo-L1 gene deletions are susceptible to infections with trypanosomes that would normally only infect domestic animals.

8.7 Immunopathology and VSG

African trypanosome infections are associated with a complex set of pathophysiological effects that are connected with a dysregulated type 1 (inflammatory) immune response. VSG is involved in the mechanisms of all of these pathologies, through the action of GPI-PLC and the release of GIP-VSG and DMG, as described above. In early infection, GIP-VSG and IFN-γ stimulate the development of classically activated (M1) macrophages (Figure 8.5). The source of early IFN-γ is likely to be from both VSG-specific Th1 cells and NK cells. This early M1 activation is important in the control of the early stages of infection, where mediators such as NO and TNF-α are involved in control of parasitaemia.

However, sustained activation of M1 macrophages and other myeloid cells (such as astrocytes in the brain), which appears to be exacerbated by interactions with DMG and LPS as infection progresses, causes a range of inflammatory pathologies. These include tissue damage in the liver, anaemia, cachexia and probably also the encephalitis that develops in the CNS. Type 1 inflammatory cytokines such as TNF-α and IFN-γ promote the transmigration of trypanosomes from the blood into the CNS across the blood brain barrier and are associated with the onset of late stage human African trypansomiasis. Once infection in the CNS is established, meningoencephalitis ensues and is invariably fatal if untreated. Meningoencephalitis can be ameliorated temporarily using anti-inflammatory agents, confirming the role of inflammatory dysregulation in this disease.

Given the role of the inflammatory response in pathology in African trypanosome infections, it is predicted that amelioration of this response may lead to less severe pathology and possibly prolonged survival. This appears to be the case, as mutant trypanosomes lacking the GPI-PLC gene (and thus defective

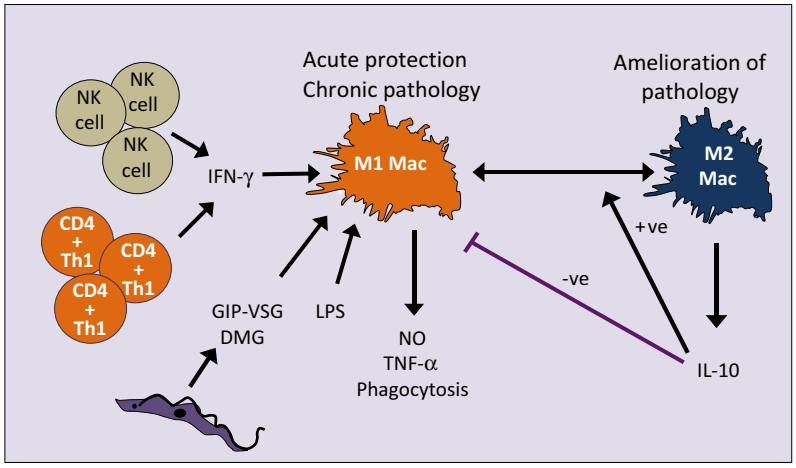

Figure 8.5 The balance of M1 and M2 macrophage activation determines the level of immunopathology in African trypanosomiasis. GPI components act as macrophage activating factors with co-stimulatory IFN-g. Survival in chronic infection requires an overall type 2 response, with IL-10 playing a critical role.
Abbreviations: DMG, Dimyristoylglycerol; GIP-VSG, Glycosylinositolphosphate residue of the GPI anchor of *Trypanosoma brucei*; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; NK, natural killer; NO, nitric oxide; TNF-α, tumour necrosis factor-α.

in M1 macrophage activation) develop very chronic infections in experimental mice that are characterised by an activation of M2 regulatory (formerly known as alternatively activated) macrophages.

Experiments using wild-type parasites, utilising experimental mouse hosts manifesting differing disease severity, suggest that the balance between development of acute (and fatal) disease and prolonged chronic infection depends on the effectiveness of a switch from the early type 1 immune response (dominated by M1 macrophage activation) to a type 2 response, with M2 regulatory macrophage activation and the production of IL-10 and other anti-inflammatory mediators (Figure 8.5). This view of the immunological context of differential disease severity is supported by cytokine expression analysis in human African trypanosomiasis patients in the field.

8.8 Summary

African trypanosome immunological interactions with the host are defined by the VSG molecule and its GPI anchor. Trypanosomes effectively evade humoral responses through a combination of antigenic variation and rapid membrane recycling of their VSG and any bound antibodies. However, the VSG and GPI anchor components are potent non-specific activators of a type 1 innate immune response that leads to immunopathology through a sustained inflammatory response syndrome. At the same time, a truncated VSG gene product, SRA, is able to neutralise the potent innate humoral factor Apo-L1 present in human serum. Antigenic variation, together with the destruction of marginal zone and follicular B cell compartments in the spleen, renders the likelihood of an effective vaccine very low.

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9 Kinetoplastids: *Trypanosoma cruzi* (Chagas disease)

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Trypanosoma cruzi is a Kinetoplastid protozoan parasite and the agent of human Chagas disease. Since *T. cruzi* has both intracellular and extracellular stages during its infection in mammals, effective immunity requires both cell-mediated and humoral immune responses. The infection is persistent, held in check by an otherwise highly effective immune response, but it is rarely cleared. In a significant number of infected individuals, the combination of parasite persistence and the immune assault controlling the infection eventually results in the life-threatening heart or gut pathology that is characteristic of Chagas disease.

The absence of routine and dependable surveys makes estimating the true impact of *T. cruzi* infection very difficult. However, it is likely that between 10–20 million people in Central and South America are infected, making *T. cruzi* infection the highest impact infectious disease in this region. Humans are only one of more than 100 mammalian species in which *T. cruzi* naturally circulates. This host species promiscuity makes eradication of *T. cruzi* virtually impossible, but is also advantageous with respect to the study of immunity to *T. cruzi*, because many of these natural hosts (including mice) are excellent models of the human infection and disease. Much of what is known about immune responses to *T. cruzi* is derived from work in mice, and is directly applicable to our understanding of the human infection.

The immune response to *T. cruzi* has been often judged as being suppressed and ineffective – or, worse, over-exuberant and disease-promoting. This chapter will take a different view, presenting the arguments and evidence that show that immunity to *T. cruzi* is, in fact, highly effective but falls just short of eliminating the infection. Attention will also be given to the immune mechanisms that are crucial for the control of *T. cruzi* infection, and why these responses

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may fail to completely clear the infection, opening the door to the eventual development of clinical disease.

9.1 Life cycle and transmission

T. cruzi is transmitted to mammals by blood-feeding triatomine insects found throughout the Americas. In Central and South America, human infection is linked primarily to poor quality housing that supports colonisation by these insects. Parasites in the insect faeces enter hosts across mucous membranes, or through scratches or abrasions. Infection by accidental ingestion of contaminated bug faeces or pulverised bugs (in unrefined foods or beverages, for example) is also increasingly recognised as a major source of human infection, and the intentional ingestion of bugs by wild and domestic animals may be the primary mechanism of infection in these hosts. Although it is generally thought to be transmitted mostly in rural settings, peri-urban and urban foci of transmission are also present. Infection in humans can also occur congenitally, or by blood or tissue transplantation, making the spread of infection possible even in areas where the insect vectors are absent.

In comparison to many other protozoans, the life cycle of *T. cruzi* is relatively simple (Figure 9.1). Trypomastigotes circulate in the blood of mammals but are non-replicating and must invade host cells in order to maintain and expand the infection. Initial entry of host cells by *T. cruzi* is within a vacuole, but the entering trypomastigotes rather quickly escape from this compartment, convert to aflagellate amastigotes, and then replicate in the cytoplasm of infected host cells. Over a 4-5 day period, amastigotes complete numerous rounds binary fission before converting back to trypomastigotes that emerge from the completely depleted host cell.

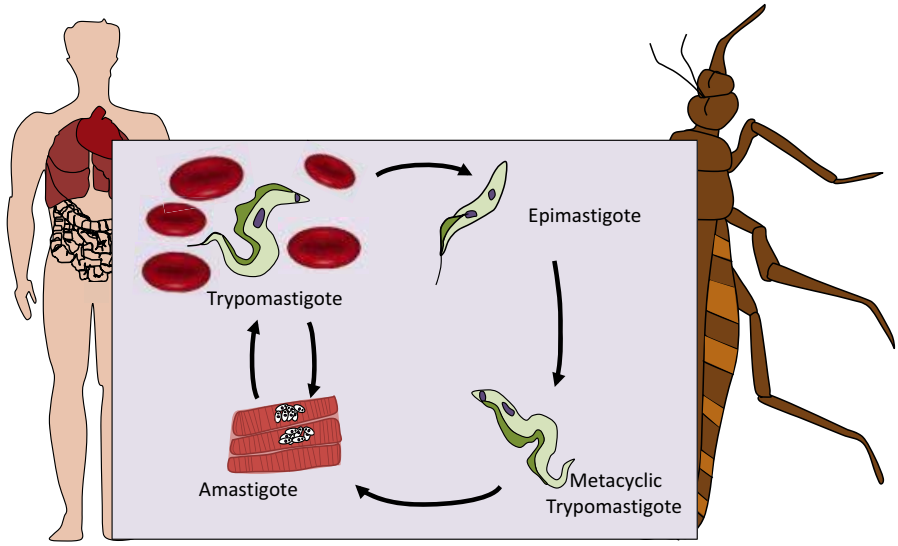

Figure 9.1 The life cycle of *Trypanosoma cruzi*.

The freed trypomastigotes either reinvade other cells, maintaining the infection and rapidly spreading it to body sites distinct from the initial site of entry, or may be ingested by insect vectors during the course of their feeding. In the gut of the triatomine, trypomastigotes convert into epimastigotes, expand in number and move posteriorly along the gut over several weeks. In the hindgut, epimastigotes differentiate into the mammalian-infective metacyclic trypomastigote stage, ready for passage out of the insect with the faeces, following the next blood meal. There is no evidence of an arrested or dormant stage in either mammals or insects, and sexual recombination appears to be extremely rare. Still, genetic diversity of *T. cruzi* is very high, probably as a result of frequent gene rearrangement and recombination within these isolated lineages.

There are more than a dozen triatomine species thought to transmit *T. cruzi*. Programmes to control vector populations within houses have been highly effective in reducing transmission of *T. cruzi* in several countries but, nevertheless, vector-borne transmission of *T. cruzi* still persists in much of Latin America. The expense and labour-intensiveness of the vector control campaigns, as well as the development of insecticidal resistance, indicates that vector control alone is not going to be successful in eliminating *T. cruzi* as a human health issue.

9.2 Immune control and disease

The symptoms of the initial period of infection with *T. cruzi* are generally unremarkable (e.g. fever, swollen lymph glands and, possibly, inflammation at the bite site), making diagnosis early in the infection rare. There are, however, exceptions to this pattern; severe, high parasitaemic infections can occur when the infective dose is particularly high, or in immunosuppressed hosts. In these later cases, acute myocarditis or meningoencephalitis are common and can be lethal.

The transition to an asymptomatic chronic infection (also referred to as the 'indeterminate phase') is marked by a decline in parasite numbers coincident with the generation of robust immune anti-pathogen responses. Diagnosis of the chronic infection is based largely upon the presence of antibodies to *T. cruzi* in blood samples, using a combination of two or more tests. Parasitological confirmation of infection employing amplification techniques (e.g. hemoculture, xenodiagnosis (feeding lab-reared triatomine the blood of subjects suspected of being infected) and PCR) fails in the majority of seropositive subjects.

One study that serially sampled a set of infected subjects for up to 21 time points over several years showed that only 20 per cent of subjects were positive at every sampling point. This result suggests a variable range and often a low number of circulating parasites in different individuals, and thus a randomness to detection of parasites in the blood by xenodiagnosis. Despite the low parasite levels, it is generally thought that spontaneous cure of *T. cruzi* infection can occasionally occur, although most infections last a lifetime.

Symptomatic Chagas disease occurs a decade or more after the initial infection in some (but not all) individuals, and it is highly variable in its severity and

rate of progression. Cardiac symptoms can range from arrhythmias and heart enlargement to the development of apical aneurysm, congestive heart failure and sudden death. Mega-syndromes in the gut are less frequently reported. Like many infections, *T. cruzi* infection results in a highly spectral disease, with both parasite and host genetics likely to be contributing to the outcome of infection.

The origins of clinical disease in human *T. cruzi* infection are still debated. Early investigations suggested that Chagas disease had an autoimmune aetiology. However, there is emerging consensus that the persistence of parasites in muscle, and the continuous immune assault on these apparently intractable parasites, is the primary cause of tissue damage in chronic *T. cruzi* infection. This damage is progressive, creating focal lesions and the eventual disruption of muscle integrity and organ function.

As outlined in greater detail below, the immune response to *T. cruzi* is multifaceted and is generally highly successful in preventing disease or death from acute infection. However, immune control is not equally effective in all cases, and even subtle shortcomings in the generation of immune responses could result in inefficient control of the infection and, when magnified by the decades long infection, the generation of more severe chronic phase disease.

9.3 Innate recognition of *T. cruzi*

The success of *T. cruzi* in mammals depends on the parasite's ability to invade and replicate in the cytoplasm of host cells. A wide variety of tissue and cells types can support the replication of *T. cruzi*, including muscle, adipose, neuronal, endothelial and epithelial cells, in addition to cells involved in innate immune recognition (e.g. macrophages and dendritic cells (DCs)). The cells that are the primary target of initial infection by *T. cruzi in vivo* have not been identified, although this may vary, depending on the site of infection. The acute infection is usually associated with parasite replication in many tissues and the presence of detectable parasites in the bloodstream. Transition to the chronic phase of infection is characterised by very low parasite load and the restriction of *T. cruzi* to a more narrow set of host cell types, most prominently, myocytes and adipocytes. Innate recognition of *T. cruzi* has been best studied *in vitro* using macrophages or (less often) DCs.

Innate immune recognition of pathogens depends heavily upon germline-encoded host receptors that recognise pathogen patterns (pattern recognition receptors – PRR) and their cognate pathogen-associated molecular patterns (PAMPs). Among the well-studied PRR are cell surface and vacuolar Toll-like receptors (TLRs), cell surface mannose receptors and cytosolic receptors of the nucleotide-binding oligomerisation domain (NOD)-like (NLR) and retinoic acid inducible gene I (RIG-I)-like receptor (RLR) families. Collectively, these PRR provide the ability to detect pathogens that are outside, within vacuoles or in the cytoplasm of host cells. Triggering of PAMPS results in endogenous activation of cells through a number of signalling pathways leading to cytokine and chemokine production and, eventually, the generation of adaptive immune responses.

Several PAMPs have been identified in *T. cruzi,* including glycosylphosphatidylinositol (GPI)-anchored members of the large mucin family of *T. cruzi* trypomastigote surface proteins and the *T. cruzi* protein *Tc*52 that activates TLR2, the CpG-rich DNA that activates TLR9, and glycoinositolphospholipid (GIPL) ceramide isolated from the insect stage epimastigotes that interacts with TLR4. Cruzipain (an abundant cysteine protease expressed by of *T. cruzi*) has also been proposed to activate DCs, through the generation of endogenous kinin peptides. The contribution of these (and other) *T. cruzi* PAMPs to immune control of the infection has received considerable attention, and data supporting a role for both TLR-dependent and TLR-independent mechanisms have been presented.

The ability of *T. cruzi* to replicate in macrophages and DCs *in vitro* is constrained by both myeloid differentiation primary response gene (88) (MyD88)- and TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways, at least in part via the induction of interferon (IFN)-β and its activation of the p47 GTPases. Not surprisingly, deficits in various components of these PRR pathways in some cases also translates to reduced cytokine production and, in turn, to decreased control of parasite load and more rapid death following *T. cruzi* infection *in vivo*. What is not yet clear from this set of studies is the portion of this increased *in vivo* susceptibility to infection that is due to host cell endogenous factors (e.g. the increased replication of *T. cruzi* within macrophages and DCs) and the portion that is due to the reduced activation of the adaptive immune responses driven by cytokines such as interleukin (IL)-12.

T. cruzi growth has been particularly well-studied *in vitro* in macrophages. Although macrophages readily support *T. cruzi* growth when quiescent, they kill *T. cruzi* via a combination of oxygen- and nitrogen-dependent mechanisms when activated by IFN-γ, tumour necrosis factor (TNF)-α and/or bacterial lipopolysaccharide. But macrophages are only one of many cell types that are infected by *T. cruzi*, and the relative importance of these various cell types to parasite survival *in vivo* is not known.

A hallmark of the process of host cell invasion by *T. cruzi* in both macrophages and other cells is its ability to infect without dramatically perturbing the host cell. Human fibroblasts and bone marrow-derived macrophages infected *in vitro* with *T. cruzi* trypomastigotes exhibit the induction of interferon (IFN)-β (albeit at a relatively slow rate) but little else and, apart from a number of interferon response genes, there is a relatively minimal alteration in the messenger RNA expression patterns.

Natural killer (NK) and NKT cells have a less prominent role in control of *T. cruzi* infection, and the specific receptors and/or parasite ligands involved in this recognition of *T. cruzi* or *T. cruzi*-infected cells is not known.

9.4 Adaptive immunity

The adaptive immune mechanisms controlling *T. cruzi* infection are specific, highly potent and multi-functional. They are largely successful in controlling

parasite replication, preventing early death in the acute phase and maintaining a low parasite burden in the chronic infection. *T. cruzi* induces (and is controlled by) a combination of cell-mediated and humoral immune responses, dominated by Th1 cytokines, lytic and opsonic antibodies and CD8+ cytolytic T cells. The absence of any one of these mechanisms results in the inability to regulate parasite growth and subsequent death of the host.

9.4.1 T helper cell responses

T. cruzi induces a relatively polarised type 1 T helper cell response with significant production of IFN-γ and TNF-α. Although low levels of type 2 cytokines are sometimes observed, blocking or inhibition of type 2 cytokine responses has essentially no effect on either control of parasite load or the intensity of inflammatory reactions. In contrast, defects in the ability to generate Th1 responses (by blocking IL-12 production, the signalling molecules involved in the generation of Th1 responses or IFN-γ production itself) prevents mice from controlling the acute infection. In fact, preventing production of IFN-γ creates one of the strongest susceptibility phenotypes in mice infected by *T. cruzi*, nearly equivalent to the effect of the absence of T cell responses in general.

CD4+ T cells are likely participants in the control of *T. cruzi* infection via a number of mechanisms, including IFN-γ enhanced macrophage-mediated trypanocidal activity, promotion of antibody production (see humoral immune responses, below), and the potentiation of inflammatory responses. Notably, CD4+ T cells are not required for the generation of functional *T. cruzi*-specific CD8+ T cells, but CD8+ T cells that are not 'helped' by CD4+ T cells are present in lower frequency and fail to control the acute phase infection.

9.4.2 CD8+ T cell responses

T. cruzi invades and replicates in a number of host cell types that lack the inducible microbicidal functions of phagocytic cells. The ability to invade muscle (and other tissues) has sometimes been considered an immune escape mechanism. However, it is clear that *T. cruzi*-infected host cells do not completely evade immune detection, and that T cells that are responsible for the recognition and destruction of pathogen-infected host cells – the CD8+ T cells – are key to the immune control of *T. cruzi* infection.

Within its cytoplasmic niche in host cells, *T. cruzi* releases proteins that are processed and presented in association with host cell surface MHC-I, the targets for recognition by *T. cruzi*-specific CD8+ T cells. CD8+ T cells responding to these *T. cruzi* epitopes exhibit cytolytic activity, produce IFN-γ (Figure 9.2), can transfer a degree of protection in mice and dominate the inflammatory sites in acute and chronically infected hosts. The presence of CD8+ T cells at these sites of inflammation has also been argued to indicate their role as drivers in the development of disease pathology. However, the finding that depletion or blockage of the functions of these cells results in *increased* parasite load and disease severity argues for a disease-protective role for CD8+ T cells, rather than a disease-promoting one.

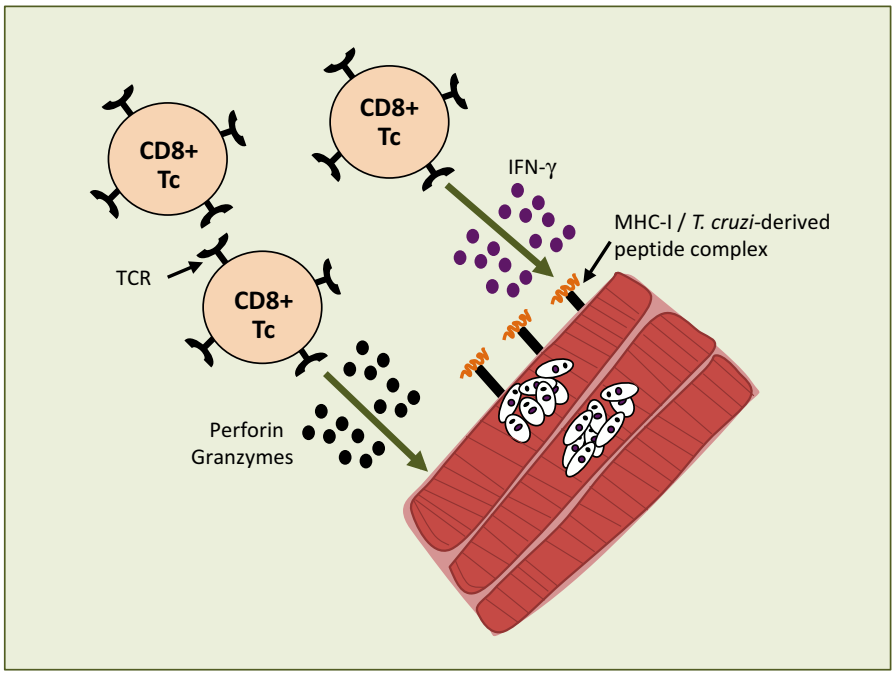

Figure 9.2 CD8+ T cells regulate the growth of *T. cruzi* in infected cells, performing a crucial role in the control of infection. The relative sizes of the CD8+ T cells and the muscle cells have been equalised for illustrative purposes but, in reality, the muscle cells are much larger than CD8+ T cells. The mechanism by which CD8+ T cells regulate parasite growth is not fully understood, but it is assumed partly to occur via lysis of the infected muscle cells. Abbreviations: IFN, interferon; MHC, Major histocompatibility complex; Tc, Cytotoxic T cell; TCR, T cell receptor.

The natural target of a significant proportion of these *T. cruzi*-specific CD8+ T cells are GPI-anchored surface proteins and, in particular, trans-sialidase (ts) proteins. In certain parasite-host strain combinations, more than 30 per cent of the total CD8+ T population at the peak of the infection is specific for a few ts peptides. This degree of immunodominance is remarkable, especially considering that the *T. cruzi* genome is large and complex, with over 12,000 haploid genes, thousands of which are in the ts family.

The high frequency of ts-specific CD8+ T cells in *T. cruzi*-infected mice has made it possible to monitor the generation and persistence of this parasite-specific response closely during infection and under various conditions. Throughout the long course of infection in mice, these *T. cruzi*-specific T cells maintain a mostly short-lived effector/effector memory phenotype, indicative of the presence of an active infection with at least occasional antigen encounter.

Upon cure of the infection using the anti- *T. cruzi* drugs, the parasite-specific CD8+ T cells assume a predominantly long-lived/central memory phenotype. Unlike many other chronic infections, the *T. cruzi*-specific CD8+ T cell response in mice does not show signs of immune exhaustion, even after more than two years of infection. This result reflects again the low antigen load in the chronic

stage of the infection, and provides additional corroboration that the immune system is successful in the long-term control of the infection.

Although the size and effectiveness of the anti-*T. cruzi* CD8+ T cell response attests to its high effectiveness, the initial generation of the response appears to be significantly delayed, relative to that seen in many other infections. This observation is consistent with the evidence, discussed above, that the infection by *T. cruzi* is relatively silent due to the absence of strong triggering of innate responses.

9.4.3 Humoral immune responses

Antibody responses during *T. cruzi* infection have often been characterised as non-specific (polyclonal), but this is not uniformly the case. It may simply reflect the vast number of antigen variants that *T. cruzi* presents to the immune system through an array of multigene families of surface proteins such as the ts, mucins, and mucin-associated proteins (MASPS). Transfer of serum, or antibody fractions of serum from infected animals, provides significant (although not total) protection to infection in naïve animals. Furthermore, animals lacking B cells are highly susceptible to infection although, interestingly, mice lacking B cells due to a knockout in the mu immunoglobulin heavy chain (μMT) are able to control acute infections somewhat longer than do mice lacking CD8+ T cells before eventually succumbing to the infection with very high parasitaemias.

The mechanism of antibody-mediated control of *T. cruzi* infection *in vivo* is not fully understood, but anti-*T. cruzi* antibodies have been demonstrated to partially block host cell invasion *in vitro*, to facilitate *T. cruzi* uptake by phagocytic cells and to induce complement-mediated and complement-independent lysis of *T. cruzi* (Figure 9.3).

Two rather unique reported activities in the pool of lytic antibodies induced by *T. cruzi* infection are anti-galactosyl antibodies that lyse *T. cruzi* without the participation of the classical or alternative complement pathway, and antibodies against a complement regulatory protein (CRP) that act by preventing the normal decay-accelerating factor-like activity of this CRP (see Chapter 1), in turn facilitating complement lysis of *T. cruzi parasites*. These lytic antibodies have been suggested as good markers of spontaneous or drug-induced parasitological cure in *T. cruzi* infection

9.5 Regulation of immune responses and parasite persistence

Early studies of experimental *T. cruzi* infections in mice suggested that the infection provoked a state of immunosuppression that prevented the development of potent anti-parasite immune responses. However, anti-*T. cruzi* immune responses are sufficiently potent to be measured easily, are highly

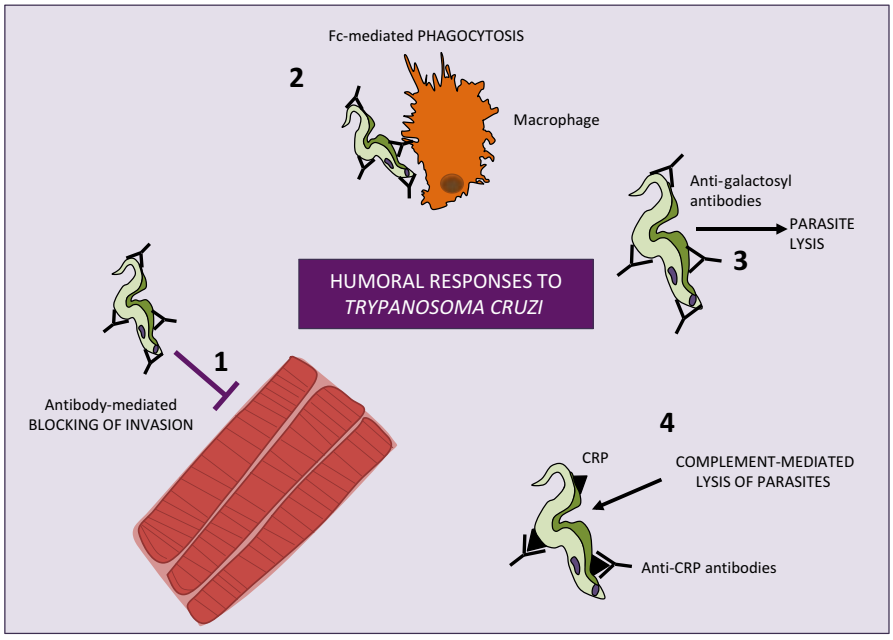

Figure 9.3 Antibodies help to control extracellular *T. cruzi* trypomastigotes in several ways. Opsonised *T. cruzi* trypomastigotes are not able to invade new cells (1) and are a target for macrophage-mediated phagocytosis (2). Furthermore, anti-galactosyl antibodies which recognise mucin-like GPI-anchored glycoproteins on the *T. cruzi* surface mediate both complement-dependent and independent parasite lysis (3). Lastly, antibodies are generated in *T. cruzi* infection, which recognise and neutralise complement regulatory protein, in turn facilitating complement-mediated lysis of parasites (4). The relative sizes of the parasites and cells have been equalised for illustrative purposes but, in reality, the Trypanosomes are much smaller than macrophages or muscle cells.
Abbreviations: CRP, complement regulatory protein.

effective and, if abrogated, lead to overwhelming parasite expansion. Among the best examples of the latter are cases of HIV exposure in individuals with chronic *T. cruzi* infection. The rapid and dramatic impact of cyclophosphamide-mediated immunosuppression in mice, in which undetectable levels of parasites become an overwhelming infection in the span of just three weeks, also emphasises just how effective the normal host immune response is in controlling *T. cruzi* infection (Figure 9.4). Nevertheless, immunity to *T. cruzi* is also rarely effective in completely clearing the infection – what is the reason for this?

The subtle character of early invasion and expansion of *T. cruzi* in host cells argues that the identified *T. cruzi* PAMPs (e.g. GPIs and DNA) are well-hidden on the invading parasites. The ability to avoid innate responses would account for successful initial establishment of the infection *in vivo*, but perhaps not for the long-term persistence of *T. cruzi* in the face of the vigorous adaptive immune responses that are generated. In experimental models or human infections, it is difficult to find strong evidence of classical regulatory mechanisms

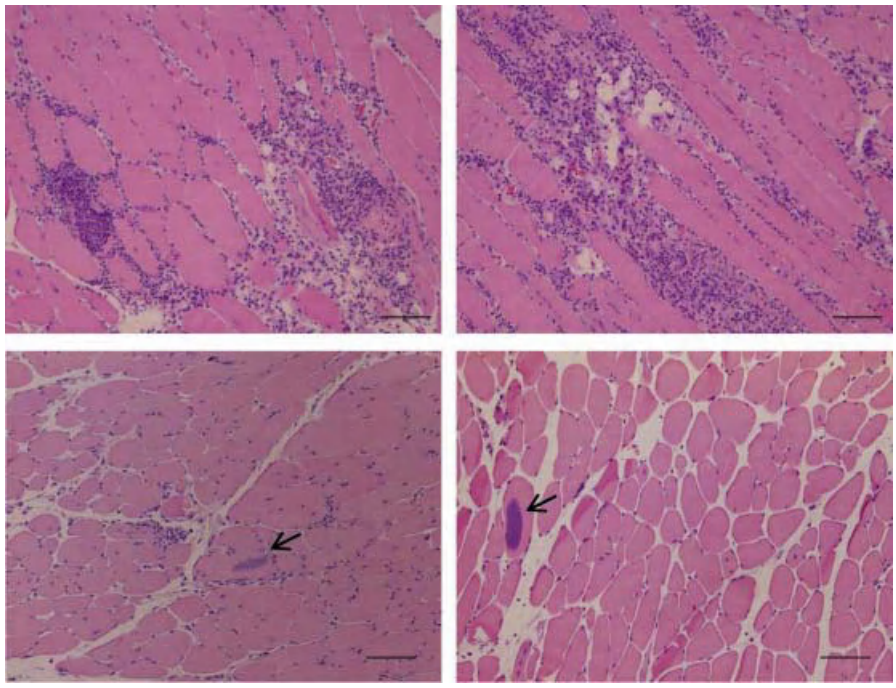

Figure 9.4 Inflammation correlates with control of parasite load. (Top) Histology sections from skeletal muscle of mice infected with *T. cruzi* CL strain at 240 dpi. (Bottom) Sections from mice infected with *T. cruzi* CL strain at 15 days post-immunosuppression with cyclophosphamide. Note the absence of inflammation and the presence of *T. cruzi* within muscle cells (arrows) following immunosuppression during the chronic infection. Scale bar: 200 mm (photo credit: Juan Bustamante).

(e.g. regulatory T cells or a high level of production of regulatory cytokines) that might thwart the effectiveness of anti-*T. cruzi* immunity. The fact that the anti-*T. cruzi* CD8+ T cell response is one of the most potent of such responses observed in any infectious disease is also a strong indication that immunoregulatory mechanisms are not a dominant force in the overall course of *T. cruzi* infection in most hosts.

Particularly in the chronic infection, *T. cruzi* would appear to spend the majority of its time inside host cells during its 4–5 day replication cycle, and relatively little time in the circulation, where antibodies appear to be highly effective in killing the extracellular trypomastigotes. How these intracellular parasites within muscle or fat cells evade the potent anti-*T. cruzi* CD8+ T cell response is not clear. The fact that ts peptides are the primary targets of *T. cruzi*-specific CD8+ T cells has attracted attention in this respect. The ts family is greatly expanded to thousands of genes in *T. cruzi*, compared with a handful of genes in other related kinetoplastids, suggesting that some strong evolutionary pressure has been exerted on this gene family. The mechanism by which the simultaneous expression of many ts proteins and the variant ts-encoded epitopes encoded by this gene family might facilitate immune evasion and parasite persistence is still unknown.

9.6 Conclusions

By whatever mechanism(s), *T. cruzi* manages to evade complete clearance and, as a result of its persistence, it sets into motion the process of tissue destruction that naturally accompanies immune responses to entities that are not easily removed. The speed with which disease develops, and its severity, are no doubt influenced by how efficiently the immune responses deal with this tenacious stimulus. The obvious solution to preventing disease is to clear the infection – something that appears to be achieved too rarely.

One of the goals of gaining a better understanding of immunity to a pathogen is to translate that understanding into a preventative or therapeutic – such as a vaccine. For *T. cruzi* infection, this may be a tall order. The known immunodominant epitopes are diverse, are variable across isolates and are capable of recombination. The existing immune response is, as repeatedly noted, highly effective, but it just falls short of parasite clearance. Simply keeping parasite load low is already achieved during the natural course of *T. cruzi* infection in humans, but this result is not sufficient to prevent disease development. Will it be possible to generate even more effective immune responses – responses that can completely clear the infection? So far, there is no evidence that the understanding of immunity to *T. cruzi* is adequate to achieve this goal.

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Section 3

Introduction to Helminth Infections

10

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The word helminth – or geohelminth – is derived from the Greek 'helmins' or 'helmithos', translating as 'worm'; it is an umbrella term commonly used to describe a group of parasitic organisms having 'worm-like' anatomical characteristics. Zoologically, 'helminth' has come to encompass several large groups of parasitic invertebrates which we now know to be unrelated' despite sharing some gross morphological features. These groups include the platyhelminths, nematodes, nematomorphs, acanthocephalids, and pentastomids. One of the common hallmarks of these organisms is their complex life cycles, often involving multiple hosts and highly adapted reproductive strategies.

Helminths have long been recognised as causative agents of disease in humans and other animals and have shaped societal habits in many parts of the world. One example of helminths shaping societal eating habits is the prohibition against eating pork products in several religious practices. 'And the swine, though he divide the hoof, and be cloven footed, yet he cheweth not the cud; he is unclean to you' (Leviticus 11: 7–8). Many believe this prohibition arose because of infections with pork tapeworm (Taeniasis, *Taenia solium*; Chapter 17) and *Trichinella spiralis* (Trichinosis; Chapter 15), which would have been common in domesticated pigs at that time.

Parasitic worms infect billions of people annually and continue to be important human pathogens. The WHO has calculated that millions of disability-adjusted life years (DALYs) are lost annually due to helminth infections. However, epidemiological studies have shown that, unlike many of the protozoal diseases, most helminth infestations are generally asymptomatic and rarely life-threatening, even in individuals harbouring heavy infections. This bespeaks either a remarkable degree of immunological trickery on the part of the parasite, or apathy by the host. Either possibility makes this a fascinating area of study for immunologists and those trying to develop vaccines to target these pathogens.

Immunity to Parasitic Infection, First Edition. Edited by Tracey J. Lamb.

This chapter serves as an introduction to the world of worms for those interested in human health and the immunology of helminth infection biology. As well as summarising general features in their anatomy, life cycles, and evolution, there is also discussion on the use of more recent innovations in genomic analysis that are reshaping our understanding of helminth biology.

10.1 Acanthocephala

Commonly known as 'thorny headed' worms, this group of organisms is comprised of approximately 1,000 species characterised by the presence of an evertable spine-bearing proboscis that pierces the intestinal wall of its hosts. These organisms have complex lifestyles, often involving a combination of hosts such as invertebrates, fish, amphibians, birds and mammals.

A number of acanthocephalids can infect humans. Infections with species such as *Macracanthorhynchus hirudinaceus* and *Moniliformis moniliformis* have been well documented and develop after the consumption of raw intermediate hosts, usually insect grubs or cockroaches. These infections are now thought to be rare, possibly because of changes in human dietary practices. However, analysis of coprolites from archaeological sites has shown that, historically, these parasites may have been common and widespread human pathogens.

Very little is known about the immunology of acanthocephalid infection. Attachment of these parasites to the host causes perforation of the intestine and inflammation. Large parasite burdens can lead to sepsis and death. Studies done in rat models of *M. moniliformis* infection indicate that protective immunity to adult stages can develop in the gut and may be mediated through mechanisms that are also important for immunity to other helminth infections (e.g. Th2 mediated inflammation and antibody-mediated immunity).

10.2 Nematodes

Also referred to as 'round' worms, nematodes are an abundant and specious group of free living and parasitic organisms. Along with the Platyhelminthes, this phylum contains those species of worms whose medical impact most seriously affects the socioeconomic development of human populations. Over 16,000 species have been described, but it is generally acknowledged that potentially hundreds of thousands more remain unidentified. Nematodes are present in almost all ecological settings and, as parasites, they have successfully colonised almost all of the large complex organisms in the animal and plant kingdoms.

Part of their success is due to their simple, yet robust, body plan which is conserved within the phylum. This consists of a tapered, bilaterally symmetrical, cylindrical body with a mouth and anus connected by a gastrointestinal (GI) tract (Figures 10.1A, B and C). It is unclear how functional the intestine is in some parasitic species. However, most nematodes actively take up and digest material from their surrounding environment.

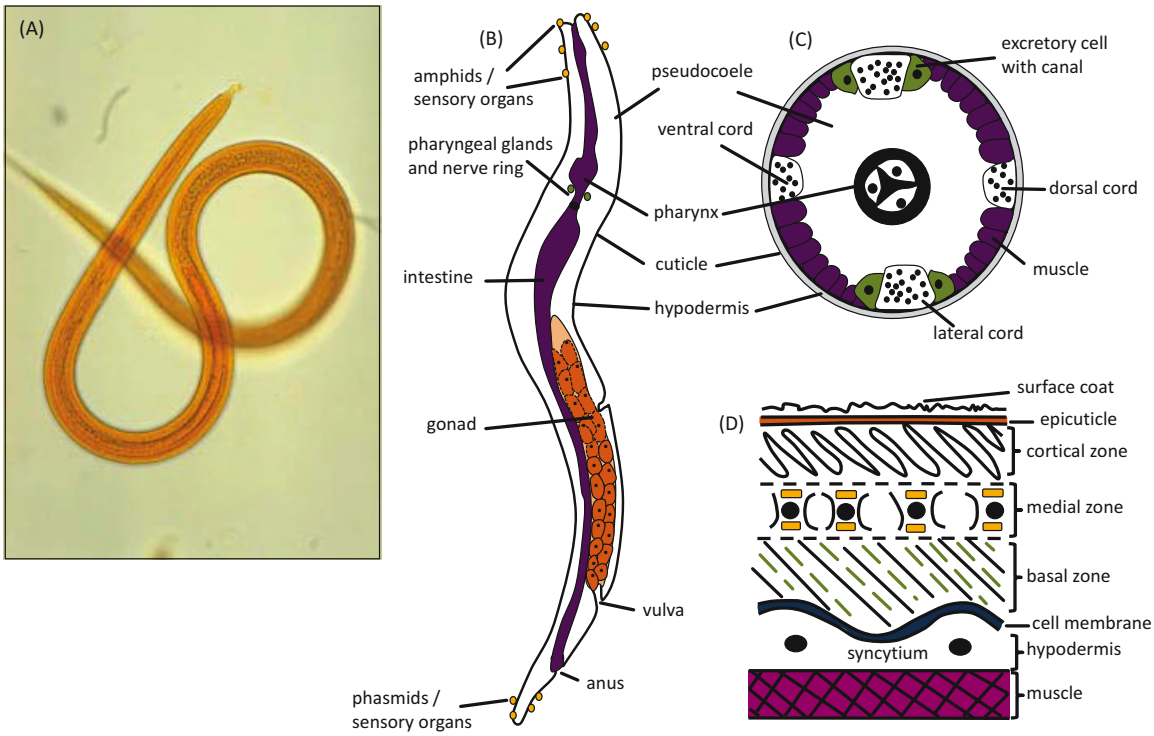

Figure 10.1 Nematodes, basic anatomy. A phase contrast image of a *Strongyloides stercoralis* filariform L_3 larvae is shown in (A). The illustration shows a section of an idealised rhabditiform nematode that has been cut either longitudinally (B) or transversely (C) near its anterior end. Major organs and features are labelled (D). This image adapted, from Page & Johnstone (2007 – The cuticle. *WormBook*, pp. 1–15) shows an idealised representation of the structure of a nematode cuticle, with the different layers labelled.

The cylindrical shape of the nematode is maintained by a body wall made up of muscle, a syncytial epithelium (hypodermis) and a tough outer surface called the cuticle (Figure 10.1C). The internal organs, such as the intestine and gonads, are housed within a fluid-filled pseudocoele that allows a considerable amount of flexibility in movement.

While it varies in thickness, the cuticle in most species is relatively inert and environmentally resistant. It is synthesised by the hypodermis and is made up of several different layers, each of which contributes to different aspects of its function (Figure 10.1D). Finally, the cuticle is covered in a surface coat of glycoproteins and glycolipids. In parasitic species, these are often highly immunogenic, with protective responses being directed against the surface coat or other cuticular components, making it one of the most important immunological features of the nematode.

The general developmental pattern of nematode life cycles is remarkably conserved. After hatching from eggs, most free-living and parasitic nematodes go through five life cycle stages, where they develop from L_1 larvae to reproductive adults. Movement through these stages is accompanied by a moult,

during which the old cuticle is replaced by a new cuticle synthesised by the hypodermis. This process is hormonally controlled and is similar to moulting in other animals, such as arthropods and *Onychophora*. Moulting is often accompanied by growth in nematode size, with the adults of some species being many times bigger (sometimes hundreds of times) than the larvae they developed from. However, unlike arthropods, growth in nematodes can occur in between moulting and is accompanied by remodelling or growth of the current cuticle.

In response to adverse environmental stimuli, many nematodes, particularly free-living species, can undergo alternative developmental programmes where, instead of becoming normal L_3 larvae, they arrest in a long-lived, hypobiotic, non-feeding stage called a dauer (L_{2d}). Dauer larvae remain highly motile, but they are extremely resistant to environmental stresses such as desiccation. In free-living species, when the environment becomes more favourable, the dauer moult to L_4 and continue their life cycle.

The biology of the dauer stage of free living species is of particular interest to parasitologists, as many parasitic species (hookworms, *Toxocara sp.*, *Strongyloides sp.*, etc.) which develop as L_1 to L_3 in settings outside of their definitive host enter a dauer-like infective stage until a new host is available.

Nematodes display the full gamut of different reproductive strategies, with both free-living and parasitic species containing examples of monoecious, dioecious and parthenogenetic species. Many free-living species, such as *C. elegans*, are hermaphroditic, with sperm derived from males being used if available, while many parasitic species are dioecious. Most female or hermaphroditic parasitic nematodes are oviparous, releasing environmentally resistant eggs that hatch and develop outside the host. However, a number of important species – such as the Filaria and trichinellids – are viviparous, with eggs completing development and hatching within the female. L_1 larvae released by these species usually take up residence in the current host and await transmission to a new definitive or intermediate host.

While a general taxonomy for the nematodes has remained rather controversial traditionally they have been broadly divided into two groups based on whether they possess posterior sensory organs called phasmids. The non-phasmid bearing species are included within the *Adenophorea* (or *Aphasmidea*), while the nematodes with phasmids are placed within the *Secernentea* (or *Phasmidea*). However, divisions based on these often cryptic anatomical characteristics have generated many disagreements within the field.

More recent molecular studies, using the 18S ribosomal DNA sequences, have roughly supported this division and have proved invaluable for understanding the relationships between species within these groups. In several instances, these molecular analyses have overturned historical groupings made using morphological characteristics or lifestyles, such as inclusion of the *Strongyloididae* within the *Rhabditida*. In addition, these studies also suggest that, within the *Nematoda*, animal parasitism has evolved independently at least four times, and plant parasitism three times. This evolutionary dynamism

Table 10.1 There are 24 species of nematodes that commonly infect humans. These species belong to six orders which are listed here, along with their mode of transmission, tissue tropism of adults and global distribution.

		Transmission	Tissue tropism of adults	Distribution
Trichocephalida				
Trichuris	*trichiura*	Ingestion of eggs	Intestine	Worldwide
Trichinella	*spiralis*	Consumption of infected host	Intestine	Worldwide
Capillaria	*philippinensis*	Consumption of infected fish	Intestine	Southeast Asia
Oxyurida				
Enterobius	*vermicularis*	Ingestion of eggs or autoinfection	Intestine	Worldwide
Spirurida				
Wuchereria	*bancrofti*	Mosquito vector	Lymphatic vessels	Tropical areas worldwide
Brugia	*malayi, timori*	Mosquito vector	Lymphatic vessels	Asia, Indonesia
Onchocerca	*volvulus*	Black fly vector	subcutaneous nodule	Mainly Africa
Loa	*loa*	Deer fly vector	Subcutaneous tissue	Africa
Mansonella	*perstans, ozzardi, streptocerca*	Biting midge vector	Peritoneal cavity, subcutaneous tissue, dermis	Africa and South America, Americas, Africa
Dracunculus	*medinensis*	Consumption of infected cyclops	Connective tissue	Small regions of Africa
Ascaridida				
Ascaris	*lumbricoides*	Ingestion of larvae	Intestine	Worldwide
Toxocara	*canis,* cati	Ingestion of larvae	Intestine of dogs, Intestine of cats	Worldwide
Anisakis	*simplex*	Ingestion of larvae in intermediate host	Intestine of marine mammals	Worldwide
Baylisascaris	*procyonis*	ingestion of larvae	Intestine of racoons	North America
Strongyloididae				
Strongyloides	*stercoralis, fuelleborni*	Skin penetration by or ingestion of larvae	Intestine	Worldwide, Central Africa
Strongylida				
Ancylostoma	*duodenale, ceylanicum*	Skin penetration by larvae	Intestine	Mainly Old World tropics, Asia
Necator	*americanus*	Skin penetration by larvae	Intestine	Mainly New World tropics
Angiostrongylus	*cantonensis*	Ingestion of larvae from intermediate or paratenic hosts	Pulmonary arteries of rats	Southeast Asia

contrasts with other highly successful helminth groups, such as the Platyhelminths, where the transition to obligate parasitism occurred only once in their evolutionary history. *Adenophorea* and *Secernentea* orders containing important human pathogens are listed in Table 10.1. These orders are assigned to clades as proposed from 18S ribosomal RNA studies (Figure 10.2).

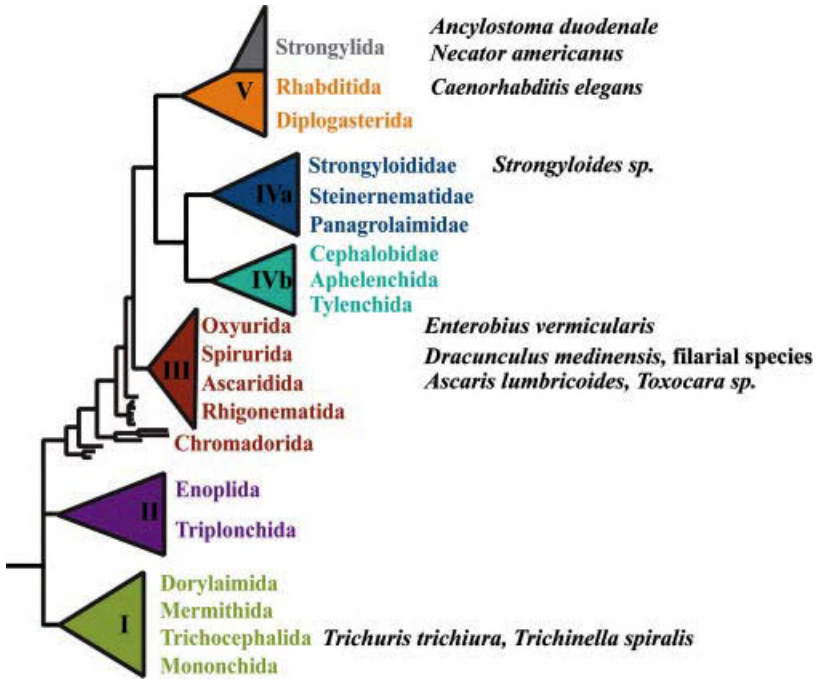

Figure 10.2 The phylogeny of the Nematoda. This illustration has been adapted from Blaxter *et al.* (1998 – A molecular evolutionary framework for the phylum Nematoda. *Nature* 392(6671), pp. 71–75) and shows the phylogenetic relationship of the different nematode orders containing parasitic species. Within these orders, the positions of the major species infecting humans are also shown.

10.2.1 *Adenophorea:* Clade I, *Trichocephalida*

This order is exclusively made up of parasites. It contains three genera of medical importance: *Trichuris* (see Chapter 14), *Trichinella* (Chapter 15) and *Capillaria*. The life cycles of these organisms are fairly diverse, with members of this group parasitising a wide variety of vertebrate hosts. Adults often live within the tissue of the intestine, although some species occupy deep tissue sites. In addition, a number of species are fairly cosmopolitan, infecting a wide range of hosts (*Trichinella* and some *Capillaria sp.*). Infection is initiated through an oral route via consumption of larvae within infected tissue or eggs passed in faeces. One notable exception is *Capillaria philippinensis*. This species can either autoinfect their host or pass eggs from the primary host in faeces, infecting an intermediate host (fish), where they develop into an infectious stage and, finally, must be consumed by a new definitive host.

10.2.2 *Secernentea:* Clade III, *Oxyurida*

The species from this order are usually called pinworms and are parasites of vertebrate and arthropod intestines. Most mammals (humans included) harbour a pinworm infection at some point in their life. One species, *Enterobius vermicularis*, is of medical importance, although a second closely related

species, *E. gregorii*, may also infect humans. Infection is transmitted via an oral-anal route, with eggs laid by gravid females on the lower bowel, anus or perianal epidermis, being re-ingested or passed on to a new host. In certain circumstances, eggs left on the perianal epidermis for long periods of time can hatch and crawl back into the intestine (retroinfection). Once in the intestine, they develop into adults, mate, and the females begin the process of migrating to the anus. Despite often having heavy infection loads, most individuals show little in the way of symptoms or pathology.

10.2.3 Clade III, *Spirurida*

Spirurids are a group of parasites that infect vertebrates. However, unlike all the other nematodes of medical importance, they utilise arthropod intermediate hosts and vectors to facilitate their transfer to new definitive hosts. The now almost extinct Guinea worm, *Dracunculus medinensis,* the filarial nematodes, which are causative agents of lymphatic filariasis and river blindness (Chapter 11), are the most important members of this order.

The life cycles of these parasites show a number of similarities. Adult females are viviparous, releasing fully motile first-stage larvae, which, in the case of filarial nematodes, are called microfilaria. These migrate to locations where they can be taken up by the biting insects that serve as their intermediate hosts. Once in these insects, the microfilaria penetrate the intestine and take up residence in the thoracic muscle, where they moult twice into infective L_3. These then move into the mouth parts of the insect, where they can be transferred to a new definitive host. In the new host, the L_3 usually go through a period of migration until they reach their chosen organ of residence, where they finish development into adults.

D. medinensis, unlike the filarial nematodes, expels its L_1 larvae directly into water via a hole that the female burrows through the skin. This rather ghoulish process is instigated by the parasite inducing a burning sensation in the afflicted area, presumably to encourage the host to place it in water. Larvae released in this way are then consumed by a cyclopoid crustacean, burrow into its hemocoel and develop into infective larvae. When drinking water contaminated with infected *Cyclops* is consumed, the parasites burrow through the intestine and migrate into the deep connective tissue, where they develop into adults and mate. Finally, gravid females then begin a process of migration to the skin, where they will release their progeny.

10.2.4 Clade III, *Ascaridida*

Species from this order are among the largest nematodes, with some species reaching in excess of 450 mm. They are typically intestinal parasites, where the adults eat the liquid contents of intestinal lumen. Although their life cycles and mechanisms for gaining entry to the intestine vary, a number of striking similarities exist. Eggs are passed from the definitive host in their faeces. These develop through their first two larval stages, but remain within the eggshell. When the egg-contained larvae are ingested, they hatch, penetrate the intestine and

migrate to the lungs, where they moult twice. Once in the lung, they then penetrate the alveoli, move up the windpipe to the pharynx and are re-swallowed. The larvae then develop into adults and mate.

Some species, such as *Toxocara sp.* and *Baylisascaris procyonis*, can use a paratenic host, such as rodents or humans, where they arrest as migrating third-stage larva and await ingestion by a definitive host. Others, such *Anisakis simplex* (which are accidental parasites of humans) cause severe damage as they attempt to exit the intestine. *Ascaris lumbricoides* (see Chapter 12) is the most important human pathogen from this group, with millions of people being affected worldwide. Worms can cause malnutrition and, in appropriate circumstances in highly infected individuals, they can fatally block the intestine.

10.2.5 Clade IV: *Strongyloididae*

The worms in this group are rather special, having a heterogonic lifestyle which alternates between free-living and parasitic reproductive cycles. While there are several species belonging to this group that can infect humans, the most important and widespread is *Strongyloides stercoralis* which has a worldwide distribution. Infection in humans is initiated either by consumption or penetration of the skin by filariform infectious L_3s. Unlike those larvae that are consumed and enter the intestine directly, those that enter via the skin make a journey similar to those made by Ascarid or hookworm larvae, migrating through the body until they reach the lungs, and subsequently transiting to the throat, where they are then swallowed. Once in the intestine, the larvae develop into adult females, who reproduce parthenogenically, laying eggs that hatch into rhabditiform larvae that pass out in the faeces during their first or second developmental stages.

Once they are in the external environment, several permutations of the life cycle can occur. The rhabditiform larvae can either develop directly into filariform infectious stages or into free-living adults, which reproduce sexually in the soil. Progeny from these free-living adults can choose between either developmental fate, and successive generations of nematodes can be produced by free-living individuals. In addition, still more diversity can be introduced into the life cycle if rhabditiform larvae manage to complete their second moult before they pass out of the host. These larvae can then penetrate the gut and go through the normal migration process before developing into adults and initiating autoinfection. The factors controlling the autoinfectious cycle are poorly understood, although immune status is a dominant factor.

Infections with *Strongyloides* are generally asymptomatic. However, under the right conditions, if left unchecked, it can develop into a life-threatening illness.

10.2.6 Clade V: *Strongylida*

This large order of parasites contains species of nematodes (the hookworms *Ancylostoma* and *Necator* – Chapter 13) which are of great medical and economic importance. The majority of this group are parasites of the intestine and

have direct life cycles, where eggs passed in faeces hatch and develop through three larval stages within the soil, where they feed on faecal matter and bacteria. After moulting to the third larval stage, they arrest and no longer feed. These larvae are highly motile and must quickly locate a definitive host to infect. Generally, larvae directly penetrate the skin of the new host, then undergo a migratory phase via the lungs similar to those performed by larval *Strongyloididae* and Ascarids. This terminates in the small intestine, where they develop into adults, mate and begin shedding eggs.

The medical problems associated with these nematodes are the adult's feeding habits. These usually involve grazing by laceration of the host mucosa and ingestion of tissue and blood. While individuals cause little damage, heavy infections can cause significant blood loss, which, while generally not fatal, can lead to anaemia and malnutrition. Other species of Strongylida, such as *Angiostrongylus cantonensis*, which infect humans as incidental hosts, can cause severe pathology due to immune reactions to the migrating L_3 larvae.

10.3 Pentastomida

Commonly known as tongue worms, this group is comprised of approximately 130 species, whose adults live within the upper respiratory tract of a wide range of terrestrial vertebrates and birds. Like other helminths, pentastomids have complex life cycles involving passage through intermediate and sometimes paratenic hosts.

Human infections with species from the genus *Linguatula*, *Armillifer* and *Porocephalus* are well documented but rare. *Linguatula serrata*, the causative agent of linguatulosis (aka Marrara or Halzoun syndrome), results from the consumption of raw offal derived from infected sheep or cattle. While *L. serrata*'s definitive host is dogs or wolves, humans can serve as both intermediate and occasional definitive hosts. Little is known about the immunology of pentastomid infection, although pentastomids appear to stimulate strong immune responses in their hosts that intuitively must be countered in some way, as they are also long-lived. Secretions from the frontal and subparietal glands which cover the surface of the cuticle may play a role in this process, as parasites deprived of these secretions are vulnerable to sustained host cytotoxic responses.

10.4 Platyhelminthes

Platyhelminthes, also commonly called flatworms, are a large phylum comprised of both free-living and parasitic species. All of the obligate parasitic species belong to the three groups Monogenea, Trematoda, and Cestoda which are members of the subphylum Neodermata. Generally, flatworms possess a bilaterally symmetrical, dorso-ventrally flattened body plan (hence their common name) and an leaf-like or oval shape (Figure 10.3A).

While most platyhelminths have a mouth and muscular pharynx, their digestive system is generally a blind sac or a set of highly branched tubes. Digested material is taken up by the gastrodermis and waste is eliminated back through

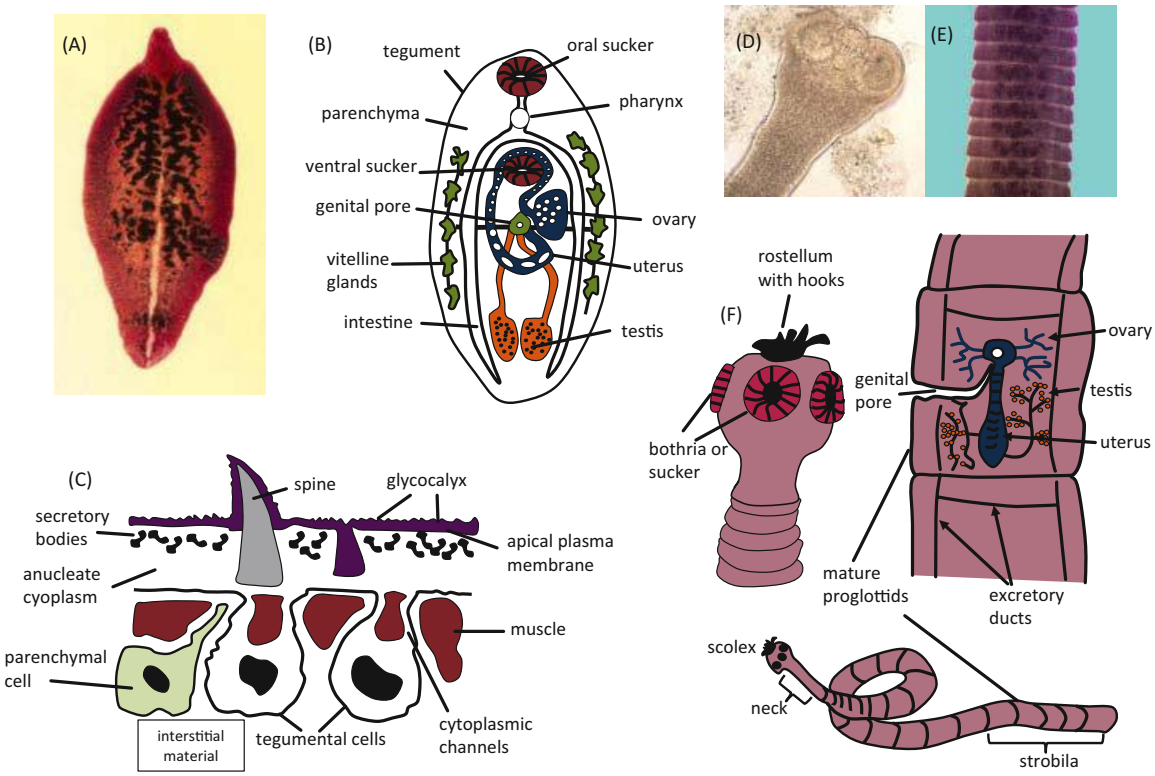

Figure 10.3 Platyhelminths, basic anatomy. (A) A light micrograph of a section of a liver fluke, *Fasciola hepatica*. (B) The illustration shows a section of an idealised trematode that has been longitudinally cut. Major organs and features are labelled. (C) This illustration, adapted from JD Smyth (1994 – *Introduction to Animal Parasitology*. Cambridge University Press, cambridge, UK), shows an idealised representation of the structure of a trematode tegument, with the different cellular features labelled. The light micrograph shows the scolex (D) and a section of proglottids (E) from the murine tapeworm, *Hymenolepis nana*. (F) The cartoons show a section of an idealised cestode body, the scolex and neck and a proglottid that has been longitudinally cut. The major reproductive organs are shown and labelled.

the mouth. While they often have very sophisticated nervous systems, flatworms lack a body cavity as seen in some other groups such as the nematodes. Instead, they have a loose network of irregularly shaped parenchymal cells and fibres of interstitial material that support their internal structures (Figure 10.3B). One striking feature of this group is their inability to synthesise fatty acids and sterols *de novo*, which may account for their predilection to parasitism.

Most platyhelminths are hermaphrodites, although there are several notable dioecious or gonochoristic genera (e.g. *Schistosoma*). While they will reproduce via self-fertilisation, exchange of sperm and cross-fertilisation is common. Egg components or vitelline cells are often formed in remote sites (such as the vitelline glands) and then transported to areas where they are incorporated into the developing eggshells. Fertilised eggs are expelled into the environment via the genital pore. For endoparasites, these then make their way to the external environment, often causing substantial damage to their host. The phases of trematode sexual reproduction in the definitive host are usually accompanied by rounds of asexual reproduction in the intermediate host. This is particularly

important for those flukes, such as schistosomids and fasciolids, that are not transmitted by direct consumption of the intermediate host.

The outer surfaces of flatworms are covered in a remarkably conserved structure called a tegument. Within these groups, the tegument is a highly dynamic structure and serves not only to protect the parasite, but also to act as an absorptive and secretory surface. The tegument is formed from a syncytial epithelium comprised of two sections with the anucleate outer layer, covered in numerous microvilli (Figure 10.3C). In some groups, such as the cestodes, these microvilli have become highly modified and are referred to as 'microtriches'. The tegument microvilli may also be interrupted by spine-like structures that may aid in attachment to the host.

The outer syncytial layer is connected via channels to a nucleated segment of cytoplasm often termed the 'tegumented cell bodies', which is buried within or beneath layers of muscle (Figure 10.3C). The surface of the tegument is covered in a carbohydrate-rich glycocalyx that is often highly immunogenic. This material has a high turnover rate and is frequently renewed, presumably to avoid recognition or haptenisation by the host's immune system. Large numbers of secretory bodies can be found in the anucleate portion of the tegument, and these are believed to contribute to the renewal of lost plasma membrane and glycocalyx components.

Because of their medical importance, understanding the evolution of parasitism within this group, and defining the relationships between classes and orders of Platyhelminths, has enjoyed a great deal of attention. Like the nematodes, taxonomies based on morphological characteristics have often been controversial. Unlike the nematodes, the adoption of a parasitic lifestyle appears to be monophyletic and basal to the radiation of the Neodermata (Figure 10.4A).

More recent molecular phylogenetic analyses have supported the Neodermata taxonomic grouping. However, support for relationships between orders within the Monogenea, Trematoda and Cestoda remain confused. Some analyses of 18S and 28S rDNAs fragments suggest a closer association between the Cestoda and Trematoda (Figure 10.4 A), but there is still a great deal of uncertainty on this topic.

10.4.1 Monogenea

This group of over 2,000 species is comprised of both ecto- and endoparasites which bear characteristic large posterior adhesive organs called haptors and anterior adhesive regions associated with their mouths. They generally have simple life cycles, with no intermediate hosts. The monogeneans are parasites of amphibians and fish, with no species infecting humans.

10.4.2 Trematoda

This highly specious group, with over 18,000 members, is also comprised of ecto- and endoparasites in two subclasses: the *Aspidogastrea* and *Digenea*. All of the important human pathogens are members of the *Digenea*.

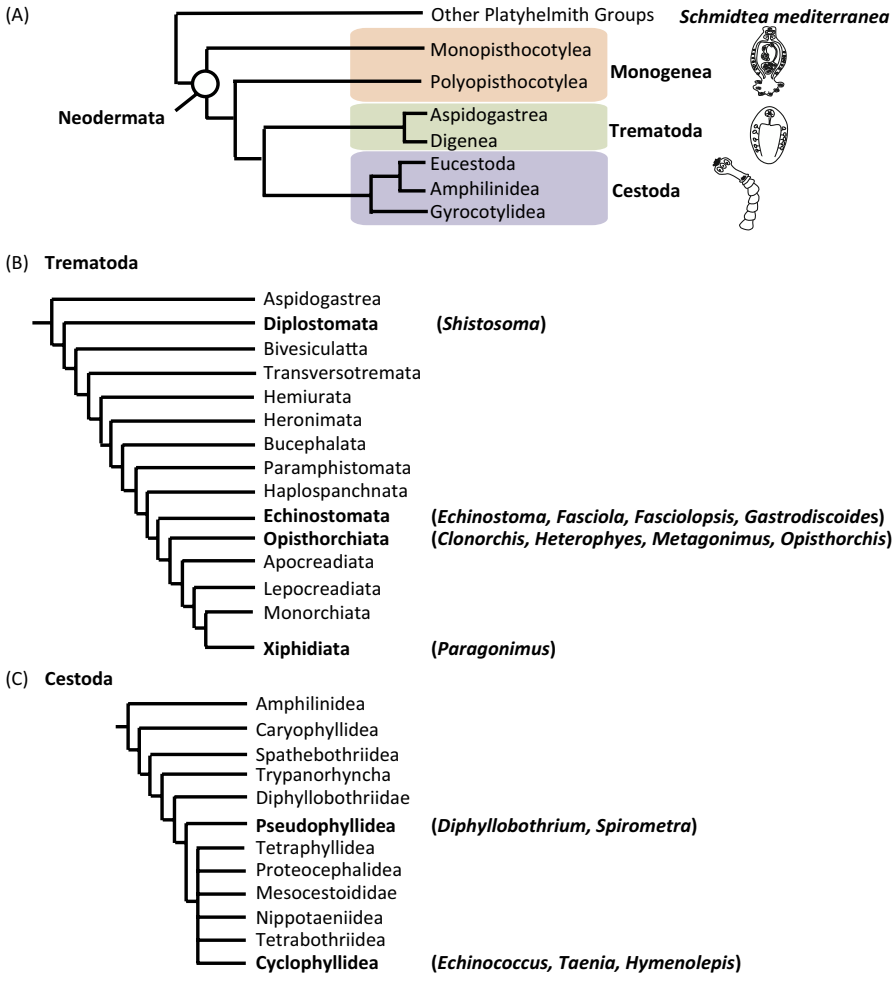

Figure 10.4 The phylogeny of the Platyhelminthes. (A) The tree adapted from Lockyer, AE *et al.*, (2003 – Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Platyhelminthes: implications and review of the cercomer theory. *Biological Journal of the Linnean Society* 78, pp. 155–171) shows a phylogenetic reconstruction of the parasitic platyhelminths groups based on the analysis of 18S rDNA. (B) The tree adapted from Olson, PD *et al.* (2003 – Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal For Parasitology* 33(7), pp. 733–755) shows a phylogenetic reconstruction of the trematode groups based on the analysis of 18S and 28S rDNA. (C) The tree adapted from Mariaux, J (1998 – A molecular phylogeny of the Eucestoda. *Journal of Parasitology* 84(1), pp. 114–124) shows a phylogenetic reconstruction of the Eucestoda groups based on the analysis of 18S rDNA. For (B) and (C), the major sub-orders and orders containing species which infect humans are shown.

Like the monogeneans, they have well-developed adhesive organs, but their tegument is absorptive and their life cycles complex, involving transitions both through intermediate and paratenic hosts. The tegument of most trematodes is bound by an unusual trilaminate plasma membrane. The blood flukes of the genus *Schistosoma* (Chapter 16) are a notable exception to this rule, with adults instead having a heptalaminate membrane covered with host proteins. Trematodes usually possess a well-developed gut, but they can also absorb

Table 10.2 There are 14 species of trematodes that commonly infect humans. These species are listed below, along with their mode of transmission, tissue tropism and global distribution. Species have been subdivided by the sub-order to which they belong.

		Transmission	Tissue tropism of adults	Distribution
Diplostomata				
Schistosoma	*mansoni, haematobium, japonicum, mekongi*	Skin penetration by cercariae	Mesenteric veins	Worldwide, Africa and Middle East, Southeast Asia, Southeast Asia
Echinostomata				
Echinostoma	*sp.*	Consumption of fresh water crustaceans and fish	Intestine	Asia
Fasciolopsis	*buski*	Consumption of fresh water plants	Intestine	Asia
Fasciola	*hepatica, gigantica*	Ingestion of fresh water plants	Biliary ducts	worldwide, Asia and Africa
Gastrodiscoides	*hominis*	Consumption of fresh water plants or fish	Intestine	Asia
Opisthorchiata				
Clonorchis	*sinensis*	Consumption of fresh water fish	Biliary ducts	Southeast Asia and North America
Heterophyes	*heterophyes*	Consumption of fresh water fish	Intestine	Egypt and Asia
Metagonimus	*yokogawai*	Consumption of fresh water fish	Intestine	Europe and Asia
Opisthorchis	*viverrini, felineus*	Consumption of fresh water fish	Biliary and pancreatic ducts	Southeast Asia, Europe and Asia
Xiphidiata				
Paragonimus	*westermani*	Consumption of fresh water crustaceans	Lungs	Asia

nutrients via their teguments. Some species have evolved specialised tegument surface areas which secrete digestive enzymes for extra corporeal digestion of tissue by contact.

There is a great deal of diversity in the organ sites that adult Digenean trematodes occupy in their definitive host. However, the chosen sites almost invariably have large amounts of protein available for the parasites to ingest as their major nutritional source. The fluke species that are important human pathogens are been listed in Table 10.2. They belong to four sub-orders (Figure 10.4B), within which human parasitism appears to have evolved a number of times. These include the blood flukes (*Diplostomata*), lung flukes (*Xiphidiata*) and liver and intestinal flukes (*Echinostomata* and *Opisthorchiata*). One hallmark of many of these species is the wide range of definitive hosts they can infect; this may have contributed to their global distribution and success as parasites.

10.4.3 *Cestoda*

This group is comprised of over 5,000 species of endoparasites with elongated bodies, usually divided into segments. The anterior end of the parasite has an adhesive organ called a scolex. The scolex is often covered in pits (bothria), hooks and other features that anchor them to the intestinal wall (Figures 10.3D–F). In the segmented cestodes, the scolex is followed by an unsegmented neck and a chain, or strobila, of reproductive segments called proglottids, which contain both male and female reproductive organs (Figure 10.3E & F). As they mature, the proglottids which contain one or more eggs break off from the central mass and pass out of the host with its faeces. In some 'primitive' groups, there is a single set of reproductive organs, or there are multiple reproductive segments with only internal segmentation. Cestodes lack an alimentary tract and instead absorb nutrients through their tegument.

This group of parasites usually have complex life cycles involving two or more intermediate hosts with adults living in the intestines or alimentary canal of the definitive host. While the parasites may use a wide range of intermediate hosts, they often infect a very specific set of definitive hosts.

There are two orders of *Cestoda* containing species that commonly cause disease in humans: the *Pseudophyllidea* and *Cyclophyllidea* (Table 10.3 and Figure 10.4C). With the exception of the fish tapeworm (*Diphyllobothrium*) species, the *Pseudophyllidea* do not use humans as their definitive hosts, and pathology is caused by the misadventure of tissue-encysted plerocercoids. The *Cyclophyllidea* contain the majority of the medically important cestode species (*Taenia* and *Echinococcus* – Chapter 17). These species are widespread and can use humans as intermediate or definitive hosts.

10.5 The evolution of parasitism within the helminths: divergent phyla with common themes

The use of the term 'helminth', while convenient as a catchall, has unfortunately led many to believe that, because of their shared worm-like characteristics, these organisms also share common biology or evolutionary histories. Helminth groups and our understanding of their placement within the *Animalia* has historically been led by morphology-based taxonomies, which placed the Platyhelminths and nematodes as 'primitive' basal groups to 'higher' animals such as chordates and arthropods. Placement of more obscure phyla, such as the Acanthocephalids and Pentastomids, was controversial and generally inconsistent.

In the early- to mid-1990s, analysis of 18S rDNA and other genes lead to the publication of a series of studies which overturned the traditional taxonomies, and which has formed the basis for our current understanding of how the helminths fit into the animal kingdom. In these studies, three major lineages within the Bilateria were observed (Figure 10.5) – the *Lophotrochozoa*, the *Ecdysozoa* and the *Deuterostoma*.

How these different groups relate to each other evolutionarily is still being resolved. However, the long-held view that organisms like the nematodes and

Table 10.3 There are approximately ten species of cestodes that commonly infect humans. These species are listed here along with their intermediate host, definitive host and distribution. They have been subdivided into the two orders to which they belong. The most important agents of human disease are found in the *Cyclophyllidea* while, within the *Pseudophyllidea*, humans generally serve as intermediate hosts and disease is rarely observed.

		Intermediate host	Definitive host	Distribution
Cyclophyllidea				
Echinococcus	*granulosus*	Humans and ruminants	Canines	Worldwide
	multilocularis	Humans and ruminants	Canines	Northern hemisphere
Taenia	*saginata*	Cattle	Humans	Worldwide
	solium	Pigs	Humans	Worldwide
	asiatica	Pigs and cattle	Humans	Asia
Hymenolepis	*nana*	Arthropod or autoinfection	Humans and rodents	Worldwide
	diminuta	Arthropod	Humans and rats	Worldwide
Pseudophyllidea				
Diphyllobothrium	*latum and other sp.*	1st intermediate: copepod 2nd intermediate: fish	Humans	Northern hemisphere
	mansonoides	1st intermediate: copepod 2nd intermediate: many vertebrates including humans	Cats and dogs	North America
Spirometra	*mansoni and other sp.*	Many vertebrates including humans	Cats and dogs	Worldwide

platyhelminths (and their body plans) are basal to the evolution of the chordates is no longer accepted. Rather, the adoption of 'simple' body plans observed in many of the invertebrates may have been part of the adaptations they underwent to exploit particular niches.

Bearing this in mind, when the four major helminth groups previously discussed are mapped to these groups, the Platyhelminthes are placed within the *Lophotrochozoa*, the Nematodes and Pentastomids (which we now believe are crustaceans) within the *Ecdysozoa*, and the Acanthocephalids are closely allied with Rotifers outside of the other main *Bilateria* groupings (Figure 10.5). Thus, it seems likely each of these groups of organisms has separately developed characteristics that have been termed helminth-like as part of their adaptation to animal parasitism.

Despite these independent origins, there are some remarkably similar themes observed in helminth life cycles and physiology. For instance, within the nematodes, many of those species which occupy the intestine as adults go through a migratory phase in the lungs as larvae, even if their initial point of entry was oral (*Ascaris sp.*, *Strongyloides sp.* autoinfection). Interestingly, schistosomes also migrate to the mesenteric veins via the lungs.

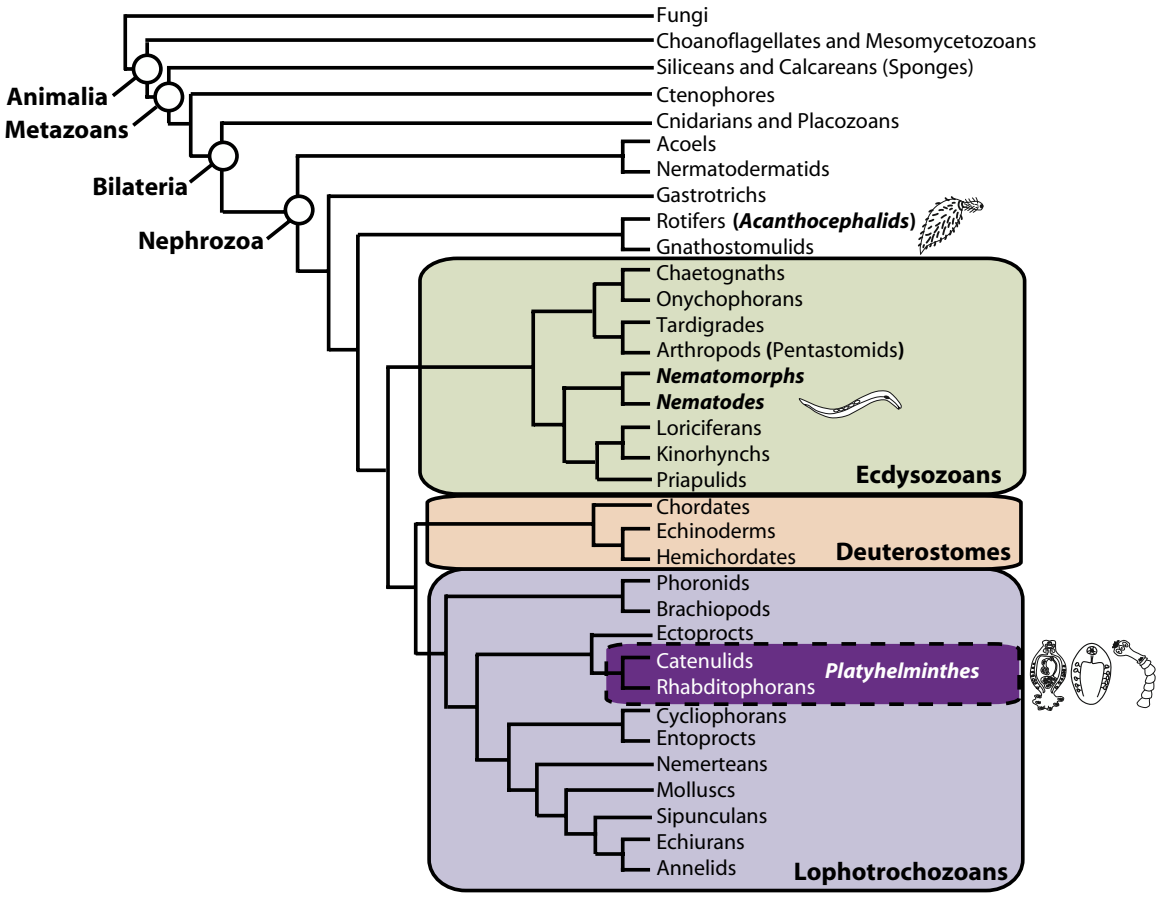

Figure 10.5 The evolution of the *Animalia* and parasitic helminths. The tree, adapted from Eernisse and Perterson (2004 – The History of Animals. In: Cracraft, J & Donoghue, MJ (eds.) *Assembling the Tree of Life*, pp. 197–208. Oxford University Press, New York.) shows a phylogenetic reconstruction of the *Animalia* based on the analysis of 18S rDNA and myosin II genes. The three major Bilaterian groups are highlighted. Phyla containing helminths that can infect humans are shown.

The lungs have been shown to be the major site of immune-mediated attrition of larval stages of these parasites, so why they traffic through this tissue remains an area of great interest, as well as the development of strategies to target larvae in this organ. The use of arthropod intermediate hosts seems a universally repeated theme in helminths, and the rarity of species that can autoinfect their hosts suggests that specific evolutionary or immunological pressures have pushed these parasites towards these common lifestyles.

Similarly, as more about the interactions of helminths with the immune system of their host is uncovered, it becomes obvious that some common approaches have been adopted by both parties. Parasites attempting to evade or resist the attentions of the immune system employ a battery of strategies, ranging from disguising themselves with host molecules to producing their own versions of host cytokines. Hosts trying to remove or segregate their unwanted passengers adopt specific cellular responses centred around Th2 cytokines.

Processes and molecules underlying the instigation of Th2 responses are still being defined. Whether they are truly protective, or the hosts' attempt at damage control, or even deliberately instigated by the parasite as part of their niche development within the host, remains unanswered.

10.6 Genomic and post-genomic exploration of helminth biology

Genomic and post-genomic technologies have ushered in a renaissance of study within the field of helminthology. In 1998, the first completed animal genome sequence of the model nematode *Caenorhabditis elegans* was published. It offered a treasure trove of potential information, not only to developmental biologists but also to those interested in using *C. elegans* as paradigm for understanding the biology of parasitic species.

Building on the success of the *C. elegans* genomic endeavour, a variety of sister projects were initiated that encompassed other nematode species, as well as those of parasitic and free-living Platyhelminths. These projects have steadily borne fruit, and the genomes or large scale transcriptome surveys of a variety parasitic helminths have been published over the past 12 years, with many more nearing completion (Table 10.4). These have offered researchers not only primary information about the genetic content of the parasites of interest, but also have prompted whole new avenues of inquiry which were completely unanticipated. One clear example of this is the rediscovery of the *Wolbachia* endosymbionts found within filarial nematodes (see Chapter 11) during the sequencing of the *B. malayi* genome. This has prompted the successful implementation of new drug therapies to treat some filarial infections, and has opened new areas of inquiry in filarial immunology.

The advent of large genomic or transcriptome datasets has helped to launch numerous post-genomic studies. From the perspective of immunologists, the most important of these are centred around firstly defining and characterising that portion of the parasite's proteome which interacts with the host's immune system, and secondly the development of transgenesis or RNA interference (RNAi) techniques that could be used to interrogate the function of specific genes in the context of infection. While the development of transgenesis or RNAi in helminth systems has been slow, proteomic techniques have succeeded in expanding our understanding of the composition of the surface and excretory/secretory (ES) products of a number of key species.

10.7 Summary

As we find out more about this unique group of organisms, it becomes clear how little we actually understand about how they have become such successful parasites. Despite being evolutionarily disparate, helminths show remarkable similarities in specific aspects of their biology and immunology. These commonalities inform us that selective pressures driving the acquisition of these traits have been felt independently by all of these parasite groups.

Table 10.4 This table lists a selection of helminth genome projects that have been completed (C) or are nearing completion (P), along with the research institution(s) contributing to the project and associated references. There have been a large number of helminth gene discovery and transcriptome-based projects that have also been published, but these are too numerous to be listed here. *Schmidtea mediterranea* is a free-living planarian that is amenable to genetic manipulation, and it has been developed as a model for organogenesis and tissue regeneration.

	Model	Status		Sequencing Institute	References
Nematoda			**Clade**		
Brugia malayi		C	III	TIGR	Ghedin, E *et al.* (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. *Science* 317(5845), 1756–1760.
Trichinella spiralis		C	I	TGC	Mitreva, M *et al.* (2011). The draft genome of the parasitic nematode *Trichinella spiralis*. *Nature Genetics* 43(3), 228–235.
Ascaris suum	Swine model of *A. lumbricoides* infection	C	III	TGC /WTSI	Jex, AR *et al.* (2011). *Ascaris suum* draft genome. *Science* 479(7374), 529–533
Nippostrongylus brasiliensis	Rodent model of hookworm infection	P	V	WTSI	
Strongyloides ratti	Rat model of *S. stercoralis*	P	IV	WTSI	
Trichuris muris	Mouse model of *T. trichiura* infection	P	I	WTSI	
Caenorhabditis elegans	Free-living model nematode	C	V	TGC/ WTSI/	*C. elegans* Genome Consortium. (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* 282(5396), 2012–2018.
Platyhelminthes			**Class**		
Schistosoma mansoni		C	trematode	TIGR/ WTSI	Berriman, M *et al.* (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature* 460(7253), 352–358.
Schistosoma japonicum,		C	trematode	CHGC	*S. japonicum* Genome Consortium (2009). The *Schistosoma japonicum* genome reveals features of host-parasite interplay. *Nature* 460(7253), 345–351.
Echinococcus granulosus		P	cestode	WTSI	
Echinococcus multilocularis		P	cestode	WTSI	

Table 10.4 (*Continued*)

	Model	Status		Sequencing Institute	References
Hymenolepis microstoma	Rodent model of tapeworm infection	P	cestode	WTSI	
Taenia solium		P	cestode	UNAM	Aguilar-Diaz, H *et al.* (2006). The genome project of *Taenia solium*. *Parasitology International* 55 Suppl. S127–130.
Schmidtea mediterranea	Free-living model planarian/ platyhelminth	P	turbellarian	TGC	Robb, SM *et al.* (2008). SmedGD: the Schmidtea mediterranea genome database. *Nucleic Acids Research* 36 (Database issue), D599–606.

Abbreviations: CHGC, The Chinese National Human Genome Centre, Shanghai China; TGC, The Genome Centre, Washington University School of Medicine, St. Louis, Missouri, USA; TIGR, The Institute for Genome Research (now J. Craig Venter Institute) Rockville, MD. USA; UNAM, Universidad Nacional Autónoma de México, Mexicao City Mexico; WTSI, Wellcome Trust Sanger Institute, Cambridge UK.

Similarly, as we uncover more about the interactions of helminths with their host's immune systems, we discover that, while they are each unique immunological entities, some common strategies have been adopted by both parties. Uncovering the hows and whys of these phenomena will ultimately give us vital information about our own immune systems and how to manipulate them successfully to resist these pathogens.

10.7.1 For general information on helminths:

More specific information on the life cycles and biology of some helminths will follow in subsequent chapters within this section of this book. However, for those wishing to obtain a more detailed view of the biology of these organisms, the textbooks *Foundations in Parasitology* by Schmidt and Roberts (WC Brown Publishers, USA) and *Introduction to Animal Parasitology*' by J.D. Smyth (Cambridge University Press, Cambridge, UK) offer an excellent starting point. The websites maintained by the USA CDC's Division of Parasitic Diseases (http://dpd.cdc.gov/dpdx/Default.htm) also offers in-depth account of all groups and species discussed.

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Nematoda: Filarial Nematodes

11

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Filarioidea are parasitic nematodes that can infect all classes of vertebrates other than fish. A common characteristic of infections with filarial nematodes is that they enter the host silently and persist many years. In a majority of infected individuals, there is a striking absence of disease. However, the minority of infected people that develop immunopathological conditions as a result of these infections represent a major health problem; in human filarial infections, the disability adjusted life years (DALYs) are estimated to total 6.3 million years. Pathology is mainly caused by adult parasites (*Loa, Brugia, Wuchereria, Dirofilaria*) or larval stages (microfilariae: *Onchocerca*). Filarial infections are also of importance in domestic and farm animals.

This chapter will discuss recent findings about the immunobiology of human filarial infections, as well as findings obtained from studies of animal infections such as *Onchocerca ochengi* in cattle and *Brugia malayi* and *Litomosoides sigmodontis* infections in laboratory rodents. An understanding of helminth-induced modulation of the host immune response is important for eradication strategies, and it may also offer new treatment options for autoimmune diseases (see Chapter 24).

11.1 The life cycle and pathogenesis of filarial nematode infections

11.1.1 Life cycle

The superfamily Filarioidea comprises the two families Filariidae and Onchocercidae, of which the Onchocercidae (the larger family) consists of almost a hundred genera. Filarial nematodes have a slender, thread-like body, a simple anterior end with inconspicuous oral lips, a cylindrical oesophagus

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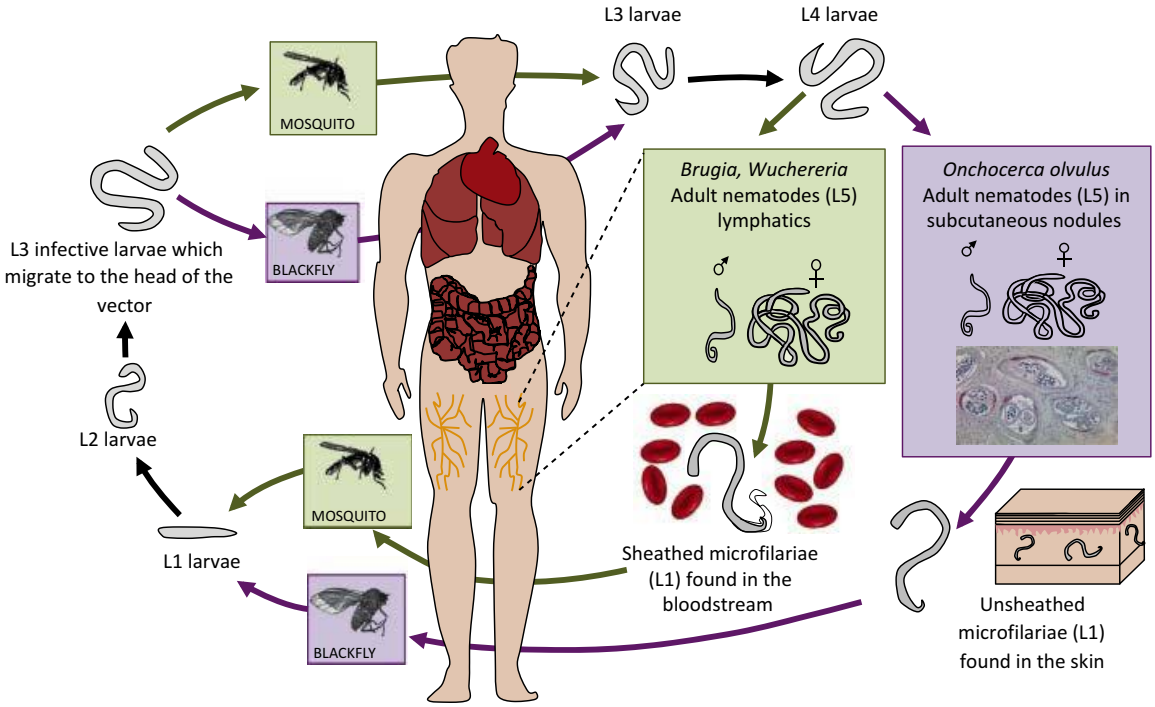

Figure 11.1 The life cycle of filarial nematodes. The purple boxes and arrows refer to *Onchoocerca*, while the green boxes and arrows refer to filarial nematodes that cause lymphatic filariasis (*Brugia*/*Wuchereria* sp.) Black arrows depict common aspects of the life cycle of these filarial nematodes.

lacking a bulbus and often unequal and dissimilar copulatory spicules in the male. A common characteristic of the life cycle of filarial nematodes is the transmission by haematophagous arthropod intermediate hosts, including biting midges (Culicoides), black flies (Simulidae), horse and deer flies (Tabanidae), mosquitoes (Culicidae), lice, mites and ticks.

The feeding vector ingests the first larval stage (L_1 microfilariae, (Mf)) that develops into the infective larval stage (L_3) after two moults (Figure 11.1). Filariae with skin-dwelling Mf (such as *Onchocerca volvulus*, the causative agent of river blindness) are transmitted exclusively by telmophagic (pool-feeding) vectors like black flies (Simuliidae). Mf migrate from the dermis into the pool of blood and tissue fluid, and they are ingested by the feeding arthropod.

Filarial species whose Mf are present in the peripheral blood may be transmitted either by pool feeders or by solenophagic (vessel feeding) arthropods (such as mosquitoes). The infective L_3 will be transmitted to a subsequent host during the next act of feeding by the vector. After a migratory period in the body, the filariae settle in host tissues and undergo two further moults, at which point they develop into mature, sexually dimorphic adults (macrofilariae), that mate and produce Mf, completing the life cycle. The migratory route, the location of adult parasites and the location of Mf within the body varies among the many

filarial nematodes, and this contributes to the diversity of pathologies observed upon infection with these parasites.

11.1.2 Clinical manifestations

11.1.2.1 Lymphatic filariasis

Lymphatic filariasis is caused by *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori*, and the adult nematode is located within the lymphatic vessels of the scrotal area or the leg. The clinical symptoms begin with filarial fever caused by the death of adult nematodes, and this typically leads to retrograde lymphangitis and lymphadenitis. This is followed by dilation of the lymph vessels (probably an early event, following antigenic stimulation) and lymphoedema and/or hydrocele.

11.1.2.2 Onchocerciasis

Onchocerciasis is caused by infection with *O. volvulus*. Disease symptoms such as keratitis, chorioretinitis and various forms of dermatitis are caused by the Mf that migrate through the skin and the eye after release from adult nematodes residing in subcutaneous nodules. In many cases, Mf elicit an inflammatory immune response when they are degenerated or moribund, or if they are exposed to the microfilaricidal drug ivermectin (this drug leads to Mf immobilisation).

In the hyperactive form of onchocerciasis (also called Sowda), a strong antibody-mediated immune reaction to healthy Mf occurs, which facilitates activation of eosinophils, macrophages and neutrophils to clear viable Mf from the body. Sowda patients present with a high-grade dermatitis and eosinophilia, the latter of which can result in the development of life-threatening cardiac fibrosis.

11.1.2.3 Loiasis

In loiasis, adult worms migrate freely through the subcutaneous tissues, occasionally crossing into sub-conjunctival tissues, where they can be visualised (hence *Loa loa* is often referred to as the 'eye worm'). Mf of *L. loa* circulate in the peripheral blood and display diurnal periodicity, retreating to the pulmonary capillaries at night. They are ingested by day-biting vector species of the genus *Chrysops* (commonly known as mango or deer flies). The major clinical sign of a bite from the *Chrysops* fly is a Calabar swelling, a localised angioedema or erythema that usually appears in the arms and legs and may last for 1–3 days. This can also be accompanied by urticaria and pruritus.

11.1.2.4 Heartworm

Of the Dirofilaria nematodes, *Dirofilaria immitis* ('heartworm') is the most widespread species due to its association with domestic animals. Living in the cardiovascular system, *D. immitis* can cause life-threatening cardiopulmonary disease in cats and dogs. In contrast, *Dirofilaria repens* resides in the

subcutaneous tissues. Mosquitoes of the genera *Aedes, Anopheles* or *Culex* are the main vectors of *Dirofilaria* species.

Human infection is incidental and non-productive (usually only one worm is transmitted, and therefore mating (and the production of transmissible Mf) does not occur). Human infection with Dirofilaria species is typically not associated with severe disease, but the clinical manifestations of human dirofilariasis are pulmonary complications and subcutaneous lesions.

11.2 Animal models of filariasis

Animal models of filariasis have significantly contributed to our current knowledge of immune responses mounted during human filarial nematode infections. However, investigation of the pathogenesis is limited because manifestations such as lymphoedema only develop in larger mammals (such as cats or dogs) infected with *Brugia spp.* The most commonly used animal models to investigate the immunology of filarial nematode infections are outlined below.

11.2.1 *Litomosoides sigmodontis* infections of rodents

L. sigmodontis, a natural parasite of the cotton rat (*Sigmodon hispidus)*, develops full patency in Mongolian gerbils (also known as jirds) (*Meriones unguiculatus*) and laboratory BALB/c mice. In these mice, circulating Mf are visible in the periphery within 55–60 days post-infection, whereas the life cycle is not completed in other mouse strains, such as C57BL/6 mice, because the filariae are progressively killed.

11.2.2 *Brugia* infections of rodents

Mice infected with L_3s of *B. malayi* (or the feline filarial nematode *Brugia pahangi*) do not develop circulating microfilariae, whereas patent infections can be established in the multi-mammate mouse (*Mastomys coucha*) upon infection with *Brugia* spp. or the filarial nematode *Acanthocheilonema viteae*. The extent to which these infections are helpful in unravelling the components of the anti-filarial immune response is limited by the lack of immunological tools for these two rodent species. Alternatively, implanting *Brugia* adult worms into the peritoneal cavity of mice has proven a suitable model for examining the immunology of filarial nematode infections.

11.2.3 *Onchocerca ochengi* infections of cattle

O. ochengi is a close relative of *O. volvulus* and it infects cattle. This infection occurs naturally in Africa and, as such, is not a model *per se*. However, as in humans infected with *O. volvulus*, cows infected with *O. ochengi* develop subcutaneous nodules which can be removed for immunological analysis or used to assess of the efficacy of candidate prevention therapies.

11.3 Immune responses mounted against filarial nematodes

In general, nematodes induce a type 2 immune response in their mammalian host, and filarial nematodes are no exception. Filarial nematode infections of mice have demonstrated the efficacy of Th2-associated cytokines and effector cells in providing protection against infection. However, the picture is more complex than this. There is a concomitant induction of the Th1 arm of the immune response in many (but not all) filarial nematode infections. It is now known that Th1 responses are mounted against mutualistic endosymbiotic bacteria of the genus *Wolbachia*, found within filarial nematodes.

Studies have shown that filarial-infected humans, cattle and mice all mount immune responses to *Wolbachia* proteins (for example *Wolbachia* surface protein (WSP) or *Wolbachia* lipoprotein (WoLP)) and produce acute-phase inflammatory proteins such as tumour necrosis factor (TNF) and interleukin (IL)-β as a result. Both Th1- and Th2-type responses contribute to inflammatory and effector mechanisms that can limit parasite invasion but also lead to pathology. Individual filarial nematodes can survive an average of ten years in their host but, in the majority of individuals, the course of the disease is asymptomatic. This is because modulation and evasion of the immune system occurs in filarial nematode infection, with concomitant suppression of both Th1 and Th2 responses that prevents tissue damage.

11.4 Innate immunity

11.4.1 Immune responses during L_3 entry

The first barrier that filarial nematodes must overcome is penetration of the skin of the host. In the *L. sigmodontis* model, innate immune responses are associated with the destruction of a majority of L_3 in the subcutaneous tissue within two days post-infection, with approximately one-third of the L_3 larvae successfully entering the host. Thus, there is prompt induction of host innate defence mechanisms, as observed when injecting *B. pahangi* L_3 into Mongolian jirds, where neutrophils can be found around the site of injection.

Upon larval stimulation, skin mast cells vigorously degranulate, increasing vascular permeability and, in turn, facilitating larval survival (Figure 11.2). It has been found that degranulation may be somewhat dependent on *Wolbachia* endosymbionts. This evasion mechanism may be counter-regulated by host production of the chemokine CCL-17, which dampens mast cell activation within the skin.

L_3 stages have also been found to down-regulate Langerhans cells (dendritic cells of the skin) in primary infections of animal models, in particular with regards to their capacity to activate CD4+ T cells (Figure 11.2). This is thought to be another mechanism that filarial nematodes use to promote the establishment of infection. The skin has also been shown to be important during vaccination trials; vaccination with irradiated L_3 (xL_3) (which can lead to protection of over 70 per cent) induces the production of IL-5 (a cytokine which

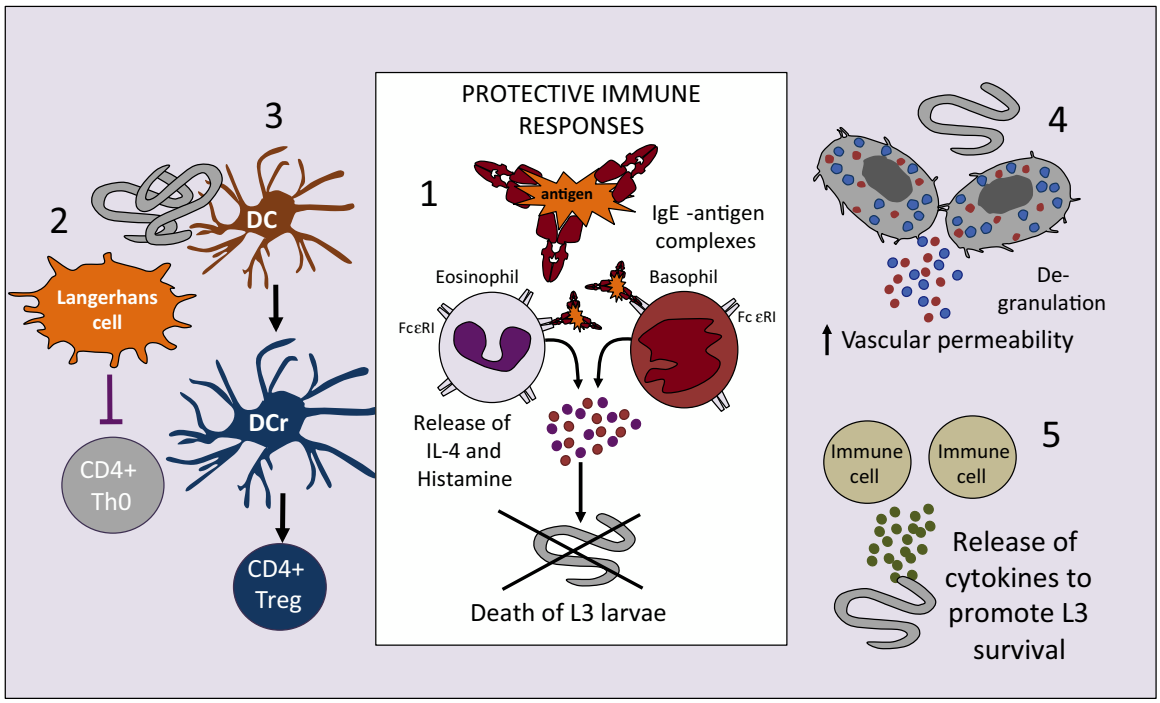

Figure 11.2 Immune responses to the infective L_3 of filarial nematode infections. From *in vitro* and *in vivo* studies of experimental models, protective immune responses against L_3 stages (shown in the white box) appears to depend on the degranulation of eosinophils and basophils in response to Fcε ligation of IgE/antigen complexes with the release of IL-4 and histamine resulting in the death of incoming L_3s (1). Immune mechanisms which promote the survival of L_3 stages (shown in the purple box) include the down-regulation of Langerhans cells in the skin (2), the activation of DCs to regulatory DCs with the promotion of Tregs (3), increase in vascular permeability via mast cell degranulation (4) and the release of cytokines (which may act as growth factors) from immune cells including activated NK cells (5).
Abbbrevation: DC, dendritic cell; DCr, regulatory DC; Ig, immunoglobulin; IL, interleukin; NK, natural killer; Th, T helper cell; Treg, T regulatory cell.

promotes the growth and differentiation of eosinophils) and activates B cells to produce anti-filarial antibodies. In vaccinated protected mice, eosinophils infiltrated into the subcutaneous tissue and degranulated in a B cell/antibody dependent mechanism. The requirement for antibodies to facilitate eosinophil degranulation (in particular IgE – see Chapter 1) is absolute, because vaccination of μMT mice (lacking mature B cells and antibody) with xL_3s does not result in protection.

11.4.2 Dendritic cells and macrophages

As with many infections, dendritic cells (DC) and macrophages react vigorously to filarial nematodes and filarial nematode-associated products, in order to activate the appropriate adaptive immune responses to clear invading nematodes. Filarial extracts have the ability to stimulate typical Toll-like receptor (TLR)-dependent inflammatory responses, leading to secretion of acute phase cytokines such as IL-6 and TNF by macrophages. *Wolbachia*-derived molecules, such as WSP and WoLP, are the main sources of such innate responses; responses to *Wolbachia*-free filarial extract produced by antibiotic

depletion of the endosymbionts, or obtained from the *Wolbachia*-free species *A. viteae*, are tenfold lower. In particular, these molecules are recognised by TLR -2, -4, -6, and the adapter molecule Myd88 is required to transduce signals from these TLRs to mediate the transcription of pro-inflammatory cytokines.

The biological relevance of the recognition of *Wolbachia*-derived molecules in the context of the anti-filarial nematode response is still unclear because, with the exception of C3H/HeJ mice, which have a natural mutation in TLR4, no strong phenotypes have been observed on the infection profile of several mouse strains deficient for components of the TLR/MyD88 signalling cascade. In addition, despite the presence of endosymbiotic bacteria, filarial-exposed DCs and macrophages can direct the immune system towards the Th2 or immunosuppressive/regulatory phenotype responses (see below).

11.4.3 Granulocytes

Granulocytes have been associated with immune responses against helminthic parasites for a long time. In filarial infections, neutrophils are present in *Onchocerca* nodules in both human and bovine filarial nematode infections. The recruitment of neutrophils to the nodules is dependent on the presence of *Wolbachia* in the infecting filarial nematode. In the absence of *Wolbachia* bacteria, eosinophil accumulation replaces the recruitment of neutrophils and activated eosinophils degranulate on the cuticle of adult worms. The degranulation of eosinophils precedes parasite death and, therefore, it is possible that the recruitment of neutrophils could be an immune evasion mechanism which keeps eosinophils away from the nematodes. However, neutrophils are not harmless for filarial nematodes, and *L. sigmodontis* infections of mice with an impaired capacity for neutrophil activation also display reduced parasite clearance, suggesting a protective role of neutrophils against filarial nematode infection.

Vaccine studies support the hypothesis that eosinophil activation is a primary host determinant for filarial life expectancy. Operating both against larval and adult stages in anatomically and temporally separate locations, filarial nematodes infecting mice which are deficient in IL-5 (and are therefore without functional eosinophils) survive and reproduce well beyond their life span in an immunologically intact host; in mice genetically modified to over-express IL-5, eosinophilia leads to enhanced parasite killing. Intriguingly, recent data indicate that larval growth may be enhanced by IL-5 production by the host, suggesting that pre-adult stages may not be a primary target for eosinophil-mediated killing.

Basophils are a cell population that are difficult to study, but they are known to release intracellular stores of histamine and IL-4 in response to ligation of FcεRs by cross-linked IgE. Cells from filarial nematode-infected individuals have been shown to release histamine and IL-4 via filarial-specific IgE, suggesting that basophils participate in the anti-filarial immune response. Studies in the *L. sigmodontis* mouse model support this idea; IL-4 is produced by basophils in *L. sigmodontis* infection, and depletion of basophils causes a significant decrease in total and parasite-specific IgE, eosinophilia and CD4+ T cell proliferation. The biological significance of basophil responses is still unclear; basophil

depletion has no effect on parasite loads in the *L. sigmodontis* model, and their main role may be to amplify Th2 responses.

11.4.4 Natural killer cells (NK)

NK cells can contribute to innate immune responses by release of cytotoxic granules as well as cytokine production (see Chapter 1). The role of NK cells in filarial nematode infection is controversial; one study has shown that NK depletion from BALB/c mice results in higher parasite numbers (protective role), whereas another has shown that stimulation of NK cells promotes parasite development.

NK cells may alter the course of filarial nematode infection through either their cytotoxic capacity (direct killing of nematodes) or through release of cytokines (enhancing the induction of adaptive immune responses). L_3 stages or Mf incubated with human peripheral blood mononuclear cells (PBMCs) results in the activation of NK cells, which subsequently release interferon (IFN)-γ and TNF. Stimulation with Mf also results in the secretion of the Th2 cytokines IL-4 and IL-5 from activated NK cells within PBMCs.

NK cell-filarial nematode interactions are complex, and NK cells may play different roles, depending on the parasite stage and phase of infection. Further studies are needed to clarify their role in filarial infections.

11.5 Adaptive immunity

The ability of helminth parasites to induce protective Th2 immune responses is well documented. Th2 responses are protective against filarial nematode infection and, in onchocerciasis, patients with a pronounced Th2 response have lower levels of circulating Mf. However, Th2 responses in onchocerciasis, when excessive, are also associated with pathogenic symptoms. An overspill of the Th2 response occurs in Sowda patients, enabling clearance of parasites at the expense of severe cutaneous pathology.

In filarial nematode infections of mice, a Th2 response is induced upon initial contact with infective L_3 (*B. pahangi*), and this can be observed in the *L. sigmodontis* model by day 12 post-infection. Depletion of Th2 associated cytokines or the CD4+ T cells that drive Th2 responses impair filarial nematode clearance in mice; IL-5 is known to control adult nematode survival, because mice lacking IL-5 harbour more parasites than immunologically intact control mice during infection. Furthermore, both IL-4 and IL-5, the predominant cytokines produced during filarial infection, have been shown to be responsible for controlling patency. In the absence of these cytokines, *L. sigmodontis*-infected mice have a higher circulating Mf count, partly because of prolonged Mf survival. These findings are consistent with studies using *Brugia* species.

Although it is generally accepted that Th2 responses can contribute to the control of filarial nematodes infections, IL-4Rα-deficient mice that are unable to

respond either to IL-4 or IL-13, or to mount a significant Th2 response, instead mount a pro-inflammatory Th1 response which reduces the survival of adult nematodes. This suggests that adult filarial nematodes may be susceptible to attack by Th1 effector mechanisms, in addition to Th2 effector mechanisms described above. The importance of Th1 responses for the defence against filarial nematodes is further highlighted by *L. sigmodontis* infections of IFN-γ deficient mice, which accommodate higher burdens of adult nematodes than immunologically intact control animals.

There is a long-standing observation that Mf induce Th1 responses, possibly a consequence of Mf degeneration and the release of *Wolbachia*. The generation of a Th1 response by Mf may function to down-regulate the Th2 responses established by the larvae and adult parasite stages, thus preventing clearance from the body and, in turn, promoting transmission.

Regulatory T cells (Tregs) are a feature of filarial nematode infection and can down-regulate both Th1 and Th2 responses. Given that, collectively, Th1 and Th2 responses function to reduce L_3 establishment, adult survival and reproduction, as well as to promote clearance of circulating Mf, immunoregulatory mechanisms such as the activation of Tregs is clearly beneficial to the parasite. One mediator of immune suppression is IL-10, and this cytokine is produced abundantly in filarial nematode infections of humans as well as in animal models. Intracellular staining of cells from *W. bancrofti*-infected individuals has revealed the presence of IL-10-producing CD4+ T cells as well as IL-4-producing CD4+ T cells.

11.6 Immune evasion

It is of interest for both the filarial nematodes and the host to limit the immune reaction in order to promote successful mating and transmission in the former, and limit immunopathology in the latter. In humans, and probably other mammalians that host filarial nematodes, immune down-regulation appears to start before birth. Studies in children born to filarial nematode-infected mothers have suggest that the immune system may be primed *in utero* to mount regulatory immune responses, which leads to fewer disease manifestations but, at the same time, leads to higher parasite loads.

Typical characteristics of this suppression include lower proliferation of T cells from umbilical blood, increased production of the immunoregulatory cytokine IL-10 and, concomitantly, a propensity of B cells to switch to produce the isotype IgG4 in response to anti-filarial antigens (IL-10 induces IgG4 production in B cells). In humans, high plasma concentrations of IgG4 have been reported from individuals with hyporesponsive immune systems and asymptomatic cases of helminth infection.

The other subclasses of IgG (IgG1, IgG2 and IgG3) can all activate immune effector mechanisms such as the complement system or antibody-dependent cytotoxicity (ADCC), but IgG4 is unable to do so. IgG4 can compete with IgE

for binding to Fcε receptors, but without inducing downstream effects elicited by ligation of cross-linked IgE. Therefore, IL-10 can suppress the immune response to filarial nematodes by inducing the production of IgG4 antibodies, in turn hindering the activation of complement and eosinophil degranulation (to inflict damage to all stages of filarial nematodes) as well as ADCC (which can clear Mf from the circulation).

IL-10-producing CD25+ Tregs have been isolated from worm-harbouring nodules in human *O. volvulus* infection, suggesting that they may play some role in promoting parasite survival. Indeed, depletion of CD25+ T cells in susceptible BALB/c mice subsequently infected with *L. sigmodontis* significantly reduced the number of adult parasites. In these experiments, chronic infection was associated with the development of hyporesponsive CD4+ cells that expressed high levels of Glucocorticoid-induced TNF receptor family-related protein (GITR) and CTLA-4, in addition to Tregs. Immune responsiveness to *L. sigmodontis* in this study could be restored by removal of Tregs, in addition to all cells expressing CTLA-4, indicating that Treg induction of hyporesponsive T cells is a mechanism of immune evasion in filarial nematode infection.

Macrophages are also recruited to sites where nematodes are located, and these are alternatively activated as characterised by the expression of arginase 1, the secreted chitinase-like lectin Ym-1 and resistin-like molecule (RELM)-α (also known as "found in inflammatory zone (Fizz) 1"). Alternatively, activated macrophages express the stereotypical macrophages surface marker F4/80, and alternative activation of macrophages occurs after exposure to the Th2 cytokines IL-4 and IL-13. Macrophage activation by Th2 cytokines is known to regulate immune responses and facilitate tissue repair, and it may contribute to the survival of filarial nematodes in the host via the release of IL-10 and transforming growth factor (TGF)- ß and consequent immunosuppression. Indeed, in *L. sigmodontis* infections, alternatively activated macrophages can suppress CD4+ T cell proliferation.

These cells may be an additional route for immune suppression in filarial nematode infections because, as already discussed above, depletion of Tregs is sufficient to prevent immune hyporesponsiveness and reduce nematode burden in *L. sigmodontis* infection, despite the presence of alternatively activated macrophages. In addition, recent data in the *Brugia* animal model has revealed that long-term exposure to Th2 cytokines and anti-inflammatory signals does not result in terminal differentiation of alternatively activated macrophages; these cells can be reprogrammed to the classically activated phenotype in response to LPS and IFN-γ.

There are few studies on alternatively activated macrophages in humans, due to the lack of specific markers. However, gene expression patterns of monocytes derived from asymptomatic patients are characterised by diminished expression of nitric oxide synthase (*NOS*)*2* (expressed by classically activated macrophages) and significantly enhanced expression of arginase (*ARG*)*1*, resistin, mannose receptor C type 1, macrophage galactose type C lectin and CCL18 – suggesting an alternatively activated phenotype. When re-stimulated with *Brugia* extract, purified monocytes from these patients produced high

levels of IL-10 and TGF-ß, which is consistent with theories explaining the immunology of asymptomatic filarial nematode infections.

11.6.1 Immune evasion molecules

The identification and characterisation of inflammatory molecules aids our understanding of host/parasite interactions and may lead the way to promising vaccine candidates. Additionally, parasite-derived immune suppressing agents may be promising candidates for treatment of autoimmune diseases or allergies.

The most well-defined immune-modulatory molecule derived from filarial nematodes is ES-62, a tetrameric glycoprotein that is linked to phosphorylcholine moieties that signals via TLR-4 in a non-classical manner and induces down-regulation of the signalling enzyme protein kinase C-α (see Chapter 24). Likewise, cystatin, a molecule released from *A. viteae*, *B. malayi* and *O. volvulus,* is efficient at reducing progression of inflammatory diseases via IL-10-dependent modulation of macrophages. Cystatin from *A. viteae* is taken up by macrophages, inducing phosphorylation of the mitogen-activated protein kinases (MAPK) ERK1/2 and p38 and modulating cytokine production.

In addition to the production and secretion of immunomodulatory molecules, filarial nematodes have evolved to express molecules that are homologous to human regulatory cytokines. A homologue of TGF-ß is produced by filarial nematodes and is strongly expressed in the hypodermis, epithelia and muscle tissue of *O. volvulus*. Two genes orthologous to mammalian TGF-ß have been cloned from *B. malayi*, *Bm*-tgh-1 and *Bm*-tgh-2). The product of *Bm*-tgh-2 is known to be secreted and can bind to mammalian TGF-ß receptors. Hypothetically, this molecule may be functional in driving host regulatory T cell differentiation.

Macrophage migration inhibitory factor (MIF) exerts wide-ranging, pro-inflammatory effects on the immune system. *B. malayi* synthesises two molecules that are homologous with mouse MIF that are able to induce pro-inflammatory cytokine secretion and up-regulation of the receptor for IL-4 in bone marrow-derived macrophages. Furthermore, when added in combination with recombinant IL-4, macrophages expressed markers of alternative activation, suggesting that MIF may promote the differentiation of alternatively activated macrophages in the context of the anti-filarial immune response.

Comparative gene expression studies in the search of novel (non-orthologous) genes that may modulate the immune system have identified the abundant larval transcript antigens (ALT) in infective L_3 stages (these comprise five per cent of the cDNA of the infective larval stage). These molecules are shared among filarial species, and a distantly related sequence exists in *Caenorhabditis elegans* (although *C. elegans* ALT lacks the acidic domain).

It has been hypothesised that ALT proteins may play a role in modulating the immune responses against L_3 stages, facilitating establishment of infection. In support of this hypothesis, ALT proteins have been shown to induce over

76 per cent protection in vaccination studies using animal models of filarial infection (see Chapter 25.4). The immunomodulatory activity of ALT proteins may be dependent on the acidic domain, since *C. elegans* is a free-living organism.

The immunomodulatory potential of the ALT proteins has been tested by analysis of the effect of heterologous expression in *Leishmania* parasites. In this system, ALT proteins up-regulated SOCS-1 and GATA-3, both molecules associated with the induction of Th2 responses observed in *in vivo* filarial infection. In doing so, they promoted intracellular survival of *Leishmania* in macrophages (possibly by impairing Th1 responses known to be required for immunity in *Leishmania* infection; see Chapter 7).

11.7 Immunopathology

In onchocerciasis, the majority of infected people have relatively high parasite loads and infected individuals have daily turnover of approximately 50,000 Mf, with over 10 microfilariae/mg skin. Despite this, most people only suffer mild dermatitis, probably because the mixed Th1 and Th2 immune response that develops (which is dominated by Th2) is counter-regulated by antigen-specific regulatory T cells, which produce high amounts of interleukin (IL)-10 and/or TGF-β and, in turn, successfully prevent excessive pathology.

However hyper-reactive onchocerciasis and Sowda is characterised by excessive production of Th2 cytokines (IL-4, IL-5, IL-13) and highly elevated IgE responses. This enhanced response, if uncontrolled, can lead to severe damage of the host, resulting in dermatitis and blindness. This has been demonstrated in an animal model of river blindness, whereby extracts of filarial nematodes were inoculated into the cornea of the mice. The mice developed a corneal hazing (indicative of damage), but only when the injected nematode extracts contained *Wolbachia*. No damage was observed when extracts from *Wolbachia*-free nematodes were injected. Therefore, it may be the case that damage of the cornea by Mf trapped there in *O. volvulus* infection is dependent on immune responses mounted against *Wolbachia* endosymbionts, rather than the filarial nematodes *per se*.

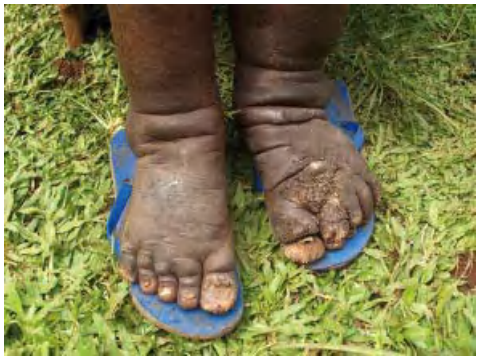

Figure 11.3 Lymphoedema caused by filarial nematode infection.

An excessive pro-inflammatory immune response may be responsible for alteration of lymphatic vessels in lymphatic filariasis. Dilation and obstruction of the vessels occurs, resulting in lymphatic pathology such as lymphoedema (Figure 11.3) or different forms of filaricele such as hydrocele, lymphocele and chylocele. A chronic immune stimulation by incoming L_3 larvae (of which a considerable percentage will die), dying adult worms and degenerated embryos and larvae released from fecund adult female worms is a low-level and constant trigger of innate immune cascades, with the end result that (lymph-) angiogenesis takes place (induced by vascular endothelial growth factors).

Both *bona fide* worm molecules, as well as molecules from *Wolbachia* endosymbionts, trigger the innate system.

Wolbachia-derived molecules, in particular, seem to play a major role in this process, since depletion of *Wolbachia* by antibiotic treatment in humans precedes a reduction of these molecules and leads to improvement of lymphatic pathology. Both lymphoedema and lymphangiogenesis develop progressively, but only a minority of affected individuals will develop the most severe manifestations of lymphatic filariasis. Evidence suggests that propensity to developing such pathology filariasis is a genetic trait, and single nucleotide polymorphisms in the promoter for the gene encoding vascular endothelial growth factor-A have been associated with lymphoedema in some studies.

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Nematoda: *Ascaris lumbricoides*

12

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12.1 Introduction

We currently understand very little of the immune response to human ascariasis, despite the widespread distribution of this parasitic nematode, particularly in comparison to other helminthiases, such as schistosomiasis and filariasis. *Ascaris lumbricoides* infects between 800 million and 1.2 billion people globally, but is predominantly prevalent in developing countries. The disease ascariasis, associated with the parasitic worm, leads to morbidity, with serious health consequences in approximately 122 million cases per year. However, ascariasis is still considered a neglected tropical disease (NTD).

Ascaris suum is a widespread parasitic nematode that causes infection in pigs, with high prevalence rates in host populations. Porcine ascariasis interferes with the health of pigs, resulting in reduced feed to gain ratios and liver condemnation, incurring economic losses. *A. lumbricoides* and *A. suum* are closely related at a phylogenetic level, as the two parasitic worm species differ by only six (1.3 per cent) nucleotides in the first internal transcribed spacer (ITS-1) and differ in sequence by 3–4 per cent in the mitochondrial genome (mtDNA).

Human and pig *Ascaris* are morphologically indistinguishable species and, while each demonstrates strong affinity for their conventional host, cross-infection has been documented. Infected human hosts were found to harbour worms of pig origin in North America and Denmark, but molecular epidemiological studies in *Ascaris* endemic regions of Guatemala and China indicate that the level of cross-infection between host species is low or absent, and that gene flow is limited between/among different genotypes.

Immunity to Parasitic Infection, First Edition. Edited by Tracey J. Lamb.

12.2 *Ascaris* infection displays an over-dispersed frequency distribution

Ascaris of both human and pig origin exhibit an over-dispersed frequency distribution, which results in the worms aggregating in few heavily infected hosts. This epidemiological pattern, in which 'wormy persons' harbour disproportionately large worm burdens, is also high within groups stratified by age and sex. A consistent variability in infection intensity is also observed between hosts, which generates the over-dispersed frequency distribution of *Ascaris* infection. Predisposition to heavy and light worm burdens has been demonstrated in longitudinal studies for ascariasis in both humans and pigs, and it can be detected over multiple rounds of chemotherapy. While predisposition to *A. lumbricoides* is observed within age cohorts, changes in the average intensity of *A. lumbricoides* infection with age are convex in form, with intensity peaking in the 5–10 year old age group. The strength of predisposition decreases with age.

Multiple studies investigating the role of exposure in influencing infection intensities and predisposition has led to different results. While some studies indicate that over half of the variability in average worm counts among households is explained by household risk factors, other studies, using population genetic analysis of *Ascaris* worms in households, have suggested that transmission is focal around households and unrelated to infection intensity.

Despite research indicating that exposure is not the entirely responsible for consistent variations in host infection intensities, the bases for heterogeneity of infection or predisposition are not yet fully understood. However, there are strong lines of evidence to indicate that the underlying mechanism of resistance/susceptibility to *Ascaris* infection is influenced by host genetics and the immune repertoire of the host.

12.3 Life cycle

Hosts contract *Ascaris* infection via the faecal-oral route. When infective eggs are ingested and hatch, *Ascaris* larvae develop in host parenteral tissues. *Ascaris* larvae undergo extra-intestinal migration (Figure 12.1), and knowledge of the pattern of *A. suum* larval migration stems from a range of studies systematically examining the intestinal, hepatic and pulmonary larval counts in both pigs and abnormal hosts. Following ingestion of infective ova, L_3 larvae (covered by the L_2 cuticle) hatch in the small intestine and migrate to the caecum and proximal colon, where they penetrate the mucosa. The larvae then migrate via the portal blood to reach the liver, where the L_2 cuticle is shed. After migration in the liver, the larvae advance to the lungs on days 6–8 post-ingestion. The larvae penetrate the alveolar space and move to the pharynx, where they are swallowed, resulting in the helminths returning to the small intestine around days 8–10 post-ingestion. *A. suum* moult again to L_4 stage larvae in the small intestine on day 10 post-ingestion, and it is the small intestine where the larvae mature, undergoing a final moult (L_5 stage larvae) on day 24 post-ingestion to reach sexual maturity.

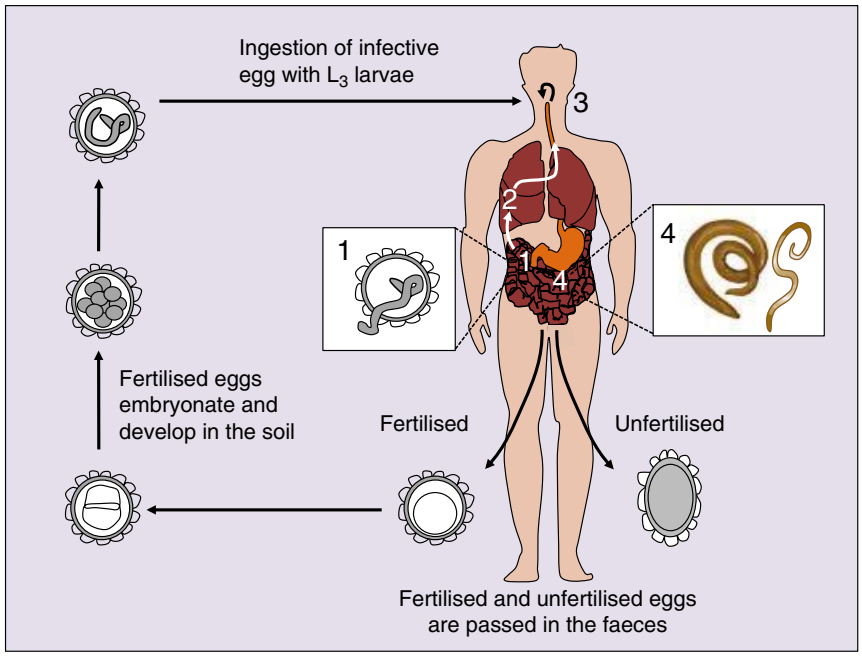

Figure 12.1 The life cycle of *Ascaris*. 1. Eggs hatch in the small intestine. 2. Larvae penetrate the large intestinal wall and migrate to the liver and lungs. 3. Larvae ascend the bronchial tree to the throat and are swallowed. 4. Worms mature and reside in the small intestine for 1–2 years.

The hepato-tracheal migration takes place over a 10 or 14 day period after the ingestion of eggs in pigs and humans respectively. Adult worms may reside in the intestines for approximately one year, but in pig infections the majority of worms are expelled by the 23rd week of infection. Male and female adult worms measure 15–25 cm and 20–35 cm respectively. Estimates of daily *Ascaris* female egg production generally are in the range of 200,000 eggs, but the number of eggs a female produces decreases with worm load. Unembryonated ova enter the environment via the faeces and can remain viable in the soil for up to 15 years; their viability is influenced by variables such as moisture, oxygen and shade. During embryonation, larvae undergo two moults in the egg to become infective.

12.4 Pathogenesis of infection

A. lumbricoides and *A. suum* both have the potential to infect humans and pigs, and are pathogenic to both of these host species. *Ascaris*-associated morbidity and mortality increase with infection intensity, so aggregation in the host population leads to the majority of those infected acquiring light worm loads and asymptomatic infection. Combined with this, age-related intensity patterns result in high rates of *A. lumbricoides*-induced morbidity in school-age children. The induced morbidity in younger hosts is compounded by their narrower

intestinal lumens, leading to a higher risk of obstructed digestive tracts from the volume of developing worms.

Symptoms of ascariasis may manifest during migration or in patent adult infection. Since *Ascaris* larvae moult and develop within a variety of host tissues, different stage-specific antigens are exposed, which may induce multiple responses and acute symptoms at the early stages of infection. The most pronounced symptoms of larval infection are concentrated at the pulmonary stages of the migratory route, and hosts tend to experience acute lung inflammation as a result of alveolar wall penetration. Larval migration in the host tissue induces pulmonary distress and dyspnoea, bronchospasm and eosinophilia, characteristic symptoms of pulmonary ascariasis collectively referred to as Löffler's syndrome.

Due to obvious ethical reasons, direct experiments on human populations are not feasible and, for this reason, our knowledge of the impact of larval migration on the pathogenesis of infection, and the immune responses elicited during larval migration or at the site of infection, is limited. While an inflammatory reaction in the liver has been documented in human infections, the hepatic response is better understood in porcine hosts and is known to be related to the immune response.

12.5 Animal models of *Ascaris* infection

Ascaris-infected monkeys (mainly cynomolgus and rhesus), pigs, cows, rabbits, guinea pigs and mice have all been studied to obtain more information about the interactions between *Ascaris* nematodes and their mammalian host.

As a natural host of *Ascaris* infection, pigs have been used to model some of the aspects of *Ascaris* infection. Although the pig is a fantastic model, it is limited by the lack of inbred strains and genetically modified lines, and the costs involved in the investigation of detailed immunological mechanisms at play in *Ascaris* infection. This has led to the development of a mouse model of *Ascaris* infection, whereby mice are infected with embryonated ova of *A. suum*.

Characterisation of the migratory patterns of *A. suum* in mice infected in this way shows many similarities to that characterised in porcine hosts. Furthermore, mice have one of the best-characterised mammalian immune systems and are easily amenable to laboratory experimentation. Thus, the mouse model of *Ascaris* infection can be used to model immune responses elicited during the migratory larval phase.

Other work on the response of the mouse immune system to *Ascaris* infection has mostly centred around the analysis of the immunogenecity of *Ascaris*-derived products, such as the excretory/secretory (ES) products. This review will incorporate results from both observational field studies in human host populations with comparative studies on porcine and murine models of *Ascaris* infection.

12.6 Immune responses generated against the migratory phase of *Ascaris*

Measurements of the immune response mounted by the human immune system during *Ascaris* infection are normally taken from the peripheral blood. This makes it impossible to pinpoint the site in the body at which immune responses against *Ascaris* is initiated, or where immune effector molecules associated with the immune response function. In turn, it has been very difficult to uncover mechanisms of resistance to infection.

Multiple locations involved in the migratory path have been suggested as sites at which the mechanism of resistance is manifested, but the assessment of local hepatic and intestinal responses to early infection is more achievable in animal models of *Ascaris* infection. In pigs infected with *A. suum*, the lymph nodes draining organs affected by the migratory larval phase do mount antigen-specific immune responses against the parasite. Furthermore, the response of lymph nodes draining specific organs exposed to migrating larvae appears to involve stage-specific antigens.

12.6.1 Hepatic manifestations of *Ascaris* larval migration: white spots

The pathological consequences of *A. lumbricoides* and *A. suum* infection have been documented in natural human infections through use of clinical data from hospital cases, but the potential for liver damage by *Ascaris* infection in humans may be underappreciated. A large body of work has presented evidence that suggests a role for the liver in resistance to *Ascaris* infection.

The characteristic hepatic inflammatory reaction in pigs infected with *A. suum*, known as white spot formation, is observed during the larval stages of porcine infections. White spots are white pathological lesions, composed of leucocytic infiltrations that form in the liver in response to the mechanical injury and inflammatory response induced by migrating larvae (Figure 12.2). Detailed descriptions of the immune cell populations involved in hepatic response to *Ascaris* infection in pigs infected with *A. suum* have been published. The early white spot lesions are composed of necrotic haemorrhagic foci, surrounded by infiltrates of numerous eosinophils, with smaller numbers of neutrophils and macrophages.

Subsequent formation of granulomatous white spots results from the infiltration of large numbers of multi-histocompatibility (MHC)-II expressing cells – mostly macrophages, with some CD3+ T cells (porcine T lymphocytes constitutively express MHC-II). CD79 a+ B cells and fibroblasts can also be seen in early granulomatous white spots. IgG-producing plasma cells can be found at the periphery of mature hepatic white spots, indicating that this immunoglobulin is important in the local response to *Ascaris* hepatic tissue invasion. However, plasma cells associated with granulomatous white spots in the liver do not generally express IgM or IgA. Larger granulomas are sometimes surrounded by fibrous connective tissue.

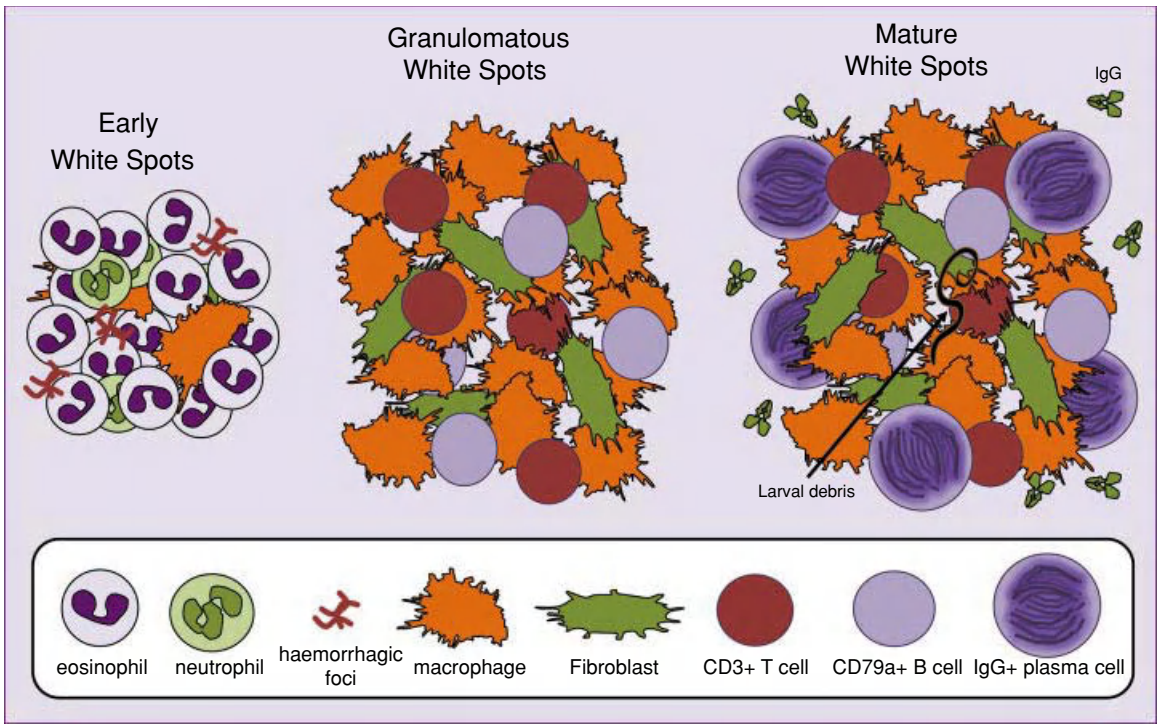

Figure 12.2 Hepatic white spot composition in *Ascaris* infection. Based on Frontera, E *et al*. (2003). Immunohistochemical distribution of antigens in liver of infected and immunised pigs with Ascaris suum. *Veterinary Parasitology* 111(1), 9–18.

White spots (also known as hepatic lesions) have been suggested to encapsulate trapped larvae and play a role in immunity to *A. suum* infection in pigs. *A. suum* larval debris has been detected within granulomatous white spots, centrally located within the granular mass. Furthermore, increased liver pathology in pigs has been shown to be related to a reduction in lung larval numbers in repeated experimental inoculations, as well as in consistently naturally-exposed herds. Coupled with this, greater numbers of lesions, containing more larvae surrounded by inflammatory cells, have been observed in infected mice and guinea pigs considered resistant due to previous exposure to *A. suum*.

Finally, the induction of pre-hepatic protective immunity, marked by a reduction in hepatic white spots and pulmonary larval burdens, has been reported by administration of twice-weekly doses of *A. suum* eggs over the course of six weeks. However, it should be noted that the induction of protective immunity in pigs by this method is controversial, and it has not always been successfully induced.

Much of the work on the hepatic stages of *Ascaris* infection has been undertaken in *A. suum* infections of pigs, where resistance and susceptibility to *Ascaris* infection is not defined. However, the mouse model of early *Ascaris* infection provides an attractive system for examining the efficacy and effector mechanism(s) behind the hepatic inflammatory response. A comparative study

of the degree of inflammatory cells in the liver of susceptible C57BL/6 J and resistant CBA/Ca mice indicates that CBA/Ca mice respond earlier and more effectively to hepatic larval invasion. Therefore, since granuloma formation is associated with trapped larvae, and appear earlier in resistant animals, the liver may be a key site in the immobilisation of migrating *Ascaris* larvae.

12.6.2 Respiratory distress

The pathology underpinning the respiratory distress experienced in *Ascaris*-infected hosts has also been investigated in several animal models. Inflammatory cells, such as eosinophils and macrophages, have been shown to increase in numbers in infected lungs, in structures not dissimilar to the granulomatous lesions (white spots) found in the liver. Several studies suggest that respiratory granulomas are more abundant in animals that are more resistant to repeated infections of *Ascaris* infection. However, although in the mouse model of *Ascaris* infection, immune responses to migrating larvae can be measured in the bronchoalveolar lavage (BAL) fluid, no evidence for a pulmonary mechanism underlying resistance to *A. suum* has been found. Immune responses in the respiratory tract and pulmonary histopathology appear to mirror larval intensity.

12.7 The cytokine response to *Ascaris lumbricoides*

Infection intensity and predisposition of certain individuals to heavy *Ascaris* worm loads tends to be a central focus of immunological studies in *Ascaris*-infected host populations. In human studies, individuals are generally characterised by their worm burden in the first instance, and this epidemiological variable can then be correlated to the immune parameters measured.

Research on cytokine responses to *Ascaris* infection has revealed that Th2 cytokines are important in mediating resistance to *Ascaris* infection, although the way in which the Th2 response coordinates this protection is still unknown. Studies detailing the innate cellular response to *A. lumbricoides* infection have focused on chronically infected hosts in hyperendemic areas. Variations in Th2 responses between studies have led authors to conclude that age- and location-related differences may impact cytokine responses to infection, adding another layer of complexity to the immunological response to *A. lumbricoides*. This is thus an additional factor to consider when implementing control strategies.

In light of research on mouse models providing evidence for a role of type 2 cytokine production in resistance to several other gastrointestinal helminth infections, much of the focus in human studies has been placed on the role of the T cell compartment in resistance to ascariasis. Often, characterisation of T cell responses in *A. lumbricoides* infection is inferred from circulating serum cytokine levels, or from re-stimulation of peripheral blood mononuclear cells (PBMCs) in response to stimulation with *Ascaris* antigen. In particular, many human studies have incorporated analysis of reinfection after individuals have been treated, to ascertain how cytokine responses to *Ascaris* correlate with the predisposition of individuals to reinfection.

Results from such studies are not always in agreement, providing contrasting correlations of resistance and susceptibility with different cytokines. However, several studies have found that infected individuals do display a highly polarised Th2 response, with significant IL-4 and IL-5 production from PBMCs stimulated with *Ascaris* antigen.

One study looked at individuals persistently susceptible to *Ascaris* infection in the Cameroon, and found that such individuals display a weak Th2 response, in particular lower levels of the Th2 cytokine IL-5, when compared with those who are more resistant to reinfection. This suggested that Th2 responses are protective against infection. Other studies have found a negative association between levels of IL-13 and general susceptibility to *Ascaris* infection, supporting this hypothesis. Older children, generally more resistant to *Ascaris* infection with lower infection intensities, have also been shown to produce higher levels of IL-4, IL-9 and IL-13, again supportive of the suggestions that Th2 responses maybe protective against *Ascaris* infection.

Some studies have found similar levels of the immunoregulatory cytokine IL-10 between infected and uninfected individuals, whereas others have looked within *Ascaris*-infected groups of individuals and found a negative association between infection intensity and IL-10 levels. Thus, it is currently unclear how IL-10 impacts on *Ascaris* infection in humans.

Children considered to be naturally immune to *A. lumbricoides* infection have been shown to have higher levels of systemic inflammatory immune responses than their susceptible counterparts. This ongoing inflammatory response, combined with heightened levels of Th2-associated parasite-specific IgE, may be an anti-parasite effector mechanism. However, it is still unknown whether this inflammatory process is more directed against a particular stage in the *Ascaris* life cycle, thus directly limiting patent infections, or is simply a widespread response to infection.

12.8 The humoral response to *Ascaris lumbricoides*

Difficulties arise in comparing studies of humoral immune responses undertaken on various infected human populations, due to a lack of consistency and standardisation with respect to antigen type, isotype of antibody response and age of individuals in the cohort. Furthermore, in endemic tropical regions, polyparasitism is commonly detected (see Section 4), so it is difficult to disentangle the measured immune responses with respect to parasitic infection in each polyparasitised individual. Early field surveys mainly focused on the relationship between specific immunoglobulin responses in *A. lumbricoides*-infected humans and intensity of infection. These studies demonstrated that *Ascaris* infection can induce antibody responses that include all isotypes of antibody (IgM, $IgG_{1\text{-}4}$, IgA and IgE). However, IgG and IgE isotypes are thought to be the prominent antibody isotypes associated with *Ascaris* infection.

Many studies have concluded that antibody responses reflect infection intensity, rather than any kind of resistance or susceptibility traits. The age-related changes in intensity patterns of *A. lumbricoides* infections observed in human

populations are generally mirrored by the antibody response levels that can be measured, indicating that the humoral immune response is dependent on burden, as opposed to being protective.

12.8.1 IgG responses to *Ascaris*

In 2005, the first age-structured analysis of the relationship between serum antibody responses to life stages of *A. lumbricoides* pre- and post chemotherapy in a Vietnamese population did not reveal any significant association between antibody responses and current infection or reinfection rates. However, there was a trend towards a negative association between antigen-specific IgG responses against larval antigens and existing infection intensity in children. The lack of evidence for a protective effect of antibody responses, coupled with marked elevation of IgG subclasses in younger hosts, has since shown consistency with studies in several different endemic populations.

In general, total IgG levels specific for *Ascaris* antigens are elevated in response to *A. lumbricoides* in infected patient groups. Breakdown of total IgG into subisotypes suggests that IgG4 is a major component of this IgG response, and some studies have found that *Ascaris*-specific IgG1 and IgG2 antibodies are positively correlated with parasite burdens. In contrast to the human studies, IgG production against *A. suum* in pigs negatively correlates with the number of larvae recovered in porcine pulmonary tissue, indicative of a protective function in this host.

12.8.2 IgE responses to *Ascaris*

High circulating levels of IgE, often in excess of 10,000 international units/ml of blood, have been consistently associated with human ascariasis. Indeed, high levels of this IgE response is antigen-specific for *Ascaris*. Although some studies have concluded that levels of specific and total IgE were related to protection, rather than exposure to infection (unlike IgG levels), protection was also associated with IgE in combination with elevated inflammatory markers such as serum ferritin, C-reactive protein and eosinophil cationic protein in naturally immune children.

In one study of Nigerian children, evaluation of the humoral responses in consistently lightly and heavily infected individuals to three different *Ascaris*-derived antigens (*Ascaris* body/pseudocoelomic fluid (ABF), a commercially prepared crude allergen extract known as *Ascaris* p1 and a recombinant protein from ABF known as *Ascaris* body wall antigen (rABA-1)), indicated that only IgE specific for ABA-1 antigen was significantly associated with predisposition status. Lightly infected individuals tended to have higher levels of recombinant ABA-1-specific IgE when compared with heavily infected individuals.

Analysis of IgG4 and IgE responses to an unfractioned crude adult antigen extract (AlAg) and rABA-1 in Cameroonian children infected with *A. lumbricoides* also suggest that IgE responses may be associated with the intensity of *A. lumbricoides* infection. Separation of participants in this study into two

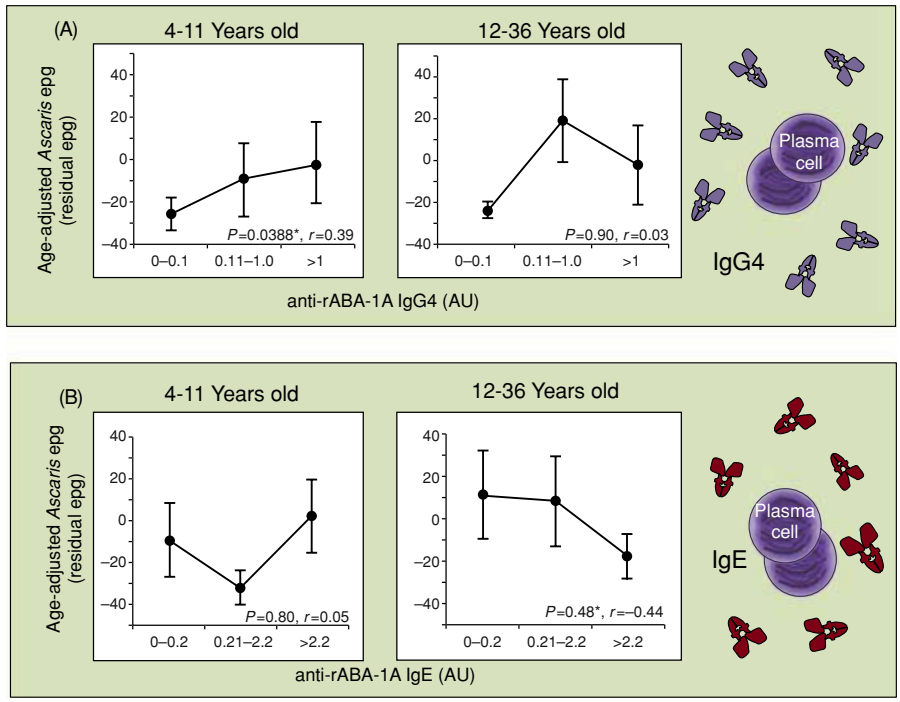

Figure 12.3 Relationships between anti-rABA-1 IgG4 (A) and IgE (B) levels and intensity of *A. lumbricoides* infection in 4-11 year olds (left hand panels) and 12-36 year olds (right hand panels). Graphs show the residual variation in eggs per gram once the age of the patients has been accounted for plotted against increasing titres of antibody. From: Turner, J *et al*. (2005). Allergen-specific IgE and IgG4 are markers of resistance and susceptibility in a human intestinal nematode infection. *Microbes and Infection* 7(7–8), 990–996.

age classes (aged 4–11 and 12–36) revealed that a significant positive relationship existed between IgG4 and infection intensity in the younger age class only (Figure 12.3A, left hand panel). However, IgE titres to rABA-1 decreased with increasing *Ascaris* intensity in 12–36 year old participants (Figure 12.2B, right hand panel), indicating a negative association between ABA-1-specific IgE and intensity of infection. The relationship between IgG4 and heightened infection intensity in younger hosts (in contrast to higher levels of IgE and lowered worm burdens in adults) is evidence of differential regulation of anti-allergen antibody isotypes relating to infection level.

Coupled with rABA-1, *Ascaris* glycolipids are also known to stimulate raised IgE in children with light or no worm burdens, indicating that these molecules may have a role in the humoral response to *Ascaris* infection. The lack of porcine anti-IgE prohibits studies on the dynamics of the serum IgE during helminth infection in porcine hosts, although biological activities related to IgE production (e.g. degranulation of intestinal mucosal mast cells – see Chapter 1) have been observed in trickle-infected pigs. However, this response is difficult to observe in single infections, indicating that repeated exposure is necessary, as might be expected if the IgE levels need to build to certain concentrations to mediate this effect.

It has been speculated that the heightened IgE response in *A. lumbricoides*-infected hosts is stimulated by larval migration, because non-invasive helminths are not associated with significant increases in IgE levels. Elevation of IgE in infected hosts has been considered to be a result of direct mitogenic effects of *Ascaris* allergens on B cells. Many allergens are secreted during larval migration and are associated with the production of large amounts of the Th2 cytokine IL-4, a driver of antibody production from B cells.

Data acquired during a genome scan project of individual human hosts from a Jirel pedigree population in East Nepal (Jiri Helminth project) has provided some support for the role of antibody responses in immunity to *A. lumbricoides*. The significant quantitative trait locus (QTL) on chromosome 13 that is thought to influence the susceptibility of humans to *A. lumbricoides* infection contains a gene called TNFSF13B (also known as BLyS). TNFSF13B is a member of the tumour necrosis factor (TNF) superfamily of cytokines, and is a major regulator of B cell activation and immunoglobulin secretion(see Chapter 1). This cytokine is also involved in promoting the survival of immunoglobulin-secreting cells. Thus, it may be active in enhancing antibody responses to *Ascaris* infection, and therefore may be a candidate gene mediating susceptibility to *A. lumbricoides* infection.

12.9 Antigens eliciting immune responses in *Ascaris* infection

Most of the information on immune responses to *Ascaris* nematodes concerns the adaptive immune response. Immunoepidemiological studies often select different *Ascaris* antigens, ranging from specific proteins in the body fluid to unfractioned crude extracts, in order to investigate the humoral or cellular responses induced in *Ascaris* infection. As *Ascaris* larvae develop, different stage-specific antigens are observed. Therefore, a number of researchers have analszed immune responses to both larval stage and adult antigen preparations. Not much is known about how innate immune responses recognise the nematodes or their ES products.

12.9.1 *Ascaris* body antigen-1 (ABA-1)

ABA-1 was the most prominent antigen detected in *A. suum*-infected pig liver, and the recombinant protein ABA-1 appears to induce the greatest adaptive immune response in human hosts. ABA-1 is a 14 kDa fatty acid-binding protein and is considered the major allergen of *Ascaris* due to the high levels of anti-ABA-1-specific IgE elicited during *Ascaris* infection. ABA-1 is the most abundant body fluid protein of the nematode and is also secreted from, and present within, the body wall. While *Ascaris* antigens tend to be species- and stage-specific, ABA-1 is present at all stages of the life cycle of the parasite and is highly conserved between *A. suum* and *A. lumbricoides*.

The immune recognition of ABA-1 in humans varies considerably, and studies of responses to ABA-1 in *Ascaris* infection have provided evidence for a

significant inverse correlation between *Ascaris* worm loads and IgE levels against ABA-1. The heterogeneity in IgE responses to ABA-1 are probably due to immune repertoire restriction by MHC-II molecules, a hypothesis which holds true in rodents experimentally infected with *A. suum*.

However, some studies suggest that IgE levels against ABA-1 may not arise solely due to an intrinsic ability of the protein to induce allergic-like responses. Other antigens in *Ascaris* body fluid may contribute to the generation of this response as *A. suum* body fluid has been shown to induce allergic-like responses against irrelevant antigens – such as the widely used model antigen ovalbumin – independently of ABA-1. Thus, the allergic response to ABA-1 may be a reflection of other molecules present in the body fluid, especially molecules that are able to induce the cytokines interleukin (IL)-4 and IL-10.

12.9.2 Phosphorylcholine-decorated molecules

Phosphorylcholine (PC) can be found associated with glycoproteins and glycolipids of *Ascaris* nematodes, and PC-specific antibodies can be measured in *Ascaris*-infected individuals. Some of the PC-decorated molecules of *Ascaris* are surface-exposed, but the exact type and function of the immune response generated by PC in *Ascaris* infection is unknown. It is possible that anti-PC immune responses may be immunoregulatory, similar to that generated by PC-decorated molecules such as ES-62 in filarial nematode infections (Chapter 11). However, the observation that children with light or no worm burdens mount an IgE response specific to *Ascaris* glycolipids suggests that immune responses mounted against PC could provide some protection against the establishment of *Ascaris* infection.

12.9.3 A. *suum* haemoglobin

In common with other nematodes, *Ascaris* contains multiple forms of haemoglobin. *A. suum* haemoglobin has been tested as a vaccine candidate for control of *Ascaris* infections. Although this was not successful at protecting against the establishment of *A. suum* infection, increased immunoreactivity against liver L_3 larvae was observed. Since freshly hatched L_3 larval stages do not produce haemoglobin, it is possible that other L_3 antigens contain molecules with some antigenic similarity to haemoglobin produced in adult nematodes. Since this increased immunoreactivity did not affect the establishment of infection, recognition of *A. suum* haemoglobin does not appear to impact adversely on *Ascaris* parasites in pigs.

12.10 Conclusions

Immunity to *Ascaris lumbricoides* infection is poorly characterised, despite the prevalence of infection and the morbidity caused in children infected with this parasite. The humoral immune response to *Ascaris* infection has been characterised in human populations, and also in naturally and experimentally infected pigs. It appears that *Ascaris*, like many other gastrointestinal helminth

infections, induces a Th2 response which may provide some protection against infection, plus an accompanying humoral response which is largely a reflection of the infection intensity in an infected individual.

The antigens from *Ascaris* eliciting the immune response are still largely uncharacterised, and there is a need to standardise the immune response to target antigens assessed in human studies. More use of animal models of *Ascaris* infection is required to tease apart the mechanisms that may confer a predisposition to resistance, particularly the cellular immune response to larvae migrating through the liver. With detailed knowledge of cellular response to *Ascaris* infection, we may be in a better position to develop immunomodulatory therapies to elicit resistance to infection.

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Nematoda: Hookworms

13

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Hookworms and humans are definitely no stranger to one another. Evidence of hookworm infections stem back to Pharaonic times in the Old World and to pre-Columbian times in the New World. Today there are approximately 740 million individuals infected with either *Necator americanus* or *Ancylostoma duodenale*, with nearly 200 million of them residing in sub-Saharan Africa. Although a strikingly large number of individuals are presumably infected, this does not truly capture the proper disease burden of hookworm, since not all of the infected individuals develop the disease. Because global hookworm prevalence is extremely high and causes very low mortality, there is a considerable degree of morbidity associated with hookworm disease. As one might expect, morbidity is related to the intensity of the infection.

Two recent events have reinvigorated interest in immunological studies on hookworm – the funding of the Human Hookworm Vaccine Initiative by the Bill and Melinda Gates Foundation (www.sabin.org/vaccinedevelopment/vaccines/hookworm) (see Chapter 25.3) and the discovery that parasitic helminths, including hookworms, can suppress inflammation associated with autoimmune diseases – a phenomenon that is embodied by the Hygiene Hypothesis (see Chapters 23 and 24). In this chapter, we will focus primarily on the immunology of human infections with *N. americanus*.

13.1 Pathogenesis of hookworm infection

Two major species of hookworm infect humans: *Necator americanus* and *Ancylostoma duodenale*. The World Health Organisation (WHO) estimates that hookworm infects 740 million people, with the largest number of cases in rural areas of sub-Saharan Africa, Latin America, South-East Asia and China (Figure 13.1). Intestinal blood loss is the primary clinical manifestation of human hookworm infections, and it has been demonstrated that blood loss in

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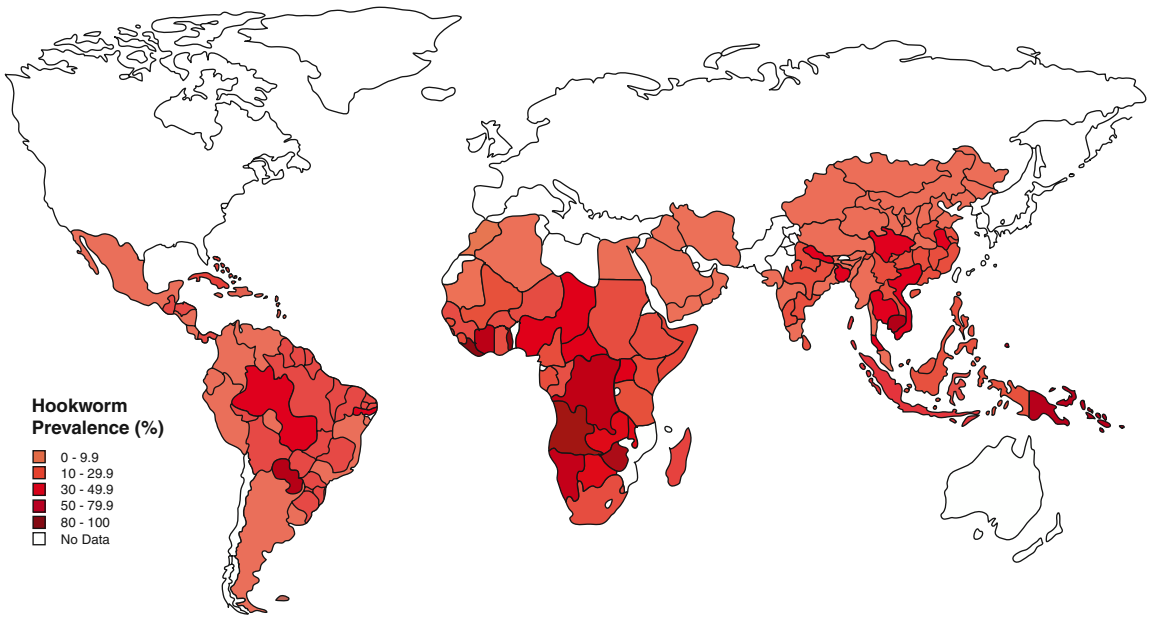

Figure 13.1 Hookworm distribution in 2003. Hookworm is mainly found in developing countries with tropical climates. Reproduced with permission from Hotez, PJ *et al*. (2005). Hookworm: 'the great infection of mankind'. *PLoS Medicine* 2(3), e67.

heavily infected individuals can reach nearly 90 ml of blood per day. Despite this tremendous blood loss, worm numbers of only 40 to 160 are sufficient to induce anaemia. Heavy infections can cause iron-deficiency anaemia, growth retardation and low birth weight.

The importance of hookworm infection has been highlighted by comparing the disability adjusted life years (DALYs) lost with those of other tropical diseases (Table 13.1). This metric highlights the enormous amount of morbidity attributable to hookworm infection, resulting in a greater number of DALYs than those attributed to schistosomiasis (see Chapter 16) or trypanosomiasis (see Chapter 8).

13.2 The life cycle of hookworms

The life cycle of the hookworm is relatively simple: eggs expelled in the faeces hatch, resulting in first-stage larvae (L_1), which then moult to become second-stage (L_2), followed by third-stage (L_3) larvae. The L_3 is the infective stage and can actively penetrate the skin of a wide range of mammalian hosts.

The larvae then enter the bloodstream, migrate through heart to the lungs, break through the alveoli, creep up the trachea and are swallowed, eventually residing in the small intestine as immature adult worms (Figure 13.2). In the gut, the maturing, dioecious, adult worms ingest blood by rupturing mucosal capillaries. Erythrocytes are lysed via pore-forming proteins in the gut of the worm, and the liberated proteins (particularly haemoglobin) are subsequently

Table 13.1 Disability adjusted life years (DALYs) lost to major human parasites.

Disease	Main Parasite	Infections (million)	DALYs* (million)	Deaths (annual)	Reference
Hookworm	*Necator americanus* *Ancylostoma duodenale*	576–740	1.5–22.1	65,000	Bethony, J *et al.* (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* 367(9521), 1521–1532.
Schistosomiasis	*Schistosoma* spp.	207	3–70	280,000	King, CH & Dangerfield-Cha, M (2008). The unacknowledged impact of chronic schistosomiasis. *Chronic Illness* 4(1), 65–79. Steinmann, P *et al.* (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *The Lancet Infectious Diseases* 6(7), 411–425.
Malaria	*Plasmodium* spp.	515	46.5	1 million	Greenwood, BM *et al.* (2008). Malaria: progress, perils, and prospects for eradication. *The Journal of Clinical Investigation* 118(4), 1266–1276. Rowe, AK *et al.* (2006). The burden of malaria mortality among African children in the year 2000. *International Journal of Epidemiology* 35(3), 691–704. Snow, RW *et al.* (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 434(7030), 214–217.

*The wide range of DALYs reflects the alternative disability weights assigned by different studies. Such differences are expected to be resolved in the following years through an initiative of the Institute of Health Metrics and Evaluation at the University of Washington, Seattle, USA.

digested via a cascade of proteolytic enzymes initiated by the hookworm protein aspartic acid protease (APR)-1. The gut of the hookworm is exposed to antibodies in the host blood during feeding, and this has become a major strategy for vaccine development (see Chapter 27). For example, antibodies blocking the enzymatic activity of APR-1 can prevent nutrient uptake in the gut of the adult worm, leading to hookworm mortality.

In the host small intestine, adult hookworms mate and produce eggs that are passed in the faeces, completing the life cycle. Eggs only hatch if environmental conditions are correct, and infective larvae generally live for a few weeks but are susceptible to desiccation if exposed to direct sunlight.

13.3 Animal models of hookworm infection

Advances in our understanding of immune responses to hookworm infection have lagged behind many other helminth parasites, due to the difficulty in maintaining the infection in laboratory animals, notably mice. Hamsters

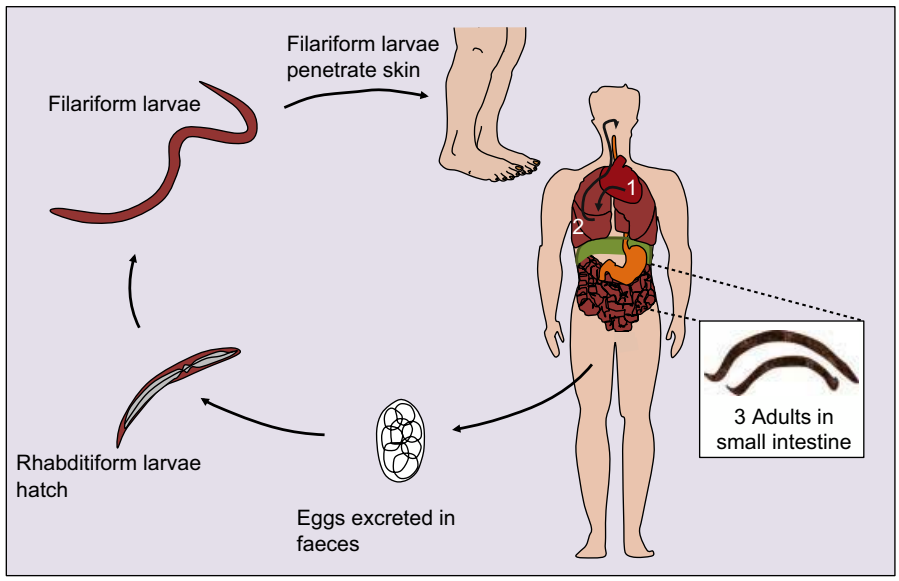

Figure 13.2 The life cycle of intestinal hookworms. Upon entering the human host, the larvae migrate through the heart and into the lungs (1), followed by a migration out of the alveoli and up the trachea (2), at which point they are swallowed, eventually residing as adult nematodes in the small intestine (3).

can yield patent infections with *N. americanus* and *Ancylostoma ceylanicum*, but adult worm burdens are relatively low (at least for *N. americanus*). In certain countries, such as Australia, hamsters are prohibited due to quarantine regulations, so studies focus on the endemic canine hookworm *Ancylostoma caninum*.

Dogs are the natural host of *A. caninum*. Although this hookworm species typically does not infect humans, *A. caninum* resembles infections with human hookworms in several ways:

1. The number of blood-feeding adult hookworms in the small intestine is directly associated with pathology.
2. Infective third-stage larvae enter their host by penetrating the skin or by oral ingestion (*A. duodenale* only).
3. Larvae undergo a short period where development is arrested during entry into the host, which is subsequently followed by feeding and development in host tissue.

Moreover, both canine and human hookworms secrete antigens during entry into the host. The release of these antigens is closely controlled by the development of the hookworm, especially when the worm transitions to a parasitic relationship with its host.

In the 1980s, *A. ceylanicum* infection in dogs was also established as a model to address human immune responses. Dogs chronically infected and then drug treated are resistant to reinfection, demonstrating that immunity can occur in

this model. *A. ceylanicum* infection of dogs is a model of human hookworm because this species of hookworm is able to infect humans.

13.4 Innate immune responses to hookworms

13.4.1 Dendritic cells

In general, hookworm infection is thought to decrease the capacity of dendritic cells (DCs) to become activated. When monocytes are purified from the blood of hookworm-infected individuals and differentiated into dendritic cells in culture, the DCs generally have decreased expression of the integrin CD11c and the monocyte marker CD14 when compared to DCs derived from monocytes of uninfected individuals. There is also a decreased expression of the co-stimulatory molecule CD86 and decreased levels of major histocompatibility complex (MHC)-I and MHC-II molecules on the DCs' surface, suggesting that hookworm infection reduces the ability of DCs to present antigen to T cells.

The reasons for this observation are unknown but, interestingly, tissue inhibitor of metalloproteases-1 from *A. caninum* (*Ac*-TMP-1), a molecule that is secreted from adult stage hookworms, has been shown to affect the maturation of mouse DCs in such a way that they promote the differentiation of CD4+ and CD8+ T cells into a regulatory phenotype.

13.4.2 NK cells

Hookworm infection increases the number of natural killer (NK) cells circulating in the bloodstream, and these cells appear to be activated, as they have been shown to produce the pro-inflammatory cytokine interferon (IFN)-γ spontaneously (without further stimulation) when cultured. However, upon stimulation, the levels of IFN-γ secreted from NK cells purified from hookworm-infected individuals are much lower than from NK cells purified from uninfected individuals. Nevertheless, spontaneous production of IFN-γ may modulate the host-protective Th2 response generated by hookworm infection (see below) and could be an immune evasion strategy.

It has been shown that *N. americanus* (but not *A. duodenale*) hookworms can release molecules such as Natural Killer cell Binding Protein (NKBP), which can attract and expand NK cells through an undefined NK receptor, supporting a role for this innate cell type in immune evasion.

13.4.3 Eosinophils

Blood eosinophilia is a common feature of hookworm infection and has been reported in many studies. Both experimental and natural (endemic) infections induce eosinophilia within four weeks of parasite exposure. Eosinophils from infected individuals have an increased expression of activation markers compared with eosinophils from uninfected individuals. In addition to their

effector capabilities, eosinophils are also competent antigen-presenting cells, and they are able to present processed antigens via MHC class II molecules to stimulate T cells. This may be important in initiating, maintaining or connecting the innate and adaptive immune responses during hookworm infection.

13.4.4 Basophils

Basophils also have gained regard as a key cell type in the initiation of Th2 immune responses. In mice, basophils are important in the early events of Th2 responses, and their depletion prior to infection with the nematode *Trichuris* results in an increased worm burden and decreased Th2 response (see Chapter 14).

In human experimental infections, basophils are activated by eight weeks post *N. americanus* infection, and they retain this status as long as five years after infection. Basophils are potentially activated by cross-linking of surface bound IgE (see Chapter 1), although IgE levels are often not detectable in experimental animal models of infection, particularly in primary infections before B cells have become activated to isotype switch. As a result, basophil activation by *N. americanus* antigens during the early phase of a primary infection may be due either to cross-linking of undetectable levels of IgE or to *N. americanus* antigen-specific surface bound IgG.

Human basophils can express the low-affinity IgG receptor CD16 and CD32, but the events following IgG binding on these cells is still unclear, since this can lead to an inhibitory, rather than a stimulatory, effect (see Chapter 1). Basophils may also act by protease activation through an as yet undefined mechanism. Naïve human basophils produce interleukin (IL)-4 and IL-13 in the presence of excretory/secretory protease-containing products of *N. americanus* (*Na*ES) in a protease-dependent manner.

Basophils are also necessary and sufficient to induce *in vitro* and *in vivo* Th2 responses to proteases allergens; once activated by proteases, they act as antigen-presenting cells and induce a Th2 response by releasing IL-4 and thymic stromal lymphopoietin (TSLP). Thus, it is likely that basophil activation in hookworm infection is caused by proteases secreted by the parasite, and that basophil activation may be important in the initiation and maintenance of Th2 responses during hookworm infection.

13.5 Adaptive immunity

Only a small number of studies have attempted to characterise the T cell and B cell immune responses to hookworm *ex vivo*. There is a small decrease in the proportions of circulating CD4+ T cells and CD19+ B cells in hookworm-infected individuals from endemic areas when compared with uninfected individuals. However, T cells, although proportionally decreased, also have increased levels of the activation markers CD69 and HLA-DR on their surface during hookworm infection.

This profile of adaptive immune cells circulating in the bloodstream of hookworm-infected individuals is not unusual, as infection with other parasites and bacteria induce similar profiles. It is most likely due to the activation and migration of T cells from the circulation into the effector site or draining lymph nodes.

13.6 Cytokine responses

The cytokine response to hookworm infection differs between experimental primary infections, natural infections and reinfections in endemic areas. Repetitive exposure to infection in particular can induce qualitative and quantitative differences in the host response. In humans, studies on cytokine profiles during hookworm infection come from experimentally or naturally (and chronically) infected people. Gastrointestinal parasitic infections have long been considered to induce polarised Th2 responses with production of IL-4, IL-5, IL-13 and IgE, all of which are necessary for their expulsion. Th2 responses have been shown to be somewhat effective against controlling hookworm infections, with elevated IL-5 positively correlating with resistance to reinfection after drug cure in humans.

There is evidence, however, that the immune response against hookworms is not as simple as a polarised Th2 response. Re-stimulation of peripheral blood mononuclear cells (PBMCs) from experimentally infected individuals with hookworm antigens results in Th2 cytokine production, although some studies with chronically infected people have also shown production of the Th1 cytokines IFN-γ and tumour necrosis factor (TNF)-α.

A negative correlation between infection intensity and IFN-γ production was detected in infected people from Papua New Guinea, although there was no association between IFN-γ production and reinfection intensity after drug cure. IFN-γ production to mycobacterial antigens was also negatively correlated with egg burden, implying systemic suppression of IFN-γ production.

In another study, in Brazil, infected people living in an endemic area were drug cured and evaluated six months after treatment. They were divided into three groups: reinfected (people that became reinfected after drug treatment); cured (people who were not reinfected after drug cure); and endemic controls (people who were not infected before or after drug treatment). Endemic controls showed the highest production of IFN-γ, IL-5 and IL-13 to hookworm antigens, indicating a protective role in a mixed Th1/Th2 response. The reinfected group had reduced production of Th1 and Th2 cytokines, but also a high level of spontaneous IL-10 production. Up-regulation of this immunomodulatory cytokine may, in turn, have down-regulated protective Th2 (or mixed Th1/Th2) response, resulting in reinfection. The cured group showed intermediate levels of IL-5 and IL-10, and thus may represent a moderately susceptible group.

The mixed Th1/Th2 response reported in endemic populations is not observed in experimental human hookworm infection. In this case, a polarised Th2 response is observed. One explanation could be that repetitive infections in an endemic setting induce a mixed Th1/Th2 response against the parasite.

However, a study using repeated experimental infection (50 larvae, followed by 50 larvae 27 months later) showed negligible levels of IFN-γ to hookworm antigens at all time points.

An alternative possibility is that other pathogens common in helminth endemic areas (e.g. malaria, bacterial or viral infections) may skew immune response towards a Th1 phenotype (see Chapter 21). Co-infection of mice with the hookworm-like nematode *Nippostrongylus brasiliensis* and Th1-inducing pathogens, such as malaria, *Toxoplasma* or the bacterium *Chlamydophila*, have been shown to result in suppression of helminth-specific Th2. However, a helminth-specific Th1 response has not been shown to occur in any of these co-infection combinations. Therefore, the factors that give rise to mixed Th1/Th2 cytokine responses in endemic populations are still not fully understood.

13.7 Antibody responses

Hookworm-specific IgG1, IgG4, IgM, IgD, IgA and IgE can be detected in people from hookworm-endemic areas, including anthropophilic infections with *N. americanus* and zoonotic infections with *A. caninum*. In experimental human infection, IgM is detected six weeks after infection, with parasite-specific IgG detectable at eight weeks post-infection. IgE responses appear to develop slowly over a number of exposures, and they are almost undetectable in primary infections. As IgE is correlated with protection in many other helminth infections, this isotype has been a strong point of interest to researchers.

In the 1970s, the role of IgE in *N. americanus* infection was studied in the highlands of Papua New Guinea. David Grove and colleagues were the first to show a relationship between IgE (both parasite-specific and total IgE) and hookworm infection. More recently, a protective role for IgE has been suggested in a study of people from endemic areas in Brazil. The levels of IgE antibodies to *N. americanus – Ancylostoma* secreted protein-2 (*Na*-ASP-2) were negatively correlated with the intensity of infection. On the other hand, IgG4 levels to the same antigen had a positive correlation with infection intensity in this population.

In other helminth infections, such as schistosomiasis and filariasis, it has been suggested that parasite-specific IgG4 correlates with a suppressed 'modified Th2' response which can be differentiated from anti-parasite (but often more pathogenic) IgG1 or IgE responses. This paradigm may also exist in hookworm infection, whereby hookworm-specific IgG4 may be the best serological indicator of infection, and a modified Th2 response may also exist in hookworm infection.

Studies in endemic areas have shown that levels of most isotypes of antigen-specific antibodies drop after drug treatment, apart from IgD, which increases, implying that hookworm infection mediates the suppression of this antibody isotype. The function of IgD has been debated for some time, but it was recently shown to bind to an unknown receptor on basophils, and cross-linking of IgD on the basophil surface induces IL-4 production. IL-4 from basophils has been

shown to be crucial in the initiation and maintenance of Th2 responses. Therefore, it is tempting to speculate that hookworm suppresses the IgD response in infected individuals to stem the development of a potentially host-protective Th2 response.

Most of the information on humoral responses to hookworm comes from blood serum studies in humans. In the context of hookworm, a parasite that resides in the gut, more studies evaluating mucosal and faecal antibody titres are required to gain a comprehensive understanding of host immunity during initial infection and subsequent reinfection. Recently, it has been shown that infection of hamsters with *A. ceylanicum* generates an IgA response that can be measured faecally, and that faecal IgA is associated with resistance to challenge infections. The animal models of hookworm infection have not yet been used to their full potential in this area.

13.8 Antigens eliciting the immune response

A large number of antigens eliciting the immune responses in hookworm infection have been characterised, in part through effort to develop a vaccine against hookworm infection (see Chapter 27). Hookworms secrete a large gamut of molecules to help establish and maintain infection in the host. These excretory/secretory (ES) products have a number of immunomodulatory functions and include a protein with anti-coagulant function (AP), enzymes that can degrade haemoglobin in the blood and collagen in the skin to facilitate migration (e.g. APR) and molecules to evade immune responses (neutrophil inhibitory factor (NIF) and calreticulin to inhibit complement function). Many of these molecules are targets for vaccination, because they perform essential functions for the successful establishment and maintenance of hookworm infection.

13.9 Memory responses

Compared with many other helminthic infections in humans, hookworm immunity does not protect against infection. Curiously, people repeatedly exposed to large numbers of infective *N. americanus* larvae over the course of their lifetime do not typically have high-intensity infections. Generally, quite the opposite occurs, where most individuals experience subclinical to moderate infections.

Evidence that memory responses to hookworm infection can be generated comes from the canine model of hookworm infection. The first hookworm vaccine, developed for the veterinary market in 1965, was based on irradiated infective larvae of *A. caninum*, and this vaccine afforded robust protection against experimental canine infections and natural field challenge. Furthermore, dogs chronically infected with *A. ceylanicum* and then drug treated are resistant to reinfection. It has been suggested that overwhelming infections are prevented due to the development of acquired immunity from previous infections through an accumulation of humoral antibodies in the circulation. However,

up to this point in time, there has been no definitive evidence to support the notion that the same specific immunity occurs in humans.

13.10 Immunoregulatory aspects of the anti-hookworm immune response

13.10.1 Hookworms and the Hygiene Hypothesis

The Hygiene Hypothesis states that as populations become more hygienic, and therefore virtually eliminate childhood parasitic infections, they experience a concurrent increase in immune dysregulatory syndromes such as autoimmunity, allergy and inflammatory bowel diseases (see Chapter 23). These diseases are substantially less common in parts of the world where helminths are endemic. Moreover, in endemic areas, the prevalence of allergic atopy is significantly lower in individuals with chronic helminth infection. Epidemiologic studies also support the notion that hookworm infection suppresses immune inflammatory diseases.

Hookworm appears to protect against asthma more than any other parasite investigated. A meta-analysis of studies in parasite endemic and non-endemic areas showed that *Ascaris lumbricoides* may have an asthma-inducing effect, while hookworm protected against asthma, despite both parasites migrating through the lung during the early stages of infection. Studies conducted after anthelmintic drug cure suggest that allergy might increase at the population level. Chemotherapy to remove intestinal helminths results, in some cases, in aggravated allergic responsiveness.

In a recent double-blinded placebo-controlled interventional trial in a hookworm endemic area in Vietnam, the drug-treated group had a significant increase in the incidence of skin allergy to house dust mite or cockroach allergens. This increase was inversely associated with a reduction in levels of the immunoregulatory cytokine IL-10 to hookworm antigens after the treatment. IL-10 induction may be one mechanism by which hookworm infection reduces the incidence of allergic reactions.

13.10.2 Therapeutic hookworm infection

The use of helminths to treat autoimmune diseases, particularly inflammatory bowel disease (IBD), has gained momentum in recent times (see Chapter 24). A clinical trial in the USA using *Trichuris suis*, the pig whipworm, to treat IBD proved very promising, with the majority of infected patients entering remission. However, humans are not fully permissive to *T. suis*, and the infection required boosting every three weeks to ensure the presence of larvae in the gut.

Using human hookworm as a treatment for diseases stemming from immune dysregulation is attractive: it is virtually asymptomatic in low level experimental infections, it poses no risk of transmission in modern sanitary environments

and it survives for years in a human host, making continual reinfection unnecessary. Hookworm therapy has been assessed in the context of hay fever, Crohn's disease and celiac disease with varying success.

13.10.2.1 Hay fever

British trials showed that hookworm infection does not exacerbate airway reactivity in allergic individuals, but no suppression of allergic responses was detected. Furthermore, no evidence of suppression of inflammatory immune responses as measured by IFN-γ and TNF-α production, or induction of immunomodulatory responses, measured by the expansion of CD4+CD25+Foxp3+ T regulatory cells or IL-10-producing CD4+ T cells. However, attempts to treat allergic rhinitis in humans with *T. suis* infection were also unsuccessful, so respiratory allergic diseases may not be particularly amenable to treatment with therapeutic nematode infection.

13.10.2.2 Crohn's disease

In Australia, a Crohn's disease trial showed a strong trend for suppression of gut inflammation after hookworm infection. However, this trial was not blinded nor placebo-controlled, and it was further confounded by continued and variable use of immune-suppressant drugs.

13.10.2.3 Celiac disease

Recently, a trial assessing the therapeutic effect of hookworms in celiac disease was conducted in Australia. Celiac disease is a dietary gluten-induced enteropathy mediated by anti-gluten Th1 and Th17 responses in the gut. Although the development of the disease is dependent on an MHC class II restriction, the absence of gluten from the diet allows celiac sufferers to stay in remission. In the Australian trial, 20 celiac sufferers were entered into a double-blinded placebo-controlled study and given hookworm infection. Twenty weeks after infection, all the participants were given an oral gluten challenge to induce celiac pathology. This trial showed a non-significant trend for less pathology in the infected group.

Spontaneous production of IL-5 from duodenal biopsies was detected in the hookworm group, with highest levels in biopsies taken adjacent to the hookworm bite site (determined by endoscopy). Interestingly, no other Th2 cytokines (IL-4 and IL-13) were spontaneously produced by duodenal biopsies in the infected group. The source of IL-5 in the mucosa is unknown, but it could be mast cells, as no other spontaneous Th2 cytokine production was observed. IL-13 and TGF-β mRNA levels, however, increased after the biopsy cells were stimulated *in vitro* with excretory/secretory products from *N. americanus* (*Na*ES).

In this study, Th1 and Th17 inflammatory cytokines from the mucosa were suppressed during hookworm infection (Figure 13.3), demonstrating immunomodulation by the parasite at the site of infection and inflammation. Furthermore, there were lower levels of IL-23 mRNA (a cytokine that drives

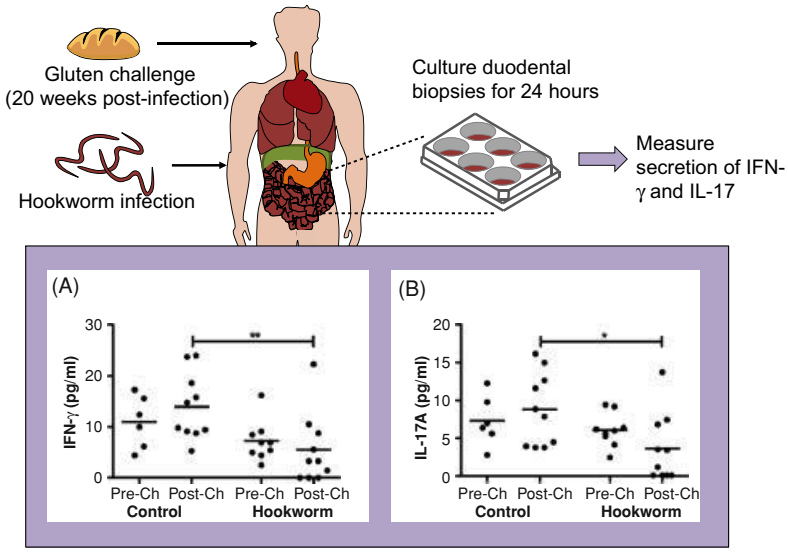

Figure 13.3 Human duodenal biopsies taken from celiac disease patients prior to gluten challenge (Pre-Ch, week 20) and post-challenge (Post-Ch, week 21) were cultured for 24 hours in tissue culture medium, then supernatants taken for cytokine bead array analysis of IFN-γ (A) and IL-17A (B). Each dot represents one biopsy. Two-way ANOVA was carried out comparing the difference between groups and time points. Unless otherwise indicated, differences are not significant. ** = $p<0.01$, * = $p<0.05$. IFN, interferon; IL, interleukin. Data taken from McSorley, H *et al*. (2011). Suppression of inflammatory responses in celiac disease by experimental hookworm infection. *PLoS One* 6(9), e24092

Th17 differentiation) in the biopsies, demonstrating that hookworm could decrease the Th17 response against gluten. Some levels of systemic suppression of inflammation were also seen, with a trend for less gluten-specific Th1 cells in the blood. Altogether, these results provide strong evidence that hookworm infection can suppress the inflammatory responses against gluten that drive the symptoms of celiac disease.

13.11 Conclusion

Human co-evolution with hookworms has reached a stage of relatively asymptomatic infection if the parasite burden is low and host nutrition is adequate. It would appear that co-evolution of host and parasite has resulted in an immune system that has adapted over millennia to deal with the presence of hookworms, and only recently have they been taken out of the equation in developed countries. Thus, in the absence of the parasite, the immune response has become 'over-exuberant', resulting in an increase in the prevalence of illnesses related to dysregulation of the immune system, especially in developed areas.

Future studies will show if we can use hookworms, or the molecules that they produce, to correct the imbalance. If a vaccine to hookworm, or to other neglected parasitic diseases, is developed and administered throughout the

developing world, an unfortunate side-effect might be increased autoimmunity and allergic diseases in these areas. However, the enormous public health benefits of such a vaccine will likely outweigh the potential problems that eliminating hookworm might precipitate.

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Nematoda: *Trichuris* 14

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14.1 *Trichuris* infection

Soil-transmitted helminths are the most prevalent infectious microorganisms of humans, with approximately two billion people infected. *Trichuris* (*Nematoda*; *Adenophorea*; *Stichosomida*; *Trichocephaloidea*; *Trichuridae*) is a soil-transmitted nematode parasite that infects humans and animals. *Trichuris trichuria* is the causative agent of human trichuriasis and infects over 800 million people. Chronic infections are common in children, and they are associated with clinical symptoms ranging from impaired nutritional status and growth retardation to anaemia, protein-losing enteropathy and intestinal obstruction. Nematode infections are also highly prevalent within the livestock industry, and infection control strategies impose a heavy economic burden. *Trichuris* infections are found predominantly in tropical climates under conditions of poor sanitation (Figure 14.1).

Anthelminthic treatment is effective, but it provides only a short-term benefit due to the stability of infective nematode eggs in the environment. Interactions between *Trichuris* and humans date back at least 5,000 years, as *Trichuris* eggs have been isolated from several mummified remains, including the Neolithic Glacier Mummy, Ötzi. *Trichuris* is the most common parasite found in prehistoric samples, having been isolated from animal coprolites (fossilised faeces) approximately 30,000 years old. Thus, the host-pathogen interaction for *Trichuris* is ancient and has undoubtedly co-evolved with its host.

This nematode is commonly referred to as whipworm, due to its thin anterior end and thick posterior portion (Figure 14.1, inset). One of the most striking features of *Trichuris* is the extreme host-parasite species specificity. While distinct species of *Trichuris* are indistinguishable at the gross morphological and biochemical levels, each mammalian species is infected by a specific *Trichuris* species, with rare cases of cross-species infectivity.

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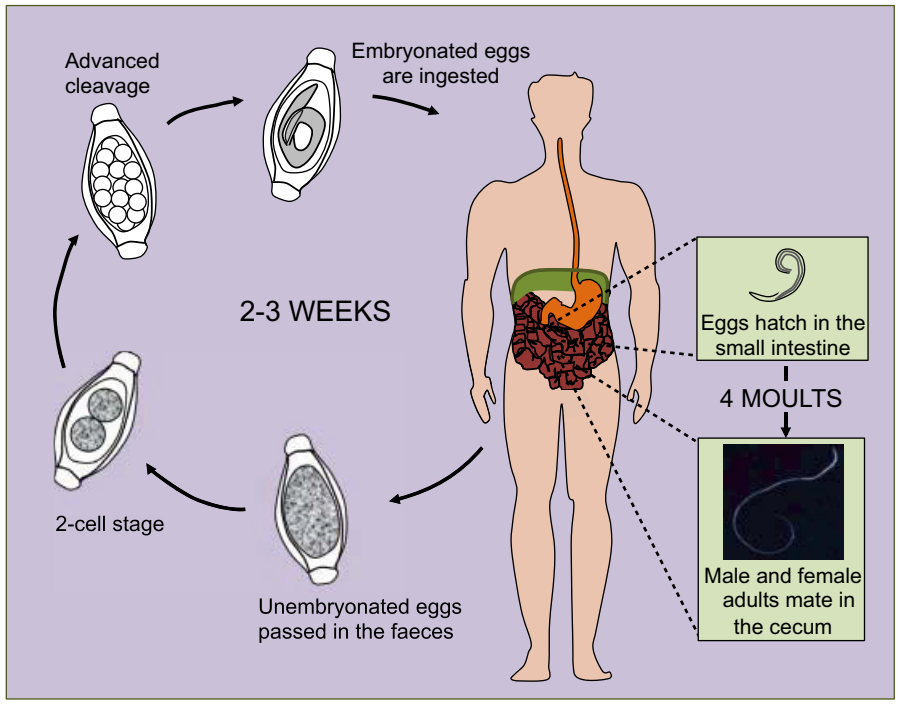

Figure 14.1 The life cycle of *Trichuris*. Inset picture shows the gross morphology of an adult parasite of *T. muris*, isolated at day 21 post-infection from the caecum of a susceptible strain of mouse (NOD-SCID).

Trichuris muris is the mouse-specific species that has been used extensively in laboratory model systems to delineate many of the immunological mechanisms associated with resistance and susceptibility to this nematode. In addition, this specificity has been exploited to treat human inflammatory bowel disease patients with *Trichuris suis*, the pig whipworm, to reduce inflammatory symptoms without resulting in infection (see Chapter 24). Overall, *Trichuris* is one of the most successful parasites of mammals and, over the past 60 years, it has been exploited as a powerful model system to understand host-parasite interactions.

14.2 Life cycle and pathogenesis

Following ingestion of contaminated water or food, embryonated eggs containing infective L_1 larvae migrate to the distal small intestine, where the larvae hatch from the eggs. The cues that trigger larval hatching are unknown, although it is thought that interactions between the eggs and the commensal bacteria of the host may be important in this regard. L_1 larvae migrate to the caecum and colon of the host, embed in the intestinal mucosa and undergo four moults to adulthood (Figure 14.1).

There is surprisingly very little tissue damage and inflammation observed during infection in immunocompetent hosts. Adult females produce between 1,000 and 10,000 eggs per day, which are excreted in the faeces. Excreted

embryonated eggs are not immediately infective, as they require approximately three weeks for larvae to develop. Infective eggs in the environment are subsequently ingested and the life cycle continues. Thus, the life cycle is entirely enteric, with no skin penetration or tissue migration phase, providing a much simpler model to analyse both the early and late immune responses that develop following infection.

14.3 Immunity to *Trichuris*

Infection of mice with *Trichuris muris* has provided a powerful model to study the intestinal immune response to nematode infection. Early studies using *Trichuris muris* were carried out by Shikhobalova, Keeling and Fahmy. Studies by Campbell in the early 1960s and Wakelin in the late 1960s were the first to begin analysing the immune response to *Trichuris* in the laboratory mouse. Since then, several groups with focus on studying the immune response during *Trichuris* infection have made significant contributions to our understanding not only of immunity to *Trichuris* infection but also of the regulation and development of mucosal immune responses in general.

14.4 Recognition by the immune system

How *Trichuris* (and all helminth parasites, for that matter) is recognised by the innate immune system is not clear. While several families of innate receptors have been identified that recognise components of viruses, fungi, bacteria and protozoans, receptors for helminth-specific products remain elusive. These receptor families can sense and respond to almost any foreign pathogen to induce an acute killing response, as well as the activation of a protective adaptive immune response. In contrast, there have been only a few reports of canonical pattern recognition receptors (PRR) binding to helminth-derived products.

During *Trichuris* infection, it has been shown that Toll-like receptor (TLR)4 and its downstream adaptor myeloid differentiation primary response gene (88) (MyD88) are required to promote susceptibility to chronic infection, possibly by activating nuclear factor-κ light chain enhancer of activated B cells (NF-κB)-dependent genes in intestinal epithelial cells in response to *Trichuris* excretory/secretory (ES) products. However, it remains unclear which antigens are responsible for activating TLR4, which cells are involved in the initial recognition and the exact molecular pathways leading to protective or non-protective responses. Whether actual PRRs exist that specifically recognise *Trichuris*-derived products is not known.

14.5 Innate immune responses

14.5.1 Intestinal epithelial cells

The surface of the gut is made up of a single layer of columnar epithelial cells (intestinal epithelial cells, IECs). *Trichuris* lives in close approximation with IECs, as the thin anterior end of the parasite burrows into the IEC layer and

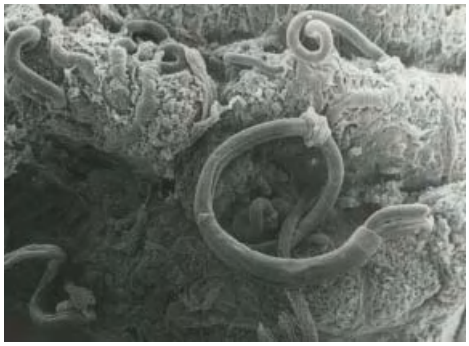

Figure 14.2 Intimate interaction between *Trichuris* and the host epithelium. Scanning electron micrograph of *T. muris* embedded in the caecal epithelium of mice. Bar, 100 μm. Figure courtesy of D. Artis and L. Tilney.

anchors itself in the mucosa (Figure 14.2). Specialised secretory cells arranged along the bacillary band on the parasite are intimately associated with IECs, suggesting possible cross-talk between the parasite and host.

Not surprisingly, IECs play a critical role during infection with *Trichuris* (Figure 14.3). Infection of mice with *T. muris* results in activation of IECs, as measured by induction of NF-κB activity. Mice with an IEC-specific defect in NF-κB activation (*Ikkb*$^{\Delta IEC}$ mice) are susceptible to *Trichuris* infection, producing decreased levels of interleukin (IL)-4, Il-5 and IL-13 and increased levels of interferon (IFN)-γ.

IECs produce several cytokines that are required for immunity to *Trichuris* infection, including thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. IEC-intrinsic,

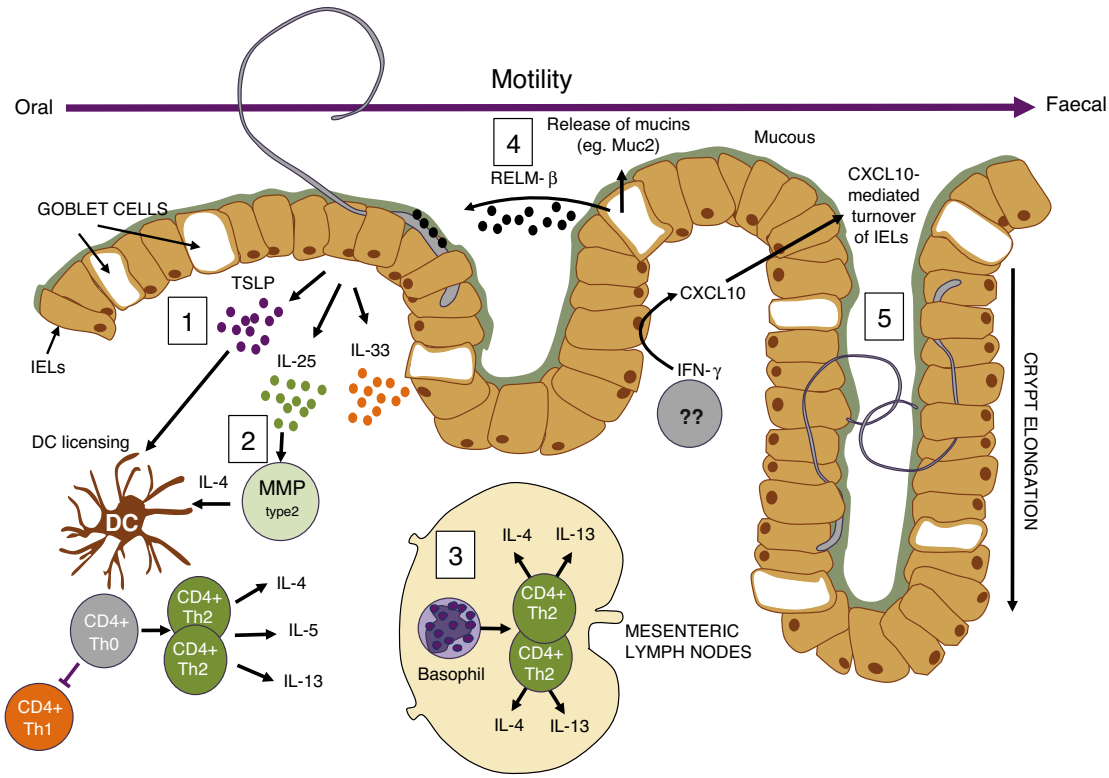

Figure 14.3 Intestinal responses to *Trichuris*. (1) IECs secrete several cytokines such as TSLP, IL-25 and IL-33 in response to *Trichuris* infection. (2) TSLP is essential for the activation of Th2 CD4+ T cells by DCs and IL-25 induces MPP^{type2} multipotent progenitor cells that secrete IL-4 to help shape a Th2 response. (3) Basophils are activated in the intestine and traffic to the mesenteric lymph nodes, where they activateTh2 CD4+ T cells. (4) Goblet cells secrete mucous and proteins, such as RELM-β, which can bind to *Trichuris* secretory structures. (5) IFN-γ induced production of the chemokine CXCL10 induces accelerated turnover of IECs and the elongation of crypts, a phenomenon that impairs clearance of the parasites from the intestine.
Abbreviations: DC, dendritic cell; IEC, intestinal epithelial cells; IL, interleukin; IFN, interferon; RELM, resistin-like molecule; Tc, cytotoxic T cell; Th, T helper cell; TSLP, thymic stromal lymphopoietin.

NF-κB-dependent production of TSLP is critical for licensing dendritic cells (DCs) to allow the development of Th2 cell responses. In addition, mice deficient in the receptor for TSLP (TSLPR KO) are susceptible to *Trichuris* infection. However, antibody blockade of IFN-γ in either *Ikkb*$^{\Delta IEC}$ mice or TSLPR knockout mice following *Trichuris* infection renders these susceptible strains resistant, demonstrating that these molecules are not required to promote protective immunity directly, but rather are critical for limiting the development of non-protective responses.

14.5.2 Epithelial cell proliferation

Epithelial cells are constantly proliferating and turning over to regenerate the intestinal epithelium. During *Trichuris* infection, increased epithelial cell proliferation and turnover leads to enhanced immunity. In susceptible strains of mice, increased epithelial proliferation is observed, but IEC turnover is limited, resulting in crypt elongation and failure to expel parasites.

Surprisingly, the control of epithelial turnover during *Trichuris* infection is controlled by a chemokine that is induced by IFN-γ, CXCL10. Antibody blockade of CXCL10 is sufficient to render susceptible mice resistant to *Trichuris* infection. Strikingly, anti-CXCL10 antibodies can render severe combined immunodeficient (SCID) mice resistant to infection. This study highlights the critical role that the intestinal epithelium plays during expulsion of *Trichuris*.

14.5.3 Goblet cells

One of the most striking physiological changes that occur in the intestine following *Trichuris* infection is the hyperplasia of goblet cells. Goblet cells are terminally differentiated IECs that secrete mucus and a variety of effector proteins that are associated with resistance to infection. For example, Muc2, the major intestinal mucin, is up-regulated during worm expulsion and is required for normal parasite clearance.

Goblet cell-specific effector proteins are some of the most highly up-regulated genes in the intestine following *Trichuris* infection. While expression of intelectin, chloride channel calcium activated 3, pancreatic lipase-related protein 2 and pancreatic colipase are up-regulated during infection, the roles of these genes remain unknown. Resistin-like molecule (RELM)-β is a goblet cell-specific gene that is induced during *Trichuris* infection, is secreted into the intestinal lumen and can bind to specific secretory structures on *Trichuris*. However, RELMβ is dispensable for resistance to infection, and it may actually impair host immunity during chronic *Trichuris* infection.

14.5.4 Mast cells and eosinophils

Innate immune cells, such as eosinophils and mast cells, have historically been associated with helminth infections. During *Trichuris* infection, there is a

significant expansion of eosinophils and mast cells in the intestine. However, mice deficient in (or with reduced numbers of) mast cells or eosinophils are resistant to infection. Thus, these cellular components of type 2 immunity are dispensable for immunity to *Trichuris*.

14.5.5 Basophils

Basophils are a rare granulocyte population (less than 0.5 per cent of circulating cells) that have been associated with nematode infections and allergies. Basophils release a variety of immune mediators following activation, including histamine, proteases, prostaglandins, leukotrienes and proteoglycans. Basophils also produce cytokines associated with Th2 cell responses, such as IL-4 and IL-13.

Recent studies have highlighted that basophils play a specific and critical role during *Trichuris* infection. Following *Trichuris* infection, basophils transiently traffic to the mesenteric lymph node (mLN), where they act as antigen-presenting cells (APCs) to activate CD4+ T cell responses.

Antibody depletion of basophils renders normally resistant mice susceptible to *Trichuris* infection. Interestingly, other helminth infections do not have an absolute requirement for basophils for resistance. Thus, in addition to providing IL-4 to promote Th2 cell differentiation, basophils also act as alternative and transient APCs early on following *Trichuris* infection.

14.5.6 Innate helper cells

Following infection with protozoan parasites such as *Toxoplasma* or *Leishmania*, innate cells such as DCs and macrophages respond by producing cytokines such as IL-12 that promote a protective Th1 cell response (see Chapters 4 and 7 respectively). However, following nematode infection, DCs and macrophages do not produce cytokines that directly promote protective Th2 cell responses, suggesting that other cells must be responding to infection to produce IL-4 and IL-13. Early on following infection with *Trichuris*, several IEC-specific cytokines are produced.

Expression of IL-25, IL-33 and TSLP are induced following infection, and these are individually required for resistance to infection. IL-25 induces a population of multi-potent progenitor cells that produce IL-4 termed $MPP^{type\ 2}$ cells. These cells express several haematopoietic stem cell markers and have the ability to differentiate into several cellular lineages, including mast cells and macrophages.

Thus, one of the earliest events following *Trichuris* infection is the activation and recruitment of uncommitted progenitor cells that produce IL-4 and, presumably, shape the nature of the subsequent immune response. It is likely that a better understanding of the development and function of these cells will be critical to the design and implementation of vaccine strategies against nematode parasites such as *Trichuris*.

14.5.7 Macrophages

Intestinal macrophages have been shown to express decreased levels of pro-inflammatory cytokines following stimulation. Furthermore, macrophages respond to cytokines by acquiring distinct cell fates. For example, TLR-stimulated macrophages differentiate into classically activated (or M1) macrophages that secrete pro-inflammatory cytokines and produce nitric oxide (NO). In contrast, IL-4 stimulation leads to alternatively activated (or M2) macrophages that produce IL-10 and TGFβ and express arginase and RELMα (see Chapter 1). *Trichuris* infection results in increased frequencies of M2 macrophages and heightened levels of RELMα. However, depletion of macrophages using clodronate liposomes during *Trichuris* infection has no effect on the development of resistance. Thus, the exact role of intestinal macrophages – specifically, M2 macrophages – remains unclear.

14.6 Adaptive immune responses

14.6.1 CD4+ T cells

Following infection, naïve CD4+ T helper (Th) cells can differentiate into several distinct lineages that differ in their production of cytokines that are required to combat the specific type of pathogen encountered. It has been demonstrated that resistance to infection with *Trichuris* in mice, pigs and humans is associated with the activation of CD4+ Th2 cells that produce the cytokines IL-4 and IL-13. The differentiation of Th2 cells is critically dependent upon IL-4/IL-13 binding to IL-4Rα on the T cell surface, leading to activation of signalling intermediates such as signal transducer and activator of transcription (STAT)6, resulting in the activation of the master transcriptional activator, GATA3. Infection of humans also results in a Th2 cell-biased immune response, with high levels of IL-4 and IL-13, immunoglobulin class-switching to IgG1 and IgA and increased epithelial cell proliferation.

14.6.2 CD8+ T cells

CD8+ T cells are critical for resistance to infections with some viruses, bacteria and protozoan parasites. CD8+ T cells function primarily by producing high levels of IFN-γ and TNF-α, as well as mediating perforin- and granzyme-dependent cytotoxicity. During chronic *Trichuris* infection, CD8+ T cells that produce IFN-γ develop. However, antibody-mediated depletion of CD8+ T cells has no effect on the outcome of infection. Thus, CD8+ T cells are not required for either the development of resistance or susceptibility to *Trichuris* infection.

14.7 Immune memory

Early studies demonstrated that following sterile cure of primary *Trichuris* infection, genetically resistant mouse strains exhibited rapid immunity upon

re-challenge. In addition, effector T cells isolated from infected mice can persist for up to six weeks and mediate resistance to reinfection following adoptive transfer into naïve mice.

Previous studies identified *Trichuris*-responsive Th2 cells that persisted in immune animals and mediated more rapid production of IL-13 and accelerated worm expulsion following infection, compared with naïve mice. Critically, production of Th2 cytokines, goblet cell hyperplasia, expression and secretion of RELM-β, and rapid expulsion were lost when CD4+ T cells were depleted in immune mice, demonstrating a critical role for CD4+ T cells in immunity to secondary infection.

CD4+ T cells are not only required, but are also sufficient to induce goblet cell hyperplasia and mediate immunity, following adoptive transfer into naïve mice. Furthermore, drug-cured *Trichuris*-infected human patients that produced lower levels of Th2 cytokines were significantly more likely to become reinfected. Thus, long-term mucosal immunity against *Trichuris* infection is mediated by memory CD4+ Th2 cells that persist in the absence of chronic infection.

14.8 Vaccines

Despite the increased knowledge of the cellular and molecular requirements for protective immunity to *Trichuris*, there is presently no vaccine against *Trichuris* infection. It has been shown in mice that subcutaneous vaccination with *Trichuris* excretory/secretory (ES) products or adult worm homogenate, in the presence of complete or incomplete Freund's adjuvant, rendered susceptible mice resistant to infection and was associated with an increased Th2 cell response. A 47 kDa antigen from adult *T. trichuria* (43 kDa in *T. muris*) is strongly recognised by human immune serum. It was further shown that this antigen can form pores in cells, but its molecular characteristics and identity remain unknown.

In humans, while no vaccination studies have been carried out, it is clear that the magnitude of the Th2 cell response during infection is directly correlated with the chance of reinfection, suggesting that vaccines that induce highly potent *Trichuris*-specific Th2 cell responses would be most beneficial during infection.

14.9 *Trichuris* as a therapeutic

One of the most fascinating developments in the biology of *Trichuris* is its use as a therapeutic treatment for inflammatory diseases of the intestine, including ulcerative colitis and Crohn's disease (see Chapter 24). Studies by Weinstock *et al*. have shown that treatment of inflammatory bowel disease (IBD) patients with *Trichuris suis*, the swine-specific species, results in a beneficial therapeutic effect. While this treatment does not result in infection, decreased severity of symptoms is noted in patients.

There is also a report of a single ulcerative colitis patient who infected himself with *Trichuris trichuria* when his disease symptoms became unbearable, resulting in a complete remission. *Trichuris*-induced disease remission is correlated with the presence of parasites and the appearance of a population of IL-22-producing CD4+ T cells, as well as a decrease in pro-inflammatory cytokines such as IL-17. Thus, future studies examining the molecular mechanisms associated with *Trichuris* therapy will potentially highlight novel drug targets in addition to optimising ongoing trials with live parasites.

14.10 Summary

Trichuris is one of the most common infections of humans and has been used extensively as a powerful model to examine the requirements for the development of protective immunity against helminth parasites. The novel use of *Trichuris* as a potential therapeutic treatment for a variety of inflammatory conditions further highlights the usefulness of this parasite.

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Nematoda: *Trichinella* 15

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Trichinellosis is a food-borne disease with an estimated yearly global incidence of 10,000 human cases. It is likely that many additional cases are not diagnosed, particularly when the infection occurs in isolated individuals rather than in the context of an outbreak. Death is rare; the mortality rate is estimated at 0.2 per cent. Although *Trichinella* species are found on all continents with the exception of Antarctica, current foci of endemic human disease are reported in Central and South America, Asia, and Eastern Europe. Trichinellosis has been included among 'Europe's neglected infections of poverty'. Table 15.1 lists the different species of *Trichinella* and their geographical distribution.

The greatest risk of infection is associated with the consumption of undercooked pork and pork products in areas where management of domestic pigs is poor and promotes exposure to wildlife. In some countries, *Trichinella* is endemic in wildlife, but cultural influences on dietary practices (e.g. prohibition of consumption of pork or the meat of carnivores) prevent transmission of the disease to humans. In other countries, the primary means of transmission is the consumption of inadequately cooked wild game meats – for example, bear or walrus in North America.

15.1 Life cycle

The life cycle of *Trichinella* is completed in one host; there is no free-living stage. The details of the life cycle described in Figure 15.1 pertain to *T. spiralis*, which is the most thoroughly characterised species. Transmission occurs when the skeletal muscle of an infected animal is consumed by a susceptible host. First-stage larvae are freed from muscle by digestion and move into the small intestine where they invade epithelial cells. The larvae actively migrate through the epithelial monolayer that lines the intestine, most often at

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Table 15.1 Species of *Trichinella*.

Trichinella species*	Human infection	Host species	Geographical distribution
Encapsulated			
T. spiralis	+	Swine, rats, seldom in carnivores	Cosmopolitan
T. nativa	+	Terrestrial & marine carnivores	Holoarctic (arctic and subarctic)
T. britovi	+	Carnivores, seldom swine	Temperate Palearctic
T. murelli	+	Carnivores	Temperate Nearctic
T. nelsoni	+	Carnivores, seldom swine	Ethiopic region
Non-encapsulated			
T. pseudospiralis	+	Birds, mammals	Cosmopolitan
T. papuae	suspected	Swine, salt water crocodiles	Papua New Guinea, Thailand
T. zimbabwensis	unknown	Nile crocodiles, Nile monitor lizards, lion	Eastern Africa

*Genotypes T6, T8, T9, and T12 have been isolated from carnivores in North America, Southern Africa, Japan and Argentina, respectively. They have not been reported to cause disease in humans or to infect domestic animals.

the crypt/villus junction, creating syncytia that die soon after the parasite exits. Larvae moult four times in 30–40 hours, and adult worms mate in the epithelium. Male worms are approximately 1 mm in length and females up to 3 mm. Eggs hatch *in utero*, and female worms release newborn larvae (NBL) as early as four days after the initial infection. NBL are approximately 110–130 micrometers in length and bear a stylet that likely facilitates their migration in tissue. In the gut, they enter the lymphatics, eventually reaching the bloodstream. In addition, NBL access the mesenteric vessels that supply the portal vein, then transit the liver and enter the systemic circulation.

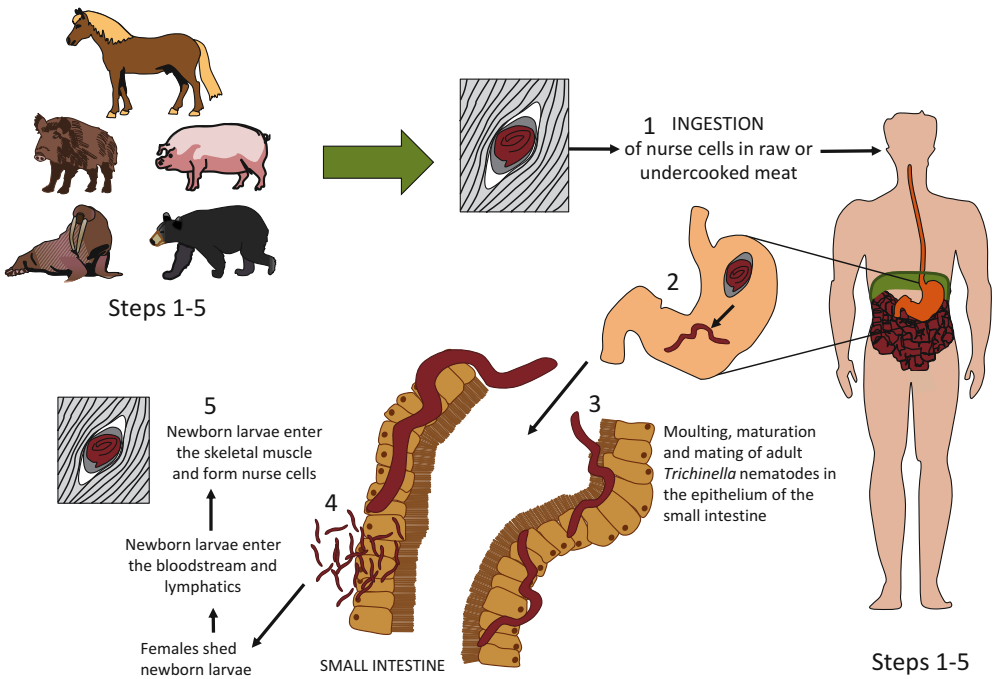

Figure 15.1 Life cycle of *Trichinella* species. The life cycle can be completed in a variety of hosts. This diagram demonstrates transmission to humans.

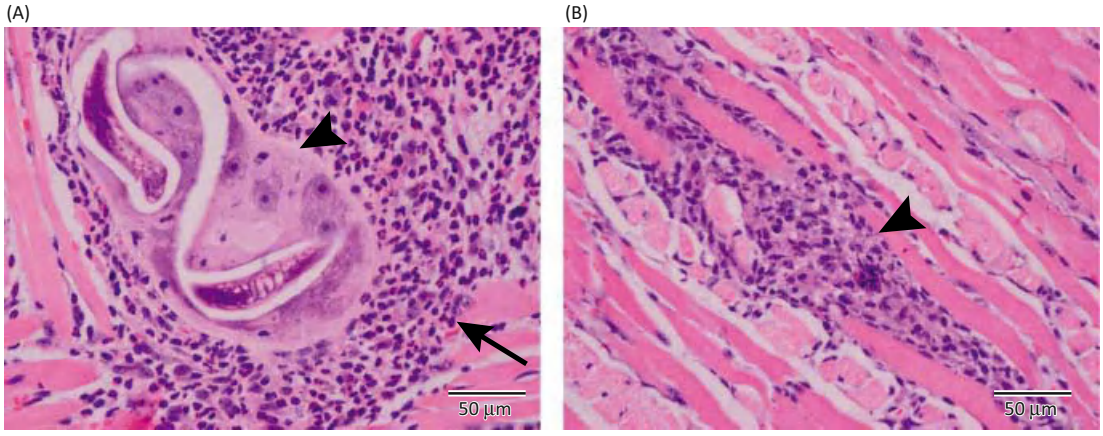

Figure 15.2 Leukocytic infiltrates at sites of *T. spiralis* infection.(A) H&E stained section of tongue from an infected C57BL/6 mouse, 22 days post-oral infection. Leukocytes (arrow) surround the capsule of nurse cell (arrowheads). Larva is large and intact. (B) In the absence of eosinophils, IL-10, or TGF-β, the nurse cell (arrowheads) is infiltrated and normal architecture is destroyed. Larva is not visible in the infiltrated nurse cell. Scale bar = 50 μm.
Abbreviations: IL, interleukin; TGF, transforming growth factor.

Larvae can extravasate in any tissue, but can only complete their development in skeletal muscle cells. Upon arrival in skeletal muscle, they invade myotubes, inducing marked alterations in gene expression as well as dramatic morphologic changes in those cells. After four days of intracellular life, the larvae begin a process of rapid growth that is completed in approximately 14 days. During this time, the myotube is transformed into a 'nurse cell' complex, characterised by the formation of a collagen capsule, local angiogenesis that forms a rete around the infected cell and infiltration of leukocytes, which surround (but are largely excluded from) the infected cell (Figure 15.2). When fully mature, each larva is approximately 1 mm long and capable of infecting a new host.

The host range for most *Trichinella* species is broad, although among susceptible hosts, the capacity to establish and be retained in the intestine or to survive in muscle may vary. For example, adult *T. spiralis* worms are expelled from the intestine by rodents in 10–15 days, but in pigs, adult worms survive and remain fecund for 4–6 weeks. The persistence of adult worms increases the number of NBL that colonise the muscle, thereby increasing the likelihood of transmission.

15.2 Pathogenesis

Traumatic injury is a significant cause of pathogenesis in trichinellosis. Larvae and adult worms (30 μm in diameter) are much larger than the intestinal epithelial cells they invade (10–15 μm), and epithelial cells die as a result of occupation. Pathological changes appear first in the proximal small intestine, moving distally as worms migrate towards the ileum. In humans, symptoms are sometimes observed during intestinal infection and include abdominal pain and/or diarrhoea. Stools contain mucus but not blood.

The NBL is the most pathogenic life-stage, causing traumatic injury as it migrates through tissues. Eosinophils and mononuclear cells are recruited to sites

of migration and exacerbate the injury. Vasculitis is prominent in human infections, and fever, oedema, myalgia and asthenia are common clinical signs at this stage. Cell damage occurs directly when NBL mechanically penetrate striated skeletal muscle cells, or indirectly when leukocytes are recruited to sites of muscle infection.

Elevated muscle enzymes and blood eosinophilia support a diagnosis of trichinellosis. Infection is confirmed by detection of larvae in muscle biopsy or by detection of rising titres of serum antibodies specific for larval glycoproteins. Once worms are cleared from the intestine and larval migration ceases, symptoms begin to abate. Individuals that develop heavy muscle infections may experience chronic muscle pain. Treatment during the acute phase of disease includes glucocorticosteroids to reduce inflammation, and mebendazole or albendazole to clear worms from the intestine.

15.3 Adaptive immunity

15.3.1 Antibody responses and target antigens

The humoral immune response to *Trichinella* is dominated by antibodies specific for glycans and glycan modifications. First-stage larvae of *T. spiralis* produce two highly immunogenic N-linked glycans: one that bears phosphorylcholine (PC) and another that bears the dideoxy-hexose, tyvelose. These are multi-antennary structures in which GalNAcβ1-4GlcNAc (LacDiNAc) constitutes the backbone of the antenna. LacDiNAc is common to many helminths. In *T. spiralis*, PC likely modifies GlcNAc in these structures. PC is widely distributed in tissues of *T. spiralis* L_1 and induces a strong antibody response in rats during the intestinal phase of infection. Nevertheless, passive immunisation of mice or rats with antibodies against PC does not protect them against infection.

In contrast, antibodies specific for tyvelose are highly protective against intestinal infection with *T. spiralis*, causing the expulsion of as many as 88 per cent of L_1 from the rat intestine within the first hour of infection. Tyvelose caps LacDiNAc and occurs in a β anomeric conformation. The distribution of tyvelose is limited to the body surface and to secretory intestinal cells called stichocytes. In contrast to the response to PC, antibodies against tyvelose are produced during the muscle phase of infection, possibly in response to the release of glycoproteins from healthy infected cells, or more likely from nurse cells that fail to mature. In rodents, tyvelose induces a dramatic Th2-dependent antibody response. Tyvelose is found in all species of *Trichinella*, and detection of tyvelose-specific antibodies is the basis for serological diagnosis of infection.

15.3.2 Intestinal immunity

Following ingestion by a susceptible host, *T. spiralis* invades the epithelium of the proximal small intestine. Clearance of adult worms is mediated by a potent Th2 response, which is characterised by dramatic increases in the numbers of lymphocytes, eosinophils, goblet cells and mucosal mast cells (Figure 15.3).

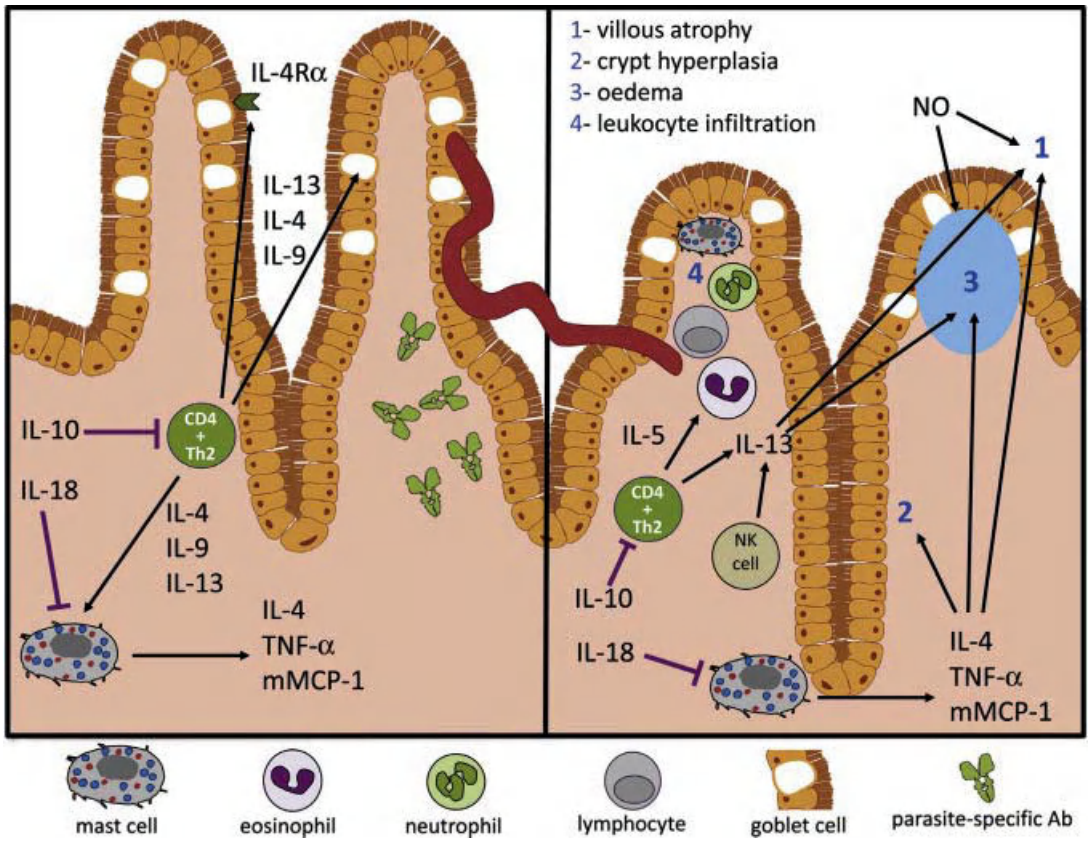

Figure 15.3 Intestinal immune response to *T. spiralis*. (A) Mechanisms required for primary clearance of adult worms. Parasites can survive for more than 80 days in the absence of Th2 cells. Expulsion is subtly delayed in the absence of IL-4Rα on both epithelial cells and bone-marrow derived cells. Efficient expulsion also requires the induction of mast cells via Th2 cell-derived cytokines and mast cell production of IL-4, TNF-α, and mMCP-1. Goblet cells and parasite-specific antibodies also contribute to clearance. (B) Mechanisms of enteropathy during T. spiralis infection. (1) Villous atrophy is dependent on IL-13, NO, and mast cell production of IL-4, TNF-α and mMCP-1. (2) Crypt hyperplasia is also dependent on mast cell products. (3) Oedema is dependent on IL-13, NO and mast cell products. (4) Leukocyte infiltration is induced by chemokines and Th2 cytokines. Tissue eosinophilia, driven by Th2 cell-derived IL-5, is shown. Th2 and NK cells are the primary sources of IL-13.
Abbreviations: IL, interleukin; mMCP, mouse mast cell protease; NK, natural killer; NO, nitric oxide; TGF, transforming growth factor; TNF, tumour necrosis factor.

Blood and tissue eosinophilia are induced by the Th2 associated cytokine interleukin (IL)-5, and local T cells and monocytes recruit eosinophils to the intestine by production of the chemokines CCL11 (eotaxin-1) and CCL24 (eotaxin-2). Despite the prominence of eosinophils in the intestinal immune response, they are not required for worm clearance.

Goblet cell hyperplasia is dependent on IL-13, with a lesser dependence on IL-4 and IL-9. Goblet cells synthesise and secrete mucins, and *T. spiralis* worms become entrapped in mucus during clearance of a primary infection, although it is unclear whether this effect is causal. The production of several goblet cell-derived molecules is increased during infection, including mucin 2, mucin 3, mucin 5ac, trefoil factor 3 (TFF3), sialyl transferase 4c (Siat4c), intelectin-2

and resistin-like molecule-β (RELM-β). Infection of gene knockout mice has shown that mucin 5ac is necessary for efficient expulsion of *T. spiralis*, while RELM-β is not required.

Intestinal mastocytosis during infection is dependent on IL-4, IL-9 and IL-13. Infection of mast cell-deficient rats and mice has revealed a role for mast cells in worm expulsion. Furthermore, mouse mast cell protease-1 (mMCP-1) and mast cell-derived TNF-α promote worm clearance. The impact of these molecules is somewhat mild, compared with the profound delay in expulsion observed in T cell-deficient mice, suggesting that mucosal mast cells and their mediators are part of a more complex mechanism. Evidence in support of this notion derives from the observation that mast cells contribute to the inductive phase of the immune response by promotingTh2 cytokine responses in the mesenteric lymph nodes (MLN).

Clearance of adult worms is severely compromised in T cell-deficient mice, and adoptive transfer of lymphocytes reverses this phenotype. The Th2 cytokines IL-4 and IL-13, which signal through a common receptor subunit (IL-4Rα), are required for efficient expulsion of adult worms. IL-4Rα is present on many cell types, and expulsion is delayed in mice in which IL-4Rα is absent from either bone marrow-derived or non-bone marrow-derived cells.

Specifically, IL-4Rα expression is not required on mast cells, CD4+ T cells or LysM+ cells (macrophages and neutrophils). However, when IL-4Rα ablation was targeted to intestinal epithelial cells, expulsion was delayed. Thus, IL-4/IL-13 stimulate epithelial cells to promote worm rejection. As IL-4Rα signalling promotes goblet cell hyperplasia and RELM-β production, it is likely that stimulation of goblet cells via IL-4Rα also increases Muc5ac production, thereby promoting worm clearance.

15.3.3 Protection against reinfection

The secondary immune response to *T. spiralis* varies among host species. Immune rats display a dramatic protective immunity, in which 90 per cent of intestinal larvae are cleared from the intestine within 30–60 minutes and 99 per cent of larvae are cleared within 24 hours – a phenomenon that has been called rapid expulsion (or rapid rejection). Secondary immunity in mice is much less robust; immune mice exhibit expulsion of adult worms that is accelerated by 2–7 days. This immunity has not been thoroughly investigated. Secondary intestinal immunity in swine resembles that of mice, although swine also manifest strong immunity to NBL.

In rats, tyvelose-specific antibodies play a key role in rapid expulsion and convey as much as 88 per cent protection upon suckling rats. In adult rats, antibody alone is not sufficient, and either immune T cells or infection with a heterologous intestinal parasite can enable anti-tyvelose antibodies to effect expulsion. Intestinal priming is not a strict requirement, as rats immunised with infections limited to the muscle phase produce anti-tyvelose IgG and display rapid expulsion.

Mucosal mast cells degranulate at the time of rapid expulsion in rats immunised with either oral or muscle infections, prompting speculation that mast

cells are central to expulsion. However, recent evidence has shown that mucosal mast cell activation is neither sufficient, nor required, for rapid expulsion. Thus, the mechanism that enables antibody-mediated protection in adult rats remains elusive.

The action of anti-tyvelose antibodies has been investigated in neonatal and adult rats, as well as in an epithelial cell culture model. In the presence of monoclonal anti-tyvelose IgG, larvae are excluded from or encumbered in the epithelium. *In vivo*, luminal larvae become entrapped in mucus, although entrapment is reversible. The evidence supports a mechanism by which antibodies directly inhibit mobility and sensory reception of larvae. All of these effects are directed solely at first-stage larvae. Following the first moult, larvae are no longer susceptible to the effects of anti-tyvelose IgG.

15.3.4 Cellular immunity in the muscle phase

Muscle infection by *Trichinella* is characterised by the development of focal inflammation at the site of the infected muscle cell (Figure 15.2A). Encapsulated species of *Trichinella* induce a more robust reaction in comparison with non-encapsulated species. The intensity of muscle inflammation is influenced by the intestinal phase of infection, as mice infected orally show greater inflammation of the muscle tissue (myositis) compared to mice infected by injections of NBL. Cellular infiltrates include macrophages, eosinophils, neutrophils and CD4+ T cells, with few CD8+ T cells or B lymphocytes. Macrophages are the most numerous cell type, and they are also observed in the cytoplasm of the nurse cell. Myositis induced by *T. spiralis* is down-modulated in intensity as the parasite matures in the muscle.

T cells are critical in orchestrating leukocyte recruitment; infiltration does not occur in athymic mice. Cytokine responses in the draining lymph nodes are mixed initially, and then they polarise to a Th2 phenotype (IL-4, IL-5, IL-10, and IL-13), which promotes a strong antibody response against tyvelose after 28 days of infection. Blood mononuclear cells recovered from human trichinellosis patients produce significant quantities of IFN-γ, IL-10, and IL-5, and they retain the ability to proliferate in response to larval antigens for as long as three years after initial infection.

Nurse cells are surrounded by alternatively activated (M2) macrophages, which are driven to an alternative phenotype by Th2 cytokines. M2 macrophages appear to protect larvae, whereas classical activation of macrophages (M1), and their production of NO, is associated with clearance of larvae from muscle (Figure 15.4).

Although M2 macrophages facilitate tissue remodelling and produce collagen, the available data indicate that it is the nurse cell itself that synthesises the collagen capsule.

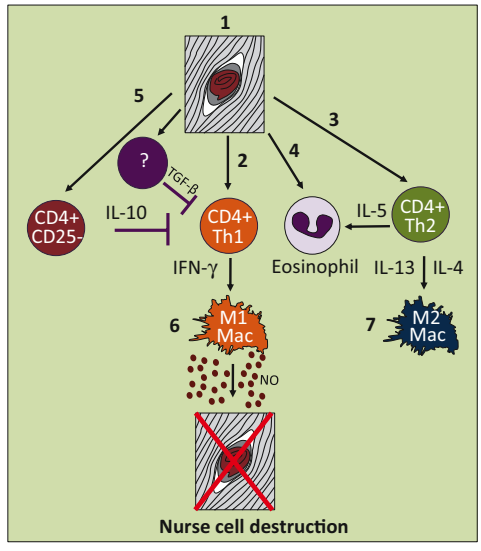

Figure 15.4 A model of immunity to *Trichinella* in the muscle. Infection of skeletal muscle cells (1) induces a mixed Th1 (2) and Th2 (3) cellular response. Tissue eosinophilia (4) and the regulatory cytokines IL-10 and TGF-β (5) are also induced by infection and contribute to the control of classically activated M1 macrophages at sites of infection (6), supporting a Th2 environment that promotes activation of alternatively activated M2 macrophages (7) and parasite survival.
Abbreviations: IL, interleukin; TGF, transforming growth factor.

M2 macrophages are disseminated systemically in *T. spiralis* infected mice, as evidenced by production of large quantities of Ym1 (a molecule with homology to the eosinophil chemotactic factor ECF-L secreted by M2 macrophages) by peritoneal macrophages during infection.

15.3.5 The regulatory response in the muscle and the role of eosinophils

Myositis is tightly regulated and generally does not lead to parasite destruction, which suggests that suppressive parasite-derived or host-derived factors are present in the muscle tissue. IL-10 and transforming growth factor (TGF)-β are known to limit myositis and suppress the production of IFN-γ and inducible nitric oxide synthase (iNOS) during the stage of infection when parasites are actively growing in the muscle (i.e. between 10–20 days post-oral infection). Muscle larvae burdens are moderately reduced in IL-10 deficient mice, and larval survival improves with iNOS inhibition, implicating NO in parasite killing.

Elimination of both TGF-β and IL-10 promotes an elevated IFN-γ response with more pronounced myositis and increased parasite death (Figures 15.2 and 15.3). Once parasite growth inside the nurse cell is complete and chronic infection is established, the Th2 response dominates and, coincidentally, regulation of inflammation switches to an IL-10 independent mechanism.

Eosinophils constitute 10–15 per cent of the infiltrating leukocytes at sites of muscle infection. The functional consequence of eosinophil deficiency on *T. spiralis* muscle infection has been tested using two mouse models of eosinophil ablation: PHIL (a mouse which expresses the diphtheria toxin receptor A chain under the control of the eosinophil peroxidase promoter, causing selective deletion of eosinophils upon expression of eosiniophil peroxidase and administration of diptheria toxin) and Δ dblGATA (deletion of the palindromic GATA-1 binding site, leading to defective GATA-1 signalling and a lack of eosinophil development). *T. spiralis* larvae die in large numbers in the absence of eosinophils, with reductions in muscle burdens ranging between 48 and 77 per cent. Parasite death correlates with enhanced IFN-γ and decreased IL-4 production in the draining lymph nodes.

Inhibition of iNOS (and therefore the ability of mice to generate NO) in these experiments improves parasite survival, further implicating NO in parasite clearance. Similarly, increasing NO production by introducing IL-10 deficiency into the PHIL background dramatically enhances NO production and increases parasite killing to 90 per cent or more. Thus, eosinophils regulate local immunity and protect the parasite during the muscle phase of infection. These findings are in stark contrast with those of *in vitro* studies that implicate the eosinophil as a mediator of *T. spiralis* destruction.

15.4 Immunopathology

Many studies have differentiated protective from pathologic responses in the intestine during *T. spiralis* infection. *T. spiralis* enteropathy includes villous

atrophy, crypt hyperplasia, oedema, mastocytosis and an increase in myeloperoxidase activity which is associated with neutrophil infiltration. Furthermore, pathological changes in smooth muscle function are induced by *Trichinella* infection, and these changes persist after worm clearance, in a syndrome referred to as post-infectious gut dysfunction.

TNF-α is a major promoter of the pathogenic symptoms of *Trichinella* infection, causing villous atrophy, crypt hyperplasia and intestinal mastocytosis. NO also plays a role in pathology, as iNOS-deficient mice display no significant enteropathy from *T. spiralis* infection. However, Th2 cytokines also contribute to pathogenesis: IL-4 production is required for crypt hyperplasia, while IL-13 mediates villous atrophy, as well as oedema.

Although pathology is dependent on IL-4 and IL-13, specific deletion of IL-4Rα signalling on CD4+ T cells or macrophages/neutrophils has no effect on the severity of lesions detected in infected mice. Therefore, other cells of the intestine are a critical source of the Th2 cytokines mediating pathology in *T. spiralis* infection. In fact, mast cell production of IL-4 (as well as TNF-α and mMCP-1) is required for enteropathy, whereas intraepithelial NK cells provide a potent, innate source of IL-13. Indeed, NK-derived IL-13 is sufficient to induce pathology in immunodeficient SCID or nude (T cell deficient) mice.

15.5 Evasion strategies

T. spiralis evades host immunity by several means. The most significant of these is sequestration in intracellular habitats. All life stages are intracellular, with the exception of migrating NBL (Figure 15.1). In addition, the parasite moults and develops rapidly in the intestine, faster than immune response clearance mechanisms become active. Immunogenic surface antigens and secreted products change with each life stage; for example, tyvelose is synthesised by first-stage larvae, but not by other larval stages or by adults. Adult worms reproduce and release NBL as early as four days after infection, ensuring that muscle infection will be established prior to immune-mediated clearance of worms from the intestine.

In the muscle, *T. spiralis* larvae can persist for months to years, consistent with evasion or suppression of host immunity. Although reactive oxygen and nitrogen species are toxic to developing larvae, mature muscle larvae express genes encoding protective antioxidant enzymes, including thioredoxin oxidase, peroxiredoxin and glutathione peroxidase. These products may protect larvae during chronic infection. Developing muscle larvae also inhibit immune-mediated destruction by stimulating production of regulatory cytokines that block Th1 immunity (Figure 15.4).

As discussed above, IL-10 and TGF-β dampen Th1 immune responses and NO production that would otherwise cause parasite death in the muscle. Eosinophils exert a similar effect, preventing local production of NO. Additional mechanisms of immune evasion will be elucidated as we improve our understanding of the requirements for maintenance of chronic infection.

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Trematoda: Schistosomes 16

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16.1 The schistosome life cycle

Of the 23+ mammalian schistosome species, five major species infect humans (Table 16.1). The tropism and migration of these five species within their definitive hosts segregate between the veins of the vesical and pelvic plexuses (*Schistosoma haematobium*) and the mesenteric veins surrounding the intestinal tract (*S. intercalatum*, *S. japonicum*, *S. mansoni* and *S. mekongi*). Accordingly, the major morbidity and pathologies associate with the bladder or the gastrointestinal (GI) tract, respectively. Blood-dwelling adult schistosome worm pairs produce hundreds of eggs per day. A significant proportion of the released eggs traverse through the wall of the intestine or bladder, depending upon the species, and exit the host via urine or faecal matter.

The precise mechanism by which eggs traverse across the endothelium, through the basement membrane and epithelium of the intestinal tract or bladder is unknown. This process requires an active immune response against the eggs, as immunocompromised hosts have significantly reduced egg release despite similar levels of egg production. Unfortunately, many parasite eggs are carried in the flow of the vasculature and become lodged within vascularised tissues and organs. Tissue-trapped eggs are the main cause of pathology following infection, rather than the adult worms themselves. The pathogenesis of schistosome infections and the morbidity associated with infection is due to a lethal combination of highly immunogenic eggs, a vigorous immune response and the various organs in which eggs become trapped.

16.1.1 Egg excretion, miracidium and snail intermediate hosts

In regions of the world where hygiene and sanitation are poorly controlled, schistosome eggs enter freely into the environment (Figure 16.1). Miracidium,

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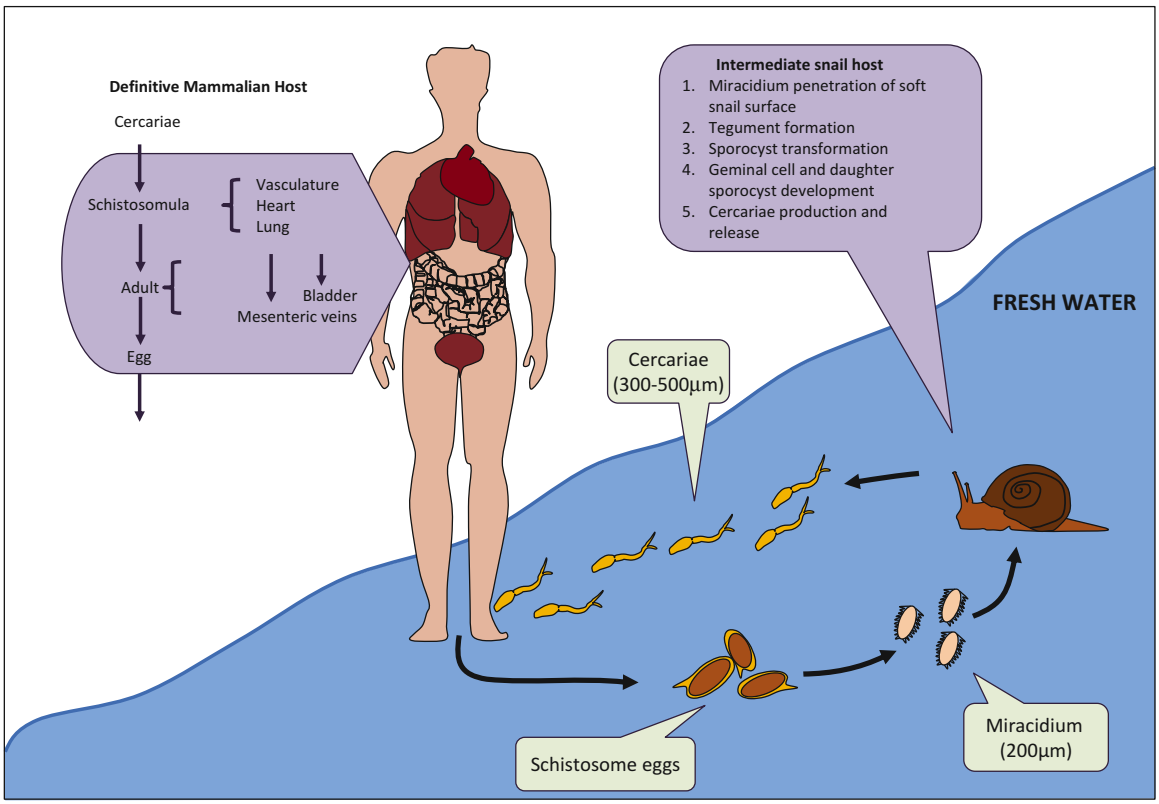

Figure 16.1 The life cycle of schistosomes.

the intermediate larval stage of the parasite, hatch from parasite eggs upon contact with a hypotonic environment in sunlight. Once released from the egg, the highly motile ciliated miracidium follow phototactic gradients, migrating towards snail secretions at the water surface.

Snails serve as the intermediate host for schistosomes, with a high degree of schistosome-snail species specificity (Table 16.1). The geographical location of the intermediate snail host largely restricts and dictates the geographic location of specific schistosome species. Once the intermediate snail host is located, miracidium penetrate through the soft exterior wall of the snail into the haemocoel, using enzyme-rich penetration glands. Within the haemocoel,

Table 16.1 Intermediate snail hosts of schistosomes.

Schistosome species	Intermediate snail host	Global distribution
Schistosoma intercalatum	*Bulinus* species	Africa
Schistosoma haemotobium	*Bulinus* species	Africa, Middle East
Schistosoma japonicum	*Oncomelania* species	Asia
Schistosoma mansoni	*Biomphalaria* species	Africa, South America, Caribbean, Middle East
Schistosoma mekongi	*Neotricula aperta*	Asia

miracidium transform into sporocysts, shedding their ciliated surface, and develop a lipid-rich tegument. Germinal cells form and mature with the production of daughter sporocysts, which generate and release thousands of cercariae. Cercariae are the second infectious larval stage, and cercarial output appears to be proportional to the size of the snail.

16.1.2 Cercariae to adult worms

As free-swimming larvae, cercariae are short-lived; their sole purpose is to travel from the intermediate snail host to the definitive mammalian host. Cercariae possess a muscular tail, which propels the larvae through the water, guided by a lipid sensory system to detect fatty acids on the skin of potential hosts. Upon detection of skin lipids, cercariae release the contents of pre-and post-acetabular glands, which are rich in enzymes for epidermal and dermal cell lysis. Following attachment and penetration, cercariae transit through a cercarial/schistosomulum transformation on the way to adulthood.

Morphological, biochemical and structural transformation enable the schistosomula to locate dermal blood vessels in the skin. These events vary in duration between schistosome species, ranging from hours to days. Once a vessel has been located and entered, schistosomula travel via the pulmonary artery to the lungs, and then on to the liver.

Within the liver, immature worms grow and develop, congregate and pair. Interestingly, without pairing, female worms remain stunted, in an arrested state of development. Similarly, in the absence of signals from the mammalian host immune system, adult worm development is stunted. These observations highlight an important interaction between parasite and host, with two-way communication regulating host immune response and parasite developmental status. Adult worm pairs finally lodge within the portal and mesenteric vessels of the small intestine, or the veins of the vesical and pelvic plexuses (depending upon the species) and produce eggs completing the life cycle.

Many schistosomes are zoonotic, with other mammals serving as reservoirs of infection. This poses a significant problem for control strategies aimed at disrupting transmission. It is important to appreciate the epidemiology and prevalence within any given population, because this information can often give clues to the presence or emergence of immunologically resistant subpopulations.

Immunological status can clearly influence, and is influenced by, infectious dose and frequency. In schistosome endemic areas, similar to other parasitic helminths, an over-dispersed distribution is frequently observed, with few individuals harbouring heavy infectious burdens. Age profiles suggest that age-dependent (or exposure-dependent, as these two factors are often inseparable) immunity can develop, with older individuals harbouring relatively fewer worms in any given endemic population. Thus, epidemiological studies which identify how some individuals develop natural immunity may hold clues for vaccinologists.

16.2 Immunological recognition of schistosomes

Schistosomes, unlike approximately 2,700 genera of digenean parasites, infect their definitive host via direct penetration of the skin. As the first line of defence, the skin provides an essential barrier between the host and pathogen. The dermal layer, in particular the basement membrane, is efficient at preventing a significant number of infective cercariae from successfully entering. However, cercariae are equipped with protease-containing pre- and post-acetabular glands capable of dermal degradation. There is evidence of dermal thickening, local vasodilatation, oedema and neutrophil infiltration into the surrounding tissue within hours of exposure to infective larvae. This indicates that there may be early recognition of invading cercariae, or that local tissue damage caused by migrating larvae induces local inflammation.

It has been technically very challenging to identify the innate and adaptive immune responses to invading cercariae in the skin. However, using human and murine skin with Franz cell technologies, and experimental animal models with radiation-attenuated (RA) cercariae, the earliest immune responses to schistosomes are being unravelled. As sentries of the immune system, the earliest cells identified within the cercarial lesion in the skin are antigen- presenting cells (APC), identified as major histocompatability complex (MHC) positive cells. MHC-II positive cells within the skin express macrophage (CD11b and F4/80) and dendritic cell (DC) (CD11c) markers along with the co-stimulatory molecule CD86 (B7-2), suggesting that the invading cercariae are detected by the innate immune system and are a potential source of antigens to prime the immune system (Figure 16.2).

To forestall the invading cercariae, innate and local stromal cells trigger an inflammatory cascade, with the release and stimulation of macrophage inflammatory protein (MIP)-1α, MIP1β, Interleukin (IL)-6, IL-1β, IL-12/23p40 and IL-18. Cercarial products that can also directly stimulate production of cytokines, such as IL-4 and IL-10, which dampen the Th1 inflammatory response via their antagonistic effects on IL-12/23p40 production.

Excretory/secretory (E/S) products such as acetabular gland contents, secreted by cercariae, are immunogenic and can recruit innate cells and activate IL-12/23p40 production. Cercariae/schistosomula transformation, which occurs soon after dermal penetration, includes the development of a double-layered tegument, essential for nutrient uptake and immune evasion. However, the schistosome tegument is also immunogenic, inducing IL-12/23 p40, tumour necrosis factor (TNF)-α and stimulating the up-regulation of CD40 and CD86 on DCs *in vitro*. Recognition of tegument antigens requires activation of the TLR-4/Myeloid differentiation primary response gene (MyD88) pathway. Thus, cercarial secretions within minutes to hours after penetration, and subsequent cercariae/schistosomula transformation, both trigger the innate immune system.

It is clear that schistosomes are recognised from their earliest interaction with the host. However, the fact that schistosomes establish chronic infections lasting many years in humans indicates that either the host response is

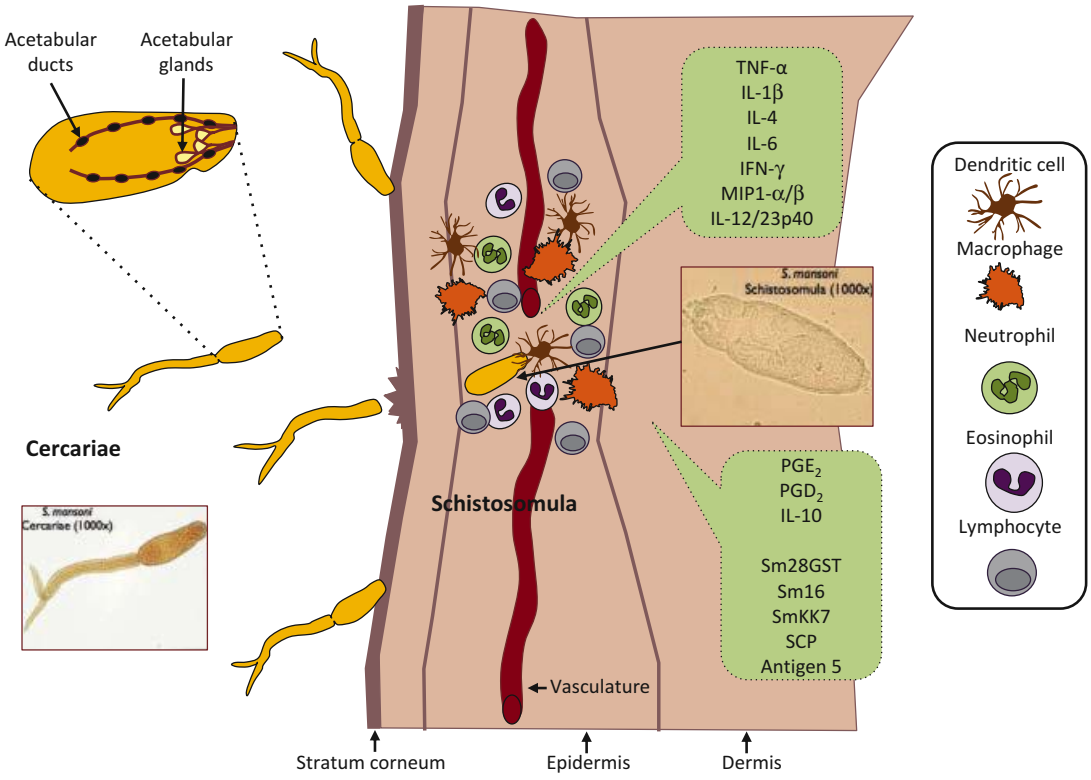

Figure 16.2 Infection of humans by *Schistosoma* cercariae.
Abbreviations: IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; PG, prostaglandin; SCP, sperm coat proteins; TNF, tumour necrosis factor.

inappropriate or subverted, insufficient, too late or suppressed (or a combination of all of these).

16.3 Innate effector mechanisms

Innate cell mobilisation and activation occurs in two distinct phases: first, following acute schistosome (and most other) infections, and second, following adaptive immune cell orchestrated events. Following cercarial penetration, eosinophils, neutrophils, macrophages, DCs and basophils have all been observed in the skin.

The initial wave of innate effector cells is effective at limiting super-infection in mice, but this has not been systematically investigated to determine the precise involvement and requirement of each cell type. The second wave of innate effector responses is instructed by the adaptive immune response and involves eosinophils, mast cells and macrophages directed towards various life cycle stages of schistosomes, from invading larvae to adult-released eggs.

The mouse model of schistosome infection has some limitations that should be appreciated. It may not be an ideal model to study resistance to reinfection, the

role of innate effector cells or the development of immunity to infection due to differences in physiology following infection, cellular receptors and molecular contents of some innate cells. For these (and many other) reasons, most of our understanding of innate effector responses to schistosomes is derived from *in vitro* studies using cells from infected humans or radiation-attenuated cercariae in mouse models.

16.3.1 Eosinophils

It is clear that activated eosinophils are potent killers of schistosomula *in vitro,* a process facilitated by antibody, complement, mast cells, neutrophils, oxygen metabolites and leukotrienes. Collectively, these studies paint a complex multifactorial picture of antibody opsonisation of schistosomula, attracting a mixed innate cell response for efficient eosinophil-mediated killing. Although human eosinophils are efficient at killing schistosomula *in vitro,* this has not been confirmed in human infections *in vivo*. However, it is noteworthy that individuals resistant to schistosome reinfection frequently have elevated circulating eosinophils.

Mouse eosinophils are not proficient at killing schistosomula *in vitro* or *in vivo*. Eosinophil-deficient mice have a similar frequency of adult worms following a primary infection, and protection from a challenge infection following irradiated cercarial vaccination was not increased in IL-5 transgenic mice which have significantly elevated eosinophils. Thus, mice cannot be used to model the role of eosinophils in protection against Schistosomiasis.

16.3.2 Neutrophils

Human neutrophils, like eosinophils, are capable of killing schistosomula *in vitro* and may synergise with eosinophils. Similar to esoinophils, neutrophil-mediated killing is enhanced by leukotrienes, antibody and complement. *In vivo* animal studies have demonstrated that neutrophils are recruited to the skin during primary infection and following irradiated cercariae vaccination.

16.3.3 Macrophages

Macrophages are recruited into inflammatory foci surrounding larvae, both in the skin and the lungs, following irradiated cercariae vaccination and post-lung lesions. One would hypothesise that macrophages are involved in parasite killing but, to date, this has not been clearly demonstrated.

16.4 Adaptive immunity

16.4.1 Effector T cell responses

Coordinating innate cell recruitment and activation, help for B cells to class switch and produce antibodies, CD4 T cells are central to almost every aspect

of the host's repertoire of responses to schistosome infection. The adaptive immune response following the early stages of acute infection is mixed with T helper 1 (Th1), Th2 and Th17 cell involvement. From a mixed Th1/Th2/Th17 response early during acute infection, egg-laying (5–6 weeks post infection) leads to a dominant Th2 response. The principal events precipitating Th2 dominance during chronic infection are schistosome egg antigens, released from eggs lodged in the liver, the gut and other vascularised organs. Immunocompetent hosts mount a strong immune response to parasite eggs, with the development of a vigorous collagen-rich granulomatous response around the eggs. This response eventually sequesters egg products, but it can also lead to severe hepatic fibrosis and portal hypertension.

Many years of research have identified essential components required for Th2 development, including the expression of CD40, CD80 and CD86 and ICOS-Ligand on APCs and the early induction of IL-6. However, one of the most integral components required for Th2 development is IL-4.

The precise source of IL-4 for Th2 differentiation eluded immunologists for a long time, with basophils recently identified as an important source, despite these cells having been observed in Th2 environments for quite some time. Recent work suggests that basophils are both necessary (as IL-4 producers) and sufficient (as both IL-4 producers and antigen presentation) to induce Th2 responses against parasite antigens. This has been challenged and is still disputed, with studies indicating that DCs, rather than basophils, are required for optimal antigen presentation and Th2 induction. Currently, it appears that a three-way interaction with basophils, DCs and T cells is most favourable for Th2 differentiation.

It has long been appreciated that adaptive immune competency is necessary for effective granulomatous reactions to develop. T cell-deficient mice, particularly CD4 helper T cell deficient mice, fail to mount an effective granulomatous response. While the relative contribution of IFN-γ-producing Th1 cells versus IL-4, IL-5 and IL-13-producing Th2 cells in the granulomatous response has long been debated, fibrosis and much of the pathology is primarily mediated by IL-13 and Th2 cytokines. In mice, signalling from the IL-4/IL-13 receptor via STAT6 is required for the development of the granulomatous response and resulting fibrosis following egg deposition. Vaccination of mice with parasite eggs and IL-12, to invoke a potent Th1 response, inhibited the Th1 to Th2 shift upon egg-laying after a challenge infection and resulted in amelioration of hepatosplenic pathology.

Mice deficient in IFN-γ signalling also display a reduction in granuloma size, suggesting that IFN-γ also contributes to granuloma formation. IFN-γ has strong anti-fibrotic properties, but excessive IFN-γ can also cause severe liver pathology in schistosomiasis, so extreme immune polarisation towards either Th1 or Th2 during schistosomiasis is detrimental, if not lethal. Indeed, upon schistosome infection, mice that lack both IL-10 and IL-4 develop an unchecked Th1 response, and 100 per cent die by nine weeks post-infection. Similarly, mice lacking IL-10 and IL-12 develop a vigorous Th2 response that is detrimental during the chronic phase of infection and display significant mortality by 12–15 weeks post-infection. Maintaining a balanced and controlled

Th1 or Th2 response is therefore critical for protective granuloma formation without excessive pathology.

16.4.2 Th17

Given the elevated levels of both IFN-γ and IL-4 at different times during infection with *S. mansoni*, and the antagonistic effects of both of these cytokines on Th17 development, IL-17 production is likely tightly controlled in schistosome infection. Immunisation with soluble egg antigens (SEA) in complete Freund's adjuvant (CFA) skews the T cell response in the Th1 direction following infection, resulting in increased inflammation and larger granulomas, extensive pathology and accelerated mortality after challenge infection. The morbidity of *S. mansoni* challenge infection after this immunisation protocol is correlated with high levels of IL-17.

Furthermore, CBA mice display a vigorous IL-17 response following infection with *S. mansoni* and develop severe liver pathology. Neutralisation with anti-IL-17 mAb reduces the granuloma size in CBA mice, supporting the notion that 17-secreting cells play an inflammatory role during acute *S. mansoni* infection if left unregulated. These studies underpin the essence of immune regulation, demonstrating that an uncontrolled adaptive immune response in any particular direction (Th1, Th2 or Th17) can be fatal.

16.4.3 Immunoregulatory pathways

Every immune response induced in schistosome infection, from innate effector responses through Th1, Th2 or Th17 cell differentiation and ultimately fibrogenesis, is tightly controlled and paralleled with an equal regulatory response. Mounting an appropriate effector immune response to an invading pathogen is important. However, turning an immune response off is equally, if not more, important. To this end, the immune system is internally calibrated by specialised cells, regulatory T cells, with the sole purpose of turning immune responses off.

Early indications of Treg activity in schistosome infections were observed in patients presenting a hypo-responsive T cell phenotype. Responsiveness, however, was recoverable *in vitro* with antibodies to IL-10 and/or TGFβ, two cytokines associated with Treg-mediated suppression. Recent studies have confirmed these initial speculations of Treg activity as a general feature of helminth infections, and Treg responses may dampen anti-helminth effector responses, allowing the establishment of chronic infections.

During schistosome infections, both natural and inducible Treg cells have been described, with varying roles including the suppression of DC activation, orchestration of the Th2 response and the regulation of Th2 effector responses, granuloma development and fibrosis.

16.4.4 Natural Tregs in schistosome infection

From four weeks post-infection with *S. mansoni*, a significant expansion of natural Treg cells, CD4+CD25+Foxp3+, develop in the mesenteric lymph nodes, with further expansion and accumulation of Tregs in the liver and spleen thereafter. Similarly, a single immunisation with *S. mansoni* eggs invokes a significant Foxp3+ Treg response, suggesting that the highly immunogenic egg antigens (SEA) may be potent inducers of both effector and Treg cells.

Lysophosphatidylserine (Lyso-PS) extracted from *S. mansoni* worms, and to a lesser degree *S. mansoni* eggs, can actively induce IL-10-secreting Treg cells, via TLR-2 signalling on dendritic cells, suggesting that Tregs can be directly induced by parasite derived molecules. Interestingly, the ratio of Tregs to effector T cells, either following infection or egg immunisation, does not appear to change, suggesting that the expansion of effector T cells are closely monitored and equalled by a regulatory T cell response. Whether Treg cells are directly induced by schistosomes, or simply develop in parallel with the effector T cell response, is not yet clear.

The requirement and importance of Treg cells during schistosomiasis has been investigated using adoptive transfer of naïve CD25-depleted (natural Treg depleted) CD4+ cells into RAG KO mice. This resulted in increased weight loss, elevated hepatotoxicity and increased mortality following infection, indicating an important requirement for Tregs to control liver pathology.

Phenotypic studies of natural Tregs during schistosome infections have identified the surface expression of CD103 and Neurophilin-1 on Tregs. CD103 is an $\alpha E\beta 7$ T cell integrin required for cell-cell contact and is highly expressed on activated Treg cells. The precise role and requirement of CD103 during *S. mansoni* infection has not yet been clearly elucidated, but targeting CD103 may dampen Treg cell function and tilt the balance in favour of effector responses. Neuropilin-1 is a receptor involved in axon guidance, angiogenesis and the activation of T cells, but the role of neuropilin-1 on Treg cells during *S. mansoni* infection is also unknown.

The mechanisms by which natural Treg cells control inflammation and immunopathology is not clear. The advent of Foxp3+ reporter mice, containing the Foxp3 green fluorescent protein (gfp) knock-in allele and 'DEREG' mice (allowing the preferential removal of Foxp3-expressing cells) will permit more detailed analysis, sorting and definitive adoptive transfer experiments to identify the development, function and specificity of Treg cells in *S. mansoni* infection.

16.4.5 Inducible Tregs in schistosome infection

IL-10 has clear regulatory roles during *S. mansoni* infection, and critically regulates liver inflammation and pathology. The majority of IL-10-producing T cells appear to be Foxp3 negative and can suppress the proliferation of naïve CD4+ T cells. Using $RAG2^{-/-}$ and $Il10^{-/-}$ $RAG2^{-/-}$ mice reconstituted with

IL-10-producing or IL-10-deficient CD4+ T cells, it has been demonstrated that CD4+ T cells cells provide a significant proportion of IL-10 during *S. mansoni* infection. However, IL-10 from natural and inducible Tregs also is important for the suppression of DC-derived IL-12 and the generation of Th1 responses during infection, helping to mould the T cell response into a Th2 phenotype. Thus, it appears that Treg cells have the capacity to control both Th1 and Th2 development.

In support of this, depletion of cells expressing the α chain of the IL-2 receptor (CD25) in an egg-immunisation model resulted in elevation of both IFN-γ and IL-4 responses following immunisation. However, it should be noted that depletion of CD25+ cells on their surface will encompass the removal of nTreg, iTreg and activated effector T cells during immunisation (all of which express CD25). Indeed, other studies have shown that suppression of IL-4 and IFN-γ appears to be independent of IL-10, indicating that both IL-10-dependent and independent pathways operate to limit effector responses.

In addition to the Treg-mediated suppression of T cell function and proliferation, Treg cells can influence the function of macrophages, CD8+ cells, B cells and eosinophils – all cells frequently elevated during schistosome infection. Therefore, Tregs may influence schistosome infection via their influence on these cell types.

16.4.6 Non-CD4 T cell responses (CD8, NKT and TCRγδ T cells)

In addition to CD4+ T cells, CD8+ T cells are also primed to respond to schistosome antigens, producing IFNγ and IL-2 upon reactivation and possessing significant cytotoxicity. It has been hypothesised that type 1 CD8+ T cells regulate CD4+ Th2 cell responses, in an IL-4-dependent mechanism. Although this hypothesis has not been clearly demonstrated, the regulatory role of IFN-γ on Th2 responses has been widely reported. IL-4 and IFN-γ-producing NKT cells are also activated following schistosome infection, with the potential both to amplify and to regulate Th1 and Th2 responses. Although TCRγδ T cells have been observed within murine schistosome egg granulomas and circulating in schistosome-infected individuals, TCR $\delta^{-/-}$ mice responded to schistosome infection in a similar way to TCR δ-sufficient mice, with no overt phenotype.

16.4.7 Effector, antibody-producing and immunoregulatory B cells

B lymphocytes play a very important role as cytokine-producing, antigen-presenting and immunoregulatory cells, in addition to more classically appreciated antibody-producing cells during schistosome infection. There are mixed reports regarding the role of B cells for the exacerbation of Th2 responses and granuloma formation. In B cell-deficient JHD mice, infection with *S. mansoni* produces more Th1 (and subsequently lower Th2) responses to schistosome antigens, albeit with a granulomatous response to liver-trapped eggs comparable to that seen with B cell-sufficient mice. However, infection of a slightly different B cell-deficient mouse (μMT) resulted in a significant increase in granuloma volume at both early and chronic stages of *S. mansoni* infection, without

any observable change in Th1/Th2 balance. B cells also appear to play an important role in early granuloma formation following *S. japonicum* infection of mice.

Although the Th cell-inducing properties of B cells are still under dispute, there is agreement about the immunoregulatory properties of B cells during schistosome infection. Several studies clearly demonstrate that schistosome-elicited B cells can actively suppress schistosome antigen and secondary immune responses.

Human observational studies have identified a positive correlation between antibody responses and resistance to reinfection, the cornerstone of vaccine efforts. In addition, the presence of a circulating CD23+ B cell subset correlates with resistance to *S. mansoni* reinfection. However, vaccination of B cell-deficient (and thus antibody-deficient) mice with irradiated cercariae resulted in protection against a challenge infection that was largely unchanged compared to that in B cell-sufficient control mice. Thus, it is probably fair to say that B cells are not critical for anti-schistosome responses or immunity, but that they play a role in the generation of optimal immune responses and down-modulation of inflammatory responses in the liver, while contributing some protection following vaccination.

16.5 Memory responses

Following treatment with praziquantel (PZQ), adult worms are efficiently eliminated, but reinfection rates are relatively high, making detailed studies of memory responses in patients living in endemic regions difficult. Returning travellers who had been infected while visiting endemic regions have measurable Th1 (IFNγ) and Th2 (IL-5 and IL-13) responses against adult worm antigens (AWA) and SEA up to eight years post-treatment. Interestingly, the magnitude of the response in the patients eight years post-treatment correlated positively with severity of acute disease eight years previously. Whether disease severity influences maintenance of memory responses is unclear and requires more detailed studies.

Animal experiments have not identified a significant central memory ($CCR7^+CD62L^+IL\text{-}2^+IL\text{-}4^-IFN\gamma^-$) response post-schistosome infection, treatment or vaccination. Effector memory CD4 T cells ($CCR7^-CD62L^-IL\text{-}2^{+/-}IL\text{-}4^+$ or IFN-γ^+), following egg-laying, are highly Th2 polarised cells and persist throughout infection. Currently, effector CD4+ cells are largely indistinguishable from the effector memory populations, making clear and focused studies difficult to design. Nevertheless, vaccination of mice with irradiated cercariae elicits a substantial population of effector memory T cells, with a Th1 bias that can persist in the lung for up to ten weeks.

In general, cellular responses in vaccinated mice post-challenge are rapidly down-regulated, suggesting that memory responses do not persist. This may be due to active suppression of antigen-experienced effector/memory T cells by regulatory T cells. However, a cell-intrinsic E3 ubiquitin ligase, Gene Related to Anergy in Lymphocytes (GRAIL) has been identified in CD4 Th2 cells following

repeated antigen exposure, and this also may contribute to the hyporesponsive state of T cells during schistosome infection.

16.6 Schistosome antigens eliciting immune responses

Identifying antigens that elicit protective immunity as potential vaccine candidates has been the quest of many researchers over many years. Whether protection can be achieved with cercarial, schistosomula, adult, egg, or a mixture of all these antigens, is still unclear. There are stage-specific antigens, with a high degree of cross-reactivity between them, which one might predict would help in developing a vaccine or new diagnostic tests. The precise cercarial, schistosomula or adult worm antigens recognised and responsible for the immune responses are slowly being defined through proteomic, mass-spec, cloning and genomic studies.

Omega-1, a T2 ribonuclease, is a specific Th2-inducing component of schistosome egg-derived antigens. Omega-1 does not stimulate IL-4 production from basophils, but rather alters the response of DCs, lowering the strength of signal and promoting Th2 differentiation. Of note, Omega-1-depleted SEA was still capable of inducing Th2 responses, suggesting that other factors within SEA are capable of driving Th2 differentiation. IPSE/α1, a highly immunogenic schistosome egg-derived glycoprotein, can induce IL-4 from basophils. Together, these two molecules, Omega-1 and IPSE/α1, provide the necessary cues for APCs to polarise naïve T cells into Th2 cells following exposure to schistosome eggs.

In experimental systems, SEAs are potent inducers of Th2 cells in the absence of any adjuvant, and they have been used as experimental allergens in models of allergic airway inflammation, inducing many of the parameters associated with human allergic asthma. This may not be uncommon. The glycosylation and structure of parasite-derived antigens is closely related to common allergens, and the rising prevalence of allergic diseases in non-endemic regions of the world may be a consequence of anti-helminth responses being replaced with anti-helminth-like allergic responses (see Chapter 23).

16.7 Immune evasion

As large multi-cellular worms residing within the blood, surrounded by immunoglobulins and leukocytes, it is essential that schistosomes evade host responses. Schistosomes have both substantial defences and the capacity to defuse host immune responses.

16.7.1 The syncytial tegument as a protective barrier

As mentioned above, the syncytial tegument of schistosomes provides a sturdy physical barrier between the environment and the internal membrane. The external surface of the tegument is decorated with host antigens, disguising the parasite within the host. Schistosomula recovered after penetration of skin, or mechanically transformed from cercariae *in vitro*, are resistant

to eosinophil, antibody or complement-mediated killing when cultured for greater than 24 hours in the presence of anti-schistosomula sera or conventional culture medium. Whether the tegument surface is covered in host factors providing protection from components of the sera, or whether this is simply an intrinsic mechanism of developing schistosomula, the schistosomula rapidly develop and are protected from host attack.

Damage or disruption to the tegument of adult schistosomes does render them susceptible to immune attack. Praziquantel (PZQ), an effective anti-schistosomal drug, binds to the β sub-unit of calcium channels within the tegument of the adult worm. PZQ-associated blisters develop, damaging the integrity of the protective tegument, exposing the adult worm to cellular and antibody-mediated responses.

16.7.2 Schistosome-derived molecules

Schistosome-derived molecules can have dramatic affects on immune cells. It has long been appreciated that schistosomes release anti-inflammatory molecules (Table 16.2). Defusing host immune responses by invading larvae is ingenious, but it may also represent an 'Achilles heal' and is currently being investigated as a potential vaccine target. SEA can mute immune responses by interfering with DC maturation and responsiveness. Whether this protects parasite eggs as they traverse out of the host, or influences host responses for successful exit, is not known. The advancement of genetically manipulated parasites will allow us to test the role of specific parasite proteins and identify their importance and function. Identifying the specific immunomodulatory properties and targets of parasite-derived molecules will not only enhance our understanding of the biology of parasitism but may also identify novel anti-inflammatory agents (See Chapter 24).

16.8 Schistosomiasis and immunopathology

The majority of schistosome infections present limited clinical symptoms but, in some cases, schistosomiasis can develop, with severe and devastating symptoms.

16.8.1 Acute schistosomiasis

Many symptoms associated with acute infection have been reported. Cercarial dermatitis causes itching, maculae, papulae, urticariae and, in some cases, oedema and fever. Following larval migration in permissive hosts, pulmonary complications, including pneumonia and local inflammatory foci within the lung, can occur. However, these should not be confused with pulmonary complications associated with chronic patent schistosome infections once egg-laying has started.

During the acute stages of infection, Katayama fever, a systemic hypersensitivity reaction caused by migrating schistosomula, can also develop. It is

Table 16.2 Immune evasion molecules secreted by schistosomes

Molecule	Life cycle stage	Effect on the immune response	Reference
Ecionsoids Prostaglandin E2 (PGE_2) Prostaglandin D2 (PGD_2) 28KDa glutathione-S-transferase (*Sm*28GST)	Cercaria	Induces the production of IL-10 from keratinocytes (skin cells)	Ramaswamy, K *et al*. (2000). A role for parasite-induced PGE2 in IL-10-mediated host immunoregulation by skin-stage schistosomula of *Schistosoma mansoni*. *Journal of Immunology* 165(8), 4567–4574.
Acetabular gland contents	Cercaria/ schistosomula transformation	PBMC proliferation inhibited	Vieira, LQ *et al.* (1986). Inhibition of human peripheral blood mononuclear cell proliferative responses by released materials from *Schistosoma mansoni* cercariae. *Parasite Immunology* 8(4), 333–343.
16kDa protein from *S. mansoni* (*Sm*16)	Cercaria	Induces apoptosis and anti-inflammatory properties in PBMCs and macrophages	Brannstrom, K *et al.* (2009). The *Schistosoma mansoni* protein *Sm*16/*Sm*SLP/*Sm*SPO-1 assembles into a nine-subunit oligomer with potential to inhibit Toll-like receptor signaling. *Infection and Immunity* 77(3), 1144–1154.
*Sm*KK7	Cercaria or Cercaria/ schistosomula transformation	Potassium channel blocker which inhibits calcium flux in T cells preventing T cell activation	Curwen, RS *et al.* (2006). Identification of novel proteases and immunomodulators in the secretions of schistosome cercariae that facilitate host entry. *Molecular & Cellular Proteomics: MCP* 5(5), 835–844. Wilson, RA & Coulson, PS (2009). Immune effector mechanisms against schistosomiasis: looking for a chink in the parasite's armour. *Trends in Parasitology* 25(9), 423–431.
23kDa molecule	Schistosomula	Induces apoptosis in T cells	Chen, L *et al.* (2002). Skin-stage schistosomula of *Schistosoma mansoni* produce an apoptosis-inducing factor that can cause apoptosis of T cells. *The Journal of Biological Chemistry* 277(37), 34329–34335.
Sperm coat domain proteins (SCP)	Cercaria or Cercaria/ schistosomula transformation	Putative immunomodulatory properties	Chalmers, IW *et al.* (2008). Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the *Schistosoma mansoni* venom allergen-like (SmVAL) gene family. *BMC Genomics* 9, 89.

Table 16.2 (*Continued*)

Molecule	Life cycle stage	Effect on the immune response	Reference
Antigen 5	Cercaria or Cercaria/ schistosomula transformation	Homologue has anti-inflammatory properties in other Platyhelminth parasitic infections (*Echinococcus*)	Dillon, GP *et al.* (2006). Microarray analysis identifies genes preferentially expressed in the lung schistosomulum of *Schistosoma mansoni*. *International Journal for Parasitology* 36(1), 1–8.
44kDa chemokine binding-protein	Egg-derived	Binds to mammalian chemokines CXCL8 and CCL3 blocking inflammatory cell recruitment	Smith, P *et al.* (2005). *Schistosoma mansoni* secretes a chemokine binding protein with anti-inflammatory activity. *The Journal of experimental medicine* 202(10), 1319–1325.

conceivable that infection-associated immunomodulation, as described above, may prevent systemic Katayama fever. However, this has not been investigated.

16.8.2 Chronic schistosomiasis

The majority of clinical symptoms associated with schistosome infection is a result of disoriented parasite eggs and, therefore, due to patent chronic infections. Schistosome eggs which mistakenly become trapped in the microvasculature of the GI tract, liver or bladder induce a vigorous granulomatous response. Subsequently, fibrosis, portal hypertension and collateral vessels can develop. These are the primary morbidities in infected individuals and in some cases they can be fatal.

The organ-associated symptoms vary with different schistosome species, due to the anatomical location of adult worms. For example, urinary schistosomiasis is caused by *S. haematobium,* with adult worms residing within the veins of the vesical and pelvic plexuses, while intestinal schistosomiasis is caused by *S. mansoni* or *S. japonicum*, with adult worms found in the mesenteric veins surround the intestine. Symptoms are not restricted to these sites with reports of diarrhoea, hepato- and hepato-splenic disease, liver abscesses, brain tumours and myeloradiculopathy, genital, pulmonary and neurological schistosomiasis.

16.8.3 Granuloma formation, macrophages and fibrosis

Schistosome eggs are highly immunogenic and, within infected hosts, eggs induce vigorous immune responses and are rapidly surrounded by inflammatory cells, creating a granuloma (Figure 16.3). Although egg-induced granulomas are detrimental to the host, it is clear that the lesions serve an important host-protective function, particularly during *S. mansoni* infection. Schistosome eggs and their secreted products provide a continuous antigenic stimulus. If these

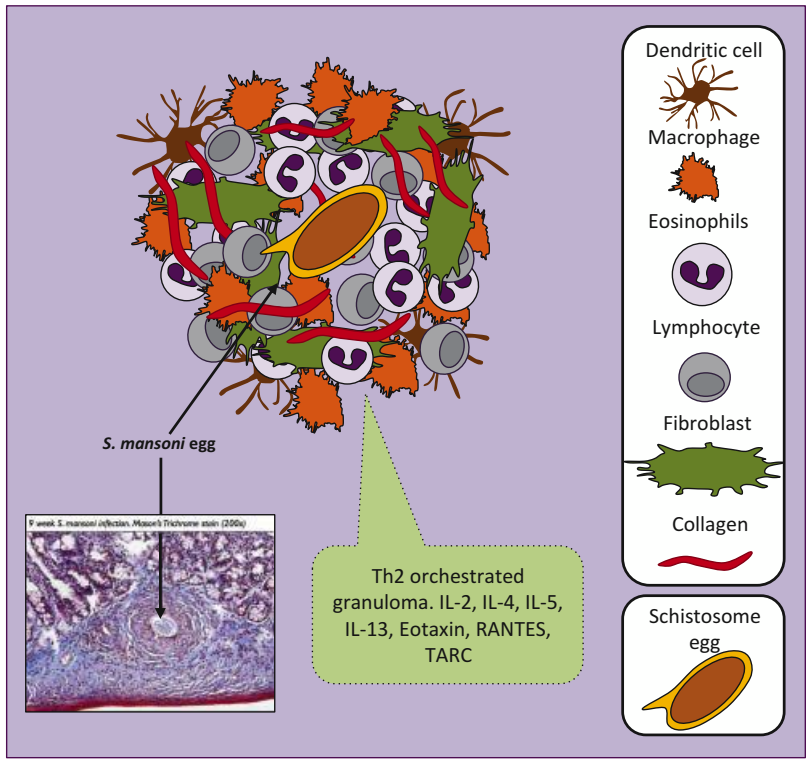

Figure 16.3 The schistosome granuloma.
Abbreviations: IL, interleukin; RANTES, regulated upon activation, normal T cell expressed and secreted; TARC, thymus and activation-regulated chemokine.

antigens are not sequestered or neutralised effectively, they can damage local tissue. T cell-deficient, nude, severe combined immunodeficiency (SCID) or egg-tolerised mice all die earlier than comparably infected immunologically intact mice, because they are unable to mount a protective granulomatous response. Schistosome granulomas are orchestrated by the CD4+ Th2 cell response induced by the egg antigens with the influx of eosinophils, mast cells, basophils and macrophages.

Macrophages are highly abundant within granulomas and are alternatively activated by the local cytokine environment. Th2 cytokines are required for the expression of arginase (Arg)-1 (a hallmark of M2 macrophages) in granuloma macrophages. Th1-skewed mice display classically activated M1 macrophages with enhanced iNOS, smaller granulomas and accelerated mortality. L-arginine is a common substrate for both M1-associated nitric oxide and M2-associated arginase. As a common denominator, L-arginine has the ability to govern the magnitude of macrophage activity, plasticity of macrophage phenotype and macrophage effector function. During alternative activation of M2 macrophages, L-arginine is hydrolysed by arginase and promotes the synthesis of collagen, providing a working model translating Th2 responses into fibrogenic responses.

16.8.4 IL-13 and IL-13Rα2

Many cytokines contribute to granuloma formation. However, IL-13 has emerged as an important pathogenic cytokine during infection. IL-13 signals through the IL-13Rα1 (which forms a complex with the signalling IL-4Rα chain) and IL-13Rα2. In *S. mansoni*-infected mice, there is a significant increase in IL-13Rα2 in the serum and liver, mirroring the Th2 response against parasite eggs; it is thought that IL-13Rα2 shedding is driven by Th2 responses via the signalling molecule STAT6. Mice with a targeted deletion of IL-13Rα2 (IL-13Rα2$^{-/-}$) develop exacerbated fibrosis compared to infected controls, and a soluble IL-13Rα2-Fc construct used to neutralise IL-13 can reverse the excessive liver fibrosis observed in infected IL-13Rα2$^{-/-}$ mice. This suggests that IL-13Rα2 acts as a potent decoy receptor for IL-13 in schistosomiasis, preventing IL-13 signalling via receptors combined of IL-4Rα IL-13Rα1.

16.8.5 Endogenous desensitisation

Several studies have shown that granulomas surrounding entrapped eggs decrease in size as infection progresses to the chronic stage. This 'endogenous desensitisation' is a hallmark of the granulomatous response during infection, and is thought to be critical for host survival in cases of persistent disease. The precise mechanisms of desensitisation are unclear, with both cell intrinsic and extrinsic pathways having been suggested.

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Cestoda: Tapeworm Infection

17

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Tapeworms are a very diverse group of parasites of the class Cestoda, the most characteristic tapeworms being classified in the subfamilies Taeniidae and Echinococcinae. In general, adult tapeworms infect the small intestine of carnivorous and omnivorous mammals, including humans, and they are distributed worldwide. *Taenia* and *Echinococcus* are globally of medical and veterinary interest as the causative agents of morbidity and mortality in humans, as well as production losses of livestock. Given this importance, we will focus on *Taenia solium, Echinococcus granulosus* and *E. multilocularis* as examples of tapeworms and discuss their life cycles, pathology, epidemiology, immune responses and evasive mechanisms, complemented with experimental data from laboratory models.

17.1 The life cycle of tapeworms

Tapeworms usually need at least two hosts to complete their life cycle; an intermediate host and a definitive host where they reach maturity. Individual organisms from different species of *Taenia* (including *T. solium* and *T. saginata*) are morphologically homogenous in their adult stage; they are generally flat and large, displaying a tape-like morphology often exceeding several meters in length, with a distinctly segmented strobila and a characteristic arrangement of rostellar hooks on their scolex (Figure 17.1). Life cycles for *Taenia* involve two mammalian hosts, including a carnivorous or omnivorous definitive host.

T. solium is the parasite that causes human and swine cysticercosis. The life cycle of this parasite includes the adult stage, the egg or oncosphere and the larval stage or cysticercus. When a person ingests raw or semi-cooked pork infected with cysticerci (the intermediate form), the scolex evaginates, adheres to the intestinal wall and transforms into a fully developed adult tapeworm that

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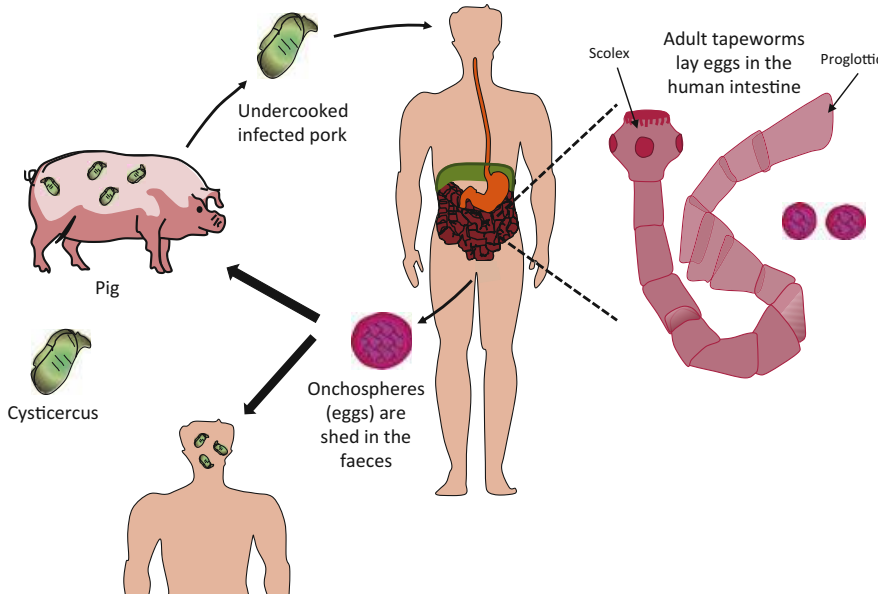

Figure 17.1 Life cycle of *Taenia solium*.

lodges in the lumen of the small intestine of the definitive host over 3–4 months. Humans are the only carriers of adult *T. solium* tapeworms. A similar life cycle has been described for *T. saginata*, whose larval form infects cattle. When this parasite attaches to the intestine and develops, it can reach 2–6 metres in length and cause taeniasis, a disease that is often asymptomatic.

The adult form (strobilate tapeworm) has a scolex, or head, with a double crown of hooks and four suckers used to attach to the intestinal mucosa. The scolex joins onto the neck and the strobila is formed by hundreds of immature, mature and gravid proglottids. The immature proglottids are the ones that are closest to the neck and have not yet developed sexual organs, whereas the gravid proglottids are furthest away and carry around 50,000 eggs each. Tapeworms are hermaphroditic organisms, with each mature proglottid containing male and female sexual organs. Gravid proglottids containing oncospheres are shed in the faeces, and can be released into environments without adequate hygienic or sanitary conditions. This adult stage (taeniasis) can last up to five years without visible symptoms.

When pigs ingest faeces with proglottids containing eggs, porcine gastrointestinal (GI) enzymes and bile induce egg hatching and the release of the oncospheres. This oncosphere penetrates the intestinal wall to migrate through the circulatory system into various organs, where it can transform into cysticerci in muscles, the eyes and the central nervous system (CNS).

Humans can become infected with this parasite stage when they ingest meat or vegetables contaminated with parasite eggs, in which case a similar process occurs, but the cysticerci preferentially go to the brain, causing neurocysticercosis (NCC), a life-threatening infection caused by the ingestion of eggs or

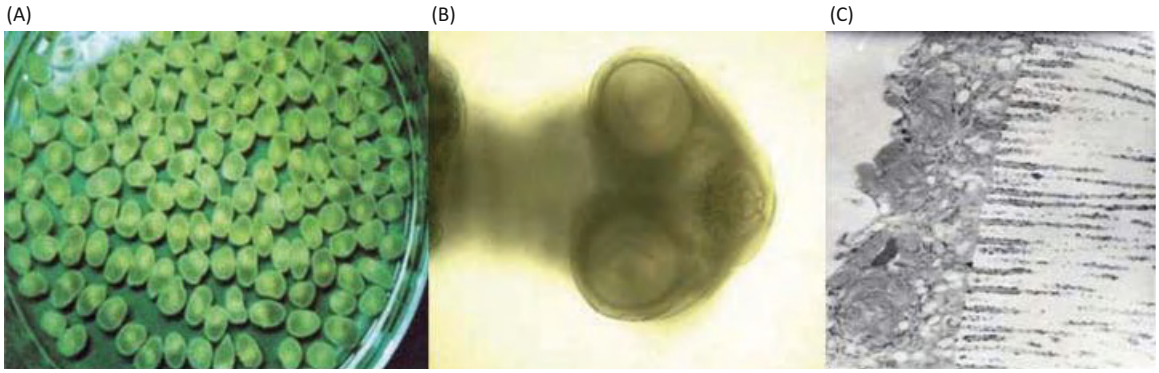

Figure 17.2 Larvae of *T. solium* obtained from infected pigs. a) Cysticerci. b) Evaginated scolex from a larva of *T. solium*. c) Electron micrograph of cysticerci tegument, note the elongations called microthriches covered with glycocalix. Photos kindly provided by Dr. Abraham Landa, Dept. of Parasitology, Faculty of Medicine, Universidad Nacional Autónoma de México.

oncospheres of the cestode *T. solium*. At this stage, the cysticerci are macroscopic vesicles (0.5–1.5 cm in diameter) filled with a clear fluid, and are composed of a vesicular membrane that folds inward as a spiral channel and ends in an invaginated scolex (Figure 17.2). Cysticerci can also develop in the eyes, muscle and subcutaneous tissue, where they can remain asymptomatic or result in vision loss or muscle hypertrophy.

17.2 Epidemiology

Cysticercosis and NCC are widely distributed throughout the world and are a public health problem in Asia (China, India, Taiwan and New Guinea), Africa (Cameroon) and Latin America (Mexico, Brazil and Peru). The prevalence of cysticercosis varies by country and also within countries, and it is influenced by the methods used for the detection of the disease. For example, the seroprevalence in northern India (measured by detection of specific serum antibodies against *T. solium* antigens) is around 3.5 per cent, but the incidence increases to 4.64 per cent in rural areas and drops to 2.32 per cent in urban zones. In Indonesia, up to 32 per cent of seropositivity in people has been reported in some regions.

In the last decade, cases of NCC have also reached dramatic numbers in developed countries, mainly due to migration from endemic countries. For example in the USA, Oregon has reported more than 135 cases of NCC between 1995 and 2000, with six deaths; in the largest public hospital in Houston, Texas, between 1997 and 2005, 111 patients were found to have NCC, with a similar number of reported cases in Kansas over the same time period.

Despite the high numbers of NCC patients, it is difficult to find adult tapeworm carriers. A recent study with 2,500 subjects demonstrated that while around four per cent tested positive for cysticercosis, only one case of taeniasis was observed. This is important, because only humans can spread this disease, and

therefore it demonstrates that a small number of people with taeniasis have the potential to infect hundreds of humans or pigs.

17.3 Pathology

In pigs, *T. solium* larvae lodge in different tissues, mainly skeletal muscle, tongue, heart and sometimes brain. In contrast, in humans, most cases of larval infection occur in the brain, resulting in NCC. Once ingested, these eggs are activated in the intestine and rapidly migrate via the bloodstream to the brain. It is unknown how this macroscopic parasite crosses the blood-brain barrier, or why these parasites preferentially lodge in the CNS. NCC is the most frequent and dangerous pathogenic neurological condition caused by a parasite, and it is the main cause of epilepsy or seizures in the developing world. NCC is considered a chronic, progressive disease, in which the severity of symptoms are closely related to the number of cysts, their anatomical location in the brain, the stage of infection and the intensity or magnitude of the inflammatory response.

NCC can be classified as parenchymal or extraparenchymal NCC. When the parasites are located inside the parenchyma, patients develop seizure disorders associated with degenerative parasites, and prognosis is good after treatment. Extraparenchymal NCC is associated with an excessive growth of the cysticerci, as well as with severe inflammation (Figure 17.3) that usually results in hydrocephalus, often requiring the need for surgery. The most severe form of this disease occurs when the cysticerci are located in the subarachnoid space at the base of the brain (subarachnoid basal NCC), although the frequency of this form is lower than that reported for parenchymal NCC.

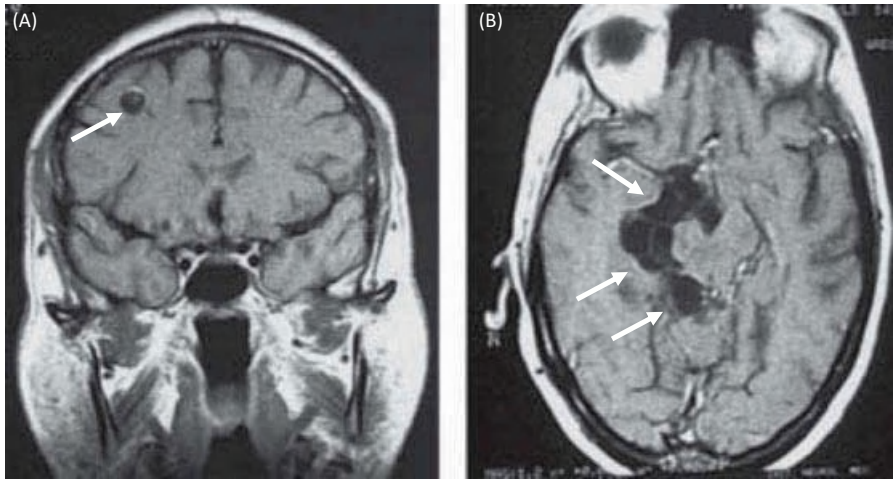

Figure 17.3 Human neurocysticercosis. a) Computer tomography scan image of a single cysticercus without anatomical alteration. b) Multiple cysticerci which displace and compress the cerebral brainstem. Reproduced with permission from: Sciutto, E *et al*. (2007) The immune response in *Taenia solium* cysticercosis: protection and injury. *Parasite Immunology* 29(12), 621–636.

Interestingly, while it is surprising that someone can harbour a parasite of such size in the brain (Figure 17.3), many patients remain asymptomatic for a long time (months to years), and the infection only becomes apparent when the parasites start to degenerate. Death of cysticerci induces a variety of symptoms, including strong headaches, epilepsy, intracranial hypertension, focal deficits, cognitive impairment and, in some cases, dementia. These symptoms – particularly dementia – are reversible in most cases (>80 per cent) after treatment or surgery. Recent reports indicate that patients with active NCC with symptoms have immune responses to parasite antigens that are qualitatively different to patients with inactive or silent NCC (without symptoms). These observations suggest that the cysticerci are effective in modulating and escaping attack by the host immune system.

17.4 Innate immunity

17.4.1 Pattern recognition receptors

As in other helminthic diseases, the innate mechanisms that initiate or participate in an immune response against the cysticerci and adult tapeworms are largely unknown. The Toll-like receptors (TLRs) appear to be important in recognising these parasites, and data obtained from laboratory models have started to elucidate this response. In the *Mesocestoides corti* mouse model of NCC, an increase in the expression of several TLRs – in particular TLR4 and TLR2 on CNS cell types such as astrocytes as well as brain-infiltrating immune cells – has been correlated with resistance. Mice deficient in MyD88 or TLR2 display a defect in the induction of the inflammatory immune response against *Taenia crassiceps* and are more susceptible to this tapeworm infection than immunologically intact mice.

A recent study of human NCC patients found an association between two different TLR4 polymorphisms (Asp299Gly or Thr399Ile) and an increased risk of NCC. These small potential variations in TLR4 may result in an altered or defective TLR4-mediated signalling that, in turn, may change the immune response against this parasite and influence the inflammatory response leading to NCC.

17.4.2 Dendritic cells

Dendritic cells (DCs), the main antigen-presenting cells of the immune system (see Chapter 1), have been analysed experimentally in models of tapeworm infection. *In vitro* assays, using excreted or secreted antigens (TES) from cysticerci of either *T. crassiceps* or *T. solium*, demonstrate that DCs are unresponsive to TES. DCs do not mature or secrete detectable levels of pro-inflammatory cytokines following such stimulation. Furthermore, the presence of TES inhibits the LPS-mediated activation of DCs, suggesting that these tapeworm-derived antigens are immunosuppressive.

DCs exposed to *Taenia* antigens, and then loaded with ovalbumin antigen, induce CD4 cells purified from DO11.10 mice (transgenic mice which express a T

cell receptor specific for a peptide epitope of ovalbumin) towards a Th2 phenotype, with a concomitant reduction of Th1 development and interferon(IFN)-γ production even in the presence of LPS. Most of these effects are related to the glycans displayed on the TES. Interestingly, *T. solium* cysticerci develop microscopic membrane elongations called microtriches (Figure 17.2c) that are rich in glycoproteins. In fact, it has been shown that live cysticerci from NCC patients release glycoproteins into the microenvironment, and it is possible that some of these molecules have specific pathogen-associated molecular patterns (PAMPs) that are able to ligate particular TLRs or lectin receptors.

Human monocytes from healthy donors are able to respond to crude extracts from *T. solium* metacestodes and release high amounts of the chemokines CXCL8 (also known as Interleukin (IL)-8), CXCL10 and CCL2, all molecules involved in recruiting inflammatory cells. This suggests that recognisable PAMPs may exist in *T. solium* cysticerci.

17.5 Adaptive immunity

17.5.1 The Th1/Th2 paradigm in tapeworm infection

Conflicting results have been reported from studies of NCC patients with regard to the type of CD4+ T helper cell responses that develop in tapeworm infection. Such differences may arise from the utilisation of different methods of analysing the immune response, different sources of parasite antigens and/or different sources of cells to be analysed across these studies. Although a well-defined Th1 or Th2 profile is not clearly associated with NCC, a more mixed Th1/Th2 response seems to be the most commonly observed result.

It is clear that CD4+ T helper cell responses do depend on whether a patient displays with asymptomatic or symptomatic NCC. Active NCC (involving some of the symptoms described earlier) is significantly associated with degenerating cysts. The development of granulomas around dying *Taenia* cysticerci is an important component of the neuropathology leading to epilepsy or other neurological symptoms. The initiation of granulomas has been attributed to a robust Th1 response (Figure 17.4). Degenerating or dead parasites trigger an intense antigen-specific cellular proliferation index, and a strong pro-inflammatory response is seen in patients involving high levels of tumour necrosis factor (TNF)-α, interleukin (IL)-12, IL-18 and interferon (IFN)-γ. It has been hypothesised that this intense inflammatory response leads to worsening symptoms in NCC.

Conversely, silent or asymptomatic NCC has been associated with the survival of cysticerci and the prevalence of a Th2 response with high levels of IL-4, IL-5, IL-13 and anti-*Taenia*-specific IgG4. Furthermore, asymptomatic NCC patients display a low proliferative cell index to cysticerci antigens. The reason for this has not been evaluated in humans but, in experimental cysticercosis, this phenomenon may be linked to the development of alternatively activated (M2) macrophages. M2 macrophages obtained from *T. crassiceps*-infected mice can directly inhibit the proliferative response of naïve T cells.

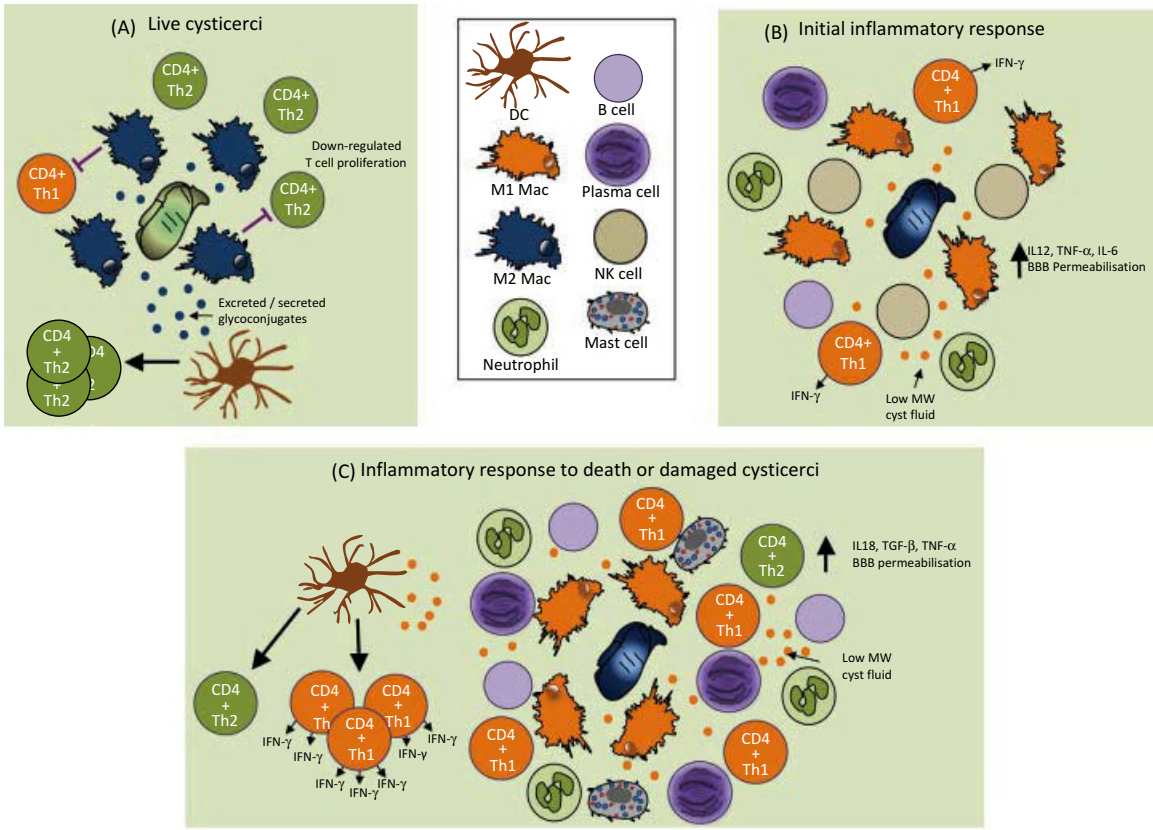

Figure 17.4 Schematic participation of immune elements during different steps in the developing of neurocysticercosis. In a quiescent condition (A), live cysticerci release glycoconjugates that can induce the development of M2 macrophages and/or modulate DC responses. M2 macrophages suppress T cell proliferation and the host is protected against exacerbated immune responses. In this state, parasites may survive for a long time in the host without causing neuropathology. At some point, cysticerci begin to die and they release immunogenic molecules that initiate an inflammatory response (B). This may lead to the generation of M1 macrophages around cysticerci, as well as the infiltration of neutrophils, NK cells and some T cells. Immune responses in this condition are characterised by the high levels of IL-12, TNF-α, TGF-β and IL-6, with some IFN-γ. The late inflammatory phase (C) is mediated by inflammatory cytokines such as TNF-α and IL-18, possibly secreted by macrophages and/or astrocytes. In this late phase, there is an increased production of IFN-γ and TGF-β; production of the latter has been correlated with fibrosis around the cysticerci. Blood-brain barrier permeability is increased, resulting in immune cell infiltration. This hyper-inflammation has been observed in severe NCC and is associated with tissue damage. Abbreviations: BBB, blood brain barrier; IFN, interferon; IL, interleukin; NK, natural killer; TGF, transforming growth factor; TNF, tumour necrosis factor.

17.5.2 Inhibition of T cell proliferation

M2 macrophages from *T. crassiceps*-infected mice are able to inhibit T cell proliferation by up-regulating the surface expression of the molecules' programmed death-ligand (PD-L)-1 and PD-L2. The ligation of PD-1 on T cells down-regulates T cell proliferation (see Chapter 1). In co-cultures of T cells with macrophages from *T. crassiceps*-infected mice, blockade of PD-L1 and PD-L2 or PD-1 by adding monoclonal antibodies significantly reduced the suppressive activity the M2 macrophages, demonstrating that the interaction of PD-L1

and PD-L2 with PD-1 on T cells is responsible for the suppressive activity of M2 macrophages in experimental cysticercosis

It is important to note that most NCC patients display normal numbers of T cells (as measured by the expression of CD3, CD4, and CD8), and that these appear to be immunologically active, as evidenced by the up-regulation of CD69. However, the numbers of B cells, eosinophils and mast cells are all elevated. Therefore, the role of M2 macrophages in the reduction of T cell proliferation *in vitro* remains to be elucidated.

17.5.3 Humoral responses

Although the measurement of antibody response has been useful as a tool for immunodiagnostics, antibodies against this parasite seem to be poorly effective in clearing the parasite. Only one *in vitro* study has demonstrated an effect of antibodies against *T. solium* oncospheres, but not against cysticerci or adult forms, indicating an early vulnerability of the larvae to humoral immunity.

Further research has confirmed that activated oncospheres from *T. ovis, T. saginata* and *T. solium* can be killed when incubated *in vitro* with sera from animals vaccinated with recombinant oncospheral antigens (called TSOL18 and TSOL45-1A), along with a source of complement. These antigens are only expressed on the surface of *T. solium* oncospheres, indicating that they are stage-specific antigens. Thus, the early events in *T. solium* development (oncospheres) are the most susceptible points of the parasite life cycle for the development of new vaccines.

17.5.4 Role of cytokines, chemokines and STATs

Cytokine activity is mediated through specific receptors, and the signal transducer and activation of transcription (STAT) molecules have a critical role in transmitting signals from cytokine receptors. STAT4 is necessary for signalling from the IL-12R to induce IFN-γ production in NK and T cells. The absence of STAT4 removes resistance to experimental cysticercosis in mice, presumably due to reduced Th1-associated effector mechanisms. These data suggest that a Th1 response is necessary to eliminate the larval *T. crassiceps* in the peritoneal cavity.

Although high levels of IL-12, TNF-α and IFN-γ can synergise with macrophages to amplify pro-inflammatory immune responses and induce effector molecules such as hydrogen peroxide or nitric oxide (NO), these cytokines may also have a direct effect on the parasite (cysticerci). Cytokine receptors or receptor-like molecules have not been detected in *T. solium* cysticerci, unlike some helminths that express receptors or even cytokine-like molecules. NO release appears to be involved in cysticerci killing, as mice treated with NO-inhibitors were highly susceptible to cysticercosis.

The absence of STAT6, a molecule critical for IL-4 signalling, induces resistance in susceptible mice. However, if cysticerci are lodged in the brain, the

absence of STAT6 exacerbates pathology, and experimental mice with NCC die. These contrasting data confirm the delicate tuning of the immune response in cysticercosis. Thus, an immune response that can clear this infection may also kill the host, which is why the treatment of NCC is always accompanied by a rigorous prescription of corticosteroids to inhibit inflammatory responses.

Chemokines play important roles in CNS inflammation, although not much is currently known about chemokines involved in the immune response in NCC. Direct stimulation of human monocytes from healthy donors with crude extracts from *T. solium* metacestodes induces the release of CXCL8/IL-8 CXCL10 and CCL2. Astrocytes in the brain do not respond directly to parasite extracts in this way, but conditioned culture supernatants from monocytes stimulated with *T. solium* extracts induce the production of the same chemokines by astrocytes in a TNF-α dependent fashion.

This astrocyte response may recruit polymorphonuclear (PMN) cells, T cells and macrophages to the site of infection to generate granulomas (Figure 17.4). Indeed, studies with NCC patients showed that low levels of CXCL8 in plasma are associated with reduced symptoms. Cyst fluid from *T. solium* cysticerci can inhibit CXCL8 production from monocytes. Thus, distinct parasite antigenic fractions may induce differential immune responses.

17.6 Antigens eliciting the immune responses

Given the multi-stage life cycle and complex physical structure of *T. solium,* there are many antigens with the potential to induce specific immune responses. The sources of antigens are diverse and include crude extracts of cysticerci, vesicular fluid, the oncosphere, scolex, cystic wall, membrane antigens and excreted/secreted antigens (TES). In tapeworm infection, the immune system can recognise both low and high molecular weight antigens (ranging from 10–200 kDa). Most of the antigens studied have been used for immunodiagnostics (from cerebral spinal fluid or plasma), and glycoproteins are known to be the main antibody targets.

Some studies have focused on the nature of the immune response that specific tapeworm antigens induce in NCC patients compared with healthy individuals. Crude lysates, cell walls of cysticerci and cyst or vesicular fluid sources of *T. solium* antigens generate very different responses. Of these three sources of antigens, only cyst fluid has been shown to stimulate cell proliferation in patients with active NCC.

Using fractionation of cyst fluid into antigens with specified molecular sizes, it has been determined that only fluid antigens with a low molecular size (<30 kDa) can induce cell proliferation. Of antigens, <30 kDa proteins between 10 kDa and 15.1 kDa can induce an anti-inflammatory response, with high levels of IL-4 and lower levels of TNF-α and IL-2, whereas antigens between 16–30 kDa induce a mixed response, with high levels of TNF-α, IL-2 and IL-10. Further studies will be required to determine whether these findings are related to pathogenesis or parasite survival.

17.7 Immunomodulation or evasive mechanisms

T. solium metacestodes are macroparasites that reach centimetres in size. An invader of such size should have a successful mechanism to avoid or resist immune attack. Parasite molecules with immunoregulatory activities have been detected in both experimental cysticercosis and natural cysticercosis. The identification and understanding of the bioactive molecules involved in this host-parasite interaction are of key importance.

T. solium cysticerci are able to secrete a variety of molecules to induce apoptosis in immune cells, in turn preventing immune attack. Cysticerci are known to release a cysteine protease that can induce apoptosis in human CD4+ T cells, in addition to annexin B1, a molecule that induces apoptosis in eosinophils. Other proteases released by *T. solium* cysticerci include a cathepsin L-like cysteine protease that is able to break down IgG and bovine serum albumin, eliminating the efficacy of the humoral response. Similarly, the formerly named 'antigen B' of *T. solium*, now recognised as paramyosin, is able to block complement activity by inactivating the C1q molecule.

T. solium also possesses enzymes, including Cu/Zn superoxide dismutase and glutathione transferase, that can protect the parasite from oxidative damage caused by superoxide anions or NO released by immune cells such as macrophages and neutrophils. *Taenia* metacestodes also express peroxiredoxin, an enzyme able to inactivate peroxides, making them highly tolerant to hydrogen peroxide.

Cestodes are rich in glycoproteins, in particular the glycocalyx surrounding the microtriches on the tapeworm tegument (see Figure 17.2c). *T. solium* cysticerci express at least six major glycoproteins, with molecular sizes from 180–45 kDa, which are found in the tegument surface of the bladder wall. In natural and experimental NCC, metacestodes can release different glycoproteins that are taken up by cells in the vicinity of the metacestode, including immune cells. In experimental cysticercosis, excreted or secreted glycoproteins released by *T. crassiceps* cysticerci *in vitro* have been showed to skew the immune response *in vivo* to a Th2 response upon injection into naïve mice, theoretically limiting the expansion of a pro-inflammatory response.

The nature of the glycoproteins (it is known that some of them are mannosylated) and the main glycans involved in immune-modulation, as well the target cells and possible receptors (TLRs or C-type lectins) for such molecules, deserves further investigation.

17.8 Echinococcosis

17.8.1 Life cycle of *Echinococcus granulosus* and *E. multilocularis*

Echinococcosis, or hydatid disease, is caused by the larval stage of tapeworms of the genus *Echinococcus*. The two species with clinical importance are *E. granulosus* and *E. multilocularis*, which cause cystic echinococcosis (CE) and

alveolar echinococcosis (AE) respectively. *E. granulosus* is the main causative agent of hydatid disease, causing 95 per cent of human cases throughout the world.

In contrast to *T. solium* and *T. saginata*, tapeworms of this genus are small; the adult *E. granulosus* reaches 3–6 mm long and resides in the small bowel of the definitive hosts – dogs, or other canids such as foxes. Gravid proglottids release eggs that are spread into the environment via faecal matter. After ingestion by a suitable intermediate host (sheep, goats, swine, cattle, horses, and camels, under normal conditions), eggs hatch in the small intestine and release an oncosphere that actively penetrates the intestinal wall and migrates through the circulatory and lymphatic system into several organs, particularly the liver and lungs. In these organs, the oncosphere develops into a metacestode (cyst) that gradually enlarges, producing protoscolices and daughter cysts that fill the interior of the cyst.

The definitive host becomes infected after ingesting the cyst-containing organs of the infected intermediate host. After ingestion, the protoscolices evaginate, attach to the intestinal mucosa and develop into adults in less than 90 days. The same life cycle occurs with *E. multilocularis* (1.2–3.7 mm), except that the definitive hosts are foxes and, to a lesser extent, dogs and cats. Its intermediate hosts are small rodents, and larval growth in the liver remains indefinitely in the proliferative stage, resulting in metastases to surrounding tissues.

Humans become infected by ingesting eggs, and the release of oncospheres in the intestine that leads to the development of cysts in various organs. As with *Taenia*, metacestodes of both species are fluid-filled vesicles protected with a tegument, with microtriches composed by a highly glycosylated laminated layer (LL); they represent the life-threatening stage of the infection.

17.8.2 Epidemiology

E. granulosus occurs worldwide, most frequently in rural areas where dogs ingest uncooked organs from infected animals. *E. multilocularis* is largely restricted to the northern hemisphere, including central and northern parts of Europe, Asia and North America. *E. granulosus* is also present in South America, and it displays up to 20 per cent seropositivity in rural areas of Peru, with only three per cent confirmed cases of CE. Both estimated human burden of disease and livestock production loss reach billions of dollars annually, indicating the necessity for increased monitoring and global control of CE.

17.8.3 Pathology

E. granulosus persists mainly in liver and lungs. It grows slowly by concentric enlargement and, as patients can harbour long infections without symptoms, this parasite appears to be well tolerated by the host. In contrast, *E. multilocularis* aggressively invades host tissues via external buds that can be released and metastasise to other organs or tissues, exacerbating disease and requiring liver transplantation in some patients. In the chronic stages of CE or AE, the

livers and lungs of infected patients generate intense fibrosis and necrosis; this can be dangerous, as such responses can obstruct bile ducts or vessels, causing the death of the host.

17.8.4 Innate immunity

As with *Taenia*, the innate immune elements involved in early responses to *Echinococcus* infections are largely unknown. It has been suggested that TLRs may be involved in early responses to *Echinococcosis*, but there are no studies to support this idea. Antigen B from *E. granulosus* impairs human DC maturation, and *Echinococcus* extract fails to activate DCs (as observed in cysticercosis) or induce nitrous oxide production by macrophages, suggesting that *Echinococcus* antigens may not have common pathogen-associated molecular patterns (PAMPs). NK cells are elevated in patients with CE, and IL-12 – an important activator of NK cells – appears to play a role in innate resistance against *Echinococcosis*.

17.8.5 Adaptive immunity

It is known that cellular immune responses against *Echinococcosis* are essential for resistance or clearance of infection. Immunosuppressed SCID mice are highly susceptible to *Echinococcus* infection, compared to immunologically intact mice. Similarly, immunocompromised humans, such as AIDS patients, suffer from rapid and irreversible growth of *E. multilocularis* following infection.

Hydatid disease induces an early, but transient Th1 response with infiltration of macrophages and eosinophils. As infection progresses, the immune response becomes dominated by a Th2 response that reduces Th1-associated anti-parasite effector mechanisms. In chronic human CE, there is an increase in the cytokines IL-4, IL-5 and IL-10 and an associated reduction in IFN-γ in the plasma. Likewise, the pro-inflammatory cytokines IL-1β, IL-18, IL-12 and TNF-α are significantly reduced in AE patients, along with an expansion of the number of T regulatory cells (CD4+ CD25+ FoxP3+). Together with abundant levels of IL-10 and TGF-β, Tregs appear to play a critical role in modulating inflammation by progressively depressing T cell responses.

The importance of Th1 responses in controlling hydatid infections is highlighted by experiments showing that boosting Th1 responses with IL-12 or IFN-γ enhances resistance. A possible effector molecule in host defence against these infections is nitrous oxide, which is mainly produced by classically activated M1 macrophages in response to both IFN-γ and TNF-α in resistant strains of mice. However, macrophages isolated from chronically infected mice are poor producers of NO.

As in cysticercosis, anti-oncospheral antibodies play a critical role in early parasite killing. Indeed, the most promising experimental vaccine is based on the anti-oncospheral induction of antibodies against the antigen EG95, and the effect of these antibodies is complement-dependent. In patients with chronic *Echinococcus* infection, IgG4 and IgE antibodies are present in the circulation, with levels decreasing after drug treatment in correlation with improvement of the patients' clinical status. These antibody subclasses disappear after surgery

to remove parasites, which indicates that the presence of viable parasites is necessary for a continuous antibody production, although anti-*Echinococcus* antibodies have no effect on the elimination of the parasite.

17.8.6 Immune evasion

Both *E. granulosus* and *E. multilocularis* have developed an intricate network of immune evasion mechanisms, which include antigenic variation, shedding of surface proteins, protease production, enzymes to avoid host-induced stress and active immunomodulation by releasing glycoproteins from the laminated layer (LL). The LL is considered to be a barrier between the parasite and the host, protecting the parasite from immune responses. However, recent studies suggest that the LL may have a modulatory role in hydatid diseases.

The LL is mainly composed of highly O-glycosylated glycoproteins and galactose. Host macrophages are able to adhere to the LL and phagocytose LL-derived particles. Such macrophages may be conditioned to become M2 macrophages that turn off the immune response through tolerogenic signals such as those mediated by PDLs or deficient M1 macrophages, as LL molecules inhibit NO production even in presence of LPS stimulation. Moreover, LL fractions strongly inhibit both antigen-specific and polyclonal splenocyte proliferation, and also induce the expansion of Treg cells.

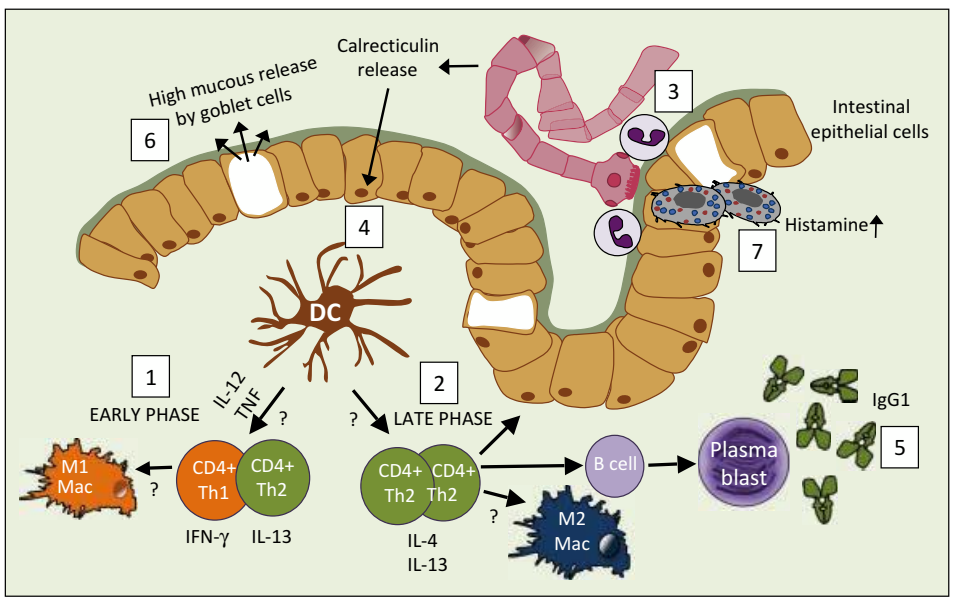

Figure 17.5 Many key points are unknown regarding immune response and immune-regulation against adult tapeworms. This is, in part, because there is not a-well established model for this infection. In experimental hamster models, a mixed Th1/Th2 response is achieved in the early phase response (1), polarising to Th2 in the late phase response (2). Eosinophils have been observed surrounding the scolex of the adult tapeworm (3), but the role of these cells remains unknown. Calreticulin released by the adult tapeworm evokes a Th2 polarised response (4), with predominant IgG1 production (5). Interestingly, gerbils are less permissive to infection showing a high goblet activity (6), recruitment of mast cells and high histamine concentration at the site of attachment (7).
Abbreviations: DC, dendritic cell; Ig, immunoglobulin; IFN, interferon; IL, interleukin; Th, T helper cell; TNF, tumour necrosis factor.

17.9 Conclusions

Along with similarities among the different tapeworms in terms of biology and transmission, the larval stages of these cestodes display similar mechanisms of immune evasion and appear to be destroyed by similar inflammatory responses that may be dangerous for host survival. Moreover, these parasites have the same Achilles heel – they are very susceptible in their early oncospheral-embryo phase to antibody- and complement-mediated responses.

The study of these tapeworms shows us that a Th2 response is not always associated with helminth protection. Therefore, a very different immune response is necessary to eliminate gastrointestinal helminths versus tissue-dwelling helminths (Figures 17.4 and 17.5).

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Section 4

18 Co-infection: Immunological Considerations

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If co-infection merely described the coincidental infection of one parasite species (micro- or macroparasites) with another, then this section of the book would be both brief and rather dull. However, co-infection describes so much more than mere coincidence. The story of co-infection is one of battles and conspiracies, of allies and deadly enemies; parasites competing for the same resources, or making an environment more accessible for another species; parasites using biological weapons against their enemies or piggy-backing upon the success of others. Co-infection requires us to take a temporary step back from the detail of this or that cytokine pathway and to consider the body as an ecosystem just like any other, with nutrients to be exploited and protected from competitors, and with predators with which to fight or from which to hide. Co-infection requires the parasitologist, immunologist and clinician to think, for a time, like an ecologist.

18.1 Co-infection is the rule rather than the exception

The vast majority of human beings will experience co-infection with multiple parasites (both micro- and macro-parasitic organisms) at the same time. In the developing world, in particular, most people will spend a large portion of their lifespan playing host to two or more parasite species. The mix of these species will vary between geographical regions, between individuals (e.g. Figure 18.1) and temporally over an individual's lifespan.

Geographical overlap of species distributions is clearly a strong contributor to the exposure patterns of people to certain groups of parasites (for example, see Chapter 19, Figure 19.1 for malaria and HIV spatial distributions). Additionally,

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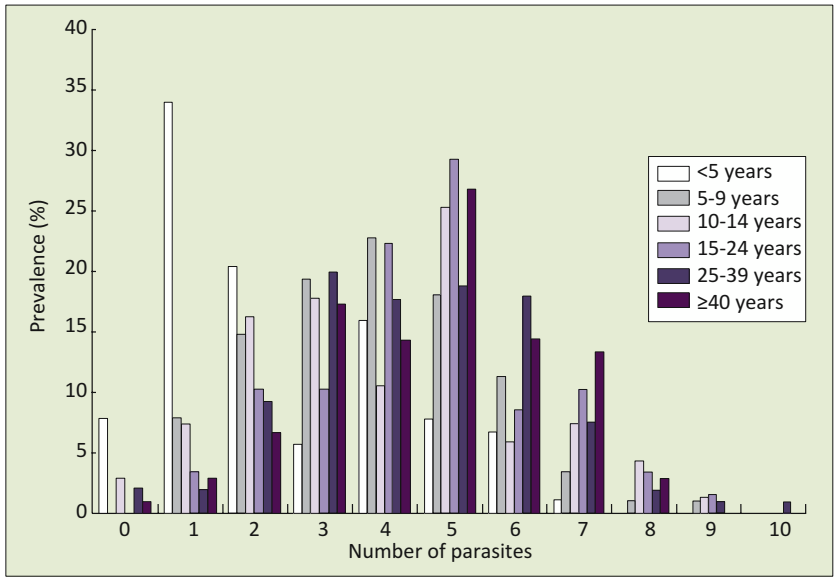

Figure 18.1 Frequency distribution of parasitic infections among 500 individuals from the village of Zouatta II, western Côte d'Ivoire, stratified by age. *Plasmodium malariae* and *P. ovale* were excluded from the analysis. Reproduced from Raso, G *et al*. (2004) *International Journal for Epidemiology* 33, 1092–1102.

differences in host genetics, socio-economic status and behaviour can all play a significant role in the level and mix of infections which people acquire. However, these factors alone cannot fully explain the degree of heterogeneity in the mix and intensity of infections acquired, nor in the differential disease progression seen between individuals. Inter-specific interactions between parasites within the host could play an essential role in these unexplained infection heterogeneities.

18.2 Interactions between co-infecting parasites

Interactions between parasites can range from the mechanical (e.g. the creation of an ulcerous wound by *Leishmania* spp. that bacterial opportunists can exploit), through to resource competition (e.g. in hookworm and malaria co-infection both pathogens utilise red blood cells), to complex indirect interactions mediated by the host immune response. It is this latter form of parasite interaction that we will focus on in this section of this book, but it is important to keep in mind the other potential forms of interaction, because multiple mechanisms can often be at play in an inter-specific parasite relationship.

Importantly, once host immune interactions are taken into account, the traditional perspective, which deems that interactions only occur between parasites inhabiting the same body compartment, becomes invalid. Often there is some degree of systemic immune response (even for localised infections), and this may affect (whether positively or negatively) parasites at distant locations from

one another. When this fact is taken into account, we see that any two species of parasites have the potential to interact with one another.

The consequence of pathogen interactions for the pathogens themselves ranges between two extremes. At one end of the continuum, a parasite species may be unable to infect a host unless that host environment has first been altered by another pathogen species; at the other extreme, one species of parasite may entirely exclude a second species from the host. In between these two extremes, parasite interactions can lead to increases or decreases in the numbers of individual parasites of each species within the host.

18.3 The Th1/Th2 paradigm in co-infection

The adaptive immune response may be categorised in many ways, but one frequently applied categorisation is the division into T cell populations and their associated cytokine profiles, i.e. Th1, Th2, Th9, Th17, Th22, T-follicular effector cells (see Chapter 1), and regulatory T cells (Tregs), the latter having a down-regulatory action upon the other cell lineages. Despite the recent description of Th9, Th17, Th22 and T-follicular cells, there remains a strong emphasis in the literature on the Th1 and Th2 lineages and on the cross-regulatory relationship between them, sometimes called the Th1/Th2 paradigm. The Th1 cell lineage and its associated repertoire of cytokines is generally considered a regulator of microparasitic infections, while the Th2 lineage is associated with control of macroparasitic infections. Applying the Th1/Th2 paradigm to co-infection, we can easily see a potential mechanism for interactions to occur between parasites.

A macroparasitic infection generally stimulates the Th2 branch of the immune response and, in so doing, can down-regulate the Th1 response. As a consequence, we might expect an increase in the numbers of a co-infecting microparasite. Such an interaction is apparent in the case of *Bordetella bronchiseptica* and the trematode *Fasciola hepatica* in the mouse. Co-infections with these two species result in both an increase in the bacterial load within the mouse and evidence of an extended infective period. Similarly, the hookworm-induced suppression of pro-inflammatory cytokine interferon (IFN)-γ (a Th1 cytokine) can have a positive effect on malarial parasites, causing increased parasitaemia.

If we could always predict that a Th2-stimulating organism would down-regulate the Th1 response, then we would have a fairly simple rule by which to predict co-infection outcome. However, the story is not so simple. Firstly, it is not always going to be the Th2 stimulating parasite that causes the bias in the host immune response. It may be that a Th1 stimulator has the strongest effect upon the host and that it is the Th2 response that is down-regulated. For example, a concurrent infection in mice with the Th1-inducing microparasite *Trypanosoma brucei* (see Chapter 8) and the Th2-inducing macroparasite *Trichinella spiralis* (see Chapter 15) results in increased *T. spiralis* numbers.

Secondly, the order of infection can alter the outcome of the interactions. Co-infection between *Giardia lamblia* (See Chapter 6) and *T. spiralis* in mice results

not only in increased susceptibility to *G. lamblia* when the infections are concurrent, but to a strong suppression of *T. spiralis* trophozoite stages when the *T. spiralis* infects prior to the *G. lamblia*. The Th1/Th2 paradigm, though important, has limitations, and therefore may not be detailed enough to truly capture the complexities of parasite interactions. Once we factor back in all the other T cell lineages, especially the Treg cells, the story becomes considerably more complicated.

The complexities of the immune response during an infection is only one confounding factor limiting the use of the Th1/Th2 paradigm as a predictor for the outcome of a co-infection scenario. The relationships between parasite species can exist on several axes, and two pathogens could interact in different ways on different axes. In the relationship between hookworm and malaria, hookworm-induced suppression of the Th1 response may benefit malaria (i.e. the immune axis), but the two parasites also have a negative association on the food/resource axis, as both require red blood cells for survival and reproduction. Therefore, the increase in malarial parasitaemia is not as high as it might be without the latter interaction.

18.4 Co-infection can alter disease severity

In addition to the consequences for the parasites themselves, co-infection obviously has implications for the health of the host. While there is a link between the effects of co-infection upon the parasites themselves and the host's health, this link is not necessarily straightforward. We might expect that, if co-infection leads to an increase in parasite numbers within the host, this will have a detrimental effect – and, conversely, that suppressed parasite numbers will result in a healthier host. However, where host damage is largely a consequence of the host immune response co-infection can sometimes decrease host damage even where parasite numbers are raised.

A possible example of this (though still contentious) is the relationship between the soil- transmitted helminth *Ascaris lumbricoides* and malaria (see Chapter 21). The most deadly form of malarial disease, cerebral malaria, is a result of Th1-induced inflammatory responses (see Chapter 3) and, as such, the down-regulation of Th1 response that results from co-infection (with the Th2-inducing helminth) consequently reduces the pathology of the disease. However, this is not all good news for the patient; malaria parasitaemia is increased in co-infected patients, resulting in greater damage to the liver and higher transmission potential. Similarly, co-infection induced suppression of parasite numbers may not necessarily be good news for the host if the immune pathway acting to reduce the parasite also increases immunopathology in the host. The majority of reported disease outcomes in co-infected patients point to increased disease severity in co-infected hosts. While we might expect this outcome, we must also be cautious and remember that clinicians are more likely to notice, and therefore to report, cases where disease severity has increased. Where disease severity is significantly decreased by co-infection,

patients may not even present themselves for treatment, so positive effects on health are likely to be under-reported.

18.5 Modelling parasite interactions during co-infection

Interspecific interactions not only influence disease severity and progression but can also alter the efficacy of parasite control. A simple model of parasite interspecific interactions between three parasite species demonstrates this point (Figure 18.2). In this model, there are three parasites, each of which stimulates an immune response against itself. The model does not specify the form of this immunity, but the immune response increases at a rate proportional to the number of parasites of that species entering the host. Each parasite interacts with one other species via the immune response. Effectively, the parasites either up- or down-regulate the immune response made against another parasite species.

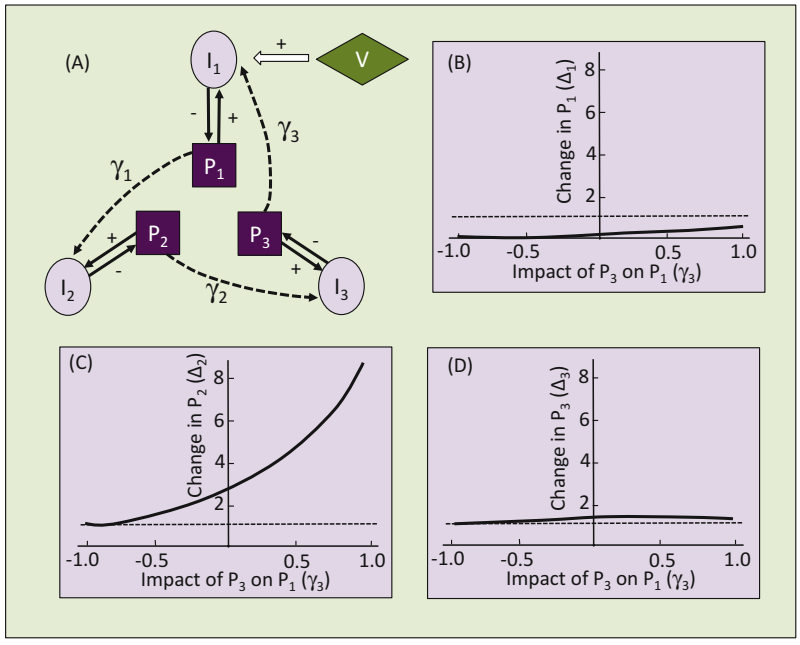

Figure 18.2 a) Schematic of three parasite species interaction model, where P_1, P_2 and P_3 are the parasite species, I_1, I_2 and I_3 are the species-specific immune responses, V is the vaccine applied against species 1 and γ_1, γ_2 and γ_3 are the parameters describing the strength of the effect of one species on another. b)–d) Proportional change (Δ_i) in equilibrium abundances of parasite species i, where i = 1 (b), i = 2 (c) and i = 3 (d), following vaccination (i.e. post-vaccination equilibrium level/pre-vaccination equilibrium) at varying levels of γ_3, the parameter describing the strength of the effect of P_3 on P_1. The dotted horizontal line represents where post-vaccination level = pre-vaccination level. Reproduced from Lello *et al*. (2004). Competition and mutualism among the gut helminths of a mammalian host. *Nature* 428, 840–844).

Parasite species 1 (P_1) always has a negative effect upon parasite species 2 (P_2), while P_2 always has a positive effect upon parasite species 3 (P_3), but the effect of P_3 upon P_1 may be altered. We can then establish the consequences of this simple interaction network upon the efficacy of a simulated vaccine. The vaccine acts only against P_1 (increasing the level of immune response against that species). If we set the efficacy of the vaccine to 50 per cent in the absence of an interaction between P_3 and P_1, we can then assess the consequences of different forms of interaction.

What this model demonstrates is that, when there is a strong negative effect of P_3 on P_1 (i.e. the immune response against P_1 is up-regulated), the vaccine's efficacy can be improved up to 99 per cent. However, if there is a strong positive effect of P_3 on P_1 (i.e. the immune response against P_1 is down-regulated), then the vaccine efficacy drops to just 15 per cent. In this case, not only is the vaccine ineffective but, unexpectedly, the numbers of P_2 increase tenfold.

This simple model demonstrates how parasite interaction networks can make the difference between a very successful and a severely detrimental vaccine strategy. Increasing evidence from laboratory and field studies also suggest that co-infection can have effects upon vaccinations. The majority of studies have demonstrated deleterious effects of helminths on vaccination efficacy. However, as demonstrated by the theoretical model, co-infection does not always result in reduced efficacy and, in fact, congenitally transmitted *Trypanosoma cruzi* was shown to increase the immune response to a range of childhood vaccinations.

18.6 Co-infection as a therapy?

Most pathogen control relies on some level of host immune response for successful clearance or control of the parasite species in question, and there is increasing evidence that even chemotherapeutic treatments can be detrimentally affected by co-infection. Of course, just as with the influence of co-infection on host health, negative effects of co-infection on pathogen control are also more likely to be reported. However, understanding co-infection could provide new insights into how the body controls individual infections, and may thereby suggest new control strategies which biologists might be able to exploit. Indeed, this is the principle behind the use of helminths in the control of autoimmune disease and allergy (see Chapter 24). Similar mechanisms could be exploited to control infectious disease.

Giving one infection to mitigate the effects of another may seem outlandish, but it is certainly not a new concept; late stage syphilis used to be treated with deliberate malaria infection before chemotherapeutic control became available. However, we may not necessarily need to co-infect patients to help control disease; we may simply be able to bias the immune response in the appropriate way (e.g. via vaccination) in order to mitigate the effects of a parasitic infection. An essential step for disease control will be that we fully understand the co-infection process and its consequences.

18.7 Consideration of co-infection in an ecological framework

We have considered the complexities of the potential timing, form and consequence of parasite interspecific interactions. We must add to this the fact that hosts are rarely infected with just two parasite species but are, in fact, assaulted by a whole community of organisms throughout their lives. Infections may not even need to be concurrent for interactions to occur, with one parasite effectively interacting through the host immune system with the 'ghost of infections past'. Factors such as host age and genetics, (e.g. different major histocompatability profiles) may further alter the outcome of any interaction. Given all this complexity it may seem that understanding co-infection is an intractable problem. Nevertheless, it is one which we must tackle if we are ever truly to understand and control disease in any natural setting.

How then can we start to address the problem of co-infection? One possible solution comes from the field of ecology, where the understanding of complex ecosystems is often approached by categorising organisms rather than trying to deal with them individually. The concepts of functional groups (or 'guilds') may be particularly helpful. These two concepts together describe organisms not on the basis of their species, but on their roles within and responses to their environment. For example, we may define organisms according to their function in the carbon cycle, e.g. plants are primary producers, fixing energy from the sun in the form of carbohydrates. These carbohydrates are then utilised by the primary consumers (herbivores) which, in turn, provide energy for the carnivores – and finally the detritivores recycle nutrients from all three of these groups back into the soil for the primary producers to utilise. The key feature here is that the interactions between the groups is understood. Therefore, by assigning a particular organism to one of these groups, we can easily see what its interaction with any other group in the ecosystem will be. Similarly, we could consider pathogens not according to species, but according to their function and response to the host ecosystem.

We have already effectively considered co-infection in terms of a simple functional grouping – by defining macroparasites as inducing and responding to Th2 cells (and their associated cytokines), and microparasites as inducing and responding to Th1 cells and their cytokines. Furthermore, we have also already defined the relationship between these two groups, in that we have said that they are reciprocally down-regulatory. Of course, we also know that we have vastly oversimplified the immune response to pathogens by assigning species only to one or other of these groups and, as we have discussed, there is a much wider set of potential T cell types and associated cytokines and a network of interactions between them (aside from other possible immunological factors). However, even taking into account this additional complexity, there are still distinct patterns to be observed.

Certain cytokines will always group together and are linked in very specific ways. If we consider parasites in term of their associations with these cytokine groupings (or similar immunological patterns), we can then begin to estimate

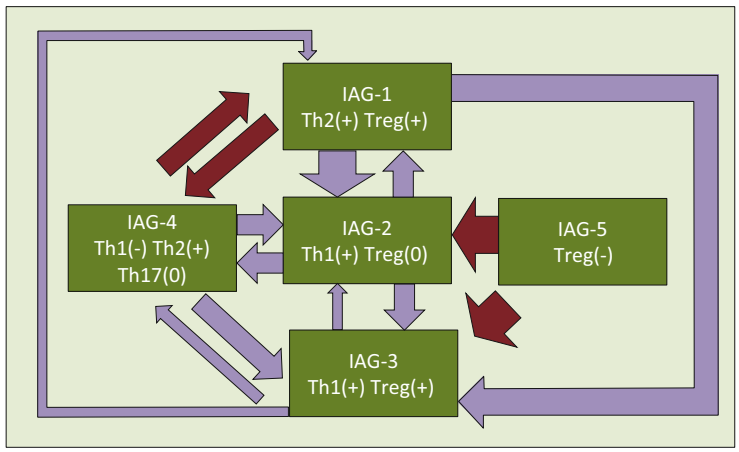

Figure 18.3 Hypothesised relationship between proposed (simplified) immune activity groups (IAG). Purple arrows indicate down-regulatory processes and red arrows indicate up-regulatory processes. The thickness of the arrow equates to the proposed strength of the action of one group upon another. T regulatory mechanisms are assumed to have down-regulatory effects on Th1 responses only. Adapted from Lello & Hussell (2008). Functional group/guild modelling of inter-specific pathogen interactions: a potential tool for predicting the consequences of co-infection. *Parasitology* 135, 825–839.

the expected interactions between them, because we know the interactions between the cytokines themselves. Each parasite can be placed into a functional group based upon two immunological activity groups (IAGs) – the IAG it invokes and the IAG it responds to. For some parasites, these two IAGs will be one and the same, whereas, for other species (e.g. those that immuno-modulate), the invoked response may not be the immune response which is effective against the parasite species.

Once characterised by their two IAGs into a functional group, the interactions between groups can be modelled (see Figure 18.3 for a simplified example of possible groups and model structure) and the likely effect of the interactions evaluated in terms of both parasite dynamics and host health. Using a simplified example of the IAGs and the subsequently derived interaction model, the outcome of concurrent co-infection with *G. lamblia* (IAG group 2, Figure 18.3) and *T. spiralis* (IAG group 1, Figure 18.3) was predicted. The model qualitatively matched the response seen in experimental infections (discussed earlier), i.e. that the *G. lamblia* trophozoites increased in number in co-infected hosts. Such simplification methods clearly offer powerful tools to enable us to understand and predict co-infection consequences.

18.8 Concluding remarks

Co-infection is ubiquitous in natural systems, and it is likely to have profound affects, both upon host and parasite populations and upon disease severity and

control. How, then, should we move forward with this difficult but fascinating topic of co-infection?

If we are to have any success at understanding and dealing with co-infection, we must seek order in the chaos; we must attack the complex ecosystem of the parasite community, just as ecologists seeks to understand the larger ecosystem. We need to find keystone species which drive the dynamics of the parasite communities, determine functional groups or 'guilds' and begin to establish overarching patterns of interspecific interactions. We must utilise modelling to make best guesses of how parasites will interact, and we must iteratively test and improve those models so that we can create a workable framework within which to control the complex network of parasites to which we are exposed.

Perhaps the most important next step is that future researchers and clinicians be made aware of the importance and complexity of the topic, because the issues of co-infection will remain with us for as long as infectious disease remain a threat to health.

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HIV and Malaria Co-infection

19

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19.1 The endemicity of HIV and malaria

The Human Immunodeficiency Virus (HIV) has spread as a global pandemic, affecting all continents. Malaria, caused by the five *Plasmodium* species known to infect man (*P. falciparum, vivax, ovale, malariae and knowlesi*) (see Chapter 3), has a more limited geographical distribution, defined by the availability of suitable mosquito vectors. The greatest burden of disease due to both HIV and malaria (predominantly *P. falciparum*) occurs in sub-Saharan Africa, although the geographical overlap of the highest risk areas for both infections is limited to central and southern Africa (Figure 19.1). Nevertheless, HIV infection persists lifelong, and *P. falciparum* can be both a chronic and a frequently recurring cause of infection, increasing the likelihood of HIV and malaria co-infections in the same individual.

Understanding the potential consequences of interaction between the two infections – including understanding their reciprocal effects on host immune responses to HIV and malaria, their combined effect on host responses to other infections, and the implications of HIV-malaria co-infection on transmission, diagnosis, treatment and prevention – is therefore of considerable importance

19.2 HIV infection

19.2.1 A short history of HIV infection

HIV is the cause of the acquired immunodeficiency syndrome (AIDS), a progressive loss of immune competence which results in susceptibility to opportunistic infection and cancer. HIV is caused by two related retroviruses – HIV-1 and HIV-2 – both of which evolved from viruses of non-human primates. It is likely that the ancestral HIV-1 virus entered the human population in the early

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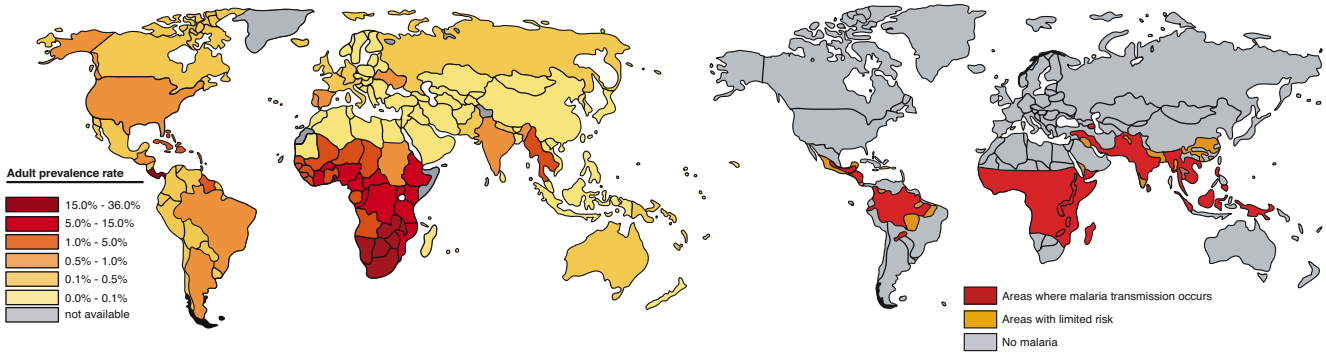

Distribution of HIV prevalence **Distribution of endemic malaria**

Figure 19.1 HIV/AIDS and malaria are highly endemic and there is wide geographical overlap in sub-Saharan Africa. Among the most severely affected countries are Cameroon, Central African Republic, Malawi, Mozambique and Zambia, where more than 90 per cent of the population is exposed to malaria, and HIV prevalence (among adults 15–49 years of age) is above ten per cent. Outside Africa, the two diseases overlap in certain at-risk groups in southeast Asia and South America, and in several Indian cities such as Mumbai. Reproduced by kind permission of The World Health Organisation.

20th century in the Democratic Republic of Congo, but AIDS was not formally recognised until its description in 1981 in the United States of America.

HIV-1 is widespread, while the HIV-2 epidemic is focused around West Africa and its migrant populations; HIV-2 infection progresses to AIDS more slowly than HIV-1. Globally, more than 30 million people are estimated to be infected with HIV, two-thirds of these in the sub-Saharan African region. This compares with 85 per cent of 240 million global malaria cases, and nearly 90 per cent of the 860,000 malaria deaths occur in the African region.

Despite this broad overlap, the burden of co-infection is unevenly distributed within the African region, and even within individual countries, due to local variation in malaria transmission and the nature of the HIV epidemic. For example, malaria is often more common in rural areas, whereas HIV is often more prevalent around urban centres. Also, immunity to malaria is acquired in childhood, before the peak of HIV acquisition in adolescence and early adulthood.

The countries most severely affected by malaria-HIV co-infection include Malawi, Mozambique, the Central African Republic, Zambia and Zimbabwe. In this chapter, we limit discussion to interactions between HIV-1 and *P. falciparum*, because there is less evidence available for interactions involving HIV-2 or the other *Plasmodium* species.

19.2.2 The HIV virus

Before considering the interactions between HIV and malaria, it is useful to consider the challenges that each infection poses on its own for host immunity, and to take note of some similarities between the two infections in the ways that they have co-evolved with the host's immune system (Table 19.1). The immunology and pathology of malaria infections has been described in Chapter 3.

HIV is a retrovirus; each enveloped viral particle (virion) contains two copies of the viral RNA genome as well as the enzymes essential for the reverse transcription of this RNA into cDNA and its integration into the host genome. The surface of the HIV virion expresses glycoproteins, which mediate binding to, and fusion with, host target cells. The most important of these are gp120, which binds to CD4, as well as the co-receptors CCR5 and CXCR4 on the host cell membrane, and gp41 which is necessary for fusion of the membranes and viral entry.

19.2.3 Cellular sources of HIV virus

Activated CD4+ T cells, which express CCR5, are the principal target for HIV invasion and the main site of viral replication, producing the majority of HIV which is detectable in the plasma (the viral load). However, HIV is also able to infect other cell types, including naïve and memory CD4 T cells, monocytes, macrophages and dendritic cells. Infection of these other cell types makes relatively little contribution to plasma viral load, but it creates a latent (i.e.

Table 19.1 Some similarities between *Plasmodium* and HIV of relevance to host immunity.

Plasmodium species	HIV
Evolutionary origin	
Zoonotic transmission from non-human primates.	Zoonotic transmission from non-human primates.
Host adaptation to invade red blood cells using specific sialic acid residues.	Adaptation to human host, e.g. HIV-1 Vpu protein antagonises the innate defence protein tetherin (which would prevent release of viral progeny from cells).
Immune evasion at infection	
Rapid transit of sporozoites to the liver limits stimulation of humoral responses.	Rapid invasion of CD4+CCR5+ cells for active replication.
	Dendritic cells and B cells carry virus to activated CD4 T cells.
	Early establishment of latently infected cells.
Immune activation	
Repeated exposure/chronic persistent parasitaemia	Persists lifelong after infection.
	Translocation of microbial products across intestinal mucosa causes immune activation.
Immune activation causes increased endothelial adhesion molecule expression, sequestration of *P. falciparum* parasitised RBCs, protection from splenic clearance and enhanced replication.	Immune activation increases viral replication.
Immune activation may cause severe disease manifestations such as cerebral malaria.	Immune activation hastens progression to AIDS.
Evasion of the humoral immune response	
Antigenic variation.	Antigenic variation.
Mutation.	Mutation.
Cryptic B cell epitopes.	Cryptic B cell epitopes.
Alternative invasion pathways, e.g. Sialic acid dependent and independent invasion of RBC.	Alternative invasion pathways e.g. CCR5 and CXCR4.
Intracellular replication cycles.	Intracellular replication cycle.
Polyclonal B cell activation diverts from specific response. (Hypergammaglobulinaemia).	Polyclonal B cell activation diverts from specific response. (Hypergammaglobulinaemia).
Aberrant memory B cell development.	Aberrant memory B cell development.
Latent infection (hynozoite) in the liver in (*P. vivax* and *P. ovale*)	Latent intracellular infection of macrophages and resting T cells.
Evasion of the cell mediated immune response	
Sporozoites down-regulate MHC I on Kupffer cells in the liver.	Viral Nef (negative regulation factor) protein inhibits MHC I expression and presentation of peptides on MHC II.
Altered peptide ligands interfere with T cell receptor interactions and activation.	Mutation of T cell epitopes interferes with T cell response to wild type immunodominant epitopes.
Intraerythrocytic replication cycle – RBCs lack MHC expression.	Viral reservoirs in privileged sites, e.g. central nervous system.
Cryptic T cell epitopes.	

non-replicating) virus reservoir, relatively protected from the immune response and from antiretroviral drugs.

Latent virus can begin to replicate following immunological activation of the host cell; for example, there are NF-κB responsive elements in the viral long terminal repeat (LTR) region, which result in transcriptional activation of the

virus by NF-κB. HIV replication in activated CD4+ T cells has a direct cytopathic effect, whereas latently infected cells may have a prolonged lifespan.

19.2.4 Transmission of HIV

HIV is transmitted between humans as cell-free or cell-associated virus in bodily fluids, principally semen, vaginal secretions and blood. It can also be transmitted from mother to child across the placenta, in the birth canal or in breast milk. The risk of transmission is closely related to the viral load in the blood and the integrity and state of inflammation of the mucosal (or placental) barriers. HIV can cross the mucosal barriers through interaction with dendritic cells or CCR5-expressing epithelial cells, with subsequent infection of CD4+ T cells in the submucosa or lymphoid tissues. Individuals with mutations that limit CCR5 expression are resistant to HIV infection.

19.2.5 The immune response against HIV

When HIV is transmitted into a new host, it establishes infection in CD4+ T cells and there is a phase of rapid viral replication, high viral load, and deletion of CCR5+CD4+ T cells from the gut and, to a lesser extent, from the peripheral blood (Figure 19.2). This acute phase manifests as an influenza-like illness in 50–80 per cent of cases. An innate immune response is initiated by binding of uridine-rich HIV RNA to Toll-like receptors 7 and 8 and triggering of

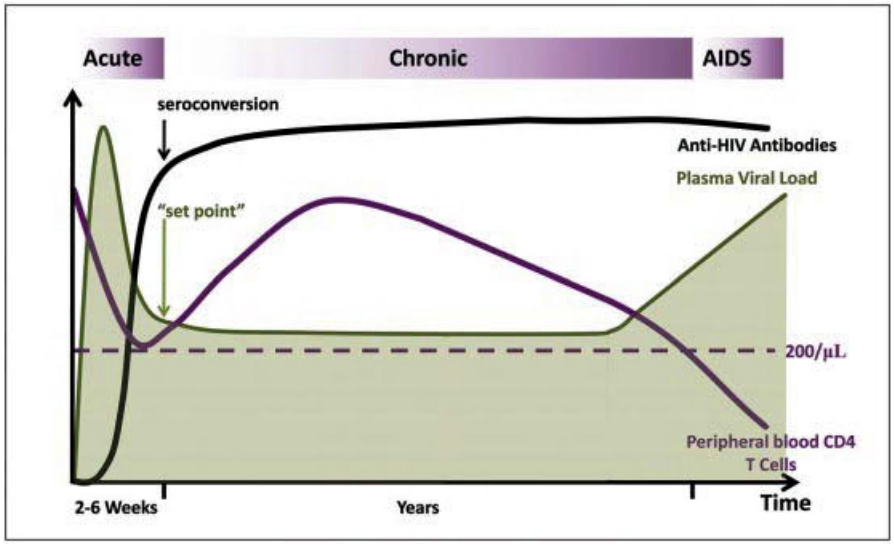

Figure 19.2 Three phases of HIV infection. Acute HIV infection is characterised by a high viral load and depletion of CD4+ T cells. Plasma viral load drops to a relatively constant 'set-point' as host immunity establishes imperfect control of viral replication, antibody seroconversion occurs, and there is recovery of CD4+ T cell numbers in the peripheral blood. In the chronic phase, there is gradual loss of functional immunity, most commonly measured by the depletion of CD4+ T-lymphocytes, and eventually a loss of control of viral replication. When the CD4+ T cell count falls below the threshold of 200 cells/μl, there is severe immunocompromise: AIDS.

interferon-α production by gp120 in monocytes and dendritic cells. It is accompanied by CD8+ T cell activation and production of antibodies to viral proteins (seroconversion). This cytotoxic CD8+ T cell response to acute infection limits, but does not eradicate, infection. Antibodies appear to be ineffective, because the humoral response is too slow to keep up with the rapid rate of mutation of HIV epitopes, such that neutralisation of intact virions is poor *in vivo*.

After 2-6 weeks, viral load falls to a 'set-point' and peripheral blood CD4+ T cell numbers rebound. In the absence of treatment, a chronic phase of infection begins; there is now a gradual decline in CD4+ T cell numbers and a gradual increase in HIV viral load, with eventual progression to AIDS. In reality, there is great variation in the time taken to progress from acute infection to AIDS, with a median of 8–10 years (although in children, progression is often faster). Some individuals progress very rapidly, while others appear not to progress (long-term non-progressors). Long-term non-progression is strongly associated with genetic variants affecting the peptide binding groove of HLA class 1, indicating that interaction of HLA class 1 with viral peptide and the quality of its presentation to CD8+ T cells is a major determinant of the effectiveness of the host response.

The acute phase of infection (seroconversion illness) is rarely identified clinically, unless there is a particular reason to expect an individual is at risk of HIV infection. The beginning of the chronic phase is asymptomatic. This means that individuals may be infected with HIV for several years without knowing that they have the virus, during which time they may transmit the virus to others. Progression to advanced stages of HIV and AIDS is defined by the onset of recurrent, severe or opportunistic infections, malignancies (cancers) or pathological effects of HIV itself (wasting syndrome or encephalopathy), or by a fall in the CD4+ T cell count below a threshold value (<200 cells/μl for adults).

19.2.6 Drug therapy against HIV infection

Highly active anti-retroviral therapy (HAART) targets the replicating virus by inhibiting the reverse transcriptase and protease enzymes necessary for the production of infective virions, but it does not affect the virus in latently infected cells, making eradication of the virus (i.e. a cure) impossible. HAART usually comprises a combination of three drugs, two nucleoside reverse transcriptase inhibitors (NRTIs) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). HAART can effectively suppress viral replication to undetectable levels, reverse immune activation and slowly allow recovery of the immune system.

In the chronic phase, treatment of HIV with HAART can prevent the progression to AIDS by suppressing viral replication and delaying the decline in peripheral blood CD4 count. Treatment of individuals after they have an AIDS defining-illness can also suppress viral load and restore peripheral blood CD4+ T cell counts over time, reversing the immunodeficiency.

Some consequences of HIV/AIDS, such as lung, kidney and neurological damage, are not reversible with restoration of the CD4 count and, unfortunately,

not all immunological dysfunction appears reversible. Notably, memory B cell numbers and function do not recover well after initiation of HAART, and this results in persisting defects in humoral immunity, which might only be avoided by initiation of HAART very early in the course of infection, before irreversible damage is done. Restoration of immune competency by HAART carries risk in those who have been very immunosuppressed, because the resurgent immune system may mount a vigorous and damaging response to covert pathogens, causing immune reconstitution inflammatory syndrome (IRIS).

Anti-retroviral drugs may also be used to prevent mother-to-child transmission (MTCT) of HIV and to prevent infection immediately following exposure to HIV (post-exposure prophylaxis). Individuals with low CD4+ T cell counts are often also given prophylactic treatment with trimethoprim-sulfamethoxazole, antibiotics which help to prevent bacterial and *Pneumocystis jirovecii* infection.

19.3 Immunopathogenesis of HIV

19.3.1 Immune activation in acute HIV infection

Although it is now accepted that HIV is the cause of AIDS, the mechanisms leading to immunosuppression remain the subject of some debate. Immunosuppression due to HIV is not a state of immunological quiescence, but quite the opposite. HIV promotes immune activation, and immune activation strongly predicts progression to AIDS (Figure 19.3).

Immune activation is identified by increased circulating pro-inflammatory mediators (chemokines and cytokines), polyclonal B cell activation, increased T cell proliferation and activated T cell phenotypes. Although only a small proportion (<1 per cent) of all CD4+ T cells are infected by HIV, the increased activation and turnover of T cells not only creates new target cells (expressing CCR5) for further viral replication, but may also ultimately exhaust the proliferative capacity of the T and B memory cell pools.

Activation and infection of important functional subsets of T cells, such as central memory CD4+ T cells, may be particularly damaging. Central memory T cells constitute a pool of precursors for effector memory T cells, and their depletion is strongly associated with development of AIDS. Similarly, depletion of polyfunctional T cells (able to secrete high levels of several cytokines) is correlated with increased viral load and progression to AIDS. Polyfunctional cytotoxic (CD8+) T cells are believed to be important in defence against HIV itself, but the capacity of these effector cells to limit HIV replication is impaired by chronic immune activation, which drives terminal differentiation towards exhausted cells, secreting lower levels of a more limited repertoire of cytokines.

Similar phenomena occur in B cells during HIV infection, with chronic stimulation leading to exhausted cell phenotypes and a reduction in their capacity to mount antibody responses to vaccination and infection. One mechanism by which chronic immune activation impairs T cell function is increased expression of the surface receptor Programmed Death-1 (PD-1) on T cells, and

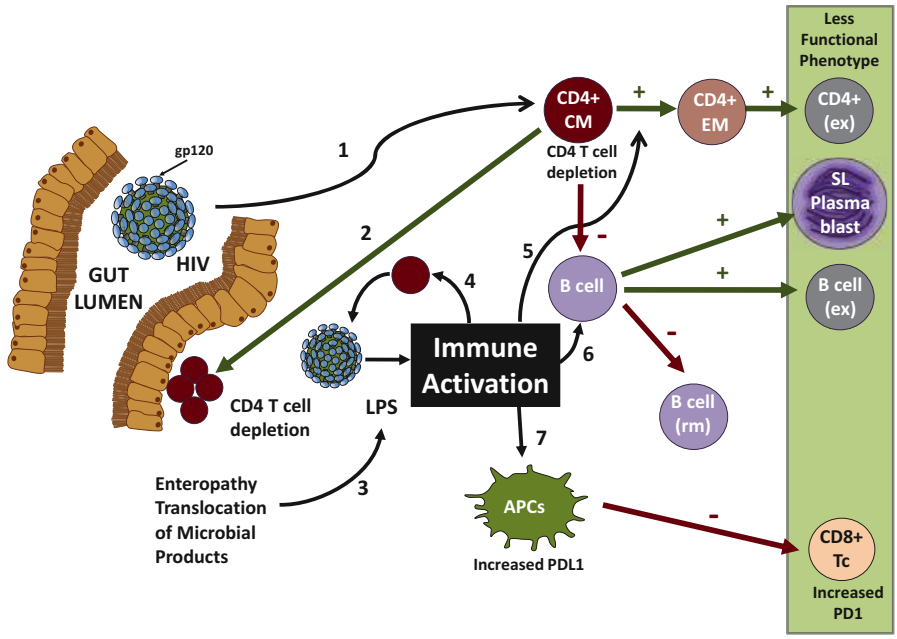

Figure 19.3 Immune activation is central to the pathogenesis of AIDS. 1. HIV preferentially infects activated CD4+ T cells expressing CCR5. 2. HIV is directly cytopathic to activated CD4+ T cells, resulting in acute depletion of CD4+ T cells in peripheral blood, particularly in the gut. 3. This causes damage to the defensive mucosal barrier of the gut, allowing translocation of bacteria and their products into the circulation. 4. Bacterial products such as lipopolysaccharide (LPS) and HIV synergise to cause chronic immune activation. 5. Consequent activation of CD4+ T cells allows increased viral replication. It promotes depletion of central memory (CM) cells and differentiation of effector memory (EM) cells to less functional terminal effector and exhausted (ex) phenotypes.6. Depletion of CD4+ T cells limits helper function for B-lymphocytes, and immune activation causes polyclonal stimulation of mature naïve B-lymphocytes, enhanced differentiation to short-lived (SL) plasmablasts and exhausted (ex) phenotypes, while irreversibly depleting resting memory (rm) B cell pools. 7. Chronic immune activation enhances the expression of Programmed Death-1 (PD-1) on CD8+ T cells and of its ligand PD-L1 on antigen presenting cells (APCs), which reduces the proliferation and enhances apoptosis of HIV-specific CD8+ T cells.

of its ligand, PD-L1, on antigen presenting cells. Ligation of PD-1 to PD-L1 reduces survival, proliferation and cytokine production of CD8+ and CD4+ T cells. Chronic immune activation also disrupts the architecture of lymphoid tissues (e.g. thymus and lymph nodes), preventing their orchestration of normal immune responses.

19.3.2 Chronic immune activation in HIV infection

The chronic immune activation that occurs during HIV infection is not attributable exclusively to the virus. It is currently believed that immune activation is largely driven by a loss of functional integrity of the gastrointestinal mucosal barrier, which allows translocation of microbial products such as lipopolysaccharide (LPS) from the gut lumen into the circulation. These microbial products are able to stimulate the innate immune response through

Toll-like receptor signalling. Furthermore, HIV infection increases the sensitivity of macrophages to Toll-like receptor ligands.

HIV infection causes inflammation of the gastrointestinal tract, with destruction of the epithelial surface and death of enterocytes. The majority of lymphocytes in the body are located in the gastrointestinal tract, and there is a dramatic depletion of CD4+ T cells from this site during acute HIV infection. This continues in the chronic phase of infection, and it is of much greater magnitude than the CD4+ T cell depletion from peripheral blood. The occurrence of opportunistic infections in HIV-infected individuals provides another stimulus to immune activation, viral replication and disease progression.

19.4 Interactions between malaria and HIV

There are many possible interactions between malaria and HIV (summarised in Figure 19.4), but good quality evidence is available to support or refute only

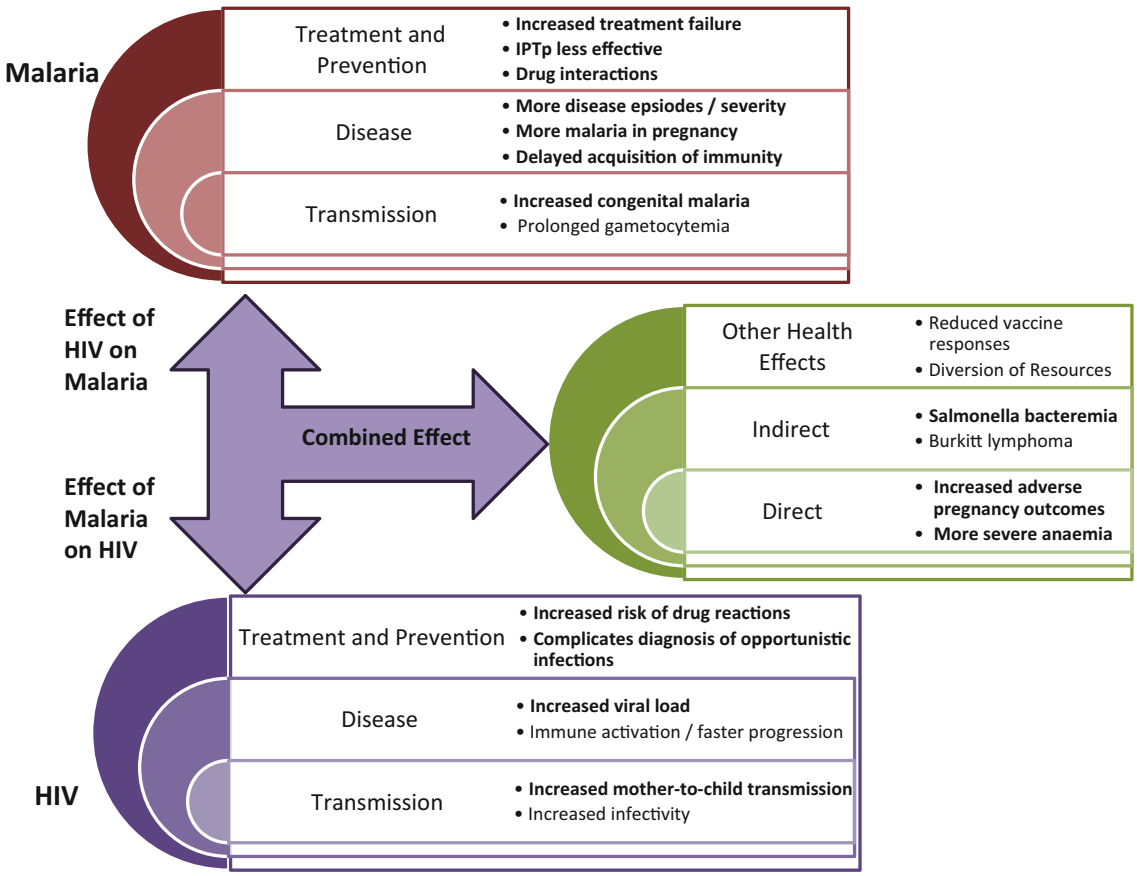

Figure 19.4 HIV and malaria have reciprocal effects on transmission, disease, treatment and prevention. The combination of HIV and malaria co-infection may have additional implications for susceptibility to other infections and poor health. Of the many potential consequences shown here, as yet only a few (shown in bold type) are supported by reliable immunological or epidemiological data.

some of these interactions. In this chapter, we will consider the likely immunological explanations for the best-established interactions between malaria and HIV, and indicate areas of potential concern that warrant further research.

19.4.1 Effect of HIV infection on the incidence and severity of clinical malaria

Assessment of the effect of HIV/AIDS on the incidence of clinical malaria, and the likelihood of severe disease, can only be meaningfully interpreted when the intensity of malaria transmission and the prior acquisition of immunity to severe disease are taken into account. As the exact nature of 'immunity' to malaria remains poorly defined, quantifying any effect immunologically is almost impossible, and thus we have to rely on inference from epidemiological studies. Even this, however, is not straightforward. Even within a defined geographical area, individuals may be exposed to different intensities of transmission of malaria and have different levels of anti-malarial immunity, regardless of their HIV status. Those who go on to acquire HIV may not necessarily be similar (genetically, immunologically, or in terms of malaria exposure) to those who remain HIV-negative. In addition, the diagnosis of clinical malaria can be difficult; the presence of parasitaemia and compatible clinical symptoms does not necessarily mean that malaria is the cause of the illness. Asymptomatic parasitaemia is common in clinically-immune individuals, and individuals with HIV are more likely to have other infections, which may be misdiagnosed as malaria if parasitaemia is present.

Thus, although many studies have claimed that HIV-infected individuals are more susceptible to clinical malaria and to severe malaria, establishing a link between HIV/AIDS and incidence and severity of malaria is problematic.

To date there are no robust data to indicate whether or not acquisition of clinical (anti-disease) or anti-parasitic immunity to malaria are impaired, differentially affected or unaffected by HIV infection. This is mainly because the very high mortality of children with vertically acquired HIV, in settings where there is also high-intensity transmission of malaria, has prevented longitudinal studies of the effect of HIV infection on acquired immunity to malaria.

However, several studies in adults who have acquired immunity to malaria prior to becoming HIV infected suggest that horizontally-acquired HIV appears to have a relatively modest effect on pre-existing anti-malarial immunity. Although the risk of clinical malaria increases several-fold, and is inversely related to the CD4+ count, the susceptibility to malaria is much less dramatic than susceptibility to bacterial infections. The risk of severe malaria does not appear to be significantly increased, indicating that HIV has relatively little effect on established anti-malarial immunity.

Importantly, however, this is not the case for adults with HIV who do not have pre-existing immunity to malaria. In settings where malaria occurs sporadically or in epidemics, the risk of severe malaria – including coma, acidosis and severe anaemia – is significantly higher in HIV-infected adults than in those without

HIV. Some severe manifestations of malaria, such as cerebral malaria, are frequently considered to be due to immunopathology, so it is an interesting observation that HIV-related immunosuppression exacerbates, rather than prevents, severe malaria. This may be reconciled by remembering that immune activation and dysregulation – rather than silencing of the immune system – is central to the pathogenesis of AIDS, and perhaps supports the concept that dysregulation is also important in the pathogenesis of severe malaria.

There has been very little research on whether HIV may increase malaria transmission, but it is conceivable that clearance of gametocytes from the blood is impaired in HIV-infected individuals, resulting in a prolonged 'carrier' state. This may be of particular relevance as a factor hindering global efforts to eliminate and eradicate malaria.

19.4.2 Effect of malaria on HIV viral load and progression

In contrast to the paucity of robust information on the effects of HIV on malaria incidence and severity, there are numerous studies demonstrating an effect of malaria on HIV infection and progression. Longitudinal studies of intercurrent malaria infections in HIV-infected individuals indicate that acute clinical malaria increases plasma viral load. This is presumed to result from immune activation by malaria, which would increase viral replication. The increase in viral load is relatively modest (less than a tenfold increase), and it resolves with anti-malarial treatment.

This is not unique to malaria, since other pathogens have also been reported to increase viral load in a similar manner (e.g. tuberculosis, herpes simplex, schistosomiasis). However, it is the possibility of frequent episodes of malaria, and of persistent asymptomatic parasitaemia affecting a large proportion of the population, that distinguish malaria as, potentially, an important cause of elevation of HIV viral load and, thus, progression to AIDS. Frequent episodes of malaria could thus hasten progression to AIDS and increase mortality. In addition, HIV plasma viral load is a major determinant of the risk of HIV transmission between individuals, and so transient increases in viral load might increase the spread of HIV.

Unfortunately, there are currently insufficient data from longitudinal studies to know if malaria is really a major force driving HIV transmission and morbidity in sub-Saharan Africa, and studies are urgently needed to address this issue. Although the immunological arguments are compelling, there are many reasons why these may not have the predicted effects. For example, adults with symptomatic malaria may be less likely to engage in sexual activity and would therefore be less likely to transmit HIV during acute malaria episodes.

19.4.3 Interactions between malaria and HIV in pregnant women

In settings with stable, high-level malaria transmission, adults are generally immune to clinical malaria and have lower levels of parasitaemia when they

become infected. This immunity is strikingly impaired by pregnancy, with a susceptibility to clinical malaria and dense infection of the placenta with *P. falciparum* being a feature of (particularly first) pregnancies. Pregnancy-associated malaria (PAM) is associated with poor outcomes for the mother (severe anaemia) and the foetus (reduced birth weight, increased neonatal mortality).

In subsequent pregnancies, the risk of PAM and adverse maternal and neonatal outcomes decreases. The most compelling explanation for this phenomenon is that PAM is caused by a subset of *P. falciparum* parasites which express variant surface antigens (VSA) on the surface of infected erythrocytes, enabling their binding to chondroitin sulphate A (CSA) on placental trophoblast and, thus, sequestration of infected erythrocytes in the placenta.

In non-pregnant individuals, acquisition of protective humoral immunity to malaria is likely to be due to acquisition of specific IgG against the predominantly expressed VSAs. CSA is rarely used as a receptor for *P. falciparum* adhesion in non-pregnant individuals, so there is no stimulus for an antibody response against the CSA-binding VSAs. In pregnancy, there is an opportunity for selection of parasite clones able to adhere to CSA, which is highly expressed in the placental intervillous space. Since there is no pre-existing immunity to CSA-binding VSAs, these clones can adhere, replicate and cause symptomatic infection. Pregnant women acquire increasing levels of antibodies to these clones during sequential pregnancies, which correlates with the acquisition of immunity to PAM.

HIV exacerbates the effects of malaria in pregnancy. Pregnant HIV-infected women suffer more frequent and more severe attacks of malaria, develop more severe anaemia and have worse neonatal outcomes than HIV-negative women. HIV is, in itself, a cause of adverse pregnancy outcomes; for example, pregnant women with HIV are particularly vulnerable to opportunistic infections. Furthermore, the remarkable perturbation of B cell function caused by HIV impairs the acquisition of protective antibodies against the pregnancy associated VSAs, and thus reduces the protective immunity that is acquired during sequential pregnancies. This means that the vulnerability to pregnancy associated malaria seen in first pregnancies also persists in subsequent pregnancies in HIV-infected women.

Maternal HIV infection also increases the risk of congenital malaria infection of the newborn, i.e. blood-stage *P. falciparum* transmitted directly across the placenta from mother to foetus. The increased risk of congenital malaria is likely a direct consequence of the increased risk of placental infection in women with HIV.

In the absence of any intervention, MTCT occurs in 35 per cent of cases. Approximately 20 per cent of these infections occur *in utero*, ≈40 per cent occur during childbirth and the remainder occur during breast-feeding. There are several reasons to think that malaria might increase MTCT of HIV. First, malaria increases HIV plasma viral load and plasma viral load is an independent predictor of MTCT. Second, placental malaria causes inflammation in the placenta, which may locally increase HIV replication and facilitate passage of

HIV across the placental barrier, increasing *in utero* transmission. It will be important for future studies to resolve the relationship between pregnancy-associated malaria, placental malaria and MTCT of HIV.

19.5 Effect of co-infection on treatment of HIV and malaria infections

There is considerable potential for co-infection to influence the treatment of malaria and HIV, both through reciprocal effects on the effectiveness of treatment and also the interaction of drugs used to treat each infection. Drug interactions can be extremely complex and difficult to predict, influencing pharmacokinetics (liberation, absorption, distribution, metabolism and elimination of the drug), pharmacodynamics (effects of the drugs on the body, HIV or the malaria parasites) and, ultimately, the effectiveness of drug treatment and emergence of resistance. Whether clinical malaria or asymptomatic parasitaemia impairs the treatment of HIV is currently unknown, but it is conceivable that, during episodes of severe malaria, there may be reduced compliance with antiretroviral drugs and changes in host factors which determine pharmacokinetics and pharmacodynamics.

The effectiveness of drug treatment of malaria is determined in part by host immunity. Adults who have acquired protective immunity, through repeated exposure to malaria, have a lower risk of treatment failure than do children in the same transmission setting, and children with higher concentrations of IgG to some parasite antigens have better treatment responses than those with lower levels. In addition, it is generally true that treatment of any infection is more likely to fail in an immunocompromised host.

If the major effect of HIV is to impair the acquisition of immunity to malaria, then it would be predicted that this would also be associated with increased rates of treatment failure in older children and adults. There is some evidence from clinical studies that HIV-infected individuals are less likely to clear their malaria infections completely after treatment, leading to recrudescence of infection after treatment and being more prone to rapid reinfection. There is also evidence that HIV infection may diminish the effectiveness of intermittent preventive treatment of malaria in pregnancy (IPTp), a strategy of providing intermittent treatment doses of sulfadoxine-pyrimethamine to pregnant women in order to eliminate subclinical malaria infections and protect against PAM.

Interactions between anti-malarial and anti-retroviral drugs have been predicted on theoretical grounds and from *in vitro* studies, but convincing evidence of clinical relevance from studies in humans is lacking. Interestingly, some of the most widely used HIV PIs have been described to have anti-malarial activity, suggesting that they may prevent or reduce the severity of malaria in HIV patients receiving PI-containing HAART. Similarly, *in vitro* studies indicate that anti-malarial drugs such as mefloquine synergise with PIs to enhance their antiretroviral activity. While such interactions might be beneficial, both anti-malarial drugs and anti-retroviral drugs have undesirable side effects, and thus further study is needed to establish that these strategies would be safe.

19.6 Combined effects of HIV and malaria on susceptibility to other diseases

Co-infection with HIV/AIDS and malaria may have cumulative effects, resulting in increased susceptibility to other diseases. Notable examples are susceptibility to non-typhoid *Salmonella* (NTS) bacteraemia, B cell lymphoma and vaccine-preventable diseases.

19.6.1 *Salmonella* bacteraemia

Malaria and HIV are independently associated with an increased risk of invasive infection with NTS, which is itself one of the most common invasive bacterial infections in children in sub-Saharan Africa. Antibodies are an important component of protection against NTS bacteraemia. Young children often lack the capacity to make antibodies against encapsulated organisms such as NTS, and dysregulated humoral immunity as a result of HIV infection or malaria infection further contributes to susceptibility. If different mechanisms are involved in the susceptibility caused by malaria and HIV respectively, then their combined effect on susceptibility to NTS may be dramatic.

19.6.2 Burkitt's B cell lymphoma

Malaria and HIV are independently associated with an increased risk of the B cell malignancy, Burkitt lymphoma. Endemic Burkitt lymphoma is a childhood cancer that occurs in malarious regions of sub-Saharan Africa, and its aetiology appears dependent on the co-incidence of repeated exposure to malaria and infection with the B-lymphotropic Epstein-Barr virus (EBV). Children repeatedly exposed to malaria have reduced control, and thus greater replication, of EBV, presumably as a consequence of polyclonal B cell stimulation by malaria antigens and suppression of T cell-mediated immunity to EBV.

EBV is an oncogenic virus which can trigger the translocation of the proto-oncogene MYC into a IgG locus within infected B cells, resulting in over-expression of the transcription factor c-myc, enhanced cellular proliferation and reduced apoptosis. HIV-related Burkitt lymphoma is less often associated with EBV (only about 40 per cent of cases), but in those EBV-positive cases it is believed that impairment of cell mediated immunity by HIV allows reactivation of EBV. While HIV and malaria are both important risk factors for Burkitt lymphoma, whether HIV increases susceptibility to endemic Burkitt lymphoma, or malaria increases susceptibility to HIV-related Burkitt lymphoma, remains to be established.

19.6.3 Vaccination

Vaccination against infectious diseases has been one of the most successful ways to reduce their global burden. Childhood vaccines recommended universally by the World Health Organisation are BCG (except in those with

confirmed HIV infection), hepatitis B, polio, diphtheria, tetanus and pertussis, *Haemophilus Influenzae* type B, pneumococcal conjugate, rotavirus, measles and human papilloma virus.

Vaccines protect from infectious diseases both at an individual and a population level. If the number of people susceptible to an infectious agent falls below a critical threshold, then the agent will not be able to spread within the population and can potentially be eradicated. Individuals who are unvaccinated, or who did not achieve a protective response to vaccination, are protected by 'herd immunity'; when the majority of individuals in the population have been vaccinated, the chance of a susceptible individual coming into contact with someone who has not been vaccinated is very small. So long as herd immunity is maintained, immunocompromised individuals are afforded some protection against the vaccine-preventable diseases.

However, herd immunity can be eroded rapidly by disruption of vaccine distribution and uptake, or by increased rates of vaccine failure. Dysregulation of B-lymphocyte function and CD4+ T-lymphocyte help, and functional impairment of CD8+ T cells in HIV-infected individuals, impairs responses to most of the vaccines studied (including polysaccharide, protein subunit and live attenuated vaccines) and leads to loss of pre-existing vaccine mediated immunity, which cannot be restored by HAART alone. Although revaccination after starting HAART generally allows effective immune responses to be mounted, these may wane more rapidly than in non-HIV infected individuals.

Children with vertically-acquired HIV may only achieve the capacity to mount normal vaccine responses if they are identified and commenced on HAART early in life, allowing preservation of their memory B cell pool. Delayed antiretroviral therapy may cause long-lasting impairment of their ability to respond to vaccination, even after commencing HAART. In areas where the HIV prevalence is high, it is likely that a large proportion of the population is susceptible to vaccine preventable diseases, and conditions are created where outbreaks may occur.

Malaria has also been reported to impair vaccine responses, most notably for T cell-independent polysaccharide antigens and possibly polysaccharide-protein conjugate vaccines administered during episodes of clinical malaria. The effect of malaria on T-dependent vaccine responses, those to protein vaccines like tetanus, and to live vaccines like measles, appears to be minimal. The mechanisms by which malaria impairs vaccine responses are uncertain, but they may include polyclonal B cell activation and disruption of normal splenic function. Although it has never been formally evaluated, the potential for erosion of the population benefits of vaccination in areas of co-existing high malaria transmission and high HIV prevalence is very worrying.

19.7 Malaria and HIV vaccines

Strenuous efforts are being made to develop vaccines against malaria and HIV, but vaccines which provide complete protection from infection remain a distant prospect. If realised, these vaccines would be of huge public health

significance, but even partially effective vaccines may have a valuable role in reducing malaria mortality and stemming the HIV epidemic, when implemented alongside other preventive measures.

Imperfect vaccines, which might reduce the likelihood of clinical and severe malaria without providing sterilising immunity, or reduce the rate of progression of HIV without preventing infection, are in development, and these may be the first generations of vaccines against these diseases to reach widespread use. However, any host factors which erode their effectiveness when used operationally (i.e. outside of clinical trials) may drastically reduce their cost-effectiveness. In other words, if HIV substantially impairs the effectiveness of a malaria vaccine, then the money spent on the vaccine might be better spent on other measures to protect from malaria in populations with a high prevalence of HIV.

19.7.1 Malaria vaccine candidate RTS,S

The RTS,S vaccine is the malaria vaccine candidate closest to achieving approval for widespread operational use (see Chapter 25). The efficacy of RTS,S in HIV-infected individuals is unknown, but there are reasons to be pessimistic: the immunogenicity of the hepatitis B surface antigen (HBsAg, which is a component of the vaccine) is diminished by HIV; the generation and longevity of antibody responses to the circumsporozoite protein are likely to be impaired; and chronic immune activation is likely to diminish the quality, quantity and durability of the polyfunctional T cell response. In order to maximise any benefit from RTS,S it may need to be employed in conjunction with measures to reduce MTCT of HIV and treat infected adults.

19.7.2 Vaccination against HIV infection

An effective vaccine to prevent HIV infection is not yet available, but much research effort has justifiably been directed into trying to identify one. HIV vaccine development has been hampered by failure of killed virus or protein subunits to elicit broadly neutralising antibodies – which appear to be necessary to prevent infection – or to generate effective CD8+ T lymphocyte responses, which might help to prevent infection and limit viral replication. This is a consequence of the rapid mutation of key viral molecules such as gp120, coupled with conformational protection of conserved regions until the moment of ligation with target cells.

Mutation of HIV CD8+ T cell epitopes also occurs rapidly, which necessitates that vaccine strategies seeking to protect through cell-mediated immunity should elicit a broad range of CD8+ T cell responses in order to be protective. However, cell-mediated responses would probably not prevent transmission of HIV and establishment of infection, even if they could attenuate viral replication and progression to AIDS. Thus, vaccines aiming to elicit a cell-mediated immune response might reduce the ‘set point’ of plasma viral load (essentially the equilibrium between replication and control) and preserve CD4+ cell counts, and these are being actively pursued.

The most successful clinical trial to date – using a six-dose prime-boost strategy based on gp120 – only showed a slight (less than one-third) reduction in risk of infection with HIV, and no effect on viral load or CD4 count in infected subjects. Given the inherent difficulties in the ability of the human immune system to mount an effective response to protect against, or control HIV, it is likely that anything that perturbs the ability to mount an optimal immune response will threaten the success of such vaccination.

There is, as yet, little evidence on which to evaluate the effect of malaria on novel vaccine technologies, so we cannot predict for certain whether malaria will hamper efforts to control HIV by vaccination, but the co-incidence of the two infections is likely to be another hurdle to be overcome. Since individuals with acute febrile illness, such as malaria, are usually not vaccinated until recovery, and there might be major operational difficulties in administering a complicated vaccine schedule in an area where there is both high malaria transmission and a high incidence of clinical malaria.

19.8 Summary

The combination of HIV and malaria has the potential to be very detrimental to health, but there is surprisingly little evidence that this is, in fact, the case. Although there are broad geographical overlaps between the greatest burdens of *P. falciparum* infection and HIV infection, the overlap at an individual level may be less dramatic. One situation where there is clear evidence that HIV and malaria have a detrimental interaction is in pregnancy, and there are obvious virological, parasitological and immunological reasons why this should be the case. Formulating safe and effective strategies to limit HIV-malaria co-infections in pregnant women is, thus, a priority.

HIV and malaria have some common effects on the host immune response, and some similar mechanisms of immune evasion, probably explaining why they represent two of the most intractable human pathogens. Lessons learned from one infection may be instructive in the future to guide strategies for tackling the other, and there is a clear pressing need for more research on the adverse effects and best management of co-infection.

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HIV and *Leishmania* Co-infection

20

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Protozoan parasites of the genus *Leishmania* are important opportunistic pathogens capable of causing lesions on the skin, the mucosal membranes and/or the visceral organs of patients infected with human immunodeficiency virus type 1 (HIV-1). The first case of leishmaniasis associated with HIV-1 infection was reported in Spain in 1985, and HIV has since spread in *Leishmania* endemic countries, resulting in a sharp increase of the prevalence of HIV-1/*Leishmania* co-infection. Cases of co-infection have been reported in 35 countries worldwide, and it has become a rapidly expanding concern of public health. Co-infected patients are highly infectious to sandflies, and this may contribute to increased spread of *Leishmania*, including drug-resistant strains.

During the first decade of co-infection, most of the cases were reported in southern Europe; between 25–70 per cent of adult human cases of visceral leishmaniasis in this area were associated with HIV-1/AIDS, although, in the late 1990s, the introduction of the highly active antiretroviral therapy (HAART) resulted in an important decrease of incidence. Nevertheless, prevalence of co-infection is steadily increasing in other areas endemic for leishmaniasis, such as East Africa or the Indian subcontinent, where there continues to be limited access to antiretroviral treatment. As an example, in northern Ethiopia, the percentage of leishmaniasis associated to HIV-1 infection had increased from 19 per cent in 1998–1999 to 34 per cent in 2006–2007.

The suppression of the immune system by HIV-1 infection not only facilitates the development of leishmaniasis from new primary infections, but also the reactivation of latent *Leishmania* infection in asymptomatic carriers. It is known that, in endemic areas, only a low proportion of immunocompetent persons infected with *Leishmania* suffer from clinical leishmaniasis; the majority of parasitised subjects live without the symptoms of this disease. Subsequent infection of these asymptomatic subjects with HIV-1 facilitates the reactivation

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and opportunistic behaviour of the *Leishmania* and increases the reports of co-infection.

20.1 *Leishmania* parasitaemia is increased in HIV-*Leishmania* co-infection

In the case of HIV-1/*Leishmania* co-infected patients, viral infection profoundly affects the course of parasite infection, particularly in the later stages of the disease. An effective immune response against *Leishmania* depends on the appropriate recognition of *Leishmania* antigen and the stimulation of CD4+ T cells to induce macrophage leishmanicidal activity mediated by interferon (IFN)-γ expression (see Chapter 7). The marked CD4+ T cell depletion induced by HIV-1 infection (Chapter 19), along with the resulting reduction in the production of IFN-γ and impaired capacity of macrophages to kill amastigotes, results in the uncontrolled replication of the parasite.

The risk of developing clinical manifestations of leishmaniasis is closely related with the quantitative level of CD4+ T cells in the peripheral blood: in 77–90 per cent of co-infected patients, the count of CD4+ lymphocytes is less than 200/mm^3 of blood; in 7–22 per cent, it is between 200 and 500/mm^3; and in only 0 to 3 per cent, the number is greater than 500/mm^3. Moreover, it has also been confirmed that HIV-1 antigens inhibit the *Leishmania*-specific T cell proliferative response induced by *L. donovani* antigens.

20.2 *Leishmania* infection increases viral replication rate

Leishmania infection induces the stimulation of host cells infected with HIV, which activates signalling pathways that may lead to increased virus replication and the progression of HIV infection towards AIDS. These particular features observed in HIV/*Leishmania* co-infected patients are not only the consequence of the concomitant presence of both pathogens in the same host, they are also the result of the specific interactions by the virus and the parasite at the cellular and systemic level.

One major problem of opportunistic parasitic infections like Leishmaniasis is that they may induce a chronic immune activation in HIV+ individuals and the activation of latent virus, which leads to increased virus load, and then the accelerated progression of AIDS that reduces life expectancy. Clinical studies have determined that *Leishmania* infection promotes an increase in the serum HIV-1 load, while experimental studies have shown that *Leishmania* promastigotes can up-regulate virus expression in monocyte-derived cell lines latently infected with HIV-1. Virus replication in T cells is promoted by parasite lipophosphoglycan (LPG) components of the parasite membrane expressed in the *Leishmania* infected-macrophage membrane. During antigen presentation, LPG is able to provoke in T cells the translocation of transcriptional factors such as the nuclear factor-κB (NF-κB), and the activation of the HIV-1 long-terminal-repeat (LTR), resulting in viral replication.

It has been proposed that the increase in cytokine production induced by the parasite may also affect virus replication both in macrophages and T

cells. Studies carried out with human tonsillar tissues and monocyte-derived macrophages has demonstrated that *Leishmania* parasites do not directly affect the virus gene expression, but instead modulate the life cycle of HIV-1 through indirect mechanisms related to the elevation of tumour necrosis factor (TNF)-α and IL-1α and the increased expression of chemokine receptors such as CCR5.

20.3 Cell specific interactions between HIV-1 and *Leishmania*

20.3.1 Interactions in macrophages

Both *Leishmania* and HIV-1 share the macrophage as their target cell, and several studies confirm that there is a multifaceted interaction between both pathogens in macrophages that can lead to an exacerbation of disease. Despite HIV-induced impairment of macrophage functions such as phagocytosis, intracellular killing, chemotaxis and cytokine production, it has been shown that *Leishmania* parasite uptake is increased in HIV-1 infected macrophages (Figure 20.1).

HIV-1-infected cells express the transcriptional transactivator (Tat) protein in large amounts, which can inhibit the IFN-γ induced leishmanicidal functions in macrophages and, in turn, promote the prostaglandin E2 (PGE2)-mediated replication of *Leishmania* parasites. Moreover, PGE2 is a potent inducer of HIV-1 replication, increasing the synthesis of transforming growth factor (TGF)-β which, in turn, enhances the growth of the parasite. It has also been observed that latent pro-virus DNA in HIV-1 infected phagocytic cells become activated when exposed to *Leishmania*-derived lipophosphoglycan (LPG) or *Leishmania*-infected mononuclear phagocytes. This is thought to be mediated by *Leishmania* parasite antigens that induce the secretion of TNF-α, a cytokine that is known to activate the expression of HIV-1 in an autocrine/paracrine manner.

The interactions between HIV-1 and *Leishmania* in macrophages are not all synergistic. TGF-β secretion is induced by *Leishmania* amastigotes inside macrophages. This cytokine can down-regulate inflammatory parasite clearance mechanisms, in turn promoting persistence of infection. However, TGF-β is also a potent suppressor of HIV-1 expression in monocytes and macrophages. After treatment for leishmaniasis, levels of circulating TGF-β decrease in patients, and the clearance of *Leishmania* parasites after anti-parasitic treatment is associated with increased HIV-1 viraemia. The reduction in TGF-β could explain the elevated replication of the virus. However this may also be explained by the recent discovery that pentavalent antimonials, the most common treatment for leishmaniasis, are able to induce HIV-1 replication directly.

20.3.2 Interactions in dendritic cells

Specific interactions between HIV and *Leishmania* have also been described in dendritic cells (DCs). Both HIV and *Leishmania* can infect DCs, and both use

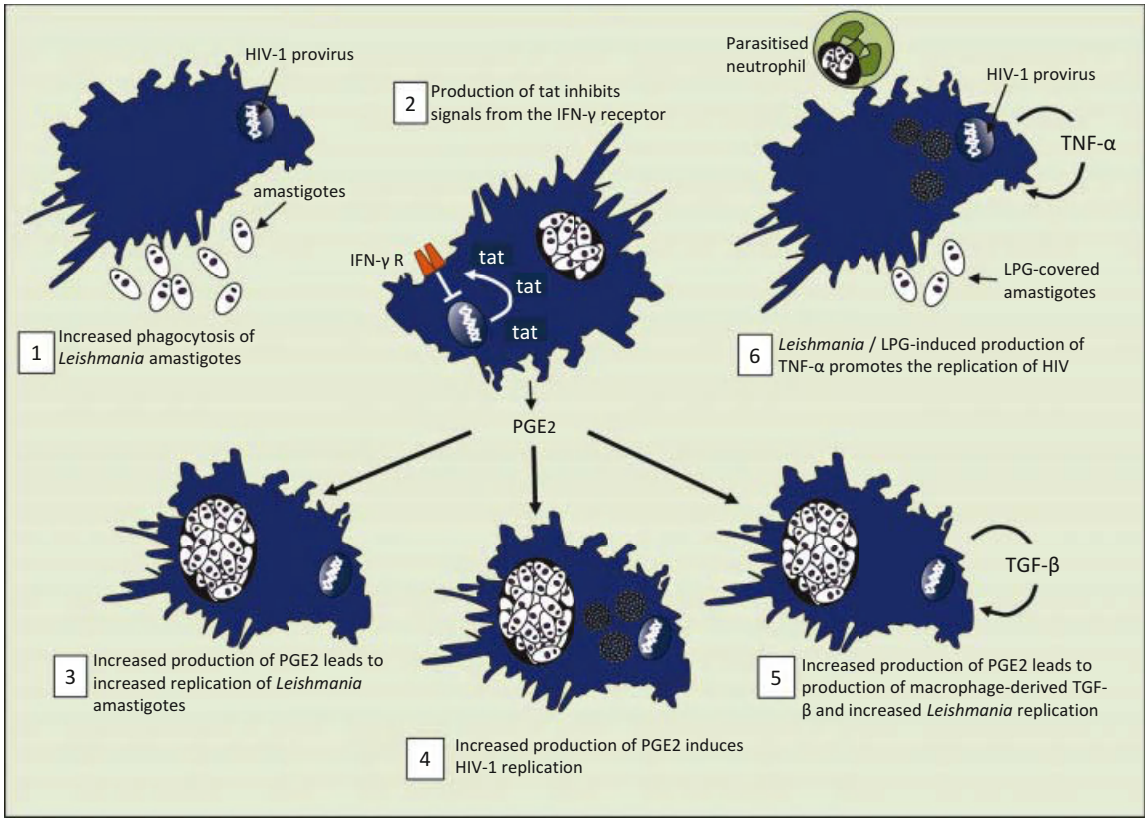

Figure 20.1 Interactions between HIV and *Leishmania* amastigotes in macrophages. Macrophages infected with HIV-1 have increased phagocytosis of *Leishmania* parasites (1). Production of Tat in HIV-infected macrophages leads to inhibition of signals from the IFN-γ R and an increase in the production of PGE2 (2) which, in turn, promotes replication of both *Leishmania* amastigotes (3) and HIV-1 provirus (4). PGE2-induced TGF-β production from macrophages also increases amastigote replication(5), while TNF-α produced upon macrophage activation by *Leishmania*-infected neutrophils, or LPG on the surface of *Leishmania* parasites, induces HIV replication (6). Abbreviations: HIV, human immunodeficiency virus; IFN-γ R, interferon-γ receptor; LPG, lipophosphoglycan; PGE2, prostaglandin E2; tat, transcriptional transactivator; TGF, transforming growth factor; TNF, tumour necrosis factor.

the same DC receptor, DC-SIGN, to gain entry into DCs. It has been shown that *Leishmania* amastigotes induce HIV-1 replication in both DCs and autologous T cells co-cultured together by inducing the secretion of IL-6 and TNF-α.

20.3.3 Pathogenesis of HIV-1/*Leishmania* co-infection

HIV-1 and *Leishmania* both cause numerous immunological disorders in humans. The severe immunosuppression induced by HIV-1 infection (see Chapter 19) leads to a poor immune response against *Leishmania* which, in turn, increases the risk of developing leishmaniasis by 100 to 2,320-fold. There is a high rate of treatment failure and recurrences of leishmaniasis in HIV-1/*Leishmania* co-infected individuals. In addition to an increased parasite load in peripheral blood and in bone marrow, co-infected patients have *Leishmania* parasites in unusual localisations such as the digestive tract or the lungs.

Furthermore, co-infection with HIV modifies the clinical spectrum of leishmaniasis, and HIV-1/*Leishmania* co-infected patients present with more severe clinical leishmaniasis, with some unusual manifestations.

Unlike immunocompetent patients, in which the frequency of localised cutaneous leishmaniasis ulcers is high, the probability of ulceration is reduced in HIV-1/*Leishmania* co-infected individuals because of the low CD4+ T cell count arising from HIV-1 infection. While the clinical presentation of cutaneous lesions is very variable, in HIV-1 co-infected individuals, it is frequently a sign of visceral involvement similar to post-kala-azar dermal leishmaniasis (PKDL).

In immunocompetent patients, the host immune response and the aetiological species of *Leishmania* involved determine the development of the different cutaneous and visceral clinical forms of leishmaniasis. The failure to control the multiplication and spread of the *Leishmania* parasite throughout the body in HIV-1/*Leishmania* co-infection is the reason for the high rate of cutaneous dissemination and visceralisation observed in co-infected patients, irrespective of the etiologic species of *Leishmania* causing infection (see Chapter 7). For this reason, HIV-1/*Leishmania* co-infection is more frequently associated with diffuse cutaneous leishmaniasis and visceral leishmaniasis than with localised cutaneous leishmaniasis.

20.4 Immune response interactions between HIV-1 and *Leishmania*

20.4.1 The immune response against *Leishmania*

The resolution of the leishmaniasis correlates with the development of an effective *Leishmania*-specific cell-mediated immunity in cutaneous and mucocutaneous leishmaniasis patients from the onset of the disease. In visceral leishmaniasis, such a response appears after successful chemotherapy. T cell responses (in particular, Th1 CD4+ T cell responses) are necessary to activate parasite-clearance mechanisms in *Leishmania* infection (see Chapter 7). In both cutaneous and visceral leishmaniasis, protective immunity to *Leishmania* parasites is determined by an adequate Th1 response, whereas susceptibility is mediated by a Th2 response.

20.4.2 HIV infection skews the CD4+ T cell response towards a Th2

Host T cells are destroyed in HIV infection, reducing the ability of the host to mount an effective Th1 response. However, in HIV-1 infection, leishmaniasis is also promoted by modulation of the Th cytokine response by HIV infection. Progressive HIV-1 infection is associated with a dominant Th2 cytokine profile, while a Th1 profile, together with reduced prevalence of virus isolation, is related to lack of progression in HIV-1 infection. Both *in vitro* and *ex vivo* assays have demonstrated that HIV-1 can modulate cytokine production in response to *Leishmania*.

The addition of HIV-1 antigens to peripheral blood mononuclear cells (PBMCs) from healthy subjects cultured with *Leishmania* resulted in decreased secretion of the type 1 inflammatory cytokines IFN-γ, interleukin (IL)-12 and IL-18, and increased secretion of IL-4 (a Th2 cytokine) and IL-10 (an immunoregulatory cytokine). Furthermore, PBMCs from HIV-1/*Leishmania* co-infected patients failed to secrete IFN-γ after stimulation with *Leishmania* promastigotes. Decreased levels of circulating IL-15, a cytokine that enhances Th1 responses and promotes the immune response to intracellular pathogens, has also been reported in HIV-1/*Leishmania* co-infected patients.

Although most of the co-infected patients can initially respond to anti-parasite treatment by generating a specific cellular anti-*Leishmania* response, this is overcome by the persistent overall Th2 skew of the immune response in these patients. This provides some explanation for the high frequency of treatment failures observed in HIV-1/*Leishmania* co-infected patients.

It can be concluded that the cytokine status of HIV-1-positive patients is altered towards a Th2 type, and that this favours the replication and reactivation of the *Leishmania* parasite. This would explain the high susceptibility of these patients to leishmaniasis, the severity of the disease symptoms and the low rate of clinical and parasitological response to treatment. In support for this, PBMCs from co-infected individuals secrete higher levels of IL-4 and IL-10 than do PBMCs from singly infected HIV-1 positive patients when stimulated non-specifically with the plant lectin phytohaemoagglutinin. This demonstrates some synergism in the development of a Th2 response in co-infection with HIV-1 infection and *Leishmania*.

20.4.3 Humoral responses

Although visceral leishmaniasis is also characterised by a strong polyclonal B cell response, HIV-1-induced immunosuppression also prevails over the humoral response against the parasite. As a consequence of the lack of CD4+ T cells able to recognise specific *Leishmania* antigen and to stimulate B cells, it results in a large proportion of co-infected patients presenting no detectable (over 40 per cent) or low levels of *Leishmania*-specific serum antibodies and low levels of parasite-specific serum antibodies. The low level of antibodies in the serum poses difficulties for the diagnosis of *Leishmania* infection in HIV-1 co-infected patients.

20.5 Immune reconstitution inflammatory syndrome in HIV-1/*Leishmania* co-infection

The initiation of routine use of HAART at the end of the 1990s resulted in a sharp decrease in the number of new cases of HIV-1/*Leishmania* co-infection in southern Europe. Since HAART is able to suppress viral replication and induce a restoration of the protective pathogens-specific immune response, the risk of developing clinical leishmaniasis in co-infected patient is significantly reduced. Furthermore, HIV-1 protease inhibitors included in the anti-viral treatment directly affect *Leishmania* proteases, inducing apoptosis and reducing the

intracellular growth of the parasite, thus increasing the therapeutic efficacy of the treatment on this type of patients.

Paradoxically, in some cases of HIV-1/*Leishmania* co-infection, there is a worsening of the clinical status after starting HAART that cannot be attributed to side-effects of therapy. These complications occur as the patient's HIV-1 load decreases and the CD4 count rises, and they have been associated with an immune reconstitution inflammatory syndrome (IRIS). This syndrome is estimated to occur in 10–32 per cent of patients who receive HAART, and it is characterised by a pathological inflammatory response to microbe antigens.

In the case of HIV-1/*Leishmania* co-infection, several cases of IRIS-associated leishmaniasis have been reported after initiation of HAART. The clinical presentation in these patients is mainly dermatological, and it has been described as diffuse cutaneous leishmaniasis and PKDL. The pathogenesis of IRIS-associated leishmaniasis has not been well defined. However, the inflammatory response and subsequent disease seems to be consequences of the previous abundant presence of *Leishmania* parasites and antigens in the patient and the enhanced response of CD4+ T cell clones able to recognise such antigens (which, as already mentioned, are necessary for development of cutaneous lesions of leishmaniasis). The main implication is that, besides the risk of reactivation of latent leishmaniasis in HIV-1/*Leishmania* co-infected patients, HAART can also lead to the activation of silent leishmaniasis in those patients who do not have a previous history of clinical leishmaniasis.

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21 Gastrointestinal Nematodes and Malaria

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21.1 Introduction

Nematodes and the vertebrate immune system have been evolving together in humans for millions of years and, with more than one billion people infected, gastrointestinal (GI) nematodes are the most prevalent parasites. There is a great overlap between endemic areas for both GI nematodes and malaria. This statistical reality has operated for thousands of years of human history and still does today in much of the tropical world.

Every year, there are still half a billion cases of malaria and more than one million deaths, mostly in children under five years old. *Plasmodium falciparum* is the deadliest human parasite by far; it emerged as a significant health burden approximately 10,000 years ago, when human communities grew to sufficient sizes to establish a substantial reservoir. Since then, it has exerted the strongest known selective pressure on the human genome, affecting erythrocyte proteins (such as haemoglobin), cytoadherence receptors and the immune system. Helminths have also shaped the human genome, with several interleukin (IL)/interleukin receptor genes being the target of balancing selection. It is therefore plausible that the genomes of all the protagonists in the GI nematode/malaria/human relationship have been shaped by this interaction, and a growing number of results from epidemiologic studies support this view.

21.2 Results from field studies in humans are conflicting

In the past decade, a growing number of publications have studied the relationship between GI nematodes and malaria. Although, in these publications, the

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Table 21.1 Some possible outcomes of co-infection on malaria and GI helminth infections.

	Malaria	GI helminths
Effects on parasites	Incidence of infection	Changes in adult parasitaemia (measured by expulsion chemotherapy)
	Alteration of prevalence within a community	Alteration of prevalence within a community
	Mixed clone/species infections (e.g. *P. falciparum* and *P. vivax* in the same host)	Alterations prevalence or ratios of co-infecting species in the same host (e.g. Hookworm and *Ascaris*)
	Gametocyte carriage (altered infectiousness to mosquitoes)	Alterations in fecundity (egg output)
Pathogenesis of infection	Protection against severe malaria	Protection against adverse consequences of GI helminths
	Exacerbation of severe malaria	Exacerbation of adverse consequences of GI helminths
	No difference in any parameters measured	No difference in any parameters measured

idea of helminth-associated immune modulation has provided motivation for carrying out the research, many studies have been epidemiological in nature, with very little data on immune mechanisms. The results of these studies have generated conflicting results, but some general trends have now become apparent. Infection parameters that may, or may not, be affected by interactions between malaria and GI helminths are outlined in Table 21.1.

21.2.1 *Ascaris lumbricoides* and malaria

In the late 1970s, nutritionists observed that malnourished patients infected with heavy worm burdens of *Ascaris lumbricoides* (see Chapter 12) seemed to be free of malaria and that, a few days after anti-helminthic treatment with piperazine, there was an increase in malaria attacks in these patients. Their assumption was that the nutritional consequences of worms suppressed malaria.

Overall, eight studies have found that *Ascaris lumbricoides* is associated with a reduction of malaria (incidence, prevalence or reduction of parasitaemia), two studies have found that *Ascaris lumbricoides* is associated with an increase in malaria prevalence, and two studies have found no relation to malaria. For severe malaria (cerebral malaria, and renal failure), two studies have identified *Ascaris lumbricoides* as the only individual worm associated with protection in adults. One study observed increased severe malaria in *Ascaris*-infected children, but the use of vomiting (which can be caused by *Ascaris*) as one of the definition criteria of severe malaria is problematical.

In summary, for *Ascaris lumbricoides*, ten studies have observed protection from malaria, three have found an increase of malaria and two have found no association with malaria.

21.2.2 Hookworm and malaria

There is a marked spatial overlap between hookworm infections (see Chapter 13) and malaria in Africa. Although hookworms have been associated with protection from severe malaria in one study, and another study found no association between the parameters of hookworm and malaria infections, the

majority of studies carried out thus far suggest that hookworms are associated with more frequent, but not severe, malaria (six studies).

21.2.3 Pooled GI nematodes and malaria

Since the immune response to different GI nematodes is similar, many studies have pooled GI helminth data. As may be predicted from the studies looking at either *Ascaris* or hookworm single infections, the results from these studies have been conflicting. It is not always clear whether incidence (the number of new cases within a population over the time period of the study) or prevalence (how common a disease is within a population at the time of the study) of malaria infections is reported in some studies. However, again results range from increased incidence of malaria in three studies, decreased prevalence of malaria in one study, no association with malaria in two studies and protection from severe malaria for three studies.

21.2.4 Other considerations

Beyond modulation of the incidence, prevalence or severity of malaria infection, GI nematodes have been related to malaria in other intriguing ways. Some of the observations are still of unknown importance. The increase of malaria gametocyte carriage or multiplicity of infection (with more than one clone or species of malaria) in nematode-infected patients, and the positive relationship between *Ascaris* fecundity and malaria-induced fever, both have potential evolutionary implications for malaria, but they await confirmation as general trends of GI nematode and malaria co-infection.

The extrapolation of findings in adults to children under five years of age must be with caution; some studies suggest important age-related differences, whereas others show results in children that are aligned with results in adults. Furthermore, most studies examine co-infection of GI nematodes with *Plasmodium falciparum* malaria, but protection from *Plasmodium vivax* malaria has been reported consistently in three different studies.

To sum up, although GI nematodes in general seem to be associated with protection from severe malaria in adults, there are some striking differences between *Ascaris lumbricoides* and hookworm in particular, with regards to incidence and prevalence data. Hookworms have been associated with increased malaria incidence in two studies and increased prevalence in four; in contrast, *Ascaris* has been reported to be associated with decreased incidence in two studies and decreased prevalence in three studies (one study found increased malaria incidence and another found increased malaria prevalence in *Ascaris*-infected persons). Some of the mechanisms behind the interactions between malaria and GI nematodes will now be discussed.

21.3 Immune responses in GI nematode and malaria co-infections

GI nematodes skew the immune responses towards a stereotypical Th2/Treg response (see Section 3). The regulatory network induced by GI nematodes can

moderate the immune response to other antigens (see Chapters 23 and 24). Key immune effectors that target malaria parasites vary according to the parasite stages involved; antibodies are central to clear sporozoites and red blood cell stages, whereas cellular immunity clears liver-stage parasites (see Chapter 3).

The control of blood-stages also relies on cellular immunity to generate antibodies with high affinity and avidity for parasite antigens. The first waves of malaria parasite amplification elicit a Th1 response that subsequently switches towards Th2, helping to facilitate the maturation of the antibody response. Blood-stage infection also induces T regulatory cells that dampen the pro-inflammatory response and reduce immune effector mechanisms that clear parasites, in turn benefiting parasite multiplication.

The severe complications of malaria are linked to parasite multiplication and the disequilibrium between the pro and the anti-inflammatory cytokines; while pro-inflammatory cytokines control the first stages of infection, they are also central in the pathophysiology of severe malaria. GI nematode immune modulation towards Th2/Treg lymphocytes would thus be expected to modify a number of facets of malaria immunity.

Surprisingly, very little is known about the actual consequences of the modulation of malaria immunity by GI nematodes in humans. Three complementary hypotheses, discussed in more detail below, have been studied to explain how GI nematodes may influence malaria parasitaemia and pathogenesis in human GI nematode/malaria co-infections:

- *Hypothesis 1*: GI nematodes induce the generation of Th2-associated antibody isotype IgE and reduce the induction of cytoadherence receptors and the triggering of FcεRII-mediated release of nitric oxide, which is parasiticidal for malaria and, therefore, protective.
- *Hypothesis 2*: The generation of T regulatory cells by GI nematodes dampens malaria-induced pathology.
- *Hypothesis 3*: GI nematodes increase the propensity of B cells to generate non-cytophilic antibodies for malaria parasite proteins, in turn reducing ADCC and increasing the incidence and severity of malaria within a community.

21.3.1 Hypothesis 1: GI nematodes, Th2, IgE complexes and the FcεRII /NO pathway

Nitric oxide (NO) is an important and versatile immune effector molecule. In malaria, it was initially flagged as a mediator of cerebral pathology but, more recently, studies in mouse models of malaria indicate that this molecule protects against malaria pathogenesis. NO is a vasodilator, a neuromediator and a regulator of several immune effector molecules as well as a cytotoxic agent against malaria parasites.

Inducible nitric oxide synthase (iNOS) oxidises the terminal guanidino nitrogen atoms of the amino acid L-arginine and releases large quantities of

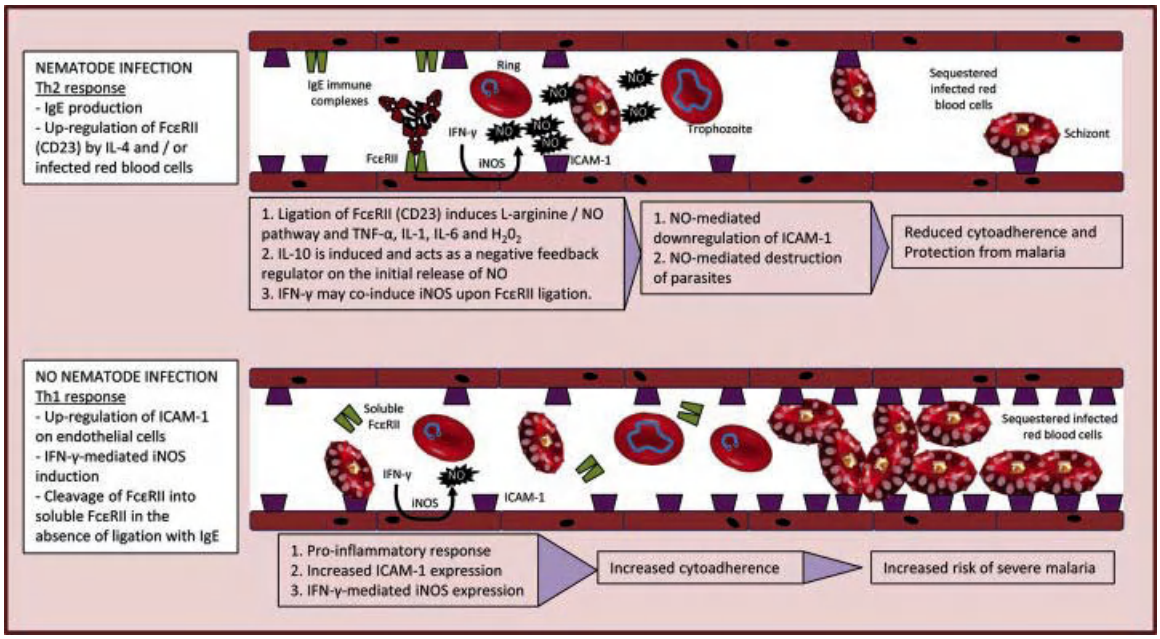

Figure 21.1 Hypothetical immunological mechanisms underlying the interactions between gastrointestinal nematodes and malaria (Hypothesis 1: The FcεRII (CD23)/nitric oxide hypothesis).
Abbreviations: CD, cluster of differentiation; FcεR, Fcε receptor; H202, hydrogen peroxide; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TNF, tumour necrosis factor.

the free radical NO. Certain polymorphisms of the iNOS gene (which result in increased production of NO in the individuals which harbour them) have also been shown to confer protection from severe malaria. The induction of iNOS is complex, with both Th1 and Th2 cytokines as potential activators. Although NO induction is often attributed to the actions of interferon (IFN)-γ, the stimulation of the low affinity receptor for IgE (FcεRII/CD23) is also a potent alternative activator of iNOS, by itself or in synergy with IFN-γ. The cross-linking of FcεRII by IgE/antigen complexes also promote secretion of IL-1, IL-6, TNF-α, and the generation of the parasiticidal molecule hydrogen peroxide (H_2O_2).

The first hypothesis for how GI nematode infections could protect against severe malaria implicates the induction IgE immune complexes by nematode infections that ligate the FcεRII, in turn activating iNOS, which leads to the release of NO and a reduction in the sequestration of parasitised red blood cells (Figure 21.1).

One study has shown a convergence of the elements involved in this first hypothesis, indicating that this could be the case. This study, carried out in Thailand, found that individuals who were co-infected with GI nematodes and malaria had significantly higher concentrations of reactive nitrogen intermediates (end-products of the short-lived NO) and a lower proportion of circulating schizonts than patients without GI nematodes.

Among the co-infected group, concentrations of reactive nitrogen intermediates were positively correlated with total IgE concentrations. Among all non-severe malaria cases, concentrations of reactive nitrogen intermediates were negatively correlated with the soluble form of FcεRII (in the absence of IgE ligation, FcεRII is cleaved into soluble FcεRII). The concentrations of reactive nitrogen intermediates was negatively correlated with the proportion of schizonts in blood smears while, amongst those with GI nematode infection, the concentration of reactive nitrogen intermediates was positively correlated with egg count.

The correlations in this data set support a model whereby GI helminth infection induces IgE, which ligates FcεRII, in turn inducing NO, which reduces malaria parasitaemia and provides protection against severe malaria.

To demonstrate that this hypothesis is feasible, an *in vitro* study showed that human lung endothelial cells incubated with malaria-infected red blood cells and the Th2 cytokine IL-4 (produced in abundance in GI nematode infection) up-regulated expression of FcεRII. The addition of IgE antibodies to the cultures to stimulate the FcεRII led to iNOS activation, the synthesis of NO and the destruction of the infected red blood cells in the culture.

Furthermore, under these conditions, the endothelial cells concomitantly down-regulated the expression of intracellular adhesion molecule (ICAM)-1, reducing the cytoadherence of the infected red blood cells. Thus, the interplay between IgE and NO is a feasible mechanism to explain how GI nematode-induced immune responses can potentiate anti-malaria immune responses.

Further support for this hypothesis can be found in work on individuals in Gabon and India, showing that IgE antibodies specific for malaria had a higher functional activity in people with asymptomatic or uncomplicated malaria compared to those with severe malaria. A second study observed an inverse correlation between placental malaria parasitaemia and IgE deposits in the placental microvasculature during pregnancy.

21.3.2 Hypothesis 2: GI nematodes, regulatory T cells and immunity to malaria

GI nematode pathogen-associated molecular patterns (PAMPs), and possibly tissue damage-associated molecular patterns (DAMPs), stimulate a variety of pattern recognition receptors (PRRs) on dendritic cells (DCs) and antigen-presenting cells. Although some differences may exist between the GI nematode species with respect to the PRRs activated on naïve DCs, the Th2 polarisation of the immune response within the context of a regulatory T cell (Treg) response is somewhat generic among nematode species. Since malaria also stimulates DCs through PRRs (see Chapter 3), alteration in the order and the timing of the stimulation of DCs in the context of co-infection could lead to differences in the nature of the immune response elicited.

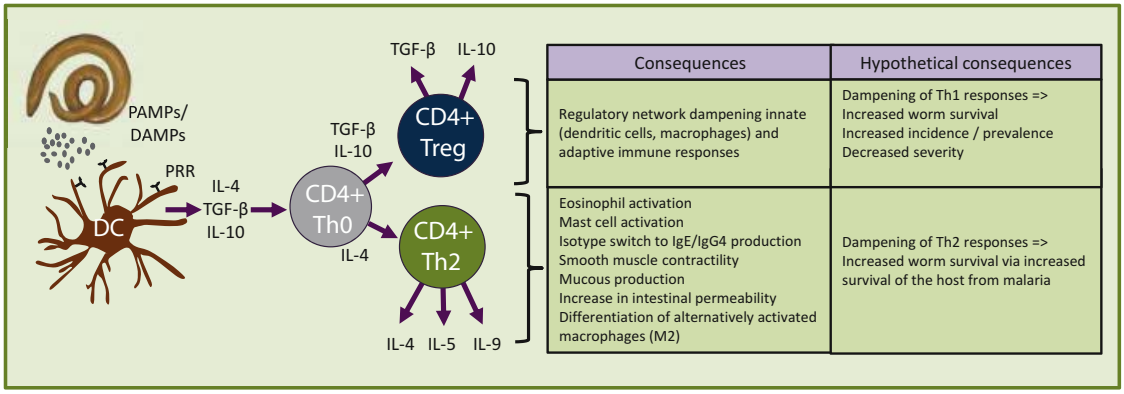

Figure 21.2 Hypothetical immunological mechanisms underlying the interactions between gastrointestinal nematodes and malaria (Hypothesis 2: The Treg hypothesis).
Abbreviations: DC, intestinal dendritic cells; DAMP, damage associated molecular patterns; PAMP, pathogen associated molecular patterns; PRR, pattern recognition receptors (e.g. Toll-like receptors, C-type lectins, NOD-like receptors, protease-activated receptors).

Natural Treg and adaptive Treg cells differ in their development and suppression mechanisms used to dampen inflammation. Activation of natural Tregs (CD25+ FoxP3+) has been described in nematode infections; activated natural Tregs can suppress immune responses through both IL-10 dependent and IL-10 independent pathways. Both malaria and helminth infections are associated with the induction of adaptive Tregs (Tr1 or Th3 cells) that can control inflammation and reduce immunopathology through IL-10 and TGF-β secretion. Hypothesis 2 (Figure 21.2) implicates GI nematode infection-induced expansion and activation of Tregs in protection against inflammatory pathology caused by malaria during co-infection.

In support of Hypothesis 2, upon stimulation with *P. falciparum*-infected red blood cells, PBMCs taken from co-infected children in a rural area of Ghana secreted higher concentrations of IL-10 than did PBMCs from children without helminth infection. The concentrations of pro-inflammatory cytokines secreted (TNF-α, IL-6, or IFN-γ) were not different between children who did or did not have co-infecting helminth infections, but increases in mRNA levels of Treg-associated genes such as FoxP3 were associated with IL-10 production.

Overall, the results suggested that T cells with a regulatory function are in higher abundance in helminth-infected patients than in those with malaria infection alone, providing some neutralisation of pro-inflammatory activity in the immune response and supporting the premise of Hypothesis 2.

In similar experiments with PBMCs from Indonesian children co-infected with GI nematodes and stimulated with *P. falciparum*-infected RBCs in culture, the presence of GI nematode infection appeared to suppress the proliferative capacity of PBMCs in response to malaria, when compared with children infected with malaria alone. This suppression was correlated with increased functional activity of the Treg populations in the PBMCs, rather than an

increase in the proportion of Treg cells circulating in the body. Depletion of Tregs (CD4+CD25^{hi} cells) from PBMCs restored the proliferative capacity (and the secretion of IFN-γ) by the other cells in the culture in response to malaria. Thus, Tregs can suppress the expansion and functionality of Th1 responses against malaria in co-infected individuals.

Although the immunological profile of co-infected individuals described above could be detrimental for the early phases of malaria and may impact on the effectiveness of any future vaccines against malaria in helminth-infected populations, theoretically this profile could also be protective against severe malaria. This fits with epidemiologic observations of increased incidence and reduced severity of malaria in GI nematode-infected individuals.

However, one could also hypothesise that the immunoregulatory network could promote parasite multiplication and severe parasite-related complications of malaria, as shown in some animal models. The studies described above did not directly investigate how immunomodulation impacted on the severity of malaria infection, nor the influence of particular GI nematodes in this regard, due to the small sample sizes examined. However, the immunomodulatory effects observed may transcend GI nematode (and possibly all helminth) infections, which is consistent with the stereotypical Th2/Treg skew of immune responses induced by these pathogens.

21.3.3 Hypothesis 3: GI nematodes, humoral immunity and antibody-dependent cellular inhibition

In endemic areas, children acquire immunity to malaria parasites progressively as they are repeatedly exposed to antigenic variants of the parasite within the community (premunition). Although premunition is slow to appear, immunity to severe malaria symptoms seems to be acquired much more rapidly. The slow development premunition possibly reflects the time necessary for each person to become immune to the varied repertoire of possible malaria antigens. Ultimately, protected by this non-sterilising immunity, such individuals will have decreased parasitaemia and subclinical malaria.

Antibodies are thought to play a central role in immunity to malaria by opsonising sporozoites preventing attachment to hepatocytes, by opsonising merozoites preventing reinvasion of red blood cells, and by neutralising glycosylphosphatidylinositol (GPI) anchors preventing excessive inflammation (see Chapter 3).

The slow development of immunity to malaria is also related to the delayed switch in the production of non-cytophilic classes of antibody isotypes (IgG2, IgG4 and IgM) towards cytophilic classes of antibody isotypes (IgG1 and IgG3) that interact with phagocytes to facilitate antibody dependent cellular cytotoxicity (ADCC) of opsonised parasites. The hypothesis that immune responses established during GI nematode infection decrease the production of cytophilic antibody isotypes against malaria, instead inducing the continued production of non-cytophilic antibody isotypes, may impact malaria parasitaemia by

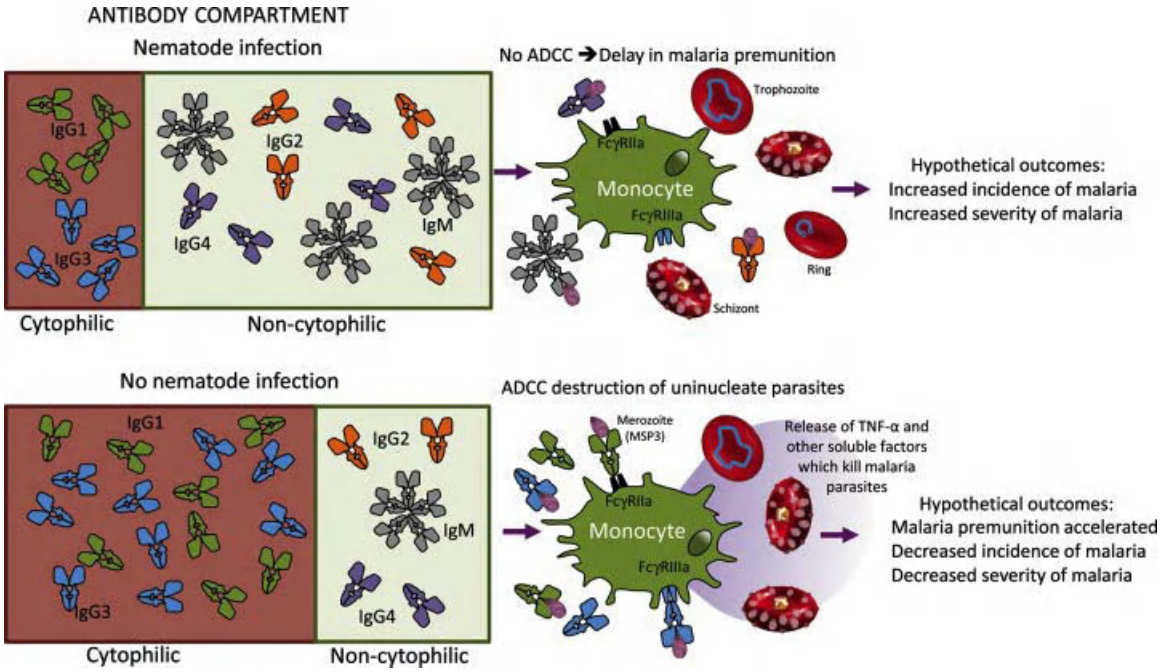

Figure 21.3 Hypothetical immunological mechanisms underlying the interactions between gastrointestinal nematodes and malaria (Hypothesis 3: The antibody-dependent cellular inhibition (ADCI) hypothesis).
Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; FcγR, Fcγ receptor; Ig, immunoglobulin; MSP, merozoite surface protein; TNF, tumour necrosis factor.

reducing the effectiveness of ADCC. This hypothesis could explain the increase in both incidence and severity of malaria in GI nematode and malaria co-infection (Figure 21.3). Antibody-dependent cellular inhibition (ADCI) is a term that relates to antibody dependent cytotoxicity, but it refers to the inhibition of parasite growth, and this extends to the development of intraerythrocytic uninucleated parasites (ring stages).

IgG1, IgG2, IgG3, and IgG4 responses against six different malaria antigens were studied in a cohort of Senegalese children. Of these, approximately half had at least one GI nematode species (mostly hookworm), while the other half had no detectable GI nematodes. As previously observed, there was an increased incidence of malaria in GI nematode-infected children, and this remained significant after adjusting for potential confounding variables. Consistent with Hypothesis 3, the children that had GI nematode infection had a shift towards non-cytophilic antibody isotypes circulating in their serum. More specifically, in GI nematode-infected children, multivariate analyses showed there was a reduction of cytophilic IgG1 and IgG3 antibody isotypes against the C-terminal region of merozoite surface protein (MSP)-3. Concomitantly, the children with GI-nematode infections had an increase in the non-cytophilic antibodies of the IgG4 isotype against epitopes of this protein.

Although the findings from this study support Hypothesis 3, they do not prove concrete evidence that the shift towards non-cytophilic antibody isotypes was responsible for the increase in malaria incidence. This hypothesis was not

tested by putting the different elements of the hypothetical causal sequence (worms → non-cytophilic antibodies → malaria) in a multivariate model. Therefore, the proposed mechanism behind Hypothesis 3 still needs to be demonstrated.

21.4 Stereotypical but different

Most studies on GI nematode infections in humans and their effect on the immune response against malaria parasites have pooled different GI nematode species into one single variable. This has been based on the premise that GI nematodes have a stereotypical action on the immune response (Th2/Treg – see Section 3), and the fact that sample sizes in these studies were too small for a detailed analysis of the individual effect from each worm species. Studies in Thailand showed that the association of GI nematodes with protection from malaria increased with the GI-nematode worm burden, as well as with the number of different GI nematodes present in an individual. However, epidemiological studies seem to suggest that there are differences in the effects that different GI nematodes have on malaria infections.

The general associations of *A. lumbricoides* with a reduction in malaria, and hookworm with an increased incidence of malaria, may have logical explanations when the biomass of the two species are considered. *A. lumbricoides* lays more eggs and arguably, therefore, it may be easier to detect. In addition, *A. lumbricoides* is the largest nematode infecting humans, and the weight of foreign biological material in a host infected with *Ascaris* is larger than for any other parasitic infection. Moreover, in laboratories around the world, *Ascaris* antigens are notoriously potent allergens. A large field study comparing IgE concentrations between different GI nematodes showed that total IgE levels were highest in *Ascaris*-infected children. The reason why this parasite would evolve to stimulate such an intense immune reaction may be related to the potential space limitations within the human intestine during infection with *A. lumbricoides*, i.e. an adaptation which limits superinfection with other nematodes competing for space would potentially be beneficial. The strong IgE responses measured in *Ascaris*-infected individuals accommodates the tenets of Hypotheses 1 and 2 (described above) to explain the observed protective effects of *Ascaris* infection towards malaria-induced pathology.

The hypothetical mechanisms for an exacerbation of malaria incidence in hookworm/malaria co-infections could be linked to a combination of immune modulation and hookworm-related anaemia. This could increase cues that are attractive for the vector (increase in lactates, increased respiratory frequency and CO_2 exhalation and increased cardiac output can all be influenced by the severity of anaemia), in turn leading to a greater probability of infective bites.

21.5 Animal models of GI nematode-malaria co-infection

Given the scarcity of immunological data on co-infections in humans, and the difficulty of carrying out such studies, animal models have been employed successfully to tease apart some of the immune mechanisms that may be at

Table 21.2 Results from animal models of GI nematode/malaria co-infections and malaria severity.

Organism	*Trichinella spiralis*	*Heligmosomoides polygyrus*	*Strongyloides ratti*	*Nippostrongyloides brasiliensis*
P. berghei	4↓	2↔	1↓	1↓
P. yoelii		2↑		
P. chabaudi		1↓ 2↑		

Symbols: ↑Aggravation; ↔ No difference; ↓ Protection.

play in GI nematode and malaria co-infection immunology. Depending on the experimental model, the malaria outcomes of the interaction are even more variable than human co-infection studies, and a range of effects, from protection to increased severity and no effect, have been reported (see Table 21.2).

Although mouse models are a necessity in gaining knowledge on the intricacies of immune responses, and are also useful in studies examining the efficacy of malaria vaccine-induced immune responses in GI nematode-infected mice, the co-infection models used are not natural infections. There is a fundamental difference between animal models and the human reality of co-infections, because the parasites in the former encounter each other in the laboratory and do not reflect the thousands of years of evolution in the latter. Thus, results from mouse studies can be difficult to extrapolate to humans, and the models used all have limitations.

Differences in the outcomes of GI nematode and malaria co-infection reported in mouse models can occur due to differences in the parasite species used, the timing of infections and the genetics of the mice examined. Recent modelling showed that the optimal balance between Th1 and Th2 responses was greatly affected by many host, micro- and macroparasite life-history traits. Studies on the effects of the timing and the order of the different infections may shed some light on the observed discrepancies.

As a case in point, the use of mouse models of GI nematode and malaria co-infections to investigate Hypothesis 2 (that GI nematode-induced Tregs can dampen pro-inflammatory immune responses generated by malaria infection, in turn increasing parasite survival) has resulted in slightly different interpretations as to the importance of this mechanism.

Mouse malaria infection is exacerbated in *Heligmosomoides polygyrus*-infected mice, but the cells responsible differ, depending on whether the mouse model used is *Plasmodium yoelii* (exacerbation of malaria by *H. polygyrus* infection can be reversed by *in vivo* depletion of Tregs) or *Plasmodium chabaudi* (exacerbation of malaria by *H. polygyrus* infection can be reversed in STAT6-/- mice, in which Th2 cells cannot be induced due an inability to signal through the IL-4 receptor).

In the latter combination, *H. polygyrus*-induced Th2 cells impaired the ability of the mice to produce IFN-γ in response to *P. chabaudi* and, as a consequence, co-infected mice were not able to generate type 1-associated malaria-specific IgG2a antibody to clear parasites to the same level as mice singly infected

with malaria. Therefore, data from mouse models needs to be interpreted with caution. The exacerbation of *P. chabaudi* malaria infection in *H. polygyrus* may involve Treg cells, but it is not necessarily dependent on this.

21.6 Conclusions

Despite recent interest, there is still scarce data on the immunological consequences of GI nematode-malaria co-infections and the precise mechanisms mediating the observed epidemiological interactions. However, after reviewing the literature, it can cautiously be suggested that GI nematodes – especially *Ascaris lumbricoides* – seem associated with protection from severe malaria but can also be associated with increased malaria incidence – notably for hookworm co-infection. Three different hypotheses have been put forward in this chapter to explain some of the observed interactions between GI nematodes and malaria, and these hypotheses do not necessarily exclude one another.

There are three practical measures that follow from these observed interactions, the first of which is the requirement for more research to disentangle the mechanisms at play, to identify the effects of different helminth species on malaria infection more clearly and to elucidate the effect of age on the outcome of co-infections. Second, caution should be exercised in vertical deworming programmes when malaria treatment facilities are lacking, because of the potential risk of a subsequent increase of severe malaria. The third (and perhaps most obvious measure) is that malaria vaccine trials and vaccination campaigns in the tropics should take nematode infections into account.

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Malaria and Schistosomes 22

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22.1 The epidemiology of schistosomiasis and malaria co-infection

The current distribution of malaria and schistosomiasis is largely restricted to the tropics, with the main burden of disease being borne by sub-Saharan African countries. As well as being co-endemic, age-infection curves show that school-aged children bear the burden of co-infection and associated morbidities in these populations. For malaria, there are three different levels of immunity (see Figure 22.1A): immunity to severe malaria; immunity to mild but symptomatic malaria; and immunity to parasitaemia, the latter of which is not complete. Immunity to severe malaria develops quickly, after only a couple of attacks, while immunity to mild malaria and parasitaemia develop much more slowly. The development of immunity to parasitaemia is the slowest to develop and, even in areas of high transmission, a large number of adolescents and adults carry asymptomatic infections.

Age-related infection intensity curves of schistosomiasis have a characteristic shape, in which the quantity of egg excretion – an indicator of the worm burden – increases throughout childhood, peaks in adolescence and then declines during late adolescence and adulthood (Figure 22.1B). For many years, there was debate as to whether this reflected changes in water contact with age, or whether it was due to the development of resistance to reinfection. However, curves of post-treatment reinfection intensities in communities where water contact by adults exceeds that of children are of a characteristic shape, indicating that resistance to schistosomiasis infection develops during adolescence and early adulthood.

From these age-infection profiles, it is clear that in many of the regions where schistosomiasis is present and stable malaria transmission occurs, those with

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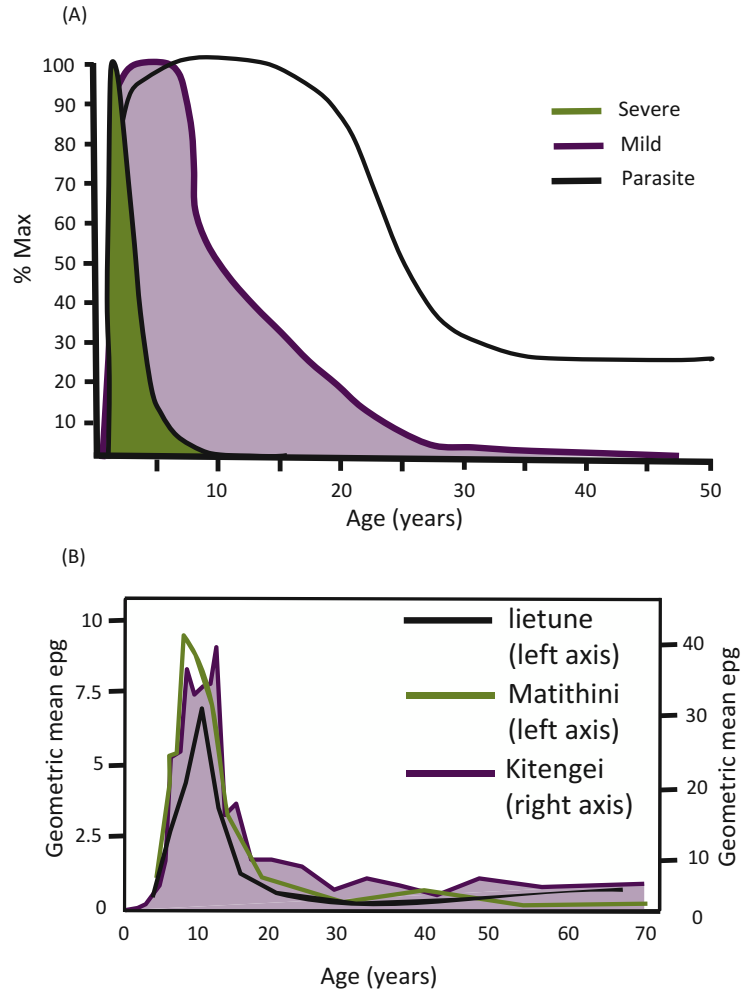

Figure 22.1 Age-infection profiles of malaria and schistosomiasis. A) Redrawn from Marsh, K & Kinyanjui, S (2006). **Immune effector mechanisms in malaria**. *Parasite Immunology* 28(1–2), 51–60 (with kind permission of John Wiley and Sons). B) From Fulford, AJC *et al*. (1998). **Puberty and age-related changes in susceptibility to schistosome infection**. *Parasitology Today* 14, 23–26 (with kind permission of Elsevier Ltd).

the highest schistosomiasis infection intensities will have been chronically exposed to malaria and are likely to still be susceptible to infection, even if they no longer suffer clinical attacks on a regular basis.

22.2 Study design for malaria/schistosome co-infection studies

Two of the most important aspects of field-based human immunology studies are to get the study design correct and to apply the correct analysis to the data. If this is not done, a completed study will not provide the answers to the

questions that have been posed. In order to design the correct study and analyse the data correctly, it is necessary to understand the epidemiology of the infections within the proposed study area – hence the reason that human community-based immunology studies are often referred to as immuno-epidemiological studies.

One of the most common study designs for malaria-schistosomiasis immuno-epidemiological studies is a case-control study in which matched groups of co- and mono-infected school-aged children are compared. Care needs to be taken in the design of these studies to avoid making the wrong conclusions. In the theoretical situation in Figure 22.2, the researchers compare the effects of co-infection with malaria and schistosomiasis to malaria infection only, on a response that is known to develop with increasing age and to be associated with protection against malaria.

22.2.1 Transmission intensity of both malaria and schistosomiasis should be similar

Due to the geospatially focal nature of schistosomiasis to obtain this cohort, the researchers in Figure 22.2 have chosen to conduct their study on schoolchildren from neighbouring villages, and the cohort age range is indicated by the box. As the villages are in close proximity (at similar altitude and with the same climatic conditions) and prevalence of parasitaemia is comparable at the time of the study, they have made the assumption that malaria transmission is also comparable. As they have sex- and age-matched children from the two schools, they think that they can apply simple statistics to their immunological data.

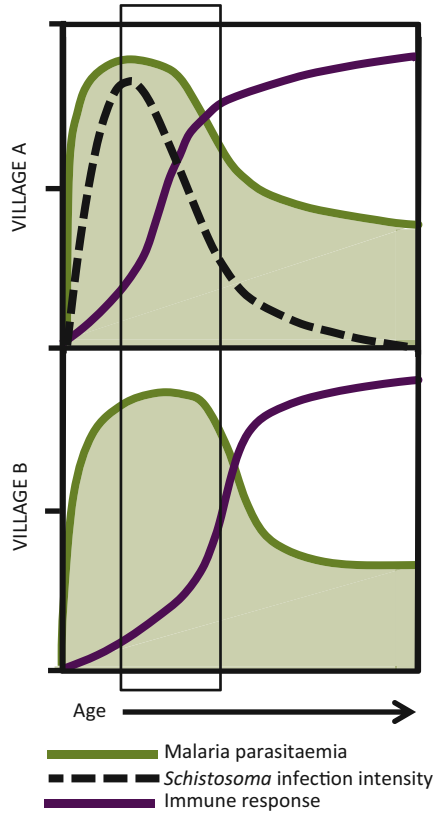

Figure 22.2 Getting the correct study design – a theoretical example. Two villages are examined, and studies will compare the effects of co-infection with malaria and schistosomiasis to malaria infection only. The cohort age range to be examined in this study is depicted by the box.

The response measured in the co-infected group is greater than that in the mono-infection group, so the researchers conclude that schistosomiasis up-regulates the protective anti-malarial response. If the transmission of malaria is more intense in the village where children are co-infected, however, and the response is driven by exposure to malaria, then the higher transmission will lead to earlier development of a protective anti-malarial immune response, so the conclusion drawn is wrong.

Finding matching groups with comparable malaria exposure is very difficult. Reported prevalence of infection can vary greatly, depending on diagnosis used. With the fluctuations in parasitaemia that are characteristic of the infection, it is possible for current infection prevalence, or levels of parasitaemia, to bear little reflection on accumulative exposure. There are also the complexities of transmission season and differing vectorial capacities to consider. Even on a microgeographical scale, species of transmitting mosquitoes may vary due to variations in environment; in one village there

could be transmission over a longer season by the poorer vector *Anopheles funestus*, and in the other a shorter transmission season by the efficient vector *Anopheles gambiae*.

22.2.2 Microgeographical variation differences in transmission can confound the results of a co-infection study

Conducting a case control within a village does not always solve these problems. Both malaria and schistosomiasis transmission can be very geospatially focal. In some endemic areas, particularly where water contact is for domestic rather than for occupational use, contact with infested water occurs more frequently for members of households close to schistosome transmission sites, leading to spatial patterns in infection intensities. Spatial patterns within a village also occur for malaria, with transmission dropping over a few hundred metres from mosquito breeding sites.

Figure 22.3 shows three theoretical situations (A, B and C), in all of which the transmission of schistosomiasis is identical. The profile of a

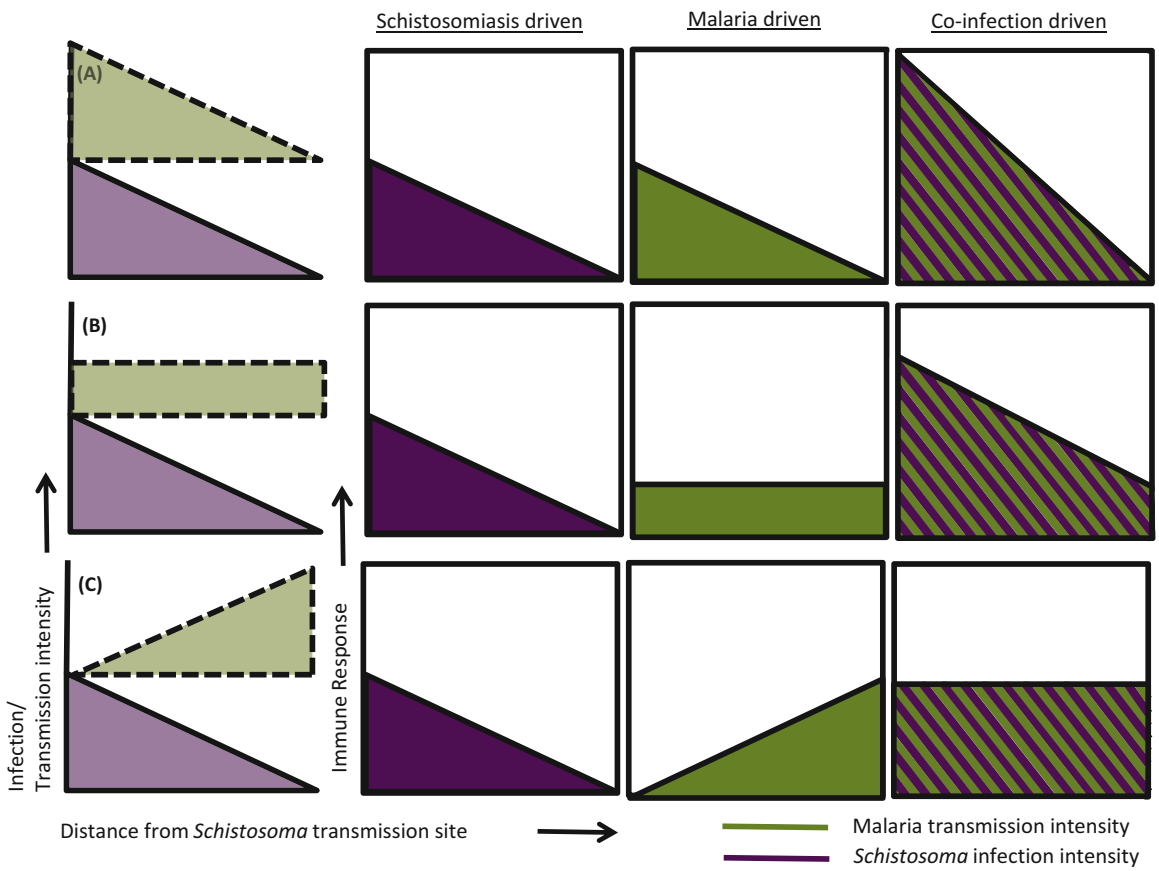

Figure 22.3 The effect of spatial patterns of transmission on immune responses during co-infection with malaria and schistosomes.

schistosomiasis-driven response is therefore the same in all three situations, decreasing in level with distance away from the transmission site. In each situation, the pattern of malaria transmission varies in relation to distance from schistosome transmission site, and the pattern of a malaria-driven response varies correspondingly.

The last column of Figure 22.3 shows the pattern of a response, such as a circulating cytokine response, to which both infections are contributing equally. This is obviously a gross over-simplification of what happens in the real world, ignoring all modulating effects, either positive or negative, but it does illustrate how differing spatial patterns of transmission for just one of the two parasites can lead to very different profiles of an immune response, both in terms of shape and magnitude, on a spatial scale.

A closer look at the situation C in Figure 22.3 can illustrate why microgeographical variations in parasite transmission can confound the results of case-control immunological studies. If, for example, situation C represents a community with only one school, and researchers design an age-sex matched case control study in which the children attending this school are allocated into groups depending on whether they have malaria, schistosomiasis or both, then a spatial bias will occur (Figure 22.4). Those children with malaria only are most likely to come from households furthest away from the schistosome transmission site, those with schistosomiasis from households closest to the transmission site, and the co-infected children are most likely to reside in the middle of the community.

If we then look at a schistosome-driven response, analysis is likely to indicate that it is highest in the schistosomiasis-only group, lowest in the malaria-only group and is somewhere in the middle for the co-infected children (Figure 22.4).

The following conclusion can be made: schistosomiasis, but not malaria, induced the immune response and, in co-infected children, the immune response is down-regulated by comparison to schistosomiasis mono-infected

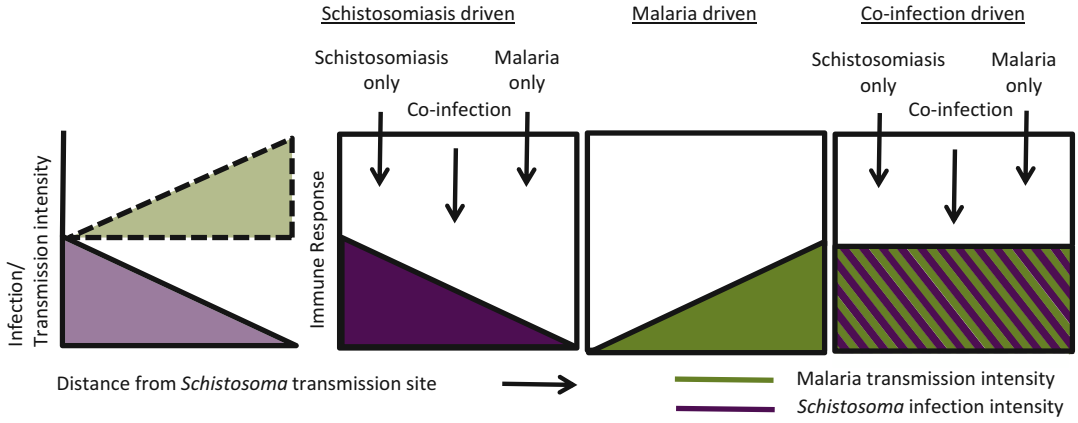

Figure 22.4 Microgeographical variations in parasite transmission can confound the results of co-infection studies.

children. If, the response were driven equally by both infections, there would be no difference between these groups, and the conclusion drawn would probably be that there was a basal level of response in this population that is not driven by either parasite. So, by ignoring spatial patterns and applying a simple case-control study design, two wrong conclusions could have been drawn.

22.2.3 Possible solutions to problems associated with infection intensities

One partial solution may be to match the schistosomiasis-only group and the co-infection group on schistosome infection intensity as well. In reality, this can be difficult: a perfect series of matched age, sex and infection intensity cases and controls is unlikely to exist, forcing the researchers to relax their matching condition, and there may still be problems with differences in exposure to malaria.

Another solution, and one which is normally feasible with the intention to measure plasma levels of antibodies and cytokines, is to conduct a larger cross-sectional study. This would involve measuring the immune response of interest in all the children attending the school and using statistical techniques, rather than study design techniques, to control for other variables such as demographics and infection prevalence or intensity.

This style of study, though powerful, can also suffer from problems when unrelated variables appear to be associated with the correlate in question, and sometimes is not possible to tease apart the relative contribution of each parasite. As an example, in Figure 22.2A, the immune response increases with age as both the prevalence of malaria parasitaemia and the intensity of schistosomiasis drop. In addition, a larger cross-sectional study is not usually feasible when the immune responses to be measured are *ex vivo* cellular responses to antigens. These studies tend to be more complicated and time-consuming to conduct, and it is in these studies that a well-designed case control in which demographics, including residence, are taken into account, can be of use.

22.3 Antibody responses

The immunology of schistosome and malaria co-infection of humans is an area where directed research is in its infancy. The few papers published mostly concentrate on the IgG3 response to the two parasites. This Ab isotype is capable of inducing complement-mediated cell lysis and mediating antibody dependent cellular cytotoxity (see Chapter 1). Immuno-epidemiological studies have shown that levels of this isotype specific for malaria antigens increase with age. Depending on the specific Ag used, they have been associated with resistance to clinical malaria attacks.

22.3.1 Cross-reactivity of IgG3 responses in schistosome-malaria co-infection

The major outcome of these papers is an ongoing debate on IgG3 cross-reactivity and/or whether co-infection results in higher production of these antibodies. Western blots of crude extracts of one parasite, probed with sera from individuals from areas where only the other parasite is endemic, have shown that, when an IgG3 response has been raised against one of the parasites, molecules from the other can be detected, i.e. there is some cross-reactivity in this response directed at the two parasites.

Blocking experiments, where serum samples from individuals exposed to both parasites are pre-absorbed with antigen preparations from one parasite prior to detecting the response to the other, indicate that levels of IgG3 to crude *Plasmodium falciparum* schizont (*Pfs*) antigen preparations are not significantly reduced by pre-incubation with either schistosome soluble egg antigen (SEA) or soluble worm antigen (SWA). However, responses to SEA and SWA are significantly reduced by pre-incubation with *Pfs* antigen. IgG3 responses to the *Pfs* antigen, therefore, appear to be mostly *Pfs*-specific (and *Pfs*-driven), while those to SWA and SEA are mostly due to cross-reactivity with the *Pfs*-driven response. However, some schistosome-driven cross-reactive IgG3 response cannot be discounted, as bands are detected on Western blots of *Pfs* preparations with schistosomiasis-only exposed serum samples.

22.3.2 IgG3 levels and protection against clinical disease

Theoretically, as IgG3 responses to malaria have been associated with protection, if cross-reactivity between schistosomiasis and malarial antigens drives higher IgG3 antibody levels in co-infected children, then co-infection could result in protective immunity developing at a younger age. This argument may be supported by the finding of some co-infection studies, that children co-infected with schistosomiasis have fewer symptomatic malarial attacks. However, IgG3 to malarial antigens are strongly driven by exposure on both macro- and micro-geographical scales and, depending on the malaria antigen chosen, levels can fluctuate with transmission season. This makes the interpretation of these results particularly vulnerable to being confounded by spatial and temporal issues, as discussed above.

Studies of clinical attacks of malaria in schistosomiasis co-infection can also suffer from being confounded by transmission intensity. Without detailed assessment of the micro-geographical pattern of transmission in the study area, differences in frequency of attack due to different transmission intensities can never be discounted. There have been reports that IgG3 levels to malarial antigen are higher in children co-infected with schistosomiasis. However, not all of the literature agrees, and further well-designed studies that take into account the complexity of transmission within the study areas are required to clarify the effects of co-infection on protective IgG3 responses to malarial Ag.

22.3.3 Antibody responses against schistosome worm antigen, schistosome egg antigen and malaria can be regulated separately

Although theoretically beneficial for the host, in terms of protection against malaria attacks, increased levels of IgG3 to schistosomiasis are unlikely to be beneficial in the same way. It is important to remember the complex life cycle of schistosomes, and not to consider anti-schistosome responses as one entity. Adult worm specific responses that are associated with resistance could well be detrimental to the host if maintained in response to the egg. Although there are a number of antigens shared between the schistosome egg and the schistosome adult worm, the human host appears to have evolved to balance the response to these different life stages.

Schistosome infections have been shown to induce Th2 responses and it is the Ab responses associated with this polarised response (IgG4 and IgE – see Chapter 1) that are observed to have the strongest associations with biologically relevant parameters. The IgE responses specific for SWA increase with age. This probably reflects the intermittent exposure to adult worm antigens that are normally hidden from the host immune system and only exposed upon worm death. SWA-IgE responses have been associated with resistance to reinfection, but a prolonged IgE-mediated response to egg antigens, which are seen by the host on a regular basis, would lead to severe immunopathology. Instead, for SEA responses, it is IgG4 that mirrors the age-infection intensity curve, potentially blocking a damaging IgE response.

In contrast, IgG3 responses to SEA and SWA are not related to age or infection intensities and, unlike IgG1, IgG4 and IgE responses to the same antigen, anti-SWA IgG3 responses are not boosted after treatment (effectively an *in vivo* dose of worm antigen as worms die and disintegrate). Interestingly, a schistosome molecule originally identified as a cross-reactive epitope using *P. berghei* infection of the rat, and its *P. falciparum* homologue identified by cDNA library screening (*Schistosoma mansoni* leucine-rich repeat protein, *Sm*LRR), is expressed by both the adult worm and egg stages. This induces IgG3 responses that are correlated with Pfs-IgG3 responses, as well as IgG4 responses that are correlated with *S. mansoni* egg counts. Therefore, *Sm*LRR drives IgG3 and IgG4 isotypes in malaria and schistosomiasis infections respectively, suggesting that there is specific regulation of cross-reactive isotypes during co-infection with these pathogens.

22.4 Cytokine responses

Cytokine responses in immuno-epidemiological studies can be measured in two compartments – the plasma, and cellular recall responses to antigen in culture.

22.4.1 Elevated levels of IL-10 and TNF receptor II indicate greater requirement for immune regulation in schistosome-malaria co-infection

To date, studies examining plasma cytokine levels in malaria/schistosomiasis co-infection indicate that co-infected individuals have higher levels of circulating interleukin (IL)-10 and soluble tumour necrosis factor receptor II (sTNF-RII) than their singly infected counterparts. sTNF-RII shedding from cells is induced by TNF-α itself as a negative feedback mechanism, while IL-10 is a regulatory cytokine involved in controlling both the pro-inflammatory response associated with the severest manifestations of malaria infection and the proliferation of cells in response to schistosome antigens (the latter likely to be important in preventing immunopathology).

IL-10 is also the major cytokine driving B cells through cell division and class-switching to produce IgG1 in the first instance, later followed by IgG3. Although IL-10 has been shown to be highly correlated with IgG3 responses to malarial antigens, the levels of this cytokine measured in the plasma of co-infected individuals are more likely to reflect the immediate need for control of potentially damaging pro-inflammatory responses. Positive correlation of anti-Pfs IgG3 levels with IL-12p70 and sTNF-RII (which initiate and shed, respectively, during a pro-inflammatory response) in addition to IL-10 support this hypothesis. There are also reported increased levels of another archetypal Th1 cytokine, IFNγ, in co-infected individuals, both in the plasma and in cell cultures stimulated with malaria antigens.

22.4.2 Th2 responses in schistosome-malaria co-infection

In general, the literature on schistosomiasis infection indicates a that polarised Th2 response is mounted, in particular once egg-laying has commenced (see Chapter 16). However, a prolonged polarised Th2 response is as detrimental to the surrounding tissue as a Th1-mediated pro-inflammatory response.

Mouse model experiments have implicated the archetypal Th2 cytokine IL-13 in promoting fibrotic deposition around the egg, and human studies have associated higher levels of IL-13 in response to SEA with presentation of the severest, but relatively rare, manifestation of infection, periportal fibrosis. However, large immuno-epidemiological studies suggest that in sub-Saharan African communities, which tend to have lower incidence of periportal fibrosis than other endemic countries, the levels of Th2 cytokines released in cellular recall responses upon culture with SEA are low, and it is in response to SWA stimulation that high levels of Th2 cytokines are observed. This is probably reflective of active regulation of this potentially damaging response to the frequently seen eggs, but an up-regulation of the polarised Th2 responses that are required to make the protective IgE response against reinfection.

Interestingly, the Th2 response to SEA, but not to SWA, is lower in children who are co-infected with malaria. Mouse model experiments confirm this finding,

with lower levels of IL-4 and IL-5 produced by splenocytes stimulated with SEA from mice infected with both *S. mansoni* and *P. chabaudi* infections, compared with mice infected solely with *S. mansoni* (see below). In addition, mice infected with *P. berghei yoelli* have schistosome egg granulomas in the liver that are smaller, with reduced eosinophil recruitment, when compared with non-malaria infected mice.

The flip side of this observed skewing away from the pro-fibrotic Th2 response could be an increase in the incidence of hepatosplenomegaly, a manifestation observed in many children in *S. mansoni* endemic areas which is associated with a pro-inflammatory immune response. The morbidity of hepatosplenomegaly is exacerbated when *S. mansoni* and malaria are co-endemic, an observation associated with higher plasma levels of pro-inflammatory and Th1 cytokines, with concomitantly lower levels of immunoregulatory cytokines secreted in ex vivo cell cultures stimulated with SEA.

22.5 Contribution of experimental models to the understanding of *Schistosoma mansoni* and *Plasmodium* co-infection

In order to provide a better understanding of the relationship between the effect of *S. mansoni* and malaria co-infection in the host, several experimental studies with different animal models have been conducted. The advantage of studying immune responses to experimental infections is the capacity to control all parameters and to obtain large number of cells for experimentation – often a limiting factor of samples from co-infected donors. However, the disadvantage of this model is that the mouse is only infected by rodent malaria species, which have an asexual erythrocytic cycle of about 24 hours when compared to the 48-hour cycle of *P. falciparum* (see Chapter 3).

Additionally, the effect of *S. mansoni* co-infection depends both on the genetic background of mice and on the strain of malaria parasite used. Patent *S. mansoni* infection renders C57BL/6 mice more susceptible to *P. chabaudi* infection, but pre-existing *S. mansoni* infection protects susceptible A/J mice from death with the same malaria strain. In BALB/c mice, patent *S. mansoni* infection contributes to an increase in the growth of a non-lethal *P. yoelii* strain, leading to the death of the co-infected animals, but on the other hand it protects C57BL/6 infected with *P. berghei* ANKA from the pathogenesis of cerebral malaria (CM) (although it is unable subsequently to prevent disease and death, due to hyperparasitaemia in this infection).

In contrast to human immuno-epidemiological studies, it has been observed that IFN-γ levels are significantly lower in *S. mansoni-P. berghei* co-infected mice. *S. mansoni* infection seems to increase the percentage of reticulocytes – young red blood cells that are preferentially invaded by *P. berghei*. Thus, *S. mansoni* not only reduces levels of an inflammatory 'killer' cytokine for *P. berghei*, but also provides malaria parasites with more target cells. These two combined actions favour *P. berghei* growth, with an eventually fatal outcome for the co-infected host.

With respect to the observed protection effect of patent schistosomiasis on CM in C57BL/6 mice infected with *P. berghei* ANKA, lower systemic pro-inflammatory cytokines were observed, suggesting that the Th2-driven response by *S. mansoni* decreases the Th1 inflammatory response induced by *P. berghei*. As a result, this may have reduced cellular adhesion molecules and lead to the recruitment of leukocytes to the brain, potentially leading to protection from cerebral pathology in co-infected mice.

Unfortunately, studies that combine field and laboratory evidence are rare and difficult to carry out. Therefore, it is necessary to be very careful when extrapolating results from experimental data to human co-infections. Nevertheless, it seems clear that the type, dynamic and magnitude of immune responses are driven in opposite directions by the infecting parasites (malaria stimulating Th1 responses while pre-existing schistosomiasis establishes Th2 responses). Hence, it is easily extrapolated that the timing of infection will very likely affect both the immunopathology and the outcome of the infection.

22.6 Conclusions

Due to the fairly recent impetus to study the immunological interactions between schistosomes and malaria in co-infections, the literature is often contradictory. However, strong themes are emerging. The debates on whether cross-reactivity/regulation of antibody responses are having an impact on the development of resistance to clinical malaria attacks, and the influence of skewed cytokine environments on parasite clearance and immunopathology, are ongoing.

It is still early days, and further well-designed studies are required to identify and understand potential confounders and modifiers in human immuno-epidemiological studies. Further consideration of the effects of malaria parasite strain, mouse genetic background and infection order and timings are required in experimental models. What is certain is that understanding these complex interactions is important; polyparasitism is the norm in endemic situations and, without the understanding of the modulating effects of co-infection, results of chemotherapeutic interventions and vaccine trials in one endemic situation cannot be directly extrapolated onto others.

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Section 5

23

Hygiene and Other Early Childhood Influences on the Subsequent Function of the Immune System

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Summary of the Hygiene Hypothesis

Helminths form a crucial part of the 'Darwinian' synthesis of the Hygiene (or 'Old Friends') hypothesis which seeks to explain the striking increase in chronic inflammatory disorders that started in Europe in the mid-19th century. This increase is *partly* attributable to dysregulation of the immune system, resulting from diminishing exposure to microorganisms that were tasked by co-evolutionary processes with establishing the 'normal' background levels of immunoregulation, a role that they perform in concert with the gut microbiota. The relevant organisms co-evolved with mammals, already accompanied early hominids in the Palaeolithic and are associated with animals, mud and faeces. They tend to establish stable carrier states, or are encountered continuously in primitive environments as 'pseudocommensals' from mud and water.

The central role of the helminths in this is emerging from epidemiology, animal experiments and clinical trials, and their immunoregulatory

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mechanisms are being revealed. These immunomodulatory organisms were *not* lost during the first epidemiological transition (settled agriculture), which might even have resulted in increased exposure to them. However the crucial organisms, including helminths, are lost progressively as populations undergo the second epidemiological transition (modern urban environment). The consequences of the loss of the 'Old Friends' are aggravated by other modern environmental changes that also lead to enhanced inflammatory responses (obesity, vitamin D deficiency, pollution (dioxins), etc). The range of chronic inflammatory disorders affected may be larger than had been assumed (allergies, autoimmunity, inflammatory bowel disease, but *also* celiac disease, food allergy, vascular disease, some cancers, and depression/anxiety when accompanied by raised inflammatory cytokines).

23.1 Introduction

In developed countries, there have been striking increases in the incidences of many chronic inflammatory disorders during the last hundred years. These disorders include allergies (hay fever, asthma, eczema), autoimmunity (type 1 diabetes (T1D), multiple sclerosis (MS)) and inflammatory bowel diseases (e.g. IBD; Crohn's disease (CrD) and ulcerative colitis (UC)). In the early 19th century, these diseases were almost unknown. Interestingly, all of these disorders show evidence of defective immunoregulation.

This suggests that, in rich developed countries, there is an increasing generalised defect in regulation of the immune system that is manifested in different individuals as a failure to terminate inappropriate responses to allergens (allergy), self (autoimmunity such as MS or T1D) or gut contents (IBD). Indeed, we know that a generalised dysfunction of immunoregulatory mechanisms can lead to simultaneous increases in these diverse types of pathology, because genetic defects of Foxp3, a transcription factor that plays a crucial role in the development and function of regulatory T cells (Treg), leads to a syndrome known as X-linked autoimmunity-allergic dysregulation syndrome (XLAAD), which includes aspects of allergy, autoimmunity and enteropathy.

Interestingly, the increases in these chronic inflammatory disorders were first noticed in the wealthier urban social classes, suggesting that environmental factors associated with modern life-styles play a major role. The 'Hygiene Hypothesis', and the more recently formulated 'Old Friends' hypothesis, suggest that the increase in immunoregulatory disorders is due to lack of contact with subsets of microorganisms and helminths that had co-evolved a role as initiators of immunoregulatory circuits.

23.2 The Hygiene Hypothesis (or 'Old Friends' hypothesis)

The epidemiological studies of allergies and family size that led to the invention of the term 'Hygiene Hypothesis' resulted briefly in a narrow focus on the

common infections of childhood, with an underlying view that these infections might regulate the Th1/Th2 balance. This view has had to be replaced by a hypothesis capable of explaining the simultaneous increases in both Th2- and Th1/Th17-mediated chronic inflammatory diseases and the presence of immunoregulatory defects in these disorders, as outlined above. The 'Old Friends' hypothesis suggests that the lack of appropriate levels of immunoregulatory activity in rich northern countries is a consequence of diminished exposure to three categories of organism:

1. The gut microbiota; the nature and balance of these are changing in the developed world.
2. Harmless organisms associated with mud, untreated water and fermenting vegetable matter that were always present, so are termed 'pseudocommensals'.
3. Infections, particularly helminths, that are still common in developing countries but are almost completely absent from rich ones.

These organisms were entrusted during our evolutionary history with the induction of immunoregulatory circuits, because they needed to be tolerated and so needed to induce immunoregulation. The helminthic parasites need to be tolerated because, although they are not always harmless, once they are established in the host, any effort by the immune system to eliminate them is futile and merely causes tissue damage. This phenomenon is an example of evolved dependence. Thus, the 'Old Friends Hypothesis' postulates that the mammalian immune system is in a state of evolved dependence on a range of immunomodulation-inducing microorganisms with which we co-evolved. We depend on genes encoded in the genomes of these organisms to set our immunoregulatory circuits at the right level.

23.3 Epidemiological transitions

Some organisms that would have been carried by Palaeolithic populations are listed in Figure 23.1, and those that have been implicated in the hygiene hypothesis are indicated. About 10,000 years ago, the shift to agriculture and husbandry created the first (Neolithic) epidemiological transition (Figure 23.1). This caused dramatic changes to humanity's microbial environment; many new virus infections evolved, but it did *not* result in loss of exposure to the organisms implicated by epidemiology in the Hygiene Hypothesis because, until the modern era, more than 97 per cent of the population still lived in rural environments, close to the mud, animals and faeces which were the sources of these organisms.

The situation did not change until the mid-19th Century. Since then, some populations have undergone a second epidemiological transition, in which public health measures and, more recently, antibiotics, have resulted in diminished (or *delayed*) exposure to many of the organisms with which the mammalian immune system evolved.

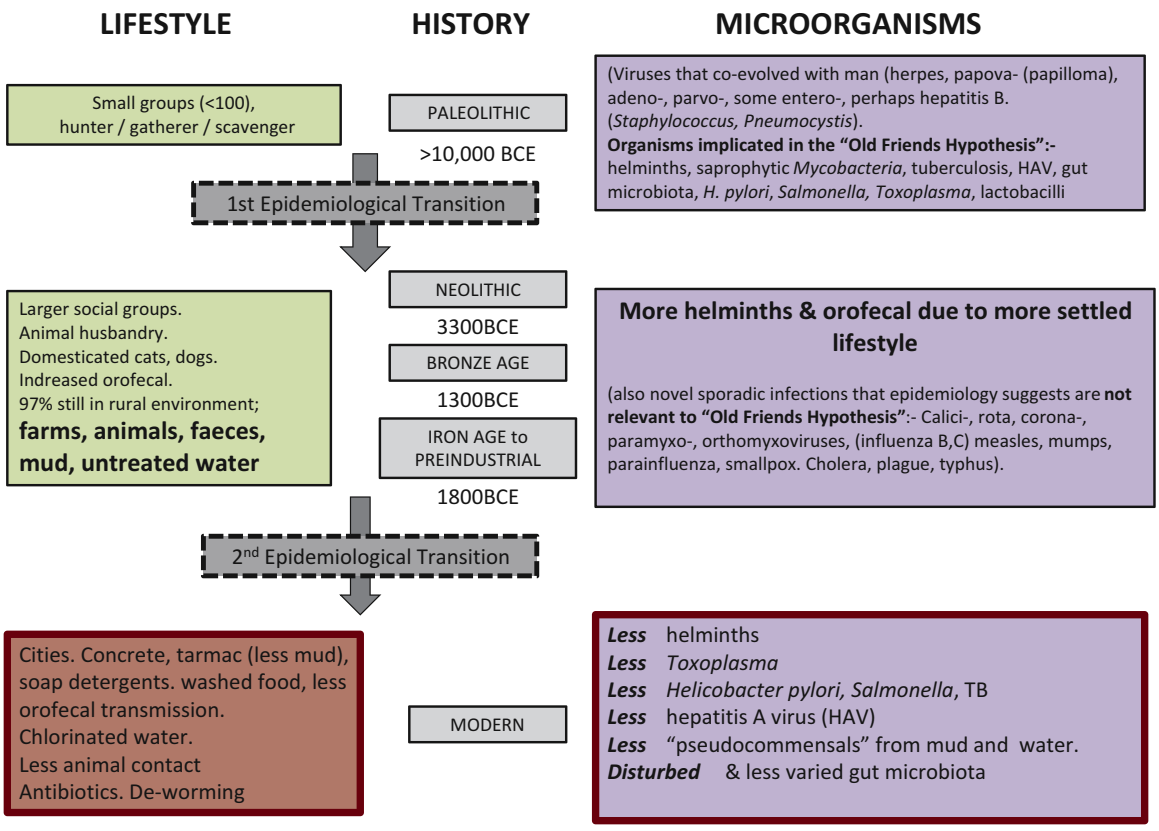

Figure 23.1 Aspects of man's microbiological history that are most relevant to the Hygiene Hypothesis. Epidemiological data, laboratory and animal models, and preliminary clinical trials investigating the Hygiene Hypothesis implicate organisms that are thought to have accompanied mammalian and human evolution. This relationship was long enough for the establishment of evolved dependence on these organisms that must be tolerated, so have developed roles in the initiation of regulatory pathways. Organisms that evolved during the Neolithic are less likely to be relevant in this context, and the first epidemiological transition did not reduce human contact with organisms associated with animals, faeces and mud. On the other hand, the second epidemiological transition has led to gene-environment misfit, as the 'Old Friends' from the Palaeolithic were progressively removed from the modern environment.

23.4 Compensatory genetic variants

A further layer of evolved dependence is revealed by genetic studies. In parts of the world where there was a heavy load of immunoregulation-inducing organisms, there has been selection for single nucleotide polymorphisms (SNP) or other variants that partially compensate for the immunoregulation. This is seen for several proinflammatory cytokines, IgE production, STAT6 (a transcription factor involved in Th2 responses), and a truncated form of the serotonin transporter that also has a marked pro-inflammatory effect.

The problem here is clear (Figure 23.2). As soon as the immunoregulation-inducing organisms are withdrawn by the modern lifestyle (second epidemiological transition), these genetic variants lead to excessive inflammation and become risk factors for chronic inflammatory disorders.

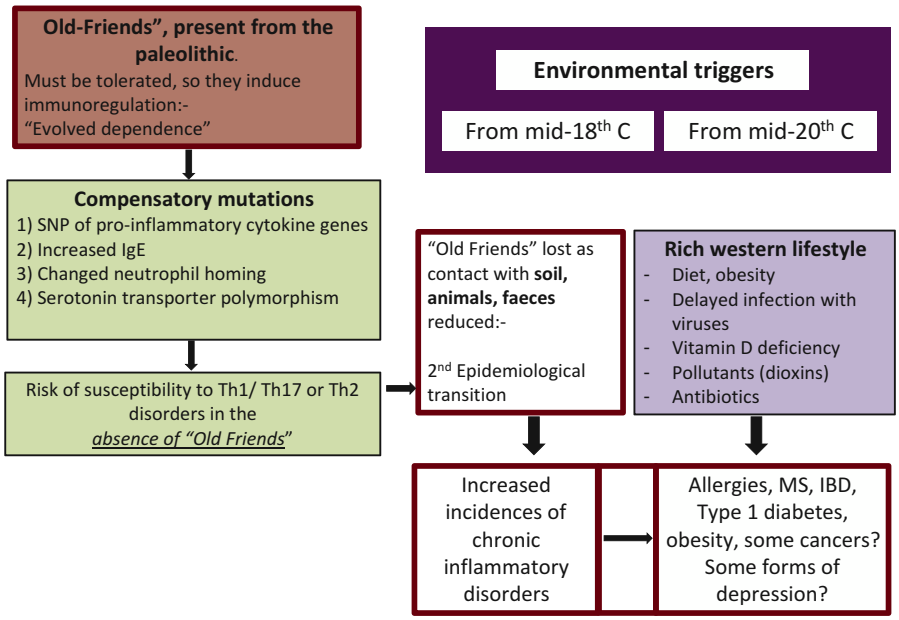

Figure 23.2 Interaction of genetics and loss of the 'Old Friends'. The Old Friends had to be tolerated, so they co-evolved roles as triggers of immunoregulatory pathways. In areas with very high loads of these and other organisms, particularly helminths, compensatory genetic variants accumulated to partially restore inflammatory responses. In the absence of the Old Friends, not only is immunoregulation inadequately primed, but also these genetic variants cause excessive inflammation and become risk factors for chronic inflammatory disorders. Genetic variants that were advantageous, and did not cause disease in the past, start to do so in the absence of the Old Friends. Several aspects of modern life are potentially exacerbating the consequences, as described in the main text.

23.5 The critical organisms and their immunological role

These considerations allow prediction of the organisms involved in the 'Old Friends' hypothesis. From a Darwinian perspective we would expect the relevant organisms to have been present, inevitably and continuously, from relatively early in the evolution of the immune system ('Old Friends'). One would also anticipate a reliable mode of transmission, such as the orofaecal route or transmission via contaminated soil in the close environment, often accompanied by the ability to establish carrier states that facilitate such transmission. The helminths are important in this context.

23.6 Helminth infections and allergic disorders

The helminths are the main focus of this chapter. In 1947, it was estimated that about 36 per cent of the population of Europe carried helminths such as *E. vermicularis, T. trichiura* and *A. lumbricoides*. Now even pinworm (*E. vermicularis*) has become a rarity in Europe. A number of studies have reported inverse correlations between indicators of helminth burden and allergic sensitisation to environmental allergens. More importantly, the risk of wheeze was reduced in

individuals with hookworm infection in Ethiopia, and *Enterobius* infestation was negatively correlated with asthma and rhinitis in primary school children in Taiwan. Similarly, it was suggested that infection with *Schistosoma mansoni* was associated with milder forms of asthma.

However, not all of the published studies are in agreement. Meta-analyses of the 33 studies included by Leonardi-Bree *et al.* in 2006 support the contention that infections with *Trichuris*, hookworm or schistosomes are negatively associated with allergen skin test reactivity, and they also indicate that hookworm infection is associated with a reduced prevalence of allergic asthma. In sharp contrast, *Ascaris lumbricoides* was associated with significantly increased risk of asthma in children in China and Costa Rica.

It seems likely that the major variables underlying these inconsistent results are the size of the worm burden and the duration of the infection. Transient or low level infections might merely contribute a Th2 adjuvant effect, so enhancing Th2 responses to other allergens. On the other hand, prolonged heavy infection triggers immunoregulatory pathways that inhibit damaging immunopathology, as discussed in relation to mechanisms below. Recent work in animal models supports these hypotheses.

23.6.1 Helminths that do not protect

Toxocara canis, the dog roundworm, may be an example of a helminth that does not protect against allergies, precisely because the worm burden is low and transient. In humans, *T. canis* cannot complete its life cycle and, although it may persist for months or even years, it is eventually eliminated. Thus, the Th2 adjuvant effects may prevail.

23.6.2 Effects of anti-helminthics

If helminths protect from chronic inflammatory disorders, the protection should be lost when the helminths are eliminated by anti-helminthic treatments. In fact, this effect constitutes strong evidence for their protective role. Short periods of treatment (<12 months) did not change the prevalence of atopy or clinical signs of allergy. In contrast, de-worming Vietnamese schoolchildren for 12 months, and still more prolonged treatment of children in Venezuela or Gabon, all led to increased allergen sensitisation and skin prick test responses. These studies did not reveal simultaneous increases in clinical allergies such as eczema, wheeze or rhinitis, but this would probably require still longer periods of treatment and follow-up.

23.7 Helminths and non-allergic chronic inflammatory disorders: human data

Gale (2002) has argued persuasively that the rise in type 1 diabetes (an autoimmune destruction of the insulin-secreting β-cells in the pancreas) in Western

Europe and the USA during the 20th Century correlates with the decline of helminth infections, particularly *Enterobius vermicularis*.

Similarly, the prevalence of MS was shown many years ago to correlate inversely with sanitation. MS is extremely rare in countries with a prevalence of *Trichuris trichiura* of more than ten per cent, but its incidence rises dramatically in areas with lower prevalence of this parasite. This is entirely correlative, circumstantial evidence. However Correale and colleagues (2007) have shown that patients with MS who become infected with helminths have a strikingly diminished rate of disease progression and develop circulating myelin-specific Treg that release IL-10 and TGF-β in response to a peptide from myelin basic protein. Helminth infection also induces a population of IL-10-secreting regulatory B cells in these patients.

As far as IBD is concerned, the epidemiological data are less strong than for allergic disorders because IBD, like MS, is much less common. Nevertheless, analyses of the available data conclude that exposure to helminths is one of the environmental factors most convincingly associated with a low risk of IBD.

23.7.1 Clinical trials that use helminth infections to treat chronic inflammatory disorders

Encouraging clinical trial results using oral administration of the eggs of *Trichuris suis* in UC and CrD further support the protective role of helminth infections. In Brisbane, Australia, a trial of hookworm has been completed in coeliac disease (see http://clinicaltrials.gov/show/nct00671138). Dose-ranging studies using hookworm (*Necator americanus*) and a preliminary clinical trial in asthma have been completed. Further trials are in progress in allergic disorders and in MS, both using *Trichuris suis* and hookworm (see Chapter 24).

23.8 Animal models of helminth infection used to test the Hygiene Hypothesis

The potential protective role of helminths in all of these types of disease is supported by numerous rodent models. Some of these are listed and referenced in Table 23.1. It should be noted that protective effects have been recorded in models of all the major types of chronic inflammatory disorder. Moreover, this is seen, whether the helminth is a natural infection of the rodent or a biologically unnatural infection with a human parasite. Importantly, the protective effect of helminths in general, or even of particular species such as *Heligmosomoides polygyrus*, is seen whether the chronic inflammatory disorder is mediated by Th1/Th17 cells (autoimmunity) or by Th2 cells (allergy).

23.9 Non-helminthic 'Old Friends'

Clearly, the helminths are not the only 'Old Friends' that can protect against chronic inflammatory disorders, and other organisms implicated are reviewed

Table 23.1 Animal models used to study the protective role of helminth infection against allergic and autoimmune inflammatory conditions.

Type of animal model*	Helminth
Allergy	*Heligmosomoides polygyrus* *Schistosoma mansoni* *Strongyloides stercoralis*
Autoimmunity: Type 1 diabetes	*Schistosoma mansoni* *Trichinella spiralis* *Heligmosomoides polygyrus*
Experimental autoimmune encephalomyelitis (EAE)	*Schistosoma mansoni* *Schistosoma japonicum* *Trichinella spiralis* *Fasciola hepatica*
Colitis	*Heligmosomoides polygyrus* *Schistosoma mansoni* *Hymenolepis diminuta*
Arthritis	*Schistosoma japonicum* *Schistosoma mansoni* *Hymenolepis diminuta*

*Reviewed and referenced by Osada & Kanazawa (2010).

elsewhere. The gut microbiota potently modulates systemic immune responses, and 'pseudocommensals' that enter regularly into the gut turn out to have immunoregulatory roles. The composition of the microbiota is changed by diet, hygiene and antibiotics. The microbiota of city-dwelling European children differs dramatically from that of rural Africans.

Similarly, the flora of the skin and milk are likely to be important, but are now often absent. The load of ectoparasites (fleas, lice, etc.) has also fallen from abundant to zero, and there is evidence that they modulate the immune system. Finally, a range of orofaecally transmitted organisms has been highlighted in recent studies of the hygiene hypothesis: *H. pylori, Salmonella*, Hepatitis A virus (HAV), enteroviruses and *Toxoplasma gondii*.

23.10 Mechanisms of immunoregulation

Numerous mechanisms of immunoregulation exist, and molecular mechanisms are discussed in Chapter 1. The underlying evolutionary principle is that the host-parasite relationship evolved so that an anti-inflammatory equilibrium is established, rather than provoking needless damaging aggressive immune responses. Some mechanisms are summarised in Figure 23.3.

Frequently there is modulation of dendritic cells (DCs), such that these drive Treg rather than Th1, Th17 or Th2 effector cells. A striking example of this in human autoimmunity is a recent experiment of nature. Patients in Argentina suffering from multiple sclerosis were followed up for 4–6 years. It was found that those who developed parasite infections (which were not treated) had

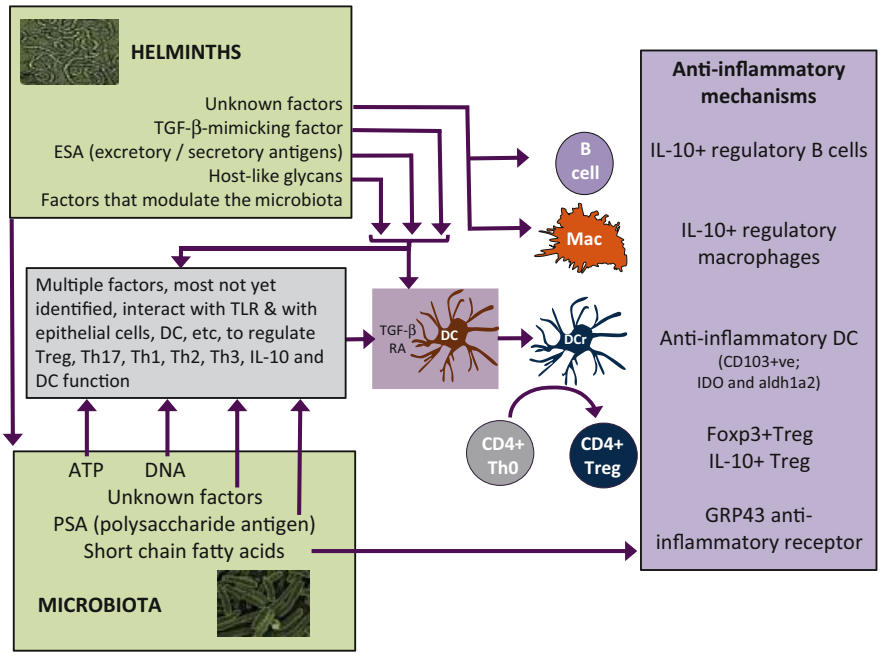

Figure 23.3 Some mechanisms involved in the immunoregulatory properties of the 'Old Friends' and microbiota. A key pathway is the modification of DCs so that they tend to drive Treg, mediated in part by retinoic acid and TGF-β derived from appropriately triggered epithelial cells. Such DCs also process self-antigens, allergens, etc., and so drive crucial specific regulatory cell populations. The intestinal helminths also exert indirect effects by modulating the microbiota. Abbreviations: ESA, excretory-secretory antigen of *Heligmosomoides polygyrus*; PSA, polysaccharide antigen of *Bacteroides fragilis*; SCFA, short chain fatty acids; RA, retinoic acid; DC, dendritic cell; DCr, regulatory dendritic cell; Mac, macrophage; GRP43, G-protein coupled receptor 43; Aldh1a2, retinaldehyde dehydrogenase 2; CD103, an integrin and marker of intestinal regulatory DC; IDO, indoleamine 2,3 dioxygenase).

significantly fewer exacerbations than those who did not. Moreover, they also developed regulatory lymphocytes that specifically responded to a peptide from myelin basic protein by releasing the anti-inflammatory cytokines, IL-10 and TGF-β. In other words, the presence of the parasite appeared to drive the development of regulatory cells that recognised the autoantigen and inhibited the autoimmune disease process. The parasites acted as 'Treg adjuvants'.

23.11 Conclusions

It would be foolish to assume that decreased exposure to microbial 'Old Friends' is the only reason for the increasing frequency of chronic inflammatory disorders in developed countries. Obesity is associated with altered gut microbiota and excessive release of pro-inflammatory cytokines. Stress also alters gut microbiota and drives corticotropin-releasing hormone (CRH), which increases permeability of the gut mucosa. Increased absorption of LPS and other microbial components drives further release of proinflammatory cytokines.

Lack of vitamin D exacerbates immunodysregulation, as does the triggering of Th17 cells by dioxins.

Meanwhile, the changes in the gut are also likely to impact on Th17 development. Viruses that used to be encountered harmlessly in early infancy (under cover, perhaps, of maternal antibody) can trigger autoimmunity if encountered for the first time later in life. Raised levels of proinflammatory cytokines trigger depression in some individuals, and this feeds back into the CRH/gut circuits.

Nevertheless, the loss of helminths is probably a significant factor, and the clinical trials discussed above should lead eventually to the discovery of the relevant immunomodulatory molecules and pathways, so that novel prophylactic and therapeutic drugs can be developed.

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24 Nematodes as Therapeutic Organisms

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24.1 Evidence that parasitic nematodes can protect humans from allergy and autoimmunity

An impressive feature of certain nematode species that parasitise humans is their longevity. For example, *Wuchereria bancrofti,* a causative agent of lymphatic filariasis, can live in excess of a decade. As humans are capable of mounting a vigorous immune response to nematode parasites, it is accepted that one major factor contributing to long-term infection is interference with this immune response. In relation to this, analysis of immune responses detectable against nematode antigens during human lymphatic filarial nematode infection indicates an impairment of the ability of lymphocytes to proliferate, with an associated increase in the production of the Th2-associated cytokine IL-4, the anti-inflammatory cytokine IL-10 and the anti-inflammatory antibody type IgG_4. Indeed, it is now accepted that a Th2-like, somewhat anti-inflammatory, immunological phenotype is a feature of all nematode infections.

An interesting characteristic of this nematode-induced phenotype is that it has been reported to extend to subsequent responses to non-nematode antigens, for example to antigens in vaccines targeting bacterial infections that normally rely on Th1 responses for efficacy. This has prompted the question as to

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whether the existence of this nematode-driven change in immunological phenotype from Th1 to Th2 could lead to reductions in susceptibility to diseases associated with aberrant Th1-associated inflammatory responses, such as the autoimmune diseases. In relation to this, there is currently great interest in the idea that the recent alarming increase in autoimmune diseases in the industrialised world, but not the Third, World, reflects increased hygiene, resulting in a reduction in infection with pathogens such as parasitic worms (the 'Hygiene Hypothesis' – see Chapter 23).

This chapter will consider whether nematodes are truly protective against development of autoimmunity by examining results obtained from both human and animal studies. Furthermore, there is evidence for an association between parasitic nematode infection and reduced allergy in addition to autoimmunity. This is, perhaps, initially surprising, because allergic disease is normally characterised as having the same immunological polarity as nematode infection, i.e. Th2. However, protection against allergy can be correlated with the ability of nematodes to induce anti-inflammatory responses such as IL-10. As is becoming increasingly evident, this ties in with the generation of various regulatory cell types such as regulatory T cells (Tregs) and alternatively activated macrophages that appear to be features of nematode infection.

24.1.1 Evidence from human studies

Although there are cases in which no association (or indeed increased susceptibility) has been observed, multiple studies support the idea that nematode infection can protect against the development of allergy, as measured by factors such as skin reactivity to allergens or wheezing and also, more consistently, by comparing symptoms before and after treatment with anti-helmintics. The major findings from original papers are listed in Table 24.1. Autoimmune disorders have not been as well studied as the allergic diseases, but there is also some evidence supporting an inverse relationship between nematode infection and common autoimmune conditions in the developed world, such as inflammatory bowel disease (IBD), multiple sclerosis (MS), rheumatoid arthritis (RhA) and type 1 diabetes (T1D).

That protection against allergy/autoimmunity is not always observed in the human studies has taken scientists somewhat by surprise, perhaps because initial enthusiasm for the hypothesis has led to widespread acceptance that it is correct. Thus, it has been suggested that observed inconsistencies with this hypothesis may reflect differences in factors such as the species of helminth under study, the degree of exposure to infection, the clinical parameter being measured and genetic factors of the human population.

With respect to asthma, most recent reviews appear to indicate that helminths are not protective in general, although there may be certain exceptions to this, such as the hookworm. However, studies can be found that clearly indicate that parasitic nematodes can offer protection against allergy/autoimmunity in humans in some situations.

Table 24.1 Studies suggesting that nematode infection may protect humans from the development of allergy.

Nematode	Finding	Reference
Various gastrointestinal nematodes	Reduced skin reactivity to house dust mite allergen with infection.	Hagel, I *et al*. (1993). Allergic reactivity of children of different socioeconomic levels in tropical populations. *International Archives of Allergy and Immunology* 101, 209–214.
Various gastrointestinal nematodes	Increased skin reactivity to house dust mite allergen and serum IgE against environmental antigens following anthelmintic treatment. Decreased reactivity with increased worm infection.	Lynch, NR *et al*. (1993). Effect of anthelmintic treatment on the allergic reactivity of children in a tropical slum. *The Journal of Allergy and Clinical Immunology* 92, 404–411.
A. lumbricoides and *N. americanus*	Inverse correlation between incidence of asthma and infection.	Selassie, FG *et al*. (2000). Total and specific IgE (house dust mite and intestinal helminths) in asthmatics and controls from Gondar, Ethiopia. *Clinical and Experimental Allergy* 30, 356–358.
A. lumbricoides and hookworms	Inverse association between skin-test reactivity to various allergens and infection.	Nyan, OA *et al*. (2001). Atopy, intestinal helminth infection and total serum IgE in rural and urban adult Gambian communities. *Clinical and Experimental Allergy* 31, 1672–1678.
Hookworm	Inverse association between recent wheeze and infection.	Scrivener, S *et al*. (2001). Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. *Lancet* 358, 1493–1499.
A. lumbricoides and hookworms	Reduced skin-test reactivity to various allergens with greater worm burden.	Cooper, PJ *et al*. (2003). Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. *The Journal of Allergy and Clinical Immunology* 111, 995–1000.
A. lumbricoides	Inverse association between recent wheeze and infection.	Dagoye, D *et al*. (2003). Wheezing, allergy, and parasite infection in children in urban and rural Ethiopia. *American Journal of Respiratory and Critical Care Medicine* 167, 1369–1373.
A. lumbricoides and *T. trichiura*	Increased skin reactivity to house dust mites with anthelmintic treatment.	van den Biggelaar, AH *et al*. (2004). Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. *The Journal of Infectious Diseases* 189, 892–900.
A. lumbricoides and hookworms	Inverse correlation between infection and skin sensitisation to house dust mites.	Flohr, C *et al*. (2006). Poor sanitation and helminth infection protect against skin sensitisation in Vietnamese children: A cross-sectional study. *The Journal of Allergy and Clinical Immunology* 118, 1305–1311.

Species: *A. lumbricoides, Ascaris lumbricoides; N. americanus, Necatur americanus; T. trichiura, trichuris trichiura.*

24.1.2 Evidence from animal studies

It must be stated at the outset that using model systems to examine whether nematodes are likely to offer humans protection against disease has the restriction of variation in exposure pattern (models usually employ a small number of exposures to a large number of nematodes, whereas humans will

generally become parasitised by nematodes, one or a few at a time, over a period of years).

Accepting this caveat, however, there has been a number of recent studies using mouse models of disease that clearly illustrate that nematodes can protect against allergic disease, in particular lung-associated disorders (Table 24.2). Indeed, the data obtained from these studies are more compelling than that from the human studies and are often used to advertise the protective power of nematodes against development of allergy. This type of approach has also provided convincing evidence for the ability of nematode infections to protect against the autoimmune diseases, arthritis, colitis, diabetes and MS (the animal model experimental autoimmune encephalomyelitis (EAA)). Overall, the employment of animal models of disease has produced a series of very persuasive findings supporting the hypothesis that nematodes may protect humans from allergy and autoimmunity.

24.2 Mechanism of action

Nematode infections are invariably chronic and are characterised by the induction of a Th2 immune response, as well as regulatory responses mediated by Tregs. It is convenient simply to view these two types of immune response in terms of cytokines generally associated with them, e.g. IL-4, IL-5 and IL-13 for Th2 responses and IL-10 and TGF-β for regulatory responses. A summary of the various immunomodulatory strategies associated with nematodes that may result in protection against allergy and/or autoimmunity is shown in Figure 24.1.

24.2.1 T cells: Th2 polarisation

Although the host may have evolved regulatory responses to deal with any persistent significant antigen challenge (regardless of source), it is clear that nematodes are particularly adept at polarising immune responses towards Th2. Their effects on dendritic cells (DCs), the sentinels of the immune system that sample and process foreign molecules for presentation to T cells, is particularly pertinent in this regard.

The induction of Th2 responses presumably reflects particular characteristics of nematode molecules. This is not necessarily advantageous to the worms, as there is evidence from rodent models of gastrointestinal (GI) nematode infection, as well as some epidemiological studies, that Th2 immune responses can lead to their elimination. Moreover, Th2 responses can be associated with inflammatory pathology during GI nematode infection and, therefore, it may be the case that induction of Th2 responses is a compromise that nematodes have had to accept, because the molecules involved possess functions that are important for worm survival.

However, it is possible to perceive that the induction of an immunological environment biased towards Th2 may provide a situation in which Th1-dependent autoimmune diseases would find it difficult to flourish. In IL-10-/- mice which

Table 24.2 Studies indicating that nematode infection can protect mice from allergic and autoimmune conditions.

Nematode species	Observation	Reference
Allergic disease		
S. stercoralis	Suppression of allergic response to ovalbumin in the lungs	Wang, CC *et al*. (2001). Infection of mice with the helminth *Strongyloides stercoralis* suppresses pulmonary allergic responses to ovalbumin. *Clinical and Experimental Allergy* 31, 495–503.
H. polygyrus	Inhibition of allergic responses to peanut extract.	Bashir, ME *et al*. (2002). An enteric helminth infection protects against an allergic response to dietary antigen. *Journal of Immunology* 169, 3284–3292.
N. brasiliensis	Inhibition of ovalbumin-induced airway eosinophilia and eotaxin levels in the lungs.	Wohlleben, G *et al*. (2004). Helminth infection modulates the development of allergen-induced airway inflammation. *International Immunology* 16, 585–596.
H. polygyrus	Inhibition of airway hyper-responsiveness to ovalbumin and Der p 1.	Wilson, MS *et al*. (2005). Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *The Journal of Experimental Medicine* 202, 1199–1212.
H. polygyrus	Suppression of ovalbumin-induced airway eosinophilia and bronchial hyper-reactivity.	Kitagaki, K *et al*. (2006). Intestinal helminths protect in a murine model of asthma. *Journal of Immunology* 177, 1628–1635.
Autoimmune disease		
H. polygyrus and *T. muris*	Inhibition of colitis development in IL-10-/- mice.	Elliott, DE *et al*. (2000). Does the failure to acquire helminthic parasites predispose to Crohn's disease? *The FASEB Journal* 14, 1848–1855.
T. spiralis	Reduced colitis induced by DNBS.	Khan, WI *et al*. (2002). Intestinal nematode infection ameliorates experimental colitis in mice. *Infection and Immunity* 70, 5931–5937.
H. polygyrus	Protection from colitis in piroxicam-treated IL-10-/- mice.	Elliott, DE *et al*. (2004). *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. *European Journal of Immunology* 34, 2690–2698.
H. polygyrus and *T. spiralis*	Protection against diabetes in the NOD mouse.	Saunders, KA *et al*. (2007). Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. *Infection and Immunity* 75, 397–407.
T. spiralis	Protection against experimental autoimmune encephalomyelitis in DA rats.	Gruden-Movsesijan, A *et al*. (2008). *Trichinella spiralis*: modulation of experimental autoimmune encephalomyelitis in DA rats. *Experimental Parasitology* 118, 641–647.
T. psuedospiralis	Protection against experimental autoimmune encephalomyelitis in mice.	Wu, Z *et al*. (2010). Infection of non-encapsulated species of *Trichinella* ameliorates experimental autoimmune encephalomyelitis involving suppression of Th17 and Th1 response. *Parasitology Research* 107, 1173–1188.
L. sigmodontis	Protection against diabetes in the NOD mouse.	Hubner, MP *et al*. (2009). Inhibition of type 1 diabetes in filaria-infected non-obese diabetic mice is associated with a T helper type 2 shift and induction of FoxP3+ regulatory T cells. *Immunology* 127, 512–522.

Species: *A. lumbricoides, Ascaris lumbricoides; H. polygyrus, Heligosomoides polygyrus; L. sigmodontis, Litomosoides sigmodontis; N. brasiliensis, Nippostrongylus brasiliensis; S. stercoralis, Strongyloides stercoralis; T. muris, Trichuris muris; T. pseudospiralis, Trichinella pseudospiralis; T. spiralis, Trichinella spiralis; T. trichiura, Trichuris trichiura.*

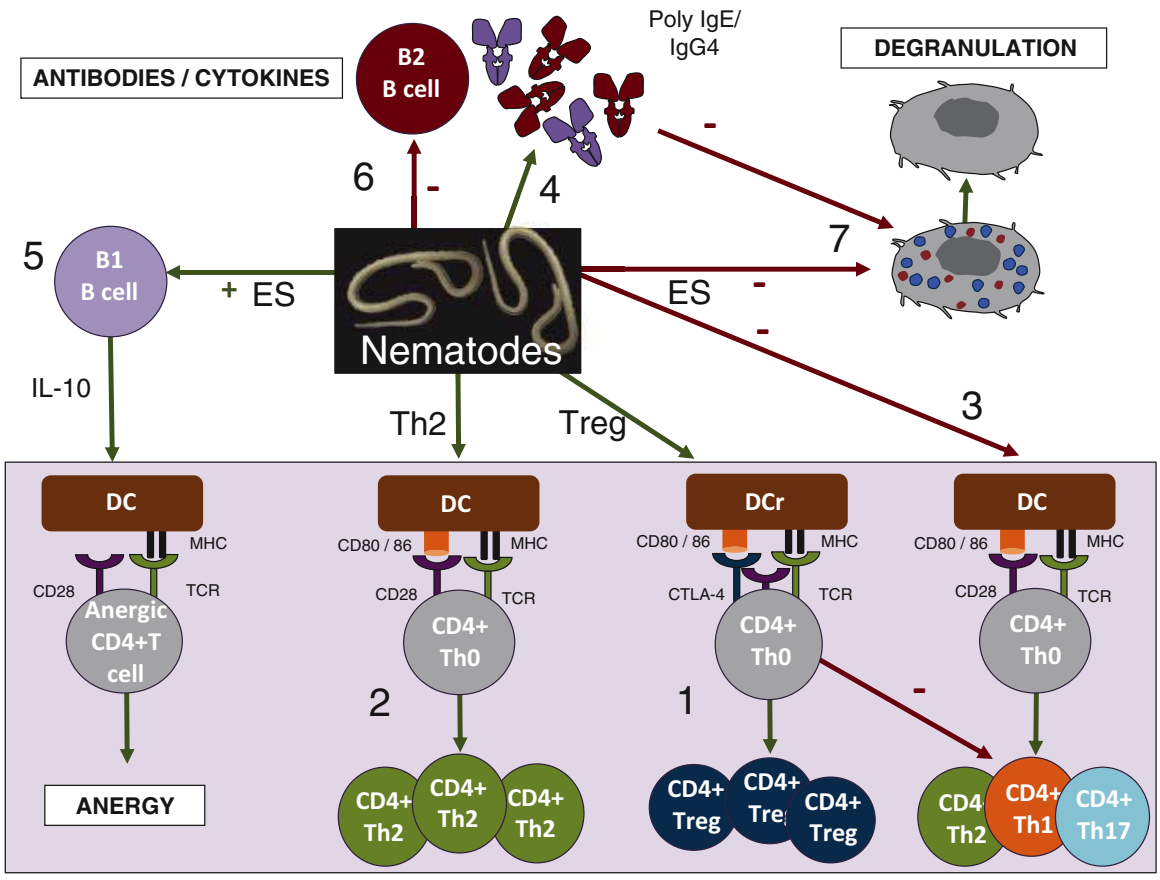

Figure 24.1 Potential Mechanisms of nematode-induced immunomodulation and suppression of inflammatory disease. Infection with nematodes can induce T cell hypo-responsiveness by directly or indirectly (through antigen-presenting cells such as DCs) suppressing T cell activation. For example, infection can result in the induction of Treg cells (1) that are refractory to antigen and can prevent maximal T effector responses of either Th1-, Th2- or Th17-based inflammation. Alternatively, the worms, or their ES products, can modulate DC maturation to polarise T cell responses towards a Th2 phenotype (2) and, hence, counteract Th1-based inflammation, or they can act on DCs to block Th1, Th2 or Th17 differentiation (3). In contrast, it has been proposed that nematodes can antagonise Th2-driven inflammation by producing polyspecific IgE/IgG4 (4) to block allergen-induced mast cell degranulation. Moreover, certain ES products have been shown to directly induce mast cell hyporesponsiveness by subverting FceRI signalling (see Figure 24.2). Similarly, worm products have been shown to directly modulate B1 (5) and B2 (6) responses. (7) Green arrows represent positive induction; red arrows represent inhibition.
Abbreviations: DC, dendritic cell; ES, excretory-secretory; Ig, immunoglobulin; IL, interleukin; MHC, major histocompatibility complex; TCR, T cell receptor; Th, T helper cell; Treg, T regulatory cell.

have colitis, the decrease in inflammation observed upon infection with *Heligmosomoides polygyrus* (an animal model of GI nematode infection) correlates with inhibition of the Th1 cytokines IFN-γ and IL-12 in the mucosa and a concomitant increase in the Th2 cytokine IL-13.

24.2.2 T cells: the role of Th17 cells

Recently it has been discovered that several inflammatory diseases, including colitis, once considered to be strictly Th1 or Th2, are in fact associated with

Th17 immune responses. Colonisation of colitis-suffering IL10-/- mice with *H. polygyrus* inhibits IL-17 production and a decrease in Th17 cells suggesting that the suppression of IL-17 release might be an alternative or additional mechanism to Th2-mediated down-regulation of Th1 responses that occur during protection of nematode-colonised mice from inflammation in colitis. However, the suppressive effect on the IL-17/Th-17 axis was investigated in normal mice, and it appears to reflect Th2 polarisation, because it is dependent on IL-4 – although, in addition, the anti-inflammatory cytokine IL-10 also plays a role.

24.2.3 T cells: T regulatory cell responses

Regulatory responses could be generated in response to any chronic inflammatory insult in an attempt by the body to exert some control. With respect to nematodes, recent evidence suggests that these responses may contribute to worm survival via inhibition of protective immunity. However, regulatory responses in nematode infections do not just inhibit inflammatory responses to nematodes but also to heterologous antigens. This means that responses to other infections and vaccines may be affected, and susceptibility to diseases associated with aberrant inflammatory responses may be altered (see Chapter 21).

The Th2 dominance associated with nematode infection provides an attractive argument to explain the ability of the helminths to inhibit Th1-mediated autoimmune diseases, but it obviously cannot explain protection against allergic diseases, which are, themselves, Th2-dependent. However, both Th1 and Th2 responses may be dampened down by anti-inflammatory regulatory responses, so the ability of nematodes to protect against allergy may be explained in this way. Consistent with this explanation, the capacity of *H. polygyrus* for blocking allergic airway hyper-responsiveness in the mouse has been shown to be dependent on CD4+CD25+ Tregs.

The nature of the *H. polygyrus*-generated regulatory response that protects against allergic airway hyper-responsiveness differs, depending on the background of the infected mice. C57BL/6 mice produce IL-10 in response to nematode infection, whereas BALB/c mice produce TGF-β. In keeping with this observation, *H. polygyrus* can still protect BALB/c mice deficient in IL-10 from allergic airway hyper-responsiveness, whereas there is no effect of this helminth infection on allergic airway hyper-responsiveness in IL-10 deficient C57BL/6 mice.

In the BALB/c strain of mice, Tregs and TGF-β have been found to mediate the ability of a rodent filarial nematode, *Litomosoides sigmodontis*, to inhibit airway hyper-responsiveness, but not the lung inflammatory response (e.g. cell infiltration) to the model antigen ovalbumin. These experiments suggest that different mechanisms may be required for targeting different components of the inflammatory response.

Tregs induced by *H. polygyrus* infection may also be playing a role in the down-regulation of inflammation in IL-10-/- mice suffering from colitis. The inhibition of Th1/Th17 cytokines in the context of increased Th2 cytokines

mentioned is associated with an expansion of Tregs in the mesenteric lymph nodes draining the GI tract, and such T cells from infected animals can transfer the protection against colitis.

24.2.4 B cells: A role of non-specific IgE and IgG_4 in allergy prevention?

The idea of regulatory responses preventing the development of allergy during nematode infection is clearly supported by experimental evidence, but there are other hypotheses to explain the inhibitory effects that have been observed. Nematodes have a tendency to induce considerable non-specific IgE secretion during infection and it has been suggested that these antibodies may block mast cell/basophil degranulation, in response to specific antigens, by saturating FcεRI sites. Certainly, an association between high-circulating non-specific IgE levels and protection against allergy has been shown in a number of studies, and increased cutaneous allergic responses following anthelmintic treatment are associated with a corresponding decrease in non-specific IgE.

However, there is also evidence which does not support this hypothesis. For example, *Nippostrongylus brasiliensis* infection (rodent hookworm infection) does not reduce mast cell degranulation in response to ovalbumin in the skin of immunised mice, despite the infection generating a massive increase in non-specific IgE production. Indeed, the generation of a small amount of IgE specific for the parasite antigen, novel *N. brasiliensis* antigen (*Nb*-Ag1), is able to facilitate mast cell degranulation in the presence of much greater levels of non-specific IgE.

Studies on samples obtained from humans infected with filarial nematodes indicate that the relative levels of polyclonal IgE to filaria-specific IgE do not usually reach that required for blockage of filaria-induced mast cell or basophil degranulation. Thus, overall, it appears that saturation of FcεRI by poly-specific IgE antibodies during nematode infection cannot, alone, explain protection from allergy.

Nematode infections can also be associated with the production of high levels of IgG4, an antibody that competes with IgE for allergen binding but does not promote mast cell or basophil degranulation. However, studies investigating whether high levels of competing IgG4 can protect against allergic responses in humans have produced conflicting results.

In conclusion, there is little current support for polyclonal IgE/IgG4 playing a role in protection against FcεRI-mediated allergy.

24.3 Nematode molecules involved in preventing allergic/autoimmune disease

Clearly, a number of different nematode species can prevent the development of a number of different autoimmune/allergic conditions in animal models. In some cases, a protective effect can be achieved by the administration of

parasite extracts and active infections are not required. For example, an extract containing the excreted/secreted products (ES) of adult stage *N. brasiliensis* can prevent the development of asthma (ovalbumin hypersensitivity model) in the lungs of mice, in a similar manner to infection with the parasite itself.

An extract of *Ascaris suum* induced a protective/anti-inflammatory effect in two different experimental animal models of arthritis – the collagen-induced arthritis model (CIA) and zymosan-induced arthritis (ZYA). The extract demonstrated clinical benefits when given prophylactically as well as therapeutically, but the active ingredient(s) of this extract are undefined and the logical next step is to define the specific molecules involved in ameliorating inflammation. This section discusses some of the nematode-derived anti-inflammatory molecules that have been characterised to date. A summary of the various immunomodulatory properties associated with nematode products that may result in protection against allergy and/or autoimmunity is shown in Figure 24.1.

24.3.1 *Ascaris suum* PAS-1

An *A. suum* extract was reported in 2002 by Lima and colleagues as inhibiting lung inflammation and hyper-responsiveness, and as suppressing IL-4, IL-5 and eotaxin production in response to ovalbumin. More recently, it was discovered that *A. suum* contains a protein – APAS-3 – that, upon immunisation and challenge, is able to induce Th2-dependent, eosinophilic airway hyperreactivity in BALB/c mice. Interestingly, it was also found that another *A. suum* product – PAS-1 – is able to block this response. Inhibition is associated with reduced secretion of chemokines (eotaxin, RANTES), Th2 cytokines (IL-4, IL-5) and the Th2 antibody isotypes IgG_1 and IgE, as well as an elevated level of IL-10 in bronchoalveloar lavage fluid. PAS-1 induces IL-10 and TGF-β production in macrophages, and this correlates with loss of pro-inflammatory cytokine production. This product is most effective when administered prophylactically at both immunisation and challenge stages.

24.3.2 *Dirofilaria immitis*-derived antigen (DiAg)

A purified 15 kD protein derived from the canine filarial nematode, *Dirofilaria immitis*, termed DiAg (*Dirofilaria immitis*-derived antigen), is known to cause polyclonal proliferation of B cells and the production of non-specific IgE. Injection of DiAg into the non-obese diabetic (NOD) strain of mouse prevents spontaneous generation of IgG anti-insulin antibodies and the associated development of Th1-dependent autoimmune diabetes. This effect may be partly mediated by the appearance of the cytokines IL-4 and IL-10, shown to be induced by DiAg upon injection into BALB/c mice.

DiAg can also inhibit passive cutaneous anaphylaxis (PCA) reactions in the rat. Interestingly, unlike the human situation mentioned earlier, this effect appears to be due to non-specific saturation of FcεRI rather than an effect on the number or viability of mast cells, or to differences in the amount of histamine released.

24.3.3 Cystatin

Another filarial nematode-derived molecule, the well-characterised protease inhibitor cystatin, can protect against ovalbumin-induced airway hyperreactivity by reducing eosinophilia, IgE and IL-4. The effect of this molecule is dependent on the actions of IL-10 for some anti-inflammatory activities, although the source of this IL-10 appears to be macrophages rather than Tregs. Indeed, cystatin was originally described as inhibiting T cell responses via its effects on macrophages. Filarial cystatin also protects against colitis that occurs in response to dextran sodium sulphate (DSS) in a mouse model.

24.3.4 Galectin-9 homologue of *Toxascaris leonina*

This molecule shows 35 per cent homology with human galectin-9 and, in recombinant form, it can reduce DSS-induced intestinal inflammation in mice. The reduction in disease correlates with increases in both IL-10 and TGF-β, raising the possibility that these anti-inflammatory cytokines could be responsible for amelioration of inflammation. It has been speculated that this nematode molecule may function like a host galectin in playing a role in regulation of immune responses, although the carbohydrate binding capacity of Galectin-9 homologue of *Toxascaris leonina* was found to be less than that of rat galectin.

24.3.5 ES-62

Excretory/secretory molecule of 62kDa (ES-62) is a phosphorylcholine (PC)-containing glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae*, which has been shown to have a wide range of immunomodulatory and anti-inflammatory properties. As a consequence of this, the molecule has been tested in mouse models of autoimmune and allergic disease and has been found to be active against CIA and type-I hypersensitivity in the skin and lungs.

With respect to CIA, ES-62 is effective both as a prophylactic and therapeutic treatment and, in both cases, the protective effects are associated with inhibition of collagen-specific pro-inflammatory (TNF-α, IL-6) and Th1 (IFN-γ) cytokines, as well as Th1 (IgG2a) antibody production. Prophylactic effects are also associated with an enhanced production of collagen-specific IL-10. A comparison amongst ES-62, PC-free ES-62 and PC, conjugated to an irrelevant protein (ovalbumin), has revealed that, in the CIA model, the anti-arthritis effect of ES-62 is dependent on its PC moiety. The mechanism by which the PC moiety of ES-62 exerts this protective effect is unknown but it is, perhaps surprisingly, not dependent on a reduction in collagen-specific IgG2a production.

ES-62 has also been found to have the ability to suppress lipopolysacharide (LPS) pro-inflammatory cytokine production by cells obtained from human synovial fluid and membranes. Human cells are also susceptible to the anti-inflammatory action of PC-ovalbumin, suggesting that anti-inflammatory effects of ES-62 on human cells are mediated by the PC moiety.

In mouse models, ES-62 has been used successfully to prevent allergic responses in both ovalbumin-induced airway hypersensitivity and immediate-type hypersensitivity to oxazolone in the skin. In the former, ES-62 can reduce peri-bronchial inflammation and mucosal hyperplasia, inhibit eosinophilia and prevent release of IL-4, the signature cytokine required for the development of airway inflammation. In the latter model, ES-62 targeted oxazolone-induced inflammation and reduced ear swelling.

This was correlated with effects of ES-62 on mast cells, in particular the prevention of mast cell degranulation and release of mediators of allergy in response to ligation of FcεRI. This is thought to occur via the inhibition of key signal transduction events involved in mast cell activation, including phospholipase D-coupled, sphingosine kinase-mediated calcium mobilisation and

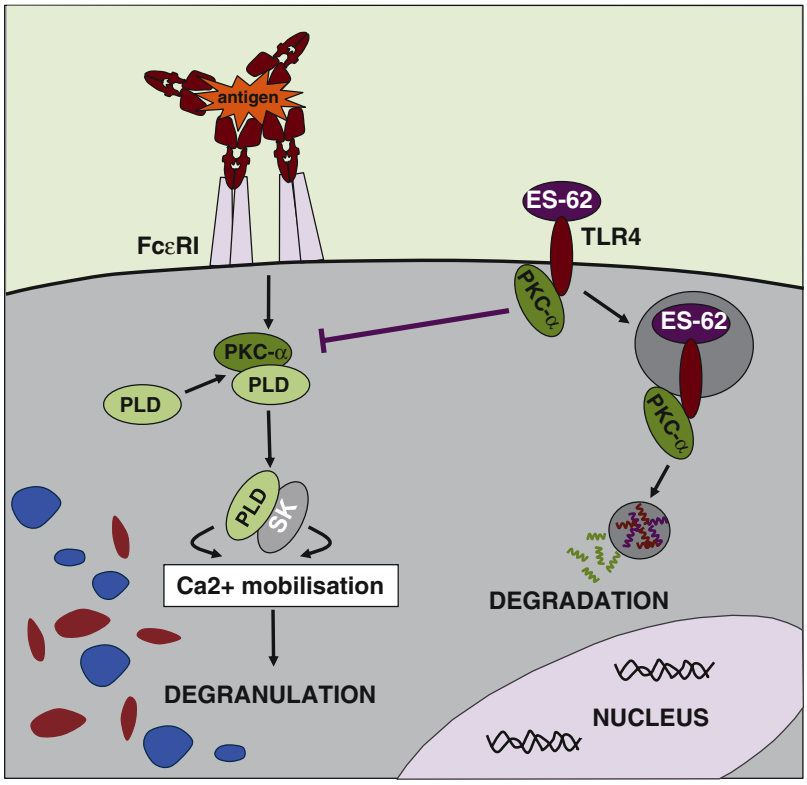

Figure 24.2 ***Acanthocheilonema viteae*-derived ES-62 induces hypo-responsiveness of mast cells by disrupting FcεRI signalling.** Ag-driven cross-linked ligation of IgE bound to FcεRI on the surface of mast cells induces degranulation and generation of pro-inflammatory mediators and cytokines. This is due to triggering of a signalling cascade involving PKC-α complexing with PLD to activate the latter, and then PLD-coupled, SK-mediated, calcium mobilisation. ES-62 directly blocks mast cell activation by subverting TLR4 signalling, such that it results in sequestration and trafficking of PKC-α for perinuclear degradation by a caveolae/lipid raft, proteosomal-independent mechanism. Such sequestration and degradation of PKC-α uncouples FcεRI-mediated mast cell activation and, hence, inhibits allergic responses. Abbreviations: Ag, antigen; ES, excretory-secretory; Ig, immunoglobulin; PLD, phospholipase D; PKC, protein kinase C; SK, sphingosine kinase; TLR, Toll-like receptor.

NF-κB activation (Figure 24.2). ES-62 mediates these effects by forming a complex with Toll-like receptor (TLR) 4, which results in the sequestration and perinuclear degradation of protein kinase C (PKC)-α, a molecule found to be critical for mast cell activation.

Since ES-62 can inhibit components of a Th2 response such as IL-4 production, ES-62 cannot be simply a nematode-derived Th-2 polarising agent which can inhibit Th1-mediated inflammation. It is possible that ES-62 may exert effects via modulation of cytokines such as IL-17, a regulator of both Th1- and Th2-type inflammation, which has been shown to play a critical role in CIA and to be inhibited by *H. polygyrus* infection. Indeed, CIA is a model of inflammation in which ES-62 was found to be protective, and the lack of correlation between effects of ES-62 on pathology and collagen-specific IgG2a antibody levels could now be re-evaluated in the context of IL-17 regulation.

Currently, work is under way to develop molecules that mimic the activity of ES-62 for employment as drugs for treating allergic and autoimmune diseases. Unlike the parent ES-62 molecule, these will be too small to be immunogenic. Given its importance in promoting ES-62 activity, the new molecules will be based around PC and will contain slight structural modifications to avoid interaction with natural anti-PC antibodies.

24.4 Clinical aspects

A large body of evidence now supports the premise that parasitic nematodes can prevent allergy and autoimmunity. Not surprisingly, this has led scientists to consider their therapeutic potential. In recent years, parasitic nematodes have been employed in clinical trials aimed at ameliorating inflammatory diseases, and it has been possible to carry out these trials due to the known or predicted lack of pathogenicity of certain species.

An example of a 'safe' species appears to be *Trichuris suis:* it is similar to the human whipworm *Trichuris trichiura* (see Chapter 14) but, following infection, it can persist only for a limited period in the human GI tract. *T. suis* has been selected for testing in human trials as a treatment for inflammatory bowel disease and, encouragingly, in a series of trials involving administration of embryonated ova, many people suffering from inflammatory bowel disease have shown improvement in symptoms. For example, in a trial of patients receiving 2,500 *T. suis* ova every two weeks for 12 weeks 43.3 per cent reported improved symptoms, compared to 16.7 per cent who were given a placebo.

The hookworm *N. americanus* has also been employed in trials. However, as this human pathogen has pathogenic potential (see Chapter 13), low numbers must be used to treat people. When infecting people suffering from Crohn's disease with 50 *N. americanus* larvae, people with moderately active (but not inactive or mild) disease showed some improvement. A trial determining the effect of hookworms on asthma has also recently been undertaken but the results do not support the existence of a protective effect.

The ultimate aim in this area of research is to employ defined nematode molecules (or derivatives thereof) in treating human disease. As a proof of concept, one need look no further than the recombinant anticoagulant protein c2 (rNAPc2), a serine protease inhibitor derived from protein secretions of the hookworm *Ancylostoma caninum*. The protein's anticoagulant activity is due to inhibition of the catalytic complex of subendothelial tissue factor/activated factor VII by a unique mechanism. This molecule has been found to inhibit thrombin generation in patients undergoing elective percutaneous coronary intervention. It can also reduce the likelihood of deep venous thrombosis in people being subjected to total knee arthroplasty and block new thrombin generation and ischemia in patients with non-ST-segment elevation acute coronary syndrome. It is hoped that nematode-derived molecules and/or their derivatives will soon be also employed in the treatment of human allergic and autoimmune diseases in a similar manner.

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Vaccination Against Malaria 25.1

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The immune response to malaria parasites is extremely complex, and non-immune individuals are highly susceptible to infection and have a high risk of developing severe malaria. The susceptibility to such clinical complications decreases after a person experiences several infections, but infected patients still display classical malaria symptoms that include fevers, chills and rigors, even with lower parasite burdens. The development of resistance to these symptoms requires frequent exposure to the parasite, suggesting that the immunological memory response to certain key disease causing parasite factors is short-lived. Although it is well known that immunity can be acquired by natural exposure to malaria parasites, providing the rationale for malaria vaccine development, the mechanisms involved in protection and pathogenesis are poorly understood.

25.1.1 Malaria vaccines: proof of concept

The first attempt to develop a malaria vaccine based on Pasteur's principles of microbe attenuation was tested in the early twentieth century using *Plasmodium relictum*-attenuated sporozoites in canaries. The protective effect using such an approach was confirmed in chickens using UV-inactivated *P. gallinaceum* sporozoites. For the first time, malaria vaccine efficacy was correlated with the induction of antibodies with the ability to agglutinate infective sporozoites. The model also defined the stage specificity of the protective immunity, as immune chickens were protected against experimental challenge with sporozoites, but not against blood-stage parasite challenge (Chapter 3).

The methodology of sporozoite inactivation was optimized using X-irradiation and the rodent malaria parasite *P. berghei*. This experimental model led to the identification of a protective effector mechanism mediated by antibodies. In essence, the antibody-mediated circumsporozoite protein (CSP) precipitation reaction was identified by incubation of viable sporozoites with sera from

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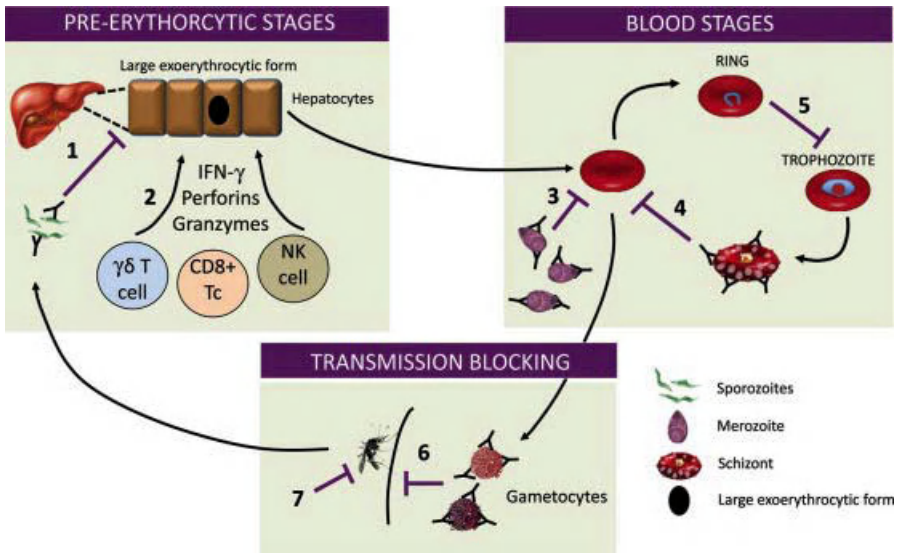

Figure 25.1.1 Vaccine targets in the malaria life cycle. Pre-existing neutralising antibodies generated by a vaccine targeted against the sporozoite stage can prevent the initiation of infection by sporozoites entering the body from the bite of a mosquito and preventing migration and/or hepatocyte invasion (1). Pre-erythrocytic malaria vaccines should ideally elicit a cellular response that can inhibit parasite development in the liver (2). Antibody responses could also be generated against blood-stages to block merozoite invasion (3) or to recognise parasite-derived erythrocytic surface antigens to modulate antibody-dependent cellular inhibition (ADCI) or complement lysis to halt any further replication cycles of the parasite (4). Erythrocytic vaccines have also been aimed to induce cellular immune responses to inhibit intra-erythrocytic parasite development (5). Lastly, vaccine aimed at preventing the successful transmission of gametocytes to mosquitoes (6), or the development of gametocyte stages once ingested (7), can reduce the pool of infected mosquitoes in the environment, in turn reducing the incidence of new infections.

protected animals (Figure 25.1.1). The fact that irradiated sporozoites could induce protection in humans was confirmed using infected mosquitoes as a delivery system. This methodology, though impractical for implementation in endemic areas, provided the proof of concept that a malaria vaccine is biologically feasible.

The first experimental evidence that an erythrocytic stage malaria vaccine is possible came in 1961, with passive immunisation experiments using gamma globulin fractions derived from adults living in malaria-endemic areas. The protein fraction containing anti-malarial antibodies was used as a therapeutic intervention measure in children infected with *P. falciparum*. Children treated with such protein fractions were able to control parasitaemia and malaria severity. These experiments showed that naturally acquired antibodies, when passively transferred into susceptible individuals, could protect against parasitaemia and clinical malaria.

Subsequent studies, using the simian malaria parasite *P. knowlesi* in rhesus monkeys and *in vitro* cultures, confirmed that the antibodies inhibited merozoite invasion and that the effect was mediated by IgG and IgM. These

findings led to successful vaccine trials involving experimental challenge of rhesus macaques, using partially purified or purified *P. knowlesi* merozoites emulsified in Freund's adjuvant, and in *Aotus* monkeys using merozoites derived from short-term *P. falciparum* cultures or parasites derived from experimentally-infected *Aotus* monkeys. The vaccine preparations tested in these early experiments were far from ideal and contained a heterologous combination of parasite and host proteins. Nevertheless, these seminal experiments also highlighted the importance of adjuvants in malaria vaccine formulations.

25.1.2 Vaccine development

The clinical pathway of testing malaria vaccine candidates can take over ten years and involves several different phases (Figure 25.1.2). Vaccine development efforts using modern technologies have been broadly focused on *P. falciparum*. However, clinical and epidemiological data indicate that *P. vivax* can also be associated with severe disease and mortality. Malaria vaccine development programmes should, therefore, also include *P. vivax*.

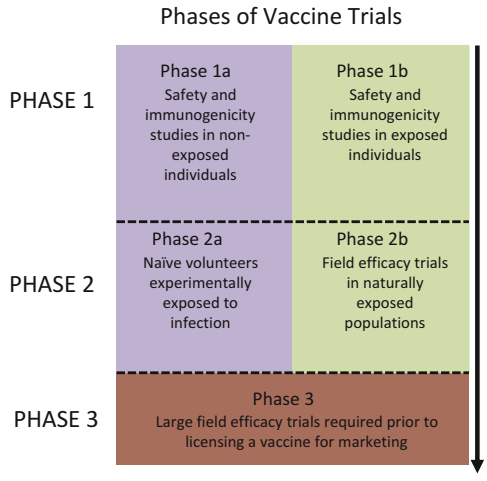

Figure 25.1.2 The path to vaccine licensing.

Vaccines targeted against the pre-erythrocytic stages of malaria are ideal for non-immune individuals who travel to endemic areas, such as tourists or military personnel, whereas vaccine-induced immune responses to target the erythrocytic cycle would be ideal to control the clinical burden of malaria by targeting the stage of the life cycle responsible for clinical complications. Alternatively, sexual stage vaccines are aimed to block transmission of the parasite to new hosts by targeting antigens expressed in the gametocyte stages or in the mosquito vector. The stages of the life cycle that could be targeted by vaccine-induced immune responses are detailed above in Figure 25.1.1.

Vaccine trials for individuals naturally exposed to *P. falciparum* have used two different endpoints – parasitological and clinical. Parasitological endpoints include infection rates, parasite density and multiplicity of infection. This is in contrast to clinical endpoints, which include rates of first episode of uncomplicated malaria, rates of all episodes of uncomplicated malaria, severe disease incidence, malaria-related admission to hospital and mortality.

Experimental evidence suggests that a malaria vaccine requires formulation with adjuvants to induce proper activation of dendritic cell subsets. It is therefore accepted that adjuvant selection is critical. However, formulation of vaccines with adjuvants is empirical, and the final potency is highly dependent on the antigen-adjuvant combination. The major obstacles against testing a variety of adjuvant formulations in clinical trials today surround intellectual property issues. It has, therefore, become a priority to develop partnerships between philanthropic and governmental agencies and pharmaceutical companies that can ensure the access of vaccine development teams to novel adjuvants that are freed of such concerns.

25.1.3 Pre-erythrocytic vaccines

25.1.3.1 RTS,S

The most advanced malaria vaccine candidate that has reached Phase 3 development status is based on the sporozoite surface protein CSP. Several CSP synthetic and recombinant vaccine constructs have been tested with limited results. However, a fusion protein developed by GlaxoSmithKline in collaboration with the Walter Reed Army Institute of Research has shown some efficacy in clinical trials. The vaccine (named RTS,S) includes the repeat region (R) and the complete carboxyl terminal region of the circumsporozoite protein, which contains several T cell epitopes (T), fused to the hepatitis B surface antigen (HBsAg) (S). When the fusion protein is mixed with additional hepatitis B surface subunits (S), the compound forms virus-like particles. To improve the cellular reactivity, this protein is formulated with an oil-in-water emulsion and a mixture of MPL (mycobacterial cell wall skeleton) and QS21 (saponin derivative from soap bark tree *Quillaja saponaria*) as an adjuvant (See Chapter 25.2 for more information on adjuvants).

The reported RTS,S vaccine efficacy to clinical episodes was 29–35 per cent, with 49–57 per cent efficacy against severe malaria. Clinical trials that included infants have indicated that the vaccine is safe and able to induce anti-CSP antibodies. However, Phase 2b trials indicated that high anti-CSP antibody titres did not predict greater protection against clinical malaria, suggesting a role of cellular immune response. A multi-centre Phase 3 trial that is underway will provide further information concerning immune correlates of vaccine-induced protection essential for further improvements of the vaccine.

The clinical development of RTS,S has confirmed the critical role of adjuvants in the formulation of subunit malaria vaccines. In fact, the initial trials of RTS,S, using alum or alum-MPL formulations, showed limited efficacy.

25.1.3.2 DNA vaccines

DNA vaccines were introduced in the field in 1993, with the purpose of combining several antigens in a single construct and to avoid the logistical constraints of expressing large amounts of recombinant protein immunogens. This vaccine platform, expressing CSP, has been tested in mice using the rodent malaria parasite *P. yoelii*, with encouraging results. The combination of CSP and the hepatocyte erythrocyte protein 17 (HEP17) in the vaccine formulation overcame the genetic restriction reported when the vaccines were tested individually.

In spite of the success in murine malaria models, the use of a DNA vaccine encoding the *P. falciparum* CSP antigen in humans showed induction of CD8+ T cells, but very poor antibody response and no protection against experimental challenge. The combination of five DNA plasmids expressing different *P. falciparum* antigens (CSP, sporozoite surface protein 2 (SSP2, also known as thrombospondin-related adhesive protein TRAP), exported protein 1 (EXP-1), liver-stage antigen (LSA)-1 and LSA-3 was safe and well-tolerated in mice and

rabbits. In clinical trials, the combination of DNA plasmids reproduced the induction of robust CTL and IFN-γ responses, but showed only weak antibody response with no evidence of protection.

25.1.3.3 Viral vector-based vaccines

The poor response obtained with DNA-based vaccines accelerated the implementation of recombinant viral vectors and the use of prime-boost heterologous immunisation strategies. Viral vectors are an optimal platform to deliver endogenous epitopes for CD8+ T cell-mediated immunity, and they have the advantage of exhibiting an intrinsic innate stimulatory effect that can be considered as a built-in adjuvant property. Viruses contain pathogen-associated molecular patterns (PAMPs) (double-strand RNA and viral DNA) that can engage the host's molecular pattern recognition receptors (PRRs), such as Toll-like receptor (TLR)3/ retinoic acid inducible gene-I (RIG)-I and TLR7/9 involved in the activation of the innate immune system. Inflammatory responses are mainly mediated by type I interferons support and enhance the adaptive immune response. Viral vectors have the additional advantage of a potential for simultaneously expressing several transgenes.

Existing viral vectors that have been modified to express malaria antigens include a variety of attenuated poxviruses, such as the modified vaccinia virus Ankara (MVA), and the fowlpox strain FP9 (Table 25.1.1). MVA was derived by passing the Ankara virus over 570 times in chicken embryo fibroblasts (CEF), resulting in a virus able to grow in CEF while demonstrating limited replication in humans cells. FP9, on the other hand, does not replicate in non-avian species. These viral vectors have been tested in humans to trial prime-boost immunisation regimens using homologous (the same viral vector used for priming and booster vaccinations) and heterologous (a different viral vector used for priming and booster vaccinations) viral vectors.

Adenovirus (Ad) vectors have been explored as an alternative viral vector to potentiate T cell priming. When used in heterologous immunisation regimens, these vectors have the advantage of inducing antibody responses and more robust CD8+ T cell responses, compared with poxvirus vectors. The vectors are also able to skew the immune response toward a Th1 phenotype. However, a major challenge to the use of recombinant adenoviruses is the potentially deleterious effect of pre-existing anti-vector immunity. This represents a significant impediment, because the seroprevalence of pre-existing Adenovirus serotype 5 (Ad5) immunity is around 30–50 per cent in the United States and Europe and as high as 95 per cent in sub-Saharan Africa and Asia. Ad-specific immune responses, with subsequent elimination of the vector, are likely to interfere with the development of a T cell response against the inserted antigen. Pre-existing immunity could also modify the outcome of Ad-vectored vaccines by participation of neutralising antibodies that may limit the efficiency of the transgene delivery.

In the search of strategies to overcome the anti-vector immunity when using Ad viral vectors, less prevalent human adenovirus vectors or simian adenovirus vectors have been characterised. These experiments have shown that

Table 25.1.1 Viral vector vaccines constructed to generate protective immune responses against malaria.

Vector	Malaria genes expressed	Results
Orthopoxvirus NYVAC	CSP TRAP LSA-1 MSP-1 SERA AMA-1 Pfs25	– Poor antibody responses with two different doses. – 90 per cent of volunteers with CTL responses to CSP, TRAP and LSA-1. – 1 out of 35 volunteers protected.
Poxviruses (various, including MVA and FP9)	Vaccine candidate: ME-TRAP A string of epitopes genetically fused to TRAP	– Induction of T cell immunity (in particular CD4+ T cell responses) that reduced parasite burdens in the liver by an estimated 90 per cent (adults) using heterologous prime-boost regimens. – Definition of threshold of T cell responses required for protection against malaria. – Weak cellular responses in children aged 1–6 years and no protection against malaria.
Recombinant adenovirus serotype 5 (Ad5)	CSP AMA-1	**Immune priming trials** – Phase 1 trial of 1×10^{10} or 5×10^{10} particle units (pu) per vector induced slight fever in the high dose group. – Low induction of antibody titres but enhanced in the high dose group. – Antigen-specific IFN-γ secreting cells peaked one month after immunisation (ELISPOT assay). **Prime-boost trials** – Homologous boosting experiments showed T cell reactivity not affected by pre-existing anti-vector immunity in a homologous challenge, but vaccine boosters had no effect on elevating cellular immunity. – No protection against experimental challenge with sporozoites using homologous prime-boost regimens. – Ad5 vectors used to boost an immune response primed by DNA vaccination induced 27 percent protection against sporozoite challenge.
Adenovirus serotype 35	CSP (*P. yoelii* rodent malaria)	Immunogenic in mice.
Simian adenovirus vectors (AdC6, AdC7, AdC9, AdCh63)	CSP (*P. berghei* rodent model)	Immunogenicity comparable to Ad5, and more robust than FP9 or MVA as viral vectors.
Adenovirus serotype 35	CSP (*P. falciparum*)	– In rhesus macaques priming and boosting with Ad5-expressing CSP. – Induced a robust cellular and humoral immune response with antibodies generated able to recognise native protein. – Vaccine trial underway in humans.

Abbreviations: Ad, adenovirus; AMA, apical membrane antigen; CSP, circumsporozoite protein; FP9, Fowlpox strain 9; IFN-γ, interferon-γ; LSA, liver-stage antigen; MSP, merozoite surface protein; MVA, Modified Vaccinia virus Ankara; NYVAC, an attenuated strain of *Vaccinia* virus; Pfs, *Plasmodium falciparum* sexual stage antigen; SERA, serine-repeat antigen; TRAP, thrombospondin-related adhesive protein.

the immunogenicity of the recombinant simian Ad was comparable to the response obtained with Ad5 and more robust than the poxviruses FP9 and MVA. The AdCh63 vector has been proposed as an optimal simian adenovirus vector for clinical development, based on its stability, the immunological profile described in rhesus macaques and the low frequency of neutralising antibodies compared to the prevalence of Ad5 neutralising antibodies in children living in malaria-endemic areas.

25.1.4 Erythrocytic vaccines

Erythrocytic or asexual blood-stage vaccines aim to protect against severe malaria, not against infection. A major constraint of such an approach is the lack of immune correlates of protection and the extensive polymorphism of erythrocytic stage antigens. The epidemiological evidence that naturally acquired immunity against severe malaria is achieved earlier in highly endemic areas, in contrast with naturally acquired immunity against uncomplicated malaria, suggests that target antigens associated with malaria severity are variable, and that some variants are more virulent than others.

25.1.4.1 Vaccines targeted against merozoite antigens

The experimental evidence that protection against clinical episodes of malaria is correlated with the antibody response to several antigens supports the premise that an effective erythrocytic vaccine should include multiple targets.

The first multi-epitope erythrocytic stage vaccine tested in clinical trials was SPf66, a polymeric synthetic peptide composed of epitopes derived from three different merozoite proteins. Several Phase 2b trials were designed to confirm protection in areas of low transmission. Subsequent studies in areas of high transmission in Africa and Thailand showed limited protective effects against the first clinical episode, but a reduction in the incidence of subsequent infections. Experimental evidence also suggested that vaccine efficacy of SPf66 would have been improved by the use of more potent adjuvants in the final formulation.

Experimental data derived from population genetic analysis of genes encoding merozoite antigens showed high levels of global diversity, suggesting extensive immune selection. Nevertheless, merozoite proteins do also contain regions conserved across malaria parasite populations, so they offer an attractive target for vaccine-induced immune response generation. The leading merozoite-targeted antigens that have progressed to clinical trials are merozoite surface protein (MSP)-1, MSP-2, MSP-3, apical membrane antigen 1 (AMA-1) and the glutamate-rich protein (GLURP) (Table 25.1.2).

25.1.4.2 *Plasmodium falciparum* Erythrocyte Membrane Protein 1(PfEMP-1) variant VAR2CSA

In *P. falciparum* malaria, infected erythrocytes containing mature forms are removed from circulation by sequestration in the post-capillary vasculature.

Table 25.1.2 Candidate vaccine antigens against erythrocytic stages of malaria.

Antigen	Location and composition	Results
MSP-1	– GPI-anchored on the surface of merozoites. – Involved in invasion of RBCs. – Undergoes several proteolytic processing steps, resulting in several protein fragments including MSP-1_{19} and MSP-1_{42}.	**MSP-1_{19}** – Poorly immunogenic in pre-clinical and clinical trials because inhibitory action of anti-MSP-1_{19} antibodies is dependent on conformational epitopes. **MSP-1_{42}** – Immunogenic inducing growth inhibitory antibodies when administered with AS02A as an adjuvant (oil in water emulsion containing MPL and QS21). – Does not provide sufficient protection against infection.
MSP-2	– GPI anchored on the surface of merozoites. – No known function.	– Included as a component of the 'combination B' vaccine candidate (a mixture of three *P. falciparum* proteins, MSP-1, MSP-2 and RESA emulsified in Montanide ISA720). – Reduction in parasite density using combination B vaccine candidate.
MSP-3	– Non-covalently associated to the surface of merozoites via an interaction with ABRA. – C-terminal end contains B cell epitopes that are targets for cytophilic antibodies to mediate ADCI (Chapter 1).	– Administration of the C-terminal end using Montanide ISA720 or Alum as adjuvants did not boost naturally acquired cytophilic antibodies against MSP-3. – Currently in a second Phase 1 clinical trials as a fusion protein with GLURP.
GLURP	– Expressed on the surface of merozoites. – Contains B cell epitopes that are targets for cytophilic antibodies to mediate ADCI.	– Phase 1 trial of vaccination with a GLURP long synthetic peptide emulsified in Montanide ISA720 or alum as adjuvants induced cytophilic antibodies. – Currently in a second Phase 1 clinical trial as a fusion protein with MSP-3.
AMA-1	– Expressed in the apical organelles of merozoites and on the surface of merozoites. – Involved in reorientation, a critical step in the invasion of RBCs.	– Vaccination of recombinant AMA-Combination 1 (AMA-C1) expressed in yeast, administered using alum as an adjuvant, induced a functional antibody response in malaria-naïve individuals but a short-lived antibody response in people living in endemic areas, – Vaccination of recombinant AMA-1 using a combination of alum and CpG7909 as an adjuvant indicated a two-fold higher antibody response than using Montanide ISA720 as an adjuvant. – Phase 1 trials of recombinant AMA-1 expressed in *E. coli* using AS02A as an adjuvant induced cellular and humoral immune responses. –PfAMA-1-FVO (AMA-1 from *P. falciparum* clone FVO) expressed in yeast and used with alum, Montanide ISA720 or AS02 as adjuvants induced a functional antibody response. – In rhesus macaques, priming of the immune response using viral vector delivery (AdCh63 and MVA – see Table 25.1.1) of two alleles of AMA-1 from *P. falciparum* clones 3D7 and FVO and boosting with the same yeast-expressed AMA-1 proteins, induced long lasting T cell responses, memory B cells and functional antibodies.

Abbreviations: 3D7, strain of *P. falciparum*; ABRA, acidic basic repeat antigen; Ad, adenovirus; ADCI, antibody dependent cellular inhibition; AMA, apical membrane antigen; FVO, strain of *P. falciparum*; GLURP, glutamate-rich protein; MPL, Monophosphoryl Lipid A; MSP, merozoite surface protein; MVA, Modified Vaccinia virus Ankara; RESA, ring-infected erythrocyte antigen.

This phenomenon is mediated by adhesive cysteine rich domains expressed by a large family of variant proteins encoded by *var* genes called *P. falciparum* erythrocyte membrane-1 (PfEMP-1) (see Chapter 3). The diversity within this gene family is extensive, and numerous *var* genes appear in the parasite population. Although each parasite typically expresses a single *var* gene, PfEMP1 expression can switch at a rate of up to two per cent per generation *in vitro*. The severity of a *P. falciparum* infection is correlated with the sequestration of these forms.

The domains of PfEMP-1 that mediate adhesion are the target of antibodies with variant-specific agglutination properties. Data obtained in areas of intense transmission have indicated that the natural acquisition of variant-specific antibodies is protective against severe malaria or complications associated with malaria during pregnancy. Primigravids are highly susceptible to clinical complication during pregnancy, in contrast to multigravid women, who exhibit resistance.

Epidemiological data have shown a clear correlation between pregnancy-associated *P. falciparum* malaria and adhesion of infected erythrocytes to chondroitin sulphate A (CSA). This adhesion, responsible for compartmentalisation of infected erythrocytes in the intervillous spaces of the placenta, is determined by the expression of the PfEMP1 variant VAR2CSA. Current efforts to develop an anti-adhesion vaccine are mainly focused on VAR2CSA, with the purpose of identifying conserved epitopes capable of inducing antibodies able to inhibit the adhesion of infected erythrocytes to CSA.

25.1.5 Transmission-blocking vaccines

The first evidence that anti-sexual blood-stage immunity plays a role in preventing mosquito infectivity was reported almost 35 years ago using the avian malaria parasite *P. gallinaceum*. Sera from chickens immunised with formalin-treated or X-irradiated gametocytes prevented the infection of mosquitoes, using a membrane-feeding apparatus. The infectivity of gametocytes was recovered by incubation with sera from normal animals. Such an effect on sexual stage development in the vector was known as transmission-blocking immunity (TBI). Transmission-blocking vaccines (TBVs) target parasite surface antigens expressed by gametocytes and gametes (pre-fertilisation antigens), or antigens expressed on zygotes and ookinetes (post-fertilisation antigens). Vector-based TBV candidates include a broad range of parasite attachment ligands that play a role in the invasion of the mosquito midgut.

The best characterised pre-fertilisation antigen belongs to the six-cysteine motif protein family characterised by the presence of several copies of structurally similar domains that contain conserved cysteine residues – Pfs48/45 contains three domains and Pfs230 contains fourteen domains. Using knockout experiments, it has been determined that Pfs48/45 is critical for microgamete fertility, but Pfs230 is not essential for fertilisation. The major obstacle in bringing these antigens to clinical trials is their structural complexity; several recombinant protein strategies have failed to produce correctly folded proteins.

Post-fertilisation antigens were identified using monoclonal antibodies developed against *P. gallinaceum* ookinetes. The antigens identified using this approach correspond to the Pfs25 and Pfs28 proteins that are critical for *P. falciparum* recognition and attachment to the mosquito midgut. Gene disruption studies have shown that these proteins have partially redundant functions. They are composed of four tandem EGF-like domains and are anchored by GPI to the parasite membrane. Monoclonal and polyclonal antibodies directed against Pfs25 block the infection of mosquitoes.

However, a major challenge for vaccine development based on Pfs25 is to express a properly folded protein. To date, only eukaryotic expression vectors have efficiently reproduced the conformational epitopes of the native protein.

Vector antigens have also been explored as potential targets for TBI, based on the evidence that the midgut invasion, an essential step in the parasite sexual stage development, requires the participation of a cascade of molecules involved in midgut cell recognition, attachment, gliding, cell entry, cell traversal and egress. Antibodies elicited against midgut antigens, such as Aminopeptidase N (APN) and carboxypeptidase B (CPB), are able to block parasite development. However, the effect is not complete, suggesting that an effective TBV requires the targeting of several mosquito ligands. The advantage of using vector antigens is that the vaccine would protect against all *Plasmodium* species, avoiding the necessity of developing species-specific vaccines.

25.1.6 Whole organism vaccines

The limited efficacy of subunit vaccines in clinical trials has convinced some researchers to employ the older whole organism approach for developing malaria vaccines using either radiation attenuated (See Chapter 3) or genetically attenuated sporozoites. The creation of a biotech company named Sanaria, whose sole aim is to establish effective immunisation regimens involving radiation-attenuated sporozoites, has fuelled research and development in this area. This approach requires the implementation of methodologies to raise pathogen-free mosquitoes, produce sterile and metabolically active irradiated sporozoites, cryo-preserve viable parasites and design proper delivery systems. Although preliminary experiments using *P. yoelii* in rodents were promising, the recent dose-escalation clinical trial showed limited protection, with immune responses lower than those previously reported in volunteers immunised with irradiated sporozoites delivered using mosquito bites.

The development of genetically modified parasites was facilitated with the availability of genomic information (Chapter 2) and stage-specific transcriptomes that allowed the identification of genes essential for development of the parasite in the liver. The first of such gene groups tested are known as the 'Up-regulated in Infectious Sporozoites' (*UIS*) genes. Deletion of the *UIS3* and *UIS4* genes led to the arrest of liver-stage parasite development, with UIS3 knockout parasites exhibiting some breakthrough infections at high doses. Deletion of genes within a second group, the *P52* and *P36* genes, also led to

arrested forms, but breakthrough infections were very common. The simultaneous deletion of *P52* and *P36* led to the complete attenuation and long-lasting protection against sporozoite challenge. Deletion of the orthologous *P52* and *P52/P35* genes in *P. falciparum* has been reported and clinical trials planned.

The main concern with the use of infected RBCs is the requirement for potent adjuvants and the safety issues associated with potential erythrocyte alloreactivity or quiescent infectious agents present in the vaccine preparation. Experimental evidence in mice and in humans suggests that sub-patent infections, induced by experimental infection followed by early drug-treatment, elicit a low frequency of antibodies reactive against variant antigens and a robust T cell response. In both systems, the adaptive immune responses generated were protective against both homologous and heterologous parasites, suggesting that conserved epitopes are involved. In human trials, protective immune responses were correlated with the ability to induce multi-functional T cells with the capacity to simultaneously produce several cytokines.

25.1.7 *P. vivax* vaccines

P. vivax is the second most common *Plasmodium* species on a global scale, and it is prevalent in wider geographic areas than is *P. falciparum*. These two species coexist in extensive areas of the planet, and both cause major illness. Although the number of fatal cases by *P. vivax* is significantly lower than *P. falciparum*, *P. vivax* produces repeated debilitating relapses, weeks or months after primary infections, due to the development of dormant parasites in the liver known as hypnozoites (Chapter 3).

The coexistence of both species, and the unique biological features of *P. vivax* – which include the production of hypnozoites, a restricted invasion of reticulocytes and early development of gametocytes – support the concept of developing a multi-species malaria vaccine effective against *P. falciparum* and *P. vivax* simultaneously. The study design for *P. vivax* Phase 2 vaccine trials is challenging, in comparison to *P. falciparum* trials reported, because some subjects will have in addition to asymptomatic parasitaemia dormant hypnozoites in the liver. A major constraint in the development of *P. vivax* vaccines is the lack of a continuous *in vitro* culture system. However, the recent availability of genomic (Chapter 2) and transcriptome information will facilitate the search of potential vaccine candidates.

25.1.7.1 CSP

The first *P. vivax* vaccine candidate that reached clinical trials was based on the CSP. This protein was expressed in *Saccharomyces cerevisiae* but, although the vaccine was well tolerated, the immune responses induced by immunisation were very weak. A chimaerical *P. vivax* CSP expressed in *E. coli* that includes the two allelic forms of the repeat domain showed promising results in mice, and this vaccine candidate is currently in clinical trials.

A third approach for vaccine development based on the *P. vivax* CSP has included the use of long synthetic peptides, produced as three different polypeptides representing the amino terminal, carboxyl terminal and the repeat domains of the protein emulsified in Montanide ISA 51 or ISA 720. Both vaccine candidate formulations were well-tolerated and immunogenic, with better antibody responses in the group immunised with Montanide ISA 51. Phase 2 clinical trials are in progress.

25.1.7.2 Duffy-binding protein

A critical step in the invasion of reticulocytes by *P. vivax* parasites is the interaction of the Duffy-binding protein (DBP) and the Duffy antigen for chemokines receptor (DARC). DBP belongs to a family of erythrocytic binding proteins that includes *P. knowlesi* DBP, *P. vivax* DBP and the *P. falciparum* 175 kDa erythrocyte-binding protein. The binding domain of the protein has been mapped within the so-called region II, an amino terminal cysteine-rich region described in all erythrocyte-binding proteins. A refolded protein has been successfully tested for safety and immunogenicity in rhesus macaques, using clinical grade adjuvants. Partial protection induced by immunisation with *P. vivax* region II emulsified in Montanide ISA 720 has been reported in *Aotus* monkeys, and clinical trials are planned.

25.1.7.3 MSP-1 and AMA-1

A conserved amino terminal fragment of the *P. vivax* MSP-1 has been tested in pre-clinical trials, with partial protection induced in *Aotus* monkeys. In contrast, the *P. vivax* MSP-1_{19} recombinant protein, expressed in *S. cerevisiae* and fused to exogenous promiscuous T cell epitopes, showed limited efficacy when tested in *Saimiri boliviensis*. Interestingly, the orthologous *P. cynomolgi* MSP-1_{42} and MSP-1_{19} protein fragments expressed in baculovirus have been evaluated in the natural host *Macaca sinica*, showing a remarkable capacity to induce protective immunity.

These results, and the phylogenetic proximity between *P. vivax* and *P. cynomolgi*, open the possibility of using *P. cynomolgi* in macaques as a surrogate model for testing *P. vivax* vaccine candidates. Similarly, the ectodomain of the *P. vivax* AMA-1 has been expressed in *P. pastoris* and tested in rhesus macaques. The heterologous challenge with *P. cynomolgi* showed evidence of partial protection. Interestingly, in this model, the antibody responses were boosted after exposure to the simian malaria parasite.

25.1.7.4 *Pv*s25

The post-fertilisation antigen *Pv*s25, expressed in *S. cerevisiae* and formulated in alum or Montanide ISA 51, has been tested in clinical trials. The vaccine formulated in alum was well tolerated and immunogenic, inducing functional antibodies as determined by membrane feeding assays. However, the clinical trial

using the protein formulated with Montanide ISA 51 was halted, due to severe adverse events.

25.1.8 Concluding remarks

For the first time, in a field plagued with many failures, a partially protective malaria vaccine (RTS,S) is may be close to licensure. However, enormous challenges will be faced in developing a second generation of vaccines that are more effective and involve multi-stage and multi-species approaches. These include the development of more efficient recombinant expression systems capable of producing *Plasmodium* proteins in their natural conformations, new immunological strategies to induce effective cross-reactive immunity, the design of tools to identify immune correlates of protection, the development of *in vitro* culture systems for liver-stage and erythrocytic-stage forms of *P. vivax* and the implementation of novel screening methodologies to evaluate efficacy for vaccine candidates resulting from the use of 'omics' technologies.

Testing the potentially high number of novel malaria vaccine candidates that will reach Phase 2a will also require the implementation of improved methods of experimental challenge for erythrocytic-stage vaccines and the use of more sensitive methodologies such as the 18S rRNA quantitative real-time polymerase chain reaction (qPCR) to evaluate of the impact of vaccination on parasite multiplication, in turn providing a more accurate evaluation of vaccine efficiency.

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25.2 Current Approaches to the Development of a Vaccine Against Leishmaniasis

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The majority of diseases caused by the parasitic protozoa *Leishmania* are characterised by a state of immunity following resolution of disease, thus demonstrating that induction of protective immunity is possible. Although there is no commercially available vaccine for leishmaniasis or any other parasitic disease, extensive pre-clinical and promising clinical results indicate that control of the disease by preventative vaccination will become a viable approach in the future. *Leishmania* parasites are transmitted by phlebotomine sandflies, and the infecting promastigote form differentiates into, and replicates as, amastigotes within macrophages in the mammalian hosts (see Chapter 7). Infected dogs represent an important reservoir of *Leishmania* infection; sandflies become infected from feeding on dogs, then later pass on the infection to humans upon a subsequent meal.

Because the parasites reside mainly within macrophages, vaccines that stimulate cellular immune responses are required for control of intracellular replication. Appropriate CD4+ T cell responses correlate with protection against

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leishmaniasis in humans and animal models. The discovery of the Th1/Th2 paradigm of CD4 responses based on cytokines produced was aided largely by studies using cutaneous leishmaniasis (CL)-resistant and susceptible inbred mouse strains (see Chapter 7). However, it is known that Th1 immune responses also play an important role in visceral leishmaniasis (VL) and that protection involves CD4+ and CD8+ T cells, as well as interferon (IFN)-γ, interleukin (IL)-12, tumour necrosis factor (TNF)-α and nitric oxide (NO).

Similar to protective Th1 immune responses against CL, inhibitory effects on the development of VL have been reported for the immunoregulatory cytokines IL-10 and transforming growth factor (TGF)-β. Thus, immune responses against all forms of leishmaniasis share similarities, and effective control of parasite growth depends on the anti-microbial mechanisms afforded by a Th1 response.

25.2.1 Vaccination against leishmaniasis

Using crude or defined antigens with appropriate adjuvants, protection against visceral and cutaneous disease has been achieved in mice, hamsters, dogs and non-human primates. Protection studies, particularly in mice, have corroborated the Th1-dependence of effective immunity against *Leishmania*. Thus, understanding how to induce protective immune responses against *Leishmania* has broad relevance to the development of T cell vaccines and vaccines against intracellular organisms. Although vaccines for diseases that need elicitation of T cell-dependent immunity for protection have been more difficult to develop than those with antibody-mediated immunity, there has been significant progress in the development of safe and effective adjuvants and delivery systems for T cell-mediated vaccines.

Effective immunisation against leishmaniasis in animal models can be achieved by delivery of Ag-encoding DNA vectors or by administration of proteins formulated with Th1-inducing adjuvants, including recombinant IL-12 or Toll-like receptor (TLR) ligands such as CpG oligonucleotides, monophosphoryl lipid A and glycopyranosyl lipid adjuvant. Vaccines targeting amastigotes (designated here as anti-amastigote vaccines) are the main focus of development. Additionally, efforts have been made to target proteins in the saliva of sandfly vectors, as these proteins have a role in establishing infection, and also to prevent transmission of the parasites (Figure 25.2.1).

25.2.2 Anti-amastigote vaccines

The history of leishmaniasis vaccine development overlaps with other diseases, beginning with live vaccines, then killed vaccines, and subsequently shifting to subunit vaccines. Evidence that individuals cured from leishmaniasis develop protection against new infections has existed for a long time and, in more recent times, the nature of the acquired immunity has been associated with Th1-type cellular immunity. Therefore, live vaccination, either by exposing one's skin to sandfly bites or by injecting the pus of an active lesion, was practised historically. With the establishment of culture conditions for *Leishmania* parasites, live cultured promastigotes have also been used for this practice.

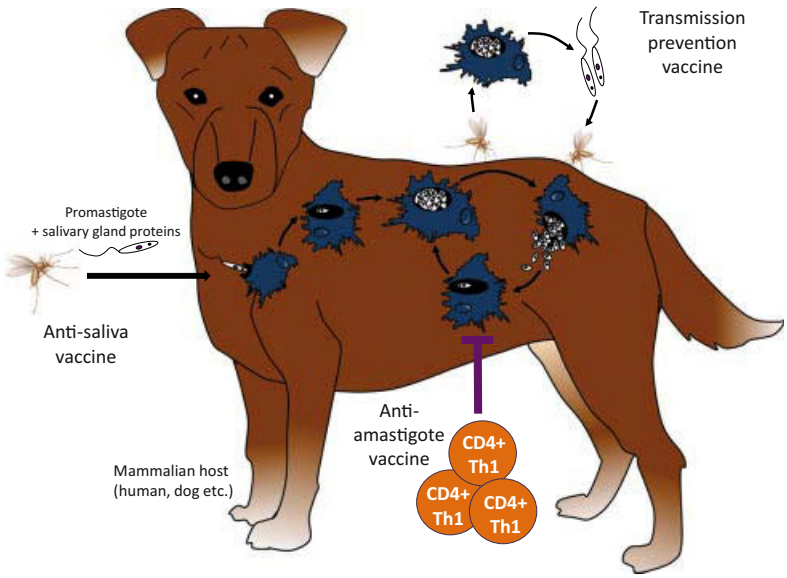

Figure 25.2.1 Vaccine targets in Leishmaniasis.

This process of 'leishmanisation' is still being used in Uzbekistan. However, drawbacks such as difficulty in the standardisation of vaccine virulence, as well as uncontrolled side effects (persistent lesions), preclude widespread use.

The principle of leishmanisation has been adapted to develop first-generation vaccines in which parasite lysates or killed parasites have been used as the vaccinogen. These vaccines have been shown to be safe and immunogenic, but standardisation has been difficult and protection has been inconsistent with only partial efficacy against leishmaniasis in a proportion of human patients vaccinated. With regards to sub-unit vaccine development, there has been progress in identifying and characterising *Leishmania* antigens that induce beneficial immune responses. These antigens (some of which are described in Table 25.2.1) confer protection against disease in different animal models of leishmaniasis.

Leish-111f is an anti-*Leishmania* vaccine produced by Infectious Disease Research Institute (IDRI: Seattle, USA) that has entered into clinical trials. This vaccine (also known as LEISH-F1) consists of a polyprotein composed of thiol-specific antioxidant (TSA), *L. major* homologue to eukaryotic stress-inducible protein (*Lm*STI1) and *Leishmania* elongation initiation factor (*Le*IF) (Table 25.2.1).

Animal models have been used extensively to characterise the efficacy of this vaccine; immunisation with this protein, formulated in the monophosphoryl lipid A in stable emulsion adjuvant (MPL-SE), protects mice and rhesus macaques against CL, and mice and hamsters against VL. The Leish-111f+MPL-SE vaccine candidate has also been evaluated in several clinical trials for safety and immunogenicity; the vaccine is safe, well-tolerated and immunogenic, both in uninfected and infected individuals. Furthermore, the Leish-111f+MPL-SE vaccine has also been shown to have efficacy as a

Table 25.2.1 Candidate vaccine antigens for an anti-amastigote vaccine.

Antigen	Expression	Function	References
Leishmania homologue of receptors for activated C kinase (LACK)	Promastigotes and amastigotes	An immunodominant antigen recognised by T cells in susceptible BALB/c mice	Mougneau, E *et al.* (1995). Expression cloning of a protective *Leishmania* antigen. *Science* 268(5210), 563–566.
Leishmanolysin (GP63)	Zinc metallo protease found on the surface of amastigotes and promastigotes	Provides the parasite with resistance to complement Facilitate attachment to macrophages and Promotes the intracellular survival of phagocytosed parasites within macrophages	Russell, DG & Alexander, J (1988). Effective immunisation against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. *Journal of Immunology* 140(4), 1274–1279.
Thiol-specific antioxidant (TSA)	Promastigotes and amastigotes	Putative antioxidant protein expressed in *Leishmania major*	Webb, JR *et al.* (1998). Human and murine immune responses to a novel *Leishmania major* recombinant protein encoded by members of a multicopy gene family. *Infection and Immunity* 66(7), 3279–3289.
Leishmania major homologue to eukaryotic stress-inducible protein (*Lm*STI1)	– –	Unknown	Campos-Neto, A *et al.* (2002). Vaccination with plasmid DNA encoding TSA/LmSTI1 leishmanial fusion proteins confers protection against *Leishmania major* infection in susceptible BALB/c mice. *Infection and Immunity* 70(6), 2828–2836.
Leishmania elongation initiation factor (LeIF)	– –	An RNA helicase homologous to eukaryotic initiation factor eIF4A Originally described as a natural adjuvant that induces an IL-12-mediated Th1 response in the peripheral blood mononuclear cells (PBMC) of leishmaniasis patients	Skeiky, YA *et al.* (1995). A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12. *The Journal of Experimental Medicine* 181(4), 1527–1537. Skeiky, YA *et al.* (1998). LeIF: a recombinant *Leishmania* protein that induces an IL-12-mediated Th1 cytokine profile. *Journal of Immunology* 161(11), 6171–6179.
Hydrophilic acylated surface protein, B family (HASPB)	A component of the surface coat of infective stage *Leishmania* parasites	*Leishmania* HASPB is a lipoprotein that is exported from the *Leishmania* parasites via an unconventional secretory pathway.	Stager, S *et al.* (2000). Immunisation with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *Journal of Immunology* 165(12), 7064–7071.

Table 25.2.1 (*Continued*)

Antigen	Expression	Function	References
Sterol 24-c-methyltransferase (SMT)	Promastigotes and amastigotes	SMT is involved in the biosynthesis of ergosterol (a membrane sterol)	Goto, Y *et al.* (2007). Protective immunisation against visceral leishmaniasis using *Leishmania* sterol 24-c-methyltransferase formulated in adjuvant. *Vaccine* 25(42), 7450–7458.
Kinetoplastid membrane protein 11 (KMP11)	Membrane associated protein expressed on the flagellum and in the flagellar pocket of promastigotes. Also expressed at lower levels in amastigotes	Immunogenic protein with unknown function; associates with lipophosphoglycan on the parasite surface.	Basu, R *et al.* (2005). Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *Journal of Immunology* 174(11), 7160–7171.
Products of the A2 gene family (A2)	A2-proteins and mRNA transcripts are developmentally expressed in the intracellular amastigote stage	Immunogenic proteins that may act as virulence factors.	Ghosh, A *et al.* (2001). Immunisation with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections. *Vaccine* 20(1–2), 59–66.
Cysteine protease genes B (CPB)	Stage regulated cathepsin-L like cysteine proteases	Multicopy genes implicated as virulence factors	Rafati, S *et al.* (2006). Prime-boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* confers protective immunity in murine visceral leishmaniasis. *Vaccine* 24(12), 2169–2175.
L110f (also known as Leish-111f and Leish-F2)	– –	Polyprotein composed of TSA, *Lm*STI1 and LeIF	Coler, RN *et al*. (2002). Immunisation with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, *Leishmania major* stress-inducible protein 1, and *Leishmania* elongation initiation factor protects against leishmaniasis. *Infection and Immunity* 70(8), 4215–4225.
KSAC	– –	Polyprotein composed of KMP11, SMT, A2 and CPB	Goto, Y *et al*. (2011). KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clinical and Vaccine Immunology* 18(7), 1118–1124.

therapeutic vaccine in humans and in dogs. The Leish-111f subunit vaccine is the first promising defined recombinant fusion protein candidate vaccine against Leishmaniasis to enter clinical trials.

IDRI has recently developed KSAC, another polyprotein vaccine antigen composed of kinetoplastid membrane protein 11 (KMP11), sterol 24-c-methyltransferase (SMT), A2 and cysteine protease B (CPB), and this has shown promise in experimental models of leishmaniasis and is being evaluated for both prophylactic and therapeutic potential in dogs (Table 25.2.1).

25.2.3 Anti-saliva vaccines

This approach targets immunomodulatory properties of sandfly saliva that have shown to exacerbate *Leishmania* infection. Prior exposure of mice to bites from uninfected sandflies confers powerful protection against *Leishmania* infection, although it can exacerbate leishmaniasis in a certain combination of sandfly and *Leishmania* species. It should be noted that immunomodulatory components of saliva do not show uniform potential as vaccine candidates, because the saliva contains antigens that, when used individually for vaccination, induce opposite effects of whole salivary extracts on disease outcome. Therefore, characterisation of protective components in the saliva is important for successful anti-saliva vaccines.

Work in this area is ongoing, but candidate antigens defined so far include maxadilan, SP15 and LJM19. Although the functions of these salivary proteins have not been fully elucidated, they are presumed to play a role in the induction of vasodilation in the area of the sandfly bite, in turn promoting the recruitment of neutrophils and macrophages to phagocytose deposited promastigotes enhancing the initiation of infection (see Chapter 7).

25.2.4 Transmission prevention vaccines

Insect vectors do not have acquired immunity. Parasites often live as extracellular forms in their insect vectors, possibly due to there being less immunological pressure in the environment than in mammalian hosts. Here, they are off-guard, not expecting a threat of antibody-mediated killing.

The idea behind a transmission-blocking vaccine is to deliver protective antibodies from the mammalian hosts at the time of bloodsucking. Interaction between promastigotes and the fly midgut is a crucial event for establishment of *Leishmania* infection in the insect vector and the resulting infection of a new mammalian host. Therefore, molecules derived either from parasite or from the insect participating in this event – including galectin, chitinase, fucose mannose ligand and lipophosphoglycan – can be targets of transmission prevention vaccines for leishmaniasis, although little focus to date has been placed on this approach.

Transmission prevention vaccines can be used to reduce the numbers of amastigotes found in asymptomatic individuals (both canine and human) in a population, in turn reducing the reservoir of parasites in a population.

25.2.5 Role of an adjuvant in vaccine development

Turning antigens into effective immunogens requires an understanding of the nature of the desired immune response and selecting delivery platforms capable of inducing such a response. A major breakthrough in the development of vaccine candidates against leishmaniasis (as well as other diseases requiring potent and directed T cell responses) occurred with the identification of adjuvants capable of inducing Th1 responses. The discovery that properly

formulated Toll-like receptor (TLR) agonists can stimulate a Th1 immune response has profoundly impacted vaccine development against intracellular pathogens like *Leishmania*.

Some TLR agonists have been evaluated by using animal models of leishmaniasis, and their usefulness as adjuvants for prophylactic and/or therapeutic vaccines has been demonstrated. They include CpG oligonucleotides (TLR9), imiquimod (TLR7), resiquimod (TLR7 and TLR8), monophosphoryl lipid A and glycopyranosyl lipid adjuvant (both TLR4 – see Table 25.2.2). In particular, the success of monophosphoryl lipid A (obtained from the cell wall of *Salmonella*) in approved vaccines for hepatitis B and human papilloma virus has

Table 25.2.2 Common adjuvants used in vaccination.

Adjuvant	Composition	Status
Alum	Aluminium compounds such as aluminium hydroxide, on which antigen can be absorbed.	Approved for use in humans.
Freund's adjuvant	**Incomplete**: paraffin oil and mannide monooleate. **Complete**: incomplete adjuvant with the addition of heat-killed and dried *Mycobacterium tuberculosis*.	Banned from use in humans.
Non-ionic block copolymers	Synthetic molecular 'blocks' of polyoxyethylene and polyoxypropylene with differing compositions. Examples include CRL1005 Titremax.	Experimental.
Monophosphoryl lipid A (MPL)	A TLR4 agonist obtained from the cell wall of *Salmonella*.	Approved for use in humans.
Glycopyranosyl lipid adjuvant	Hexa-acylated lipid A derivative.	Under clinical development
Montanides (ISA51, ISA720)	A mix of non-mineral vegetable oils and a surfactant from the monooleate family.	Under clinical development
Saponin derivatives: Quil-A ISCOMatrix QS-21 AS-02 AS-01	**QS-21:** saponin derivative from soap bark tree (*Quillaja saponaria*). **ISCOMatrix**: Immune stimulating complexes which are cage-like structures (40 nm in diameter).	Experimental. Under clinical development.
Combination adjuvant	**AS01**: MPL and QS-21 in liposomes. **AS02**: MPL and QS-21 emulsion. **AS04**: MPL absorbed in aluminium salts.	Under clinical development. Under clinical development. Approved for use in humans.
Immunostimulatory oligonucleotides	Purified TLR9 ligands such as CpG-DNA.	Under clinical development.
Bacterial products	Purified flagellin; TLR5 agonist.	Under clinical development.
Imiquimod	Topical application; TLR7 agonist.	Approved for use in humans.
Resiquimod (R-848)	Topical application; agonist for TLR7 and TLR8.	Approved for use in humans.

Abbreviations: CPG, C-poly G; MPL, monophosphoryl lipid A; TLR, Toll-like receptor.

demonstrated the safety and efficacy of engaging TLR4 without the induction of excessive inflammation.

To date, MPL is the only TLR agonist in approved vaccines for humans, and thus is the only TLR agonist with an extensive history of safety and efficacy as an adjuvant. As described in section 25.2.2 above, the Leish-111f vaccine has successfully utilised monophosphoryl lipid A as an adjuvant. Monophosphoryl lipid A is a strong enough adjuvant to elicit Th1immune responses against the epitopes in the Leish-111f fusion protein to protect mice, hamsters and dogs.

25.2.6 Future directions

The discovery of protective antigens, and the development of safe and effective adjuvants, opens up the possibility of developing a vaccine for leishmaniasis. However, there are challenges posed by the fact that 'leishmaniasis' is actually a variety of diseases caused by a variety of different organisms. Therefore, for practical reasons, candidate vaccine antigens should be selected that are conserved between species and are thus cross-protective against different *Leishmania* parasite species (listed in Chapter 7). Examples of such antigens include Sterol 24-c-methyltransferase or the antigens included in the Leish-111f vaccine. Recent completion of genome sequencing of multiple *Leishmania* species, causing diverse forms of leishmaniasis, will facilitate systematic analyses for other candidate antigens.

In addition to the prevention of leishmaniasis, another application of vaccines is for treatment of leishmaniasis, particularly in forms of human CL and mucosal leishmaniasis (ML) that may respond poorly to chemotherapy alone. Subunit vaccines have already shown their therapeutic potential in dogs (VL) and humans, although further improvements can be achieved through new adjuvant formulations (e.g. targeting several TLRs in synergy may result in enhanced responses).

Related to this, we need to further our understanding on immune correlates of protection in both prophylaxis and treatment of leishmaniasis. Recently, multi-functionality of Th1 cells (that is to say the capability of Th1 cells to produce more than one Th1 cytokine) has been shown to correlate with protective efficacy of a vaccine against *L. major* infection. Taken together, the success of leishmaniasis vaccine development relies on expansion of current basic knowledge on immune parameters relevant to vaccine development – in particular, the factors influencing the induction of Tregs and the development of T cell memory.

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25.3 Vaccination Against Hookworms

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25.3.1 The need for a vaccine

Human hookworms are one of the world's most common parasites, and they are highly prevalent in regions of South America, sub-Saharan Africa and East Asia. Until half a century ago, hookworms were fairly familiar in southern US states, Europe and Australia. However, with the use of drug therapies, hygiene practices and education, hookworms essentially no longer generate cause for alarm.

In 1947, Norman Stoll (see Introductory chapter) determined the earliest global estimate of individuals infected with human hookworm. He revealed that nearly one third of the world's population was infected with hookworm, making helminth infections one of the most common human infections. Over the next half century, efforts were put forth to reduce the prevalence of endemic hookworm in regions of North America, Asia, and Latin American, through economic improvements including clean water and sanitation.

Today, endemic hookworm has been essentially eradicated from the United States. Economic development in countries such as Taiwan, Japan, and South Korea during the 1960–1970s has resulted in significant control of hookworm infections and other nematode infections, with national prevalence levels below 1 per cent. Additionally, over the past 20 years, China has made successful

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efforts to decrease their national hookworm infection prevalence levels. On the other side of the globe, Latin America and the Caribbean have seen noticeable decreases in the prevalence of hookworm infections over time, with the implementation of various national treatment programmes coupled with economic growth. Unfortunately, prevalence rates in sub-Saharan Africa today are essentially still equal to the estimates put forth by Stoll in 1947.

In an effort to capture the true global impact, most health conditions are typically evaluated using the disability adjusted life year (DALY) metric (see Chapter 13). In reference to hookworm disease, DALYs are primarily evaluated on disability weights assigned to cognition and anaemia. While some believe that there is a necessity to revise the criteria used to calculate DALYs, one fact that remains constant is that sub-Saharan Africa currently possesses the greatest hookworm disease burden, and this is where most of the future challenges for controlling the disease lie ahead. The need for a hookworm vaccine is clearly present but whether a vaccine that can be developed to achieve sterilising or even partial immunity has proved to be challenging.

Almost all pathology and morbidity related to human hookworm infections result from intestinal blood loss and consequently low haemoglobin levels. It would, therefore, appear optimal to develop a vaccine that inhibits the development of a L_3 to a blood-feeding adult hookworm (see Chapter 13) and/or inhibit the attachment, survival and fecundity of adult hookworms in the host's intestines. Creating a vaccine that successfully inhibits both maturation and propagation would ultimately require at least a bivalent vaccine created with a major L_3 antigen and an adult hookworm protease.

25.3.2 The Human Hookworm Vaccine Initiative

In 2000, the Human Hookworm Vaccine Initiative (HHVI) was formed thanks to the appreciable funding from the Bill and Melinda Gates Foundation. The HHVI is the first and sole programme dedicated to reducing human suffering caused by hookworms, and it is a product development public-private partnership of the Sabin Vaccine Institute in Washington, DC.

The HHVI created a system of scoring potential hookworm vaccines for over 20 different recombinant antigens. This scoring system enables the HHVI to determine the efficacy of human hookworm vaccines based on endpoints such as the known function/structure of an antigen, human immunoepidemiological studies and reduction in the number of adult worms present in the small intestine, in host blood loss and in eggs per gram of faeces. The potential vaccine candidates are then tested and scored in both canine and hamster models of hookworm infection.

Before describing current candidate vaccine antigens, this chapter will examine some of the noteworthy milestones in the development of a hookworm vaccination to date.

25.3.3 The history of hookworm vaccines: experiments in dogs

In the late 1920s, observations concerning immunity and specific reactions of the host in infections with *Ancylostoma brasiliensis* and *Ancylostoma caninum* were described, concluding that 'creeping eruptions' (skin infections) result from toxic secretions from larvae. During the same time, experiments that involved administering four doses of hookworm larvae to dogs every two months suggested that superimposed infections might confer some increased resistance, and young dogs were shown to be more susceptible to cutaneous hookworm infections than older dogs. This evidence suggested that acquired immunity to hookworm in dogs existed.

The presence of acquired immunity to hookworm infections of dogs was also confirmed with the successful immunisation of dogs repeatedly exposed to different doses of *A. caninum*, which resulted in a decrease in worm burden and resistance to the development of subsequent larvae, albeit to a variable level from dog to dog.

Infections of dogs with greater doses of canine hookworm, but at intervals further apart, also demonstrated that, if the hookworm burden is too high early on in life, then whatever immunity was built up is overwhelmed. This indicated that acquired immunity is limited in providing protection against reinfection. Indeed, the limitations of immunity were also suggested by repeated skin infections at the same area of skin in white mice – this had no impact on the number of hookworm larvae that could be recovered from the site of inoculation. However, immunisation with subcutaneous injections of hookworm antigen caused some retention of larvae in the skin during penetration, again suggesting that immunity does exist.

25.3.4 Antibody production against canine hookworm

The limitations of acquired immunity to hookworm in dogs was puzzling. At the time, acquired immunity against other nematodes was known to exist, so it seemed unlikely that the host reaction to hookworm would be essentially unlike other nematode infections. In 1939, Otto and Kerr demonstrated that dogs sensitised to hookworms developed a generalised immunity and, Otto found that the sera of dogs actively immunised via superimposed infections of larvae contained rather effective antibodies against the hookworm larvae. Otto further concluded that some metabolic, excretory or secretory product of the hookworms contained the antigen. He also discussed that the phenomenon of specific acquired immunity plays a role in canine hookworm infection, and that 'consideration must be given to this same phenomenon in our study of human hookworm disease'.

In 1941, Otto discovered that oral administration of hookworms to dogs induced immunity against the worms as quickly as subcutaneous injections of larvae. He further concluded that it appeared that the level of immunity was

directly related to the number of larvae administered, rather than the route of infection. Otto also observed that immunity never achieved a level of perfect completeness, meaning that the dogs did not acquire sterilising immunity. Consequently, although the total burden of worms in the dogs decreased, anaemia still developed.

25.3.5 Vaccination against hookworm with irradiated larvae

In 1964, Miller demonstrated sterility in female larvae that were irradiated with x-rays and attenuation of *A. caninum* larvae. Furthermore, he observed that increasing amounts of radiation resulted in decreased pathogenicity of x-ray attenuated larvae to the host and a high degree of resistance to the challenge burden of normal worms, in terms of haematological, clinical and coprological changes in hosts immunised with a single subcutaneous vaccination. The arrest of irradiated larvae in some somatic locations on their migratory route (primarily the lungs), after subcutaneous inoculation, generated a more intense immunogenic stimulation of the host. In fact, nearly 75 per cent of irradiated larvae became 'arrested' before, or died prior to, reaching the intestines, the location where the hookworm causes substantial host blood loss.

Further experiments by Miller revealed that double oral vaccination was not as effective as double subcutaneous vaccination in dogs that were 3–4 months old, as measured by the establishment of adult hookworms after challenge with infective larvae. However, immunisation by both routes provided resistance to the pathogenic effects of hookworm infection upon subsequent challenge infections. Furthermore, immunity to natural hookworm infection lasted for several months after vaccination, possibly because oral vaccination could reduce the amount of migration.

The characteristics of attenuation consisted of three separate aspects that were all interconnected:

1. A reduction in larval infectivity.
2. A reduction in the pathogenicity of the worms that eventually reached the small intestine.
3. A sterilising effect on female worm's fecundity as measured by eggs per gram of faeces (epg).

Until this time, successful passive immunisation for helminth parasites exhibited variable and contradictory results, primarily because of experimental techniques. A few years after coining the 'characteristics of attenuation', Miller conducted a series of experiments revealing that the immunity of double-vaccinated dogs with X-irradiated *A. caninum* larvae could be passively and adoptively transferred to susceptible pups using serum and/or lymphoid cells, allowing description of the mechanism of resistance as an immunological phenomenon rather than a mechanical event.

The three 'characteristics of attenuation' suggested by Miller provided beneficial direction towards developing, manufacturing and licensing of a gamma-irradiated infective *A. caninum* L_3 vaccine for canines in 1973. The new,

commercially available vaccine was field tested by nearly 1,500 practising veterinarians across the entire continental USA. Overall, the vaccine proved to be safe for the dogs, and it had a considerable shelf life of approximately six months. Even though the vaccine was highly efficacious, it was quickly discontinued in 1975 due to its commercial failure. Most vets were unwilling to incorporate it into their vaccination programmes because it did not provide sterilising immunity.

25.3.6 Lessons from vaccination with irradiated larvae

Today, the majority of vaccines are sterilising in nature which involves the induction of a rapid and specific immune response that kills the entire pathogen. Such a characteristic is vital to combat certain diseases caused by pathogens that reproduce asexually, such as varicella (chickenpox) and measles. Additionally, vaccines directed at microparasites typically generate sterilising immunity, in terms of decreasing the incidence of disease in the individual receiving the vaccination and also generating so-called 'herd immunity.'

It is unlikely that a vaccine against human hookworm, or any other helminth, would ever establish a sterilising effect. In 2001, Bungiro *et al.* subcutaneously immunised LVG strain Syrian golden hamsters with non-living hookworm extracts that were obtained by homogenising previously frozen adult hookworms. They found that the immunisation provided partial resistance to the major clinical sequelae of hookworm infection (weight loss and anaemia). Importantly, the vaccination contained alum, which is the only adjuvant currently approved for use in humans. This study placed considerable emphasis on the potential for future vaccinations to prevent hookworm disease, rather than infection.

To this point in time, essentially every study concerning hookworm infections and immunisation against the worms have demonstrated that worm burdens can be reduced, but not eliminated. Hookworms are rather large, multi-cellular organisms that would be extremely difficult to completely eliminate from the host with pure immunological mechanisms. Therefore, the fact that the burden of adult hookworms can be significantly reduced in various models can be beneficial. If the hookworm burden is reduced enough with the use of a vaccine, then the prevalence of hookworm disease could be dramatically reduced as well.

A critical characteristic of the irradiated canine hookworm vaccine developed by Miller was that the efficacy of the vaccine depended on the viability of the larvae. More specifically, the attenuated larvae in the canine vaccine were able to live and generate antigens that would be secreted upon entry in the host. The importance of this viability factor, easily overlooked with the early irradiated larval vaccines for canines, has proved to be extremely informative for the development of a recombinant human hookworm vaccine. While immunising canines with live attenuated hookworm larvae appears to have a low risk-benefit ratio, immunising children with live attenuated hookworm larvae proves to be both unpractical and insecure for hookworm control.

The approach for the development of a human hookworm vaccine would most likely be designed so as to drastically reduce the total worm burden, ultimately resulting in an associated decrease in morbidity around the globe. Miller believed that the irradiated canine hookworm vaccine was discontinued due to 'the failure of veterinarians to differentially diagnose hookworm infection from hookworm disease.' As one might expect, the degree of pathology generated from hookworm is directly related to the number of worms residing within the host. The difference between hookworm infection and disease is not trivial: a human hookworm vaccine could significantly diminish the risk for heavy worm burdens and the associated blood loss and clinical sequelae.

25.3.7 Research identifying target proteins for an anti-hookworm vaccine

The mechanism behind the observed immunity to *A. caninum* was initially investigated by John Stumberg in the 1930s. By drying larval and adult canine hookworms from dog faeces *in vacuo* over sulphuric acid or calcium chloride, Stumberg was able to grind the remains into a powdery substance, and he utilised various alkaline and acid solutions as extracting fluids to obtain 'antigens' referred to as larval (DoL) and adult (DoA) antigens. However, dogs immunised with these antigens did not produce antibodies to either DoL or DoA up to seven weeks post-infection.

Later work in the early 1980s by Carroll and Grove demonstrated that immunisation with soluble *A. ceylanicum* antigens emulsified in Freund's adjuvant was able to reduce total intestinal adult hookworm numbers and faecal egg output in dogs given a challenge infection. Today, while all of the proteins that are vaccine candidates (Table 25.3.1) can be successfully isolated from living hookworms, the amount of protein needed for vaccine development requires recombinant vector expression in either prokaryotic and/or eukaryotic host systems.

25.3.7.1 Anti-coagulants (*Ac*-AP)

Much of the work put forth by Miller and others ignited an era of antigen discovery to identify major proteins secreted by hookworm larvae. In 1993, anticoagulant activity was observed from soluble adult *A. caninum* protein extracts, and this activity was associated with the inhibition of blood clotting factor Xa. This potent inhibitor of clotting factor Xa (*Ancylostoma caninum* anticoagulant peptide, *Ac*-AP) is a major anticoagulant generated by *A. caninum* adult hookworms. Since a significant proportion of morbidity associated with hookworm infections results from intestinal blood loss due to adult worms feeding, this anticoagulant could potentially serve as a feasible target for vaccine development aimed at decreasing the burden of hookworm infections.

Vaccination of dogs with recombinant fusion protein containing Ac-AP (this protein is 110 amino acids long), precipitated in alum, was only successful in generating an IgG1 response to *Ac*-AP in 17 per cent of recipient dogs. The vaccination was improved by formulating *Ac*-AP in calcium phosphate,

Table 25.3.1 Candidate vaccine antigens in hookworm vaccination.

Antigen	Stage	Function	References
Ancylostoma-secreted protein (ASP)	L_3 secreted	Released during invasion of the mammalian host. Involved in the establishment of infective L_3 stages.	Hawdon, JM *et al.* (1996). Cloning and characterisation of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. *The Journal of Biological Chemistry* 271(12), 6672–6678. Hawdon, JM *et al*. (1999). *Ancylostoma* secreted protein 2: cloning and characterisation of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. *Molecular and Biochemical Parasitology* 99(2), 149–165.
Metalloprotease (MTP)	L_3 secreted	Involved in tissue migration of L_3 stages.	Hotez, PJ *et al*. (2003). Effect of vaccination with a recombinant fusion protein encoding an astacin-like metalloprotease (MTP-1) secreted by host-stimulated *Ancylostoma caninum* third-stage infective larvae. *The Journal of Parasitology* 89(4), 853–855. Williamson, AL *et al*. (2006). *Ancylostoma caninum* MTP-1, an astacin-like metalloprotease secreted by infective hookworm larvae, is involved in tissue migration. *Infection and Immunity* 74(2), 961–967. Zhan, B *et al*. (2002). A developmentally regulated metalloprotease secreted by host-stimulated Ancylostoma caninum third-stage infective larvae is a member of the astacin family of proteases. *Molecular and Biochemical Parasitology* 120(2), 291–296.
Neutrophil inhibitory factor (NIF)	Excretory/ secretory protein	An integrin antagonist that can inhibit CD11b/18 dependent leukocyte function. NIF might allow the parasite to evade or dampen this neutrophilic inflammatory response.	Ali, F *et al*. (2001). Vaccination with neutrophil inhibitory factor reduces the fecundity of the hookworm *Ancylostoma ceylanicum*. *Parasite immunology* 23(5), 237–249. Hotez, PJ *et al*. (1999). Experimental approaches to the development of a recombinant hookworm vaccine. *Immunological Reviews* 171, 163–171. Kalkofen, UP (1970). Attachment and feeding behavior of *Ancylostoma caninum*. *Zeitschrift fur Parasitenkunde* 33(4), 339–354.
anti-coagulant peptide (AP)	Adults	Also called factor Xa, AP is a serine protease inhibitor that can prevent coagulation of blood.	Cappello, M *et al*. (1993). *Ancylostoma* factor Xa inhibitor, artial purification and its identification as a major hookworm-derived anticoagulant in vitro. *The Journal of Infectious Diseases* 167(6), 1474–1477. Hotez, PJ *et al*. (2002). Effect of vaccinations with recombinant fusion proteins on *Ancylostoma caninum* habitat selection in the canine intestine. *The Journal of Parasitology* 88(4), 684–690.
Aspartic acid protease (APR)	L_3	Cathepsin D-like proteases that can play a role in host-specific digestion of haemoglobin (feeding) and degradation of collagens in the skin, playing a role in tissue migration.	Williamson, AL *et al*. (2002). Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 16(11), 1458–1460. Williamson, AL *et al*. (2003). Hookworm cathepsin D aspartic proteases: contributing roles in the host-specific degradation of serum proteins and skin macromolecules. *Parasitology* 126(Pt 2), 179–185.

(*Continued*)

Table 25.3.1 (*Continued*)

Antigen	Stage	Function	References
Metalloendopeptidase (MEP)	Adults	A zinc MEP found localised to the microvilli of the worm's gastrointestinal tract, suggesting a potential function in the digestion of host blood.	Jones, BF & Hotez, PJ (2002). Molecular cloning and characterisation of *Ac*-mep-1, a developmentally regulated gut luminal metalloendopeptidase from adult *Ancylostoma caninum* hookworms. *Molecular and Biochemical Parasitology* 119(1), 107–116.
Tissue inhibitor of metalloproteinase (TMP)	Adults	Induces strong antibody responses.	Boag, PR *et al*. (2003). Characterisation of humoral immune responses in dogs vaccinated with irradiated *Ancylostoma caninum*. *Veterinary Immunology and Immunopathology* 92(1–2), 87–94. Zhan, B *et al*. (2002). Molecular cloning and purification of Ac-TMP, a developmentally regulated putative tissue inhibitor of metalloprotease released in relative abundance by adult Ancylostoma hookworms. *The American Journal of Tropical Medicine and Hygiene* 66(3), 238–244.
Cysteine protease (CP)	Adults	Cathepsin B cysteine proteases localised to the intestine of the nematodes and potentially involved in digestion of host blood-derived haemoglobin.	Harrop, SA *et al*. (1995). Characterisation and localisation of cathepsin B proteinases expressed by adult *Ancylostoma caninum* hookworms. *Molecular and Biochemical Parasitology* 71(2), 163–171. Loukas, A *et al*. (2004). Vaccination of dogs with a recombinant cysteine protease from the intestine of canine hookworms diminishes the fecundity and growth of worms. *The Journal of Infectious Diseases* 189(10), 1952–1961.
Glutathione-S-Transferase	Adults	Localised in the parasite's hypodermis, muscle tissue with some expression in the intestines. Can bind to haematin with high affinity.	Zhan, B *et al*. (2005). Biochemical characterisation and vaccine potential of a heme-binding glutathione transferase from the adult hookworm *Ancylostoma caninum*. *Infection and Immunity* 73(10), 6903–6911.
Ac-16	L_3 and adults	Located on the surface of the L_3 stage.	Fujiwara, RT *et al*. (2007). Reduction of worm fecundity and canine host blood loss mediates protection against hookworm infection elicited by vaccination with recombinant *Ac*–16. *Clinical and Vaccine Immunology: CVI* 14(3), 281–287.
Calreticulin	L_3 stage	Previously identified as a hookworm allergen. Can bind to and inhibit C1q, a human complement component. Induces strong Th2 responses and calreticulin-specific IgE antibodies.	Kasper, G *et al*. (2001). A calreticulin-like molecule from the human hookworm *Necator americanus* interacts with C1q and the cytoplasmic signalling domains of some integrins. *Parasite Immunology* 23(3), 141–152. Pritchard, DI *et al*. (1999). A hookworm allergen which strongly resembles calreticulin. *Parasite Immunology* 21(9), 439–450. Winter, JA *et al*. (2005). The assessment of hookworm calreticulin as a potential vaccine for necatoriasis. *Parasite Immunology* 27(4), 139–146.

resulting in a decrease in the adult hookworm burden upon challenge of dogs with *A. caninum*.

25.3.7.2 Tissue matrix metalloproteinase inhibitor 2

It follows that antigens that cause a strong antibody response may be good candidates for vaccine antigens. Western blotting of crude extracted *Ancylostoma* nematode proteins, and sera dogs that have been immunised with irradiated L_3, allowed researchers to identify which antigens had corresponding antibodies from the immunisation. They found that vaccination with irradiated L_3, followed by a challenge with infective L_3 hookworms, resulted in an antibody response primarily to antigens that were less than 20 kDa from excretory/secretory (ES) products from adult hookworms.

After conducting immunoscreening of an adult *A. caninum* cDNA library with antisera from the experiment, there were three clones that had the strongest immunoreactivity. The three clones were identical and encoded for a peptide with high similarity to tissue matrix metalloproteinase inhibitor 2 (TIMP-2), which belongs to the family of mammalian tissue inhibitors of metalloproteinases and was termed *Ac*-TMP. Studies such as this one can provide beneficial information concerning the development of a potential vaccine against hookworm. For example, it would be useful only to develop a vaccine with antigens that generate a robust antibody response. Vaccination of a recombinant fusion protein encoding Ac-TMP successfully generated a strong anti-*Ac*TMP IgG1 response, which was correlated with a reduction in adult hookworm burden in dogs challenged with *A. caninum*.

25.3.7.3 *Ancylostoma*-secreted protein

Developmentally arrested third-stage infective larvae resume development upon entry into a host via factors present in host serum, together with methylated glutathione derivatives. *Ancylostoma*-secreted protein (ASP) is a 40 kDa protein with zinc metalloprotease (MTP) activity, rapidly released during invasion of the host and activation. The *A. caninum* ASP (*Ac*-ASP-1) has homologues in the human hookworms *Ancylostoma duodenale* (*Ad*-ASP-1) and *Necatur americanus* (*Na*-ASP-1). This protein it shows a significant degree of partial homology to previously observed allergens of insect venoms, which suggests that it might be involved in immunobiology and pathobiology events of hookworm infections.

Vaccination of mice with alum-precipitated recombinant ASP-1 (rASP-1) protein protects against challenge infection of 500 infective larvae in a mouse model for ancylostomiasis, reducing the worm burden by up to 79 per cent. This alum-precipitated rASP-1 vaccine was of particular significance because it utilised alum, which is the only US-approved human vaccine adjuvant in use (see Chapter 25.2). Subsequent experiments demonstrated that the reduction in the burden of hookworms by alum-precipitated *A. caninum* rASP-1 (*Ac*-rASP-1) is antibody-mediated. Immunised mice display elevated levels of all anti-*Ac*-ASP-1 antibody classes, in particular IgG1. Increased levels of IgG and IgE are

thought to mediate hookworm burden reductions in the GI tract and muscle in mice. Together, these findings suggest that ASP could be a prospective recombinant vaccine candidate against human hookworm.

A second ASP (*Ac*-ASP-2) is a protein composed of 219 amino acids that is released as an ES product from *Ancylostoma* hookworms detectable upon feeding of infective L_3 stages *in vitro*. It appears that *Ac*-ASP-2 is released in response to specific host signals, but is less abundantly released than Ac-ASP-1. Nevertheless, if ASP-2 plays a role in the infective process, interference of this protein could potentially inhibit infection and the development of hookworm disease. With the identification of these two ASPs, the ideation of combining both antigens into a single vaccine could be extremely effective at limiting hookworm infections. Indeed, immunisation of dogs with rASP-2, formulated with AS03 adjuvant from GlaxoSmithKline, generated high antibody titres and successfully provided some protection against *A. caninum* L_3 migration through the tissue in immunised dogs. Expression of ASP-2 was observed in the oesophagus and body channels exiting the cuticle of hookworms.

To observe whether ASP antigens from different species of hookworm can cross-protect against different species of hookworm, a recombinant vaccine was developed using alum-precipitated ASPs from *A. caninum*, *A. duodenale* and *N. americanus*, and the efficacy against challenges with *A. caninum* larvae was assessed. Cross-species protection against *A. caninum* exists when using rASP-1 from human hookworms *N. americanus* and *A. duodenale*. The level of protection is directly related to the amino acid sequence homology between the ASP immunogens used for the vaccine and the *A. caninum* ASP-1 generated by the larvae used for the challenge. This information may be helpful in the development of immunogenic peptide vaccines. Unlike *Ac*-ASP-1, *Ac*-ASP-2 did not elicit vaccine protection. ASPs appear as promising vaccine candidates for nematode infections in general, and are also a vaccine candidate against filarial nematode infection (see Chapter 25.4).

25.3.7.4 Metalloprotease

When *A. caninum* infective larvae are stimulated *in vitro*, they release MTP-1, a 62 kDa zinc astacin-like metalloprotease involved in tissue migration. Intramuscular vaccination of dogs with the recombinant fusion protein Ac-MTP-1 elicits antigen-specific IgG2 titres that have been found to be inversely associated with hookworm burden and egg counts. This suggests that Ac-MTP-1 might be a potential recombinant vaccine candidate for the future.

25.3.7.5 Neutrophil inhibitory factor

Neutrophil inhibitory factor (NIF) was identified utilising whole hookworm extracts while searching for anti-haemostatic agents. It has been suggested that neutrophils gather at the site where the parasite attaches in the gut, and that NIF might allow the parasite to evade or damper this neutrophilic inflammatory response. Creating a neutralising immune response by immunising with NIF may protect the host from hookworm attachment and colonisation.

However, immunisation of hamsters with an active recombinant NIF, emulsified in Freund's adjuvant, did not support this hypothesis. Neither worm burdens nor levels of anaemia were affected upon challenge infection of *Ancylostoma ceylanicum* in vaccinated hamsters, despite the presence of a measurable antibody response against NIF. Rather, immunisation resulted in a strong anti-fecundity effect, as seen in a decrease in egg output. It may be that the generation of the anti-NIF response may be more effective under the Th2-intiating conditions of the alum adjuvant, rather than the Th1-inducing conditions of Freund's adjuvant.

25.3.7.6 Calreticulin

Calreticulin is a hookworm allergen that induces a strong Th2 response with calreticulin-specific IgG1 and IgE antibodies (see Chapter 1). This molecule is thought to behave like an immune evasion molecule, because recombinant *N. americanus* calreticulin can bind to, and inhibit, the human complement component C1q. Intraperitoneally delivered *N. americanus* calreticulin produces a strong and specific IgG1 response in BALB/c mice, and also a high titre of IgE when the calreticulin is encapsulated in polylactide-co-glycolide (PLGA) microparticles. Despite the induction of IgE in vaccination with encapsulated calreticulin, no protection against *N. americanus* is observed, and worm burdens remain unaffected by the immune response generated.

Interestingly, vaccination with non-encapsulated calreticulin can reduce the worm burden by 43–49 per cent in the lungs of challenged mice. The reason for this observation is unknown, but suggests that it may be unrealistic to search for a completely 'protective' immunological phenotype from the vaccine development viewpoint.

25.3.7.7 Cathepsin B cysteine protease

The blood-feeding stage of *A. caninum* secretes a cathepsin B cysteine protease (CP), known as *Ac*-CP-2, that is thought to be involved in the hookworm's digestion host blood-derived haemoglobin. Vaccination of canines with catalytically active recombinant *Ac*-CP-2 has been shown to provide partial protection against hookworm infection, as measured by reduced faecal egg counts, stunting of adult worms, a decreased proportion of female to male worms and generation of protease neutralising antibodies.

A comparison using different adjuvants to increase the level of protection afforded by *Ac*-CP-2 (alum, AS03, AS02A, ISA 70 – see Chapter 25.2) demonstrated an increased production of IgG1 using AS03 and IgG2 using AS02A. The vaccine formulated with alum generated an antibody response, with the longest duration which could be measured 60 days following the initial vaccination. Using immunohistochemistry, it has been shown that anti-*Ac*-CP-2 antibodies localise to the intestine of blood-feeding hookworms, demonstrating that *Ac*-CP-2 is being expressed in the hookworm gut and supporting the hypothesis of a function in digesting host haemoglobin.

25.3.7.8 Aspartic proteases

N. americanus expresses two aspartic proteases (APR) – a cathepsin-D like enzyme known as *Na*-APR-1 and a second enzyme, *Na*-APR-2, which is more similar to nematode-specific proteases ('nemepsins') than to cathepsin D. *Na*-rAPR-2 is able to cleave human haemoglobin and serum proteins more efficiently than Na-APR-1, possibly because only a quarter of *Na*-APR-2 cleavage sites within human haemoglobin are shared with those of *Na*-APR-1. The digestion of haemoglobin is an essential nutrient source for hookworms, and the fact that *Na*-rAPR-2 is able to cleave human haemoglobin and serum proteins about twice as efficiently as orthologous substrates from dogs, a non-permissive host for *N. americanus*, may partly explain host permissiveness.

Na-APR-2 was found to be localised primarily to the gut and to a smaller extent in the amphidial and excretory glands of the adult hookworm. Biochemical analysis of these APRs has revealed that *Na*-APR-1 operates optimally at a pH of 5.5, whereas *Na*-APR-2 functions ideally at a pH of 5.0. It has been hypothesised that *Na*-APR-1 may be responsible for initial cleavage of substrates in the gut, while *Na*-APR-2 is responsible for degradation of the blood meal after acidification. Blocking the function of *Na*-APR-2 via purified mouse IgG from mice immunised with *Na*-APR-2 reduced the migration of *N. americanus* L_3 larvae in the skin by up to 50 per cent. This demonstrated that this molecule performs an essential function in hookworm biology, and highlighted this enzyme as a promising vaccine target.

Indeed, vaccination of dogs with a recombinant fusion protein encoding Ac-APR-1 from *A. caninum* generated a strong IgG1 response and a decrease of 18 per cent in adult hookworm burden upon challenge infection with *A. caninum*. However, this decrease did not have a knock-on effect to hookworm fecundity, which remained the same as for unvaccinated dogs Furthermore, vaccination with *Ac*-APR-1 changed the location of adult hookworms in the vaccinated dogs, whereby the hookworms were found to be residing in the colon (rather than the small intestines) – perhaps because the high antibody titres against the vaccine antigens created an environment in the small intestine that was poor for survival.

25.3.7.9 Glutathione-S-transferase

A novel glutathione S-transferase from *A. caninum* adult hookworm, known as *Ac*-GST-1, is a 24 kDa protein that is thought to be expressed in the hypodermis, muscle tissue and intestine of the parasite. Recombinant *Ac*-GST-1 exhibits a high affinity binding to haematin, suggesting that *Ac*-GST-1 might play a role in the parasite detoxification of haem during the process of haemoglobin digestion. Vaccination of dogs with *Ac*-GST-1 can reduce both the mean adult hookworm burden and the faecal egg counts when compared to non-vaccinated control animals

However, hamsters have also been immunised with *Ac*-GST-1 and, interestingly, there was a statistically significant reduction in the hookworm burden

following challenge with *N. americanus*. These findings suggest that GST-1 in *A. caninum* and *N. americanus* share enough similar identity to generate immunological cross-reactivity.

25.3.7.10 *Ac*-16

Ac-16 is an immunodominant surface antigen of *A. caninum* expressed during the major developmental stages of hookworms, including the larvae and the adult forms, making it an attractive candidate for a canine hookworm vaccine. Intramuscular immunisation of beagles with three doses of *Ac*-16 in AS03 adjuvant inhibited larval migration through dog tissue and was associated with elevated titres of IgG1, IgG2 and IgE antibodies, in response to challenge with *A. caninum* L_3. The reduction in adult hookworms in dogs immunised with *As*-16, in turn also reduces hookworm egg counts and anaemia. In contrast to hookworm antigens such as *Ac*-ASP-1, *Ac*-APR-1 and *Ac*-GST-1, *Ac*-16 is the only effective antigen found to date that is present in both the larval and adult hookworm forms. Consequently, *Ac*-16 could be a potential monovalent vaccine candidate targeted against human hookworms.

25.3.7.11 Combination protein vaccines: *Ay*-ASP-2 and *Ay*-MTP-1

Vaccination of Syrian golden hamsters with a combination of recombinant fusion proteins *Ay*-ASP-2 and *Ay*-MTP-1 from L_3 *A. ceylanicum* has been shown to enhance the protection afforded by vaccination of either of these antigens alone. Hamsters vaccinated with both antigens had a greater reduction in the burden of adult hookworms and an associated reduction in faecal egg counts, despite no significant effects on the antibody titres in recipient hamsters.

Furthermore, the combination vaccine drastically improved the haemoglobin values and body weights of the host, compared to hamsters vaccinated with either of the single antigens. From this study, it appears that the combination of two, or possible more, antigens may represent an effective vaccine development strategy to increase protection and, consequently, decrease disease symptoms experienced by humans.

25.3.8 A human hookworm vaccine phase 1 clinical trial based on *Na*-ASP2

Of all the antigens described above, *Na*-ASP-2 was identified as a promising candidate vaccine antigen for use in the development of a human hookworm vaccine. It was demonstrated in 2005 that this protein could be produced as a recombinant protein in large quantities using the yeast *Pichia pastoris,* and that this recombinant protein had a high immunogenic potency when formulated with Alhydrogel® as an adjuvant (as measured by the strength of the antibody response and effectiveness at preventing larval migration through the skin of immunised rats).

This study proved to be a significant vaccine landmark, which helped to establish a fundamental platform for high-yield production of *Na*-ASP-2 in clinical vaccine development. Moreover, the technological processes used in this study were considerably simple, which helps with transferring the development to manufacturers in middle-income countries where hookworm infection is endemic, such as China, India and Brazil. It was reasoned that vaccination with *Na*-ASP-2 would be expected to reduce the number of infective larvae that enter the human gut, consequently reducing the number of hookworms and faecal egg counts at the individual level.

Recombinant Na-ASP-2 vaccine was manufactured and tested in a phase 1 clinical trial in healthy adults (see Chapter 25.1) that were hookworm-naïve from a non-endemic area. The trial was conducted to assess the safety, tolerability and immunogenicity of the *Na*-ASP-2 hookworm vaccine. The *Na*-ASP-2 gene was cloned and expressed in *P. pastoris* and the vaccine was produced at the Walter Reed Army Institute of Research Pilot Bioproduction Facility. The vaccine was formulated with vials containing 500 μg of *Na*-ASP-2 and 3,000 μg of Alhydrogel® in 1.0 ml Phosphate Buffered Saline (PBS) without any stabilisers. Prior to injection of the vaccine, the vials were diluted to the proper concentrations using Alhydrogel® adjuvant. The trial was designed as a randomised, double-blind, placebo-controlled dose-escalating study. A total of 36 volunteers aged between 18 and 45 years old in Washington, DC were enrolled for the study.

After a series of three injections, the vaccine was found to be tolerated well. Individuals experienced injection site pain, swelling, erythema and pruritus, but these were short-lived side effects. The immunisation resulted in a strong, antigen-specific IgG response (IgG1 and IgG4 isotypes) when compared to the placebo group, with considerable levels of IgE specific to *Na*-ASP-2. Individuals receiving the vaccination displayed lymphoproliferative responses to *Na*-ASP-2 antigen, clearly demonstrating that significant humoral and cellular immune responses could be induced using this specific antigen.

After promising results in individuals from a hookworm non-endemic area, the HHVI decided to shift the phase 1 clinical trial down to Brazil to study the *Na*-ASP-2 vaccine in a hookworm endemic area from 2007–2008. However, individuals in Brazil receiving a single dose of the vaccine unexpectedly developed generalised urticaria (hives) due to previously existing *Na*-ASP-2 specific IgE (unpublished observation). Due to these findings, the phase 1 clinical trial was quickly terminated and the HHVI decided to focus on other antigens for vaccine development.

25.3.9 The HHVI takes a different approach

After the phase 1 clinical trial of *Na*-ASP-2 in Brazil, the HHVI decided to shift its efforts towards antigens that play a significant role in the blood-feeding abilities of adult hookworms. A hookworm-infected individual can lose up to about 1 ml of blood per day from 25–30 adult hookworms, and a vaccine which is able to prevent adult hookworms from efficiently ingesting blood could be an alternative target to the inhibition of L_3 larvae migration for a hookworm vaccination.

Na-ASP-1, *Na*-CP-3 and *Na*-MEP-1 are all proteases that are expressed in the intestine of adult *N. americanus* hookworms that can bind promiscuously to haemoglobin. *Na*-APR-1 is found in the alimentary canal of the adult hookworm and is involved in haemoglobin digestion. *Na*-GST-1 forms homodimers that are sufficiently large enough to accommodate haem, haematin and related molecules, and therefore its main purpose appears to be to detoxify haem. Furthermore, immune responses generated against the haem-binding GSTs can induce partial protective immunity to *N. americanus*. Thus, multiple targets exist to block access of adult hookworms to nutrients.

Na-APR-1 and *Na*-GST-1 have been identified as two key antigens targeting the nutritional and metabolic requirements of adult hookworms. Logically, since *Na*-APR-1 is an enzymatically active protease, it would not be wise to immunise individuals with the protease. However, *Na*-APR-1 cloned from *N. americanus*, but inactivated by the introduction of two site-directed mutations at two different aspartic acid residues during cloning, can still induce neutralising antibodies. This molecule is still under development as a vaccinogen against hookworm infection,

Na-GST-1 expressed in *P. pastoris* has undergone successful process development and current good manufacturing practices (cGMP). Therefore, it should soon be expected to undergo regulatory submission and, potentially, phase 1 clinical trial testing. The HHVI has allocated significant time and resources towards these two antigens, which will potentially be combined into a bivalent hookworm vaccine. Brazil has the most hookworm cases in the western hemisphere, and so much of the product and clinical human hookworm vaccine development will be conducted in this country.

25.3.10 Developments through the last century and the future

Hookworm and hookworm vaccine developments have been occurring through the past century. Much of the hookworm vaccine developments took place in the 1930s, 1960s, 1990s and 2000s. Over these years, the focus has shifted from irradiated L_3 vaccines to targeting larval and adult surface antigens, and then to focusing on haemoglobin proteases. Ultimately, an ideal hookworm vaccine would prevent hookworm infection. However, it is unlikely that this goal will be achieved, given the complexity of the immune response necessary to eliminate hookworms from the body. Consequently, a successful vaccine is more likely to reduce the overall prevalence of hookworm disease rather than hookworm infections. Hookworm vaccine development has not proven to be a simple feat thus far.

However, while recent technological advances will aid the discovery and characterisation of new potential vaccine targets against hookworm infection, processing development, pre-clinical development and clinical development all represent three potential roadblocks for future human hookworm vaccine candidates.

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25.4 Current Approaches to the Development of a Vaccine Against Filarial Nematodes

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25.4.1 Introduction to anti-filarial nematode vaccines

The value of developing a vaccine against *Onchocerca volvulus* was recognised by The Edna McConnell Clark Foundation (EMCF) in the 1980s. In 1985, it established a 15-year long research programme aimed at the development of a recombinant protein-based vaccine against the infective stage larvae of *Onchocerca*. The specific mission for the vaccine under development was to induce protection (not necessarily sterile) against the incoming infective third-stage larvae (L_3), moulting L_3 (mL_3) and fourth-stage larvae (L_4). The rationale behind this strategy was that immune responses which kill any of these stages would prevent the development of adult worms and, thereby, production of their offspring (the microfilariae (Mf)), which are responsible for the manifestation of the disease and transmission (See Chapter 11).

The efforts to develop a vaccine against Mf, which would have blocked transmission but not infection, were not a part of the research plan. It was hypothesised that the continual natural exposure of the immunised individuals to infective larvae would sustain the protective immunity induced by immunisation

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with recombinant proteins, and that ultimately the combination of vaccination with long-term ivermectin chemotherapy could accelerate the eradication of onchocerciasis.

The programme supported:

1. immunological studies in animals;
2. immunological studies in humans;
3. development of animal models for screening candidate vaccine antigens;
4. discovery of vaccine candidates; and
5. an easy access to parasite material – L_3s, cDNA libraries, sera samples from immune and infected humans (Figure 25.4.1).

The programme ended in 1999, by which point the EMCF had appropriated US$21.6 million for onchocerciasis research. The anti-L_3 vaccine approach taken by the EMCF was endorsed in 2003 by the Conference on the Eradicability of Onchocerciasis and also by a conference on the global programme to eliminate lymphatic filariasis in 2004, which also recommended the development of a vaccine against lymphatic filariasis (LF).

In essence, anti-L_3 vaccines will not depend on mass drug administration (MDA), the present tool used to control both diseases and their transmission, but they will be administered in communities that have been already going

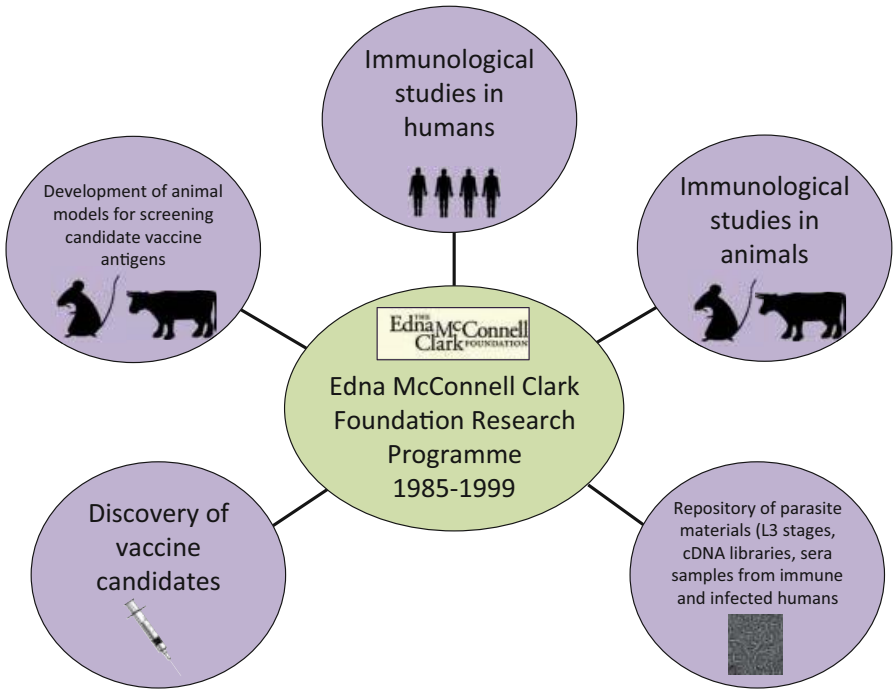

Figure 25.4.1 Activities of the Edna McConnell Clark Foundation Research Programme against filariasis. Based on the review Cook, JA *et al.* (2001). Towards a vaccine for onchocerciases. *Trends in Parasitology* 17(12), 555–558.

through many rounds of MDA. Therefore, such vaccines will complement these control measures and support the same goal: elimination of onchocerciasis as a public health problem in Sub-Sahara Africa and elimination of LF globally.

Similar approaches are also currently being pursued by other helminth vaccine initiatives, such as those for hookworm (see Chapter 25.3) and schistosomiasis. Vaccines are still the most economical, efficient and effective tool to control infectious diseases, and will be the only way to guarantee elimination of onchocerciasis and LF as a public health problem. Anti-helminthic vaccines represent a key tool for achieving UN Millennium Development Goals related to poverty reduction, education, preservation of child and maternal health from infectious diseases.

This chapter will review the current vaccine candidates that are being studied/pursued for the development of anti-L_3 vaccines against *Onchocerca volvulus* (the causative agent of onchocerciasis) and against *Wuchereria bancrofti* and *Brugia malayi* (the causative agents of LF). The animal models that have been (and can be) used to test their validity for future pre-clinical and clinical development will also be described.

25.4.2 Anti-*O. volvulus* and anti-LF vaccines are a valid approach to advance control measures against onchocerciasis and lymphatic filariasis

25.4.2.1 Protective immunity exists naturally in some individuals

The feasibility of anti-*Onchocerca* L_3 vaccines is supported by the existence of a small population of putatively immune (PI) individuals that can be identified in highly endemic areas (1–5 per cent). Although these individuals have been exposed to infection with L_3, they do not develop a patent infection. In bovine onchocerciasis (infection with *Onchocerca ochengi*), putative immunity has been also reported under conditions of natural exposure. The observation that infected individuals (humans and cows) are possibly protected from further new infections by concomitant immunity (whereby the newly introduced L_3 are eliminated, while adult worms and Mf are left unaffected) also lends weight to the idea that protective immune responses can be generated. The concept of concomitant immunity was recently verified experimentally using the *Acanthocheilonema viteae* jird model.

Protective immune responses to human lymphatic filariae and the epidemiologic outcomes of exposure to the two LF-causing filarial nematode species are similar to those described for *O. volvulus*. They include:

1. the description of individuals without signs of patent Mf positive infection – the so-called endemic normal (EN) or PI, in areas endemic for *W. bancrofti* or *B. malayi* infections;
2. the age-acquired resistance to super-infection with adult worms; and
3. the existence of anti-L_3 concomitant protective humoral and cellular immunity in infected individuals.

25.4.2.2 Vaccination against *Onchocerca* sp.

It has been repeatedly shown, using various animal models, that significant protection can be induced by immunisation with *Onchocerca* sp. x-ray attenuated L_3 (xL_3). In mice, 30–60 per cent protection was observed in the *O. volvulus* diffusion chamber model (measuring survival of L_3 and/or L_3 to L_4 moulting). In cattle, xL_3 immunisation confers a reduction in *O. ochengi* adult load (up to 53 per cent) and consequently results in significant reductions in microfilarial prevalence and density in cattle exposed to severe and prolonged field challenge to L_3. Cattle immunised with xL_3 also exhibit significant levels of protective immunity against experimental challenge with L_3 (up to 84 per cent reduction in mean adult load). Furthermore, calves immunised with live *O. volvulus* L_3s and challenged with *O. ochengi* L_3s have reduced worm burden, demonstrating a level of cross-reactive protection between species of *Onchocerca*.

It is believed that protective immunity in humans against *O. volvulus* L_3 is associated with Th1 and Th2 responses and antibody-dependent cellular cytotoxicity (ADCC – see Chapter 1). The function of antibodies in protective immunity originally provided the basis for the most commonly used strategy to clone putative *O. volvulus* antigens for use in vaccine studies (immunoscreening of cDNA libraries using immune sera from human or animal hosts). Of the protective *O. volvulus* recombinant antigens identified to date, most were discovered by immunoscreening with antibodies from PI subjects and/or xL_3-vaccinated hosts. Significant protection (35–69 per cent reduction in L_3 survival) can be induced when these recombinant proteins are immunised in alum (Th2/IgG1), complete Freund's adjuvant (CFA) (Th1/IgG2a), or using the block copolymer adjuvant (see Chapter 25.2 for details of adjuvants commonly used in vaccination).

Ov-CHI-1 can induce protection using DNA immunisation. The characteristics of the parasite proteins corresponding to protective *O. volvulus* vaccine antigens are not discussed here, but have been reviewed previously (see references for further reading). Only one additional antigen with protective properties – *Ov*-glyceraldehyde-3-phosphate dehydrogenase (GAPDH – cloned using immunoscreening) – has been reported recently.

Studies in the *O. ochengi* cow model (sponsored by the EMCF) vaccinated calves with eight *O. ochengi* recombinant antigens (orthologues of the protective *O. volvulus* recombinant proteins: *Ov*-ALT-1, *Ov*-B8, *Ov*-RAL-2, *Ov*-TMY-1, *Ov*-CPI-2, *Ov*-B20, *Ov*-RBP-1, *Ov*-FBA-1) in the presence of alum or CFA and exposed them to natural parasite transmission by the blackfly vectors of *Onchocerca*. Only 58 per cent of vaccinated calves had patent infection, compared with 100 per cent of the unvaccinated control animals, indicating that vaccination to prevent patent infection may be an achievable goal in onchocerciasis, reducing both the pathology and transmissibility of the infection. The impact for a future vaccine, even if partially efficacious on adult worms, is still high.

The *O. volvulus* diffusion chamber mouse model has proven to be not only highly successful in identifying which of the identified recombinant *O. volvulus* antigens will result in the induction of protective anti-larvae immunity, but also in the study of the potential effector mechanisms. It is noteworthy that the

protective mechanisms evoked by the recombinant antigens in *O. volvulus* experiments appear to be similar to those found in humans exposed to L_3 infection (Th1 and/or Th2, IgG1 and IgG3 antibody responses – see Chapter 11) and different from those described for the *O. volvulus* xL_3 mouse model (Th2, IgE and eosinophils). Antigen-specific antibodies may have a role in ADCC effector mechanisms against *Onchocerca* sp., as evidenced by a study showing that human neutrophils can inhibit moulting of L_3 by 96–100 per cent in the presence of purified human antibodies against the vaccine candidate *Ov*-CPI-2.

25.4.2.3 Lymphatic filariasis vaccines

25.4.2.3.1 The use of jirds in vaccine studies for lymphatic filariasis

The Mongolian gerbil (or jird), *Meriones unguiculatus*, is susceptible to *Brugia* infections, and this model of lymphatic filariasis has been useful in studies of protective immunity and vaccination. Jirds mount a significant protective immune response following immunisation with *Brugia* xL_3 and recombinant or purified antigens from filarial nematodes causing LF. Jirds vaccinated with xL_3 of *B. malayi* (or the related feline filarial nematode, *Brugia pahangi*) are protected against challenge infections with healthy L_3s injected through either the intraperitoneal (IP) or the subcutaneous (SC) route. Protection induced following SC infections is more marked, supporting the use of this route of infection in future vaccine studies. In these studies, antibody-to-surface antigens and associated ADCC reactions were associated with protective immunity.

Several LF vaccine antigens (including *Bm*-ALT-1, *Bm*-ALT-2, *Bm*-SXP-1, *Bm*-SL_3, *Bm* A-2 and *Bm*-chitinase) were discovered using cloning strategies similar to that described above for *O. volvulus* antigens; human sera was taken from individuals described as protected (EN or PI) to immunoscreen cDNA expression libraries. A rational approach, combining the use of the extended EST *B. malayi* database of stage-specific transcripts with bioinformatic comparison with the vaccine candidates already identified in *O. volvulus* and proteomic approaches, led to the discovery of *Bm*-CPI-1, *Bm*-ASP-1 (also known as *Bm*-ALT or *Bm*-VAH), *Wb*-GST, myosin, *Bm* Mf S-7 and 175 kDa collagenase as possible LF vaccine antigens.

The protective effects of these antigens has been demonstrated using the *Brugia* jird model (Table 25.4.1), in which the development or survival of the different stages of the LF-causing filarial nematode life cycle can be quantified. Protection can be elicited by targeting the infective L_3 stages (thus also reducing the establishment of infection by adult nematodes) or against the circulating transmissible Mf stages (reducing transmission). Diffusion chamber models have demonstrated that *Bm*-ALT-2 or *Bm*-ASP-1 reduced the viability and survival of *Brugia* L_3 stages by 72 per cent and 62 per cent respectively, and therefore these antigens can reduce successful establishment of adult worms by inducing immune responses that destroy infective L_3.

Often, adult numbers are used as a read-out for protection in *Brugia* jird vaccination studies, although it is unclear whether the vaccine-induced response has targeted the L_3 infective stage larvae, the L_4 stage larvae, the moulting

Table 25.4.1 Common vaccine candidates of *O. volvulus* and lymphatic filariae.

O. volvulus (accession/ gene name)	% Protection (model, adjuvant)	Lymphatic filariae (accession/ gene name)	% Protection lymphatic filariae models (animal model, adjuvant)	Protection in other helminth models (model, adjuvant)
Ov-CPI-2 (M37105)	43–49% (mice, alum/BC)	*Bm*-CPI-1 (AAC47623)	***Ls*-cystatin**: 50% reduction in patent infection (Ls mouse model, alum and Pam3Cys)	***Ac*-cystatin**: 22% reduction in worm burden (Ac dog model, AS03) (Hotez, PJ, unpublished)
Ov-ASP-1 (AF020586)	44% (mice, alum/CFA)	*Bm*-ASP-1 (Bm-VAL-1 /WbVAH) (Bm1_14040)	***Bm*-ASP-1**: 62% reduction in survival of L_3 in chamber (jird, alum) ***Bm*-ASP-1+*Bm*-ALT-2**; 79% reduction in worm burden (jird, alum)	***Ac*-ASP-2**: 26% reduction in worm burden; 69% reduction in eggs output; 60% reduction in L_3 migration *in vitro* using serum from the vaccinated dogs (Ac dog model, AS03)
Ov-RAL-2 (U00693)	51–60% (mice, BC/CFA)	*Bm*-SXP-1 (Bm1_42870)	**r*Wb*-SXP/*Bm*14**: 30% reduction of L_3 survival within chambers (mice, CFA) **r*Bm*-SXP-1**: >90% reduction in microfilaremia and 35% in adult worm burden (jird, CFA or alum)	**r*As*16**: 64% reduction in *A. suum* L_3 (mice, cholera Toxin (CT)) **r*As*16**: 58% reduction in *A. suum* lung-stage L_3s (pigs, CT) **r*Ac*-16**: 25% reduction in worm burden, 64% reduction in egg count (Ac dog model: AS03) (Hotez PJ, unpublished)
Ov-ALT-1 (U96176)	39–62% (mice, alum)	*Bm*-ALT-1 (AF183572) *Bm*-ALT-2 (U84723)	***Bm*-ALT-1**: 76% reduction in worm burden (jird, CFA) ***Bm*-ALT-2**: 72% reduction in survival of L_3 in chamber (jird, alum) ***Bm*-ALT-2 + *Bm*-ASP-1**: 79% reduction in worm burden (jird, alum)	ALT-1 is a filariae specific protein
Ov-103 (M55155)	69% (mice, alum)	Bm1_01550	ND	***Ac*-SAA-1**: antibodies inhibited (46%) migration of L_3 (Ac, CFA)
Ov-B20 (L41928)	39% (mice, alum)	Bm1_05890	49–60% (*Av* jird, CFA)	ND
Ov-RBP-1 (L27686)	42% (mice, BC)	Bm1_45045/ Bm1_33055	36–55% (*Av* jird, CFA)	ND
Ov-CHI-1 (U14639)	53% (mice, DNA)	***Bm*-chitinase** (Bm1_28620 /Bm1_17035)	48% reduction in worm burden and >90% in Mf (*Av* jird, CFA and alum)	ND
Ov-GST	was not protective	***Wb*-GST** (AY195867)	61% reduction in worm burden (*Bm* jird, alum)	***Na*-GST-1**: 32–39% reductions in adult hookworm burdens (Na, hamster, alum) ***Ac*-GST-1**: 39% worm burden reduction (Ac, dog, alum) ***Ac*-GST-1**: 51 to 54% worm burden reduction (Na, hamster, alum)

Species: Ac, *A. caninum*; ALT, abundant larval transcript; ASP, activation associated secreted protein family; *Av*, *A. viteae*; BC, block copolymer; *Bm*, *B. malayi*; CFA, Complete Freund's adjuvant; *Ls*, *L. sigmodontis*; Na, *N. americanus*; ND, not done; *Ov*, *O. volvulus*; *Wb, W. brancofti*. Abbreviations: CHI, chitinase; CPI, cysteine proteinase inhibitor; GST, glutathione-S-transferase; RBP, retinol binding protein; SAA, surface associated antigen; VAH, venom allergen antigen homologue; VAL, VAH/ASP-like.

process (L_3 to L_4 or L_4 to adult) or the adult nematodes themselves. Nevertheless, challenge infections of *Brugia* after immunisation with a collagenase purified from *Setaria cervi* (a bovine filarial nematode), *Wb*-GST (GST from *W. bancrofti*) or *Bm*-ALT-1 (ALT-1 from *B. malayi*) all resulted in protection of between 60 and 80 per cent against *Brugia* challenge infection.

Many antigens are shared between the different life cycle stages of filarial nematodes and can simultaneously target different stages of infection. For instance, it has been shown that *Bm*-37 antigen protects against establishment of infective L_3 stages as well as the Mf stages released during a patent infection. It is sometimes difficult to separate effects of vaccination against different parasite life cycle stages. For example, it has been shown that vaccination with recombinant myosin from *B. malayi* can result in a 54–58 per cent lower adult worm establishment and a 76 per cent reduction in microfilarial burden, the latter of which is not unrelated to the former. However, using Mf as a readout for protection has revealed significant protective effects of immunisation with a microfilarial soluble 38kDa protease isolated from *B. malayi* (*Bm*-38) and immunisation with a zinc-containing 175 kDa collagenase, as well as more than 90 per cent reduction in Mf production after immunisation with *Bm*-SXP-1 or *Bm*-chitinase.

25.4.2.3.2 Shared protective antigens between *O. volvulus*, filarial nematodes causing lymphatic filariasis and other helminth infections

Interestingly (and importantly for future development of vaccines against *O. volvulus* and/or LF) is the observation that seven of the 15 *O. volvulus* antigens that are protective against *O. volvulus* infection are also protective in vaccination models for LF (*B. malayi* infections in mice and/or in jirds, or *A. viteae* infections in jirds and *Litomosoides sigmodontis* infections in mice) see (Table 25.4.1). This indicates that there is a certain amount of cross-reactivity between homologous molecules of filarial nematodes.

Furthermore, homologues of some of these molecules can be found in other non-filarial nematode species, and they are able to elicit reduction in worm burden or other protective measures (examples include hookworm infection in dogs and *Ascaris* in pigs) (Table 25.4.1 and Chapter 25.3). Cross-nematode homologues include *Na*-ASP-2 (*Necator americanus* hookworm homologue of *Ov*-ASP-1 and *Bm*-ASP-1), *Ac*-cystatin (*Acylostoma caninum* hookworm homologue of *Ov*-CPI-2 and *Ls*-cystatin) and r*Ac*-16 and r*As*16, the respective hookworm and *Ascaris suum* homologues of *Ov*-RAL-2 and *Bm*-SXP-1.

Glutathione-S-transferase (GST) in particular, appears to be a common antigen that can protect against nematode infection in general. The hookworm homologues of *Wb*-GST (*Ac*-GST-1 and *Na*-GST-1) have been shown to induce protection and have reduced worm burden against challenge infection with both canine and human hookworms (Table 25.4.1) – so much so that GST is now the top vaccine candidate for the prevention of human hookworm infection and is being made ready for clinical trials in 2013 (see Chapter 25.3). However, it

must be noted that the *O. volvulus* homologue was not protective in previous studies.

25.4.3 Future directions for vaccine development

The most promising vaccine candidates for both filarial infections are the nine antigens summarised in Table 25.4.1. Comparatively, vaccine studies confirm that functionally conserved nematode L_3 proteins essential for the establishment of infection by the filarial nematodes in the final host are the ideal targets for protective immune responses against filarial nematode infection.

In the absence of a perfect small animal model system for *O. volvulus* (which presently can only support testing of the effects of vaccination on L_3 survival/moulting), the most promising approach for the identification of further effective vaccine antigens will be the use of two animal models in parallel (the *O. volvulus* mouse model and the *B. malayi* jird model), each testing relevant species-specific vaccine candidates. This will allow the selection of the most effective vaccine antigens, based on their ability to reduce the establishment of *O. volvulus* infective L_3 stages, which can be extrapolated to subsequent effects on the adult worm and microfilariae burdens, using the *B. malayi* model as a surrogate model for *O. volvulus*. Furthermore, the development of pathology can be studied more easily in the *B. malayi* jird model, allowing confirmation that the vaccine antigens do not exacerbate pathology.

It is predicted that the level of parasite killing measured in the *O. volvulus* diffusion chamber mouse model is less than that observed in natural infections. This is based on the observation that cows immunised with *O. ochengi* xL_3 had a higher level of protection (84 per cent reduction in worm burden) after experimental challenge with L_3 than that observed with xL_3s using the diffusion chamber model (30–60 per cent).

A parallel observation was also made in dogs immunised against the canine filarial worm *Dirofilaria immitis* or jirds immunised with recombinant *Bm*-ALT-2 and/or recombinant *Bm*-ASP-1 and challenged with *B. malayi* L_3. In both systems, although there was a significant killing in the diffusion chambers, the percentage killing was enhanced when adult worm survival was measured *in vivo* (*D. immitis*: 63 per cent vs. 98.2 per cent; *B. malayi*: 62–72 per cent vs. 79 per cent).

The mouse diffusion chamber model has advantages and disadvantages when compared to natural infections in the *B. malayi* jird model, but a combination of the two models will result in a unique robust pre-clinical protective efficacy testing system. As the feasibility of testing vaccine candidates using the *O. ochengi* cattle system has already been demonstrated, one might anticipate the possible use of cows as a confirmatory infection model during the pre-clinical development of the most promising recombinant *O. volvulus* vaccines before moving to human trials.

To date, experimental filarial nematode vaccine models have used primarily alum or CFA as an adjuvant. However CFA is not appropriate for clinical use

and in future pre-clinical development of vaccines against filarial nematodes alum probably is likely to be the leading adjuvant of choice. However emphasis should be also given to other experimental adjuvants for comparison, in order to identify those that will elicit the most effective protective immunity in humans. Some of the adjuvants being developed in clinical testing include monophosphoryl lipid A (MPL), Montanides (ISA51, ISA720), saponin derivatives (Quil-A, ISCOM, QS-21, AS02 and AS01), immunostimulatory oligonucleotides (CpG), flagellin, and derivatives and combinations of some of these adjuvants, such as MPL® formulations or glycopyranosyl lipid adjuvant (GLA) (see Chapter 25.2).

25.4.4 Discovery of new vaccine candidates

Infections of the filarial nematodes are successful because they have the ability to initiate regulatory pathways (Chapter 11). Bypassing this regulation may be the key to development of a vaccine and future disease control. This will require a thorough understanding of how the parasite induces regulation, and identification of the targets and processes that mediate a protective (but non-pathological) response. A large-scale proteomic analysis of the excretory/secretory (ES) products of the L_3, L_3 to L_4 moulting, adult male, adult female and Mf stages of *B. malayi* provides extended insight into the host-parasite interaction. Recent reports, indicating that there is an abundance of a number of previously characterised immunomodulatory proteins in the ES of Mf, demonstrates how proteomic analysis can direct research efforts in this area by identifying novel vaccine candidates.

The recent availability of comprehensive genomic, proteomic and transcriptomic datasets from filarial nematodes (both human- and non-human-infecting filarial nematodes) provides a fresh start by permitting a more rational approach to vaccine discovery. Proteome-wide screening of antibody and T cell reactivity, using specimens from individuals exposed to filariae but protected from chronic infections and disease, might provide information that can be mined to identify new vaccine candidate antigens.

The existence of the *B. malayi* genome, other filaria and, hopefully soon, that of *O. volvulus* (www.sanger.ac.uk/sequencing/Onchocerca/volvulus/; www.broadinstitute.org/annotation/genome/filarial_worms/GenomesIndex.html), when combined with technology platforms such as protein arrays, high throughput protein production and epitope prediction algorithms, may in the future provide an opportunity to identify antigens that function as targets of natural acquired immunity against filariae. This approach permits investigators to perform large-scale sero-epidemiological, longitudinal, and sero-surveillance analyses, in addition to assessment of immunoreactive responses at various stages of the infectious process in a manner not possible with other technologies.

'Immunomic' approaches, which enable the selection of the best possible targets by prioritising antigens according to clinically relevant criteria, may overcome the problem of poorly immunogenic, poorly protective vaccines that has plagued anti-parasite vaccine developers for years.

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Abbreviations Used in This Book

α-GalCer	α-galactosylceramide
ABA	*Ascaris* body antigen
ABRA	acidic basic repeat antigen
Ad	adenovirus
ADCC	antibody dependent cellular cytotoxicity
ADCI	antibody dependent cellular inhibition
AIDS	acquired immunodeficiency syndrome
Aldh1a2	a gene encoding retinaldehyde dehydrogenase-2
ALT	abundant larval transcript
AMA-1	apical membrane antigen-1
AMA-C1	vaccination candidate for malaria containing correctly folded ectodomain portions of recombinant AMA-1 from two different *P. falciparum* clones (FVO and 3D7)
AP	anti-coagulant protein
APAS-3	allergenic protein from *Ascaris suum*-3
APC	antigen presenting cell
APR	aspartic protease
Apo-L1	apolipoprotein L1
ARG-1	the gene encoding arginase
AS02	an oil in water emulsified adjuvant containing MLA and QS21
ASP	*Ancylostoma* secreted protein
ATP	adenosine triphosphate
AWA	adult worm antigens
BBB	blood brain barrier
BCG	Bacillus Calmette-Guérin vaccine against *Mycobacterium tuberculosis*
Bcl-2	B cell lymphoma-2
BCR	B cell receptor
BIR	baculovirus inhibitor of apoptosis protein repeat
C1-INH	complement component 1-inhibitor
CARD	caspase activation and recruitment domain
CCL	chemokine with two adjacent N- terminal cysteine (C) residues
CCK	cholecystokinin
CCR	chemokine receptor that binds to CCL chemokines
CCTH	cryptomonads, centrohelids, telonemids and haptophytes

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CD	cluster of differentiation
cDNA	complementary DNA
CGMP	current good manufacturing practices
CEF	chicken embryo fibroblasts
CFA	complete Freund's adjuvant
CHI	chitinase
CIA	collagen-induced arthritis
CL	cutaneous leishmaniasis
CM	central memory
CM	cerebral malaria
CNS	central nervous system
CP	cysteine protease
CPB	cysteine protease B
CPG DNA	-C-phosphate-G- DNA
CPI	cysteine proteinase inhibitor
CrD	Crohn's disease
CRH	corticotropin-releasing hormone
CRP	complement regulatory protein
CSA	chondroitin sulphate A
CSL	circumsporozoite-like antigen
CSP	circumsporozoite protein
CTL	cytotoxic lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
CVID	common variable immunodeficiency
CWP	cyst wall protein
CXCL	chemokine with two N-terminal cysteine (C) residues that are separated by another amino acid (X)
CXCR	chemokine receptor that binds to CXCL chemokines
DAF	decay accelerating factor
DALYs	disability adjusted life years
DAMP	tissue damage-associated molecular patterns
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEREG	depletion of regulatory T cells
DiAg	*Dirofilaria immitis*-derived antigen of 15kDa
DMG	dimyristoylglycerol
DNA	deoxyribonucleic acid
DoA	dog hookworm adult antigens
DoL	dog hookworm larval antigens
DSS	dextran sodium sulphate
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalitis
EBV	Epstein-Barr virus
EGF	epidermal growth factor
ELISA	enzyme linked immuno-assay
ELISPOT	enzyme-linked immuno-SPOT assay
EM	effector memory cells
EMCF	Edna McConnell Clark Foundation
EN	endemic normals
ER	endoplasmic reticulum

Erk	extracellular signal-regulated kinases
E/S	excretory/secretory
ES-62	excretory/secretory molecule of 62kDa from the filarial nematode *Acanthocheilonema vitea*
ESA	excretory-secretory antigen of *Heligosomoides polygyrus*
ESAGs	expression site-associated genes
EXP-1	exported protein-1
FBA	fructose 1,6 bisphosphate aldolase
Fizz-1	found in inflammatory zone-1
FoxP3	forkhead box protein-3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA-3	transcription factor-3 able to bind to the DNA sequence 'GATA'
GIP-VSG	glycosylinositolphosphate residue of the GPI anchor of *Trypanosoma brucei*
GI	gastrointestinal
GITR	glucocorticoid-induced TNFR-related protein
GLA	glycopyranosyl lipid adjuvant
GlcNAc	N-Acetyl-Glucosamine
GLURP	glutamate-rich protein
GP63	glycoprotein of 63kDa
gp120	glycoprotein of 120kDa
GPI	glycosylphosphatidyl inositol
GPI-PLC	glycosylphosphatidyl inositol-specific phospholipase-C
Gr-1	granulocyte differentiation antigen-1
GRAIL	gene related to anergy in lymphocytes
GRO-α	growth-regulated oncogene
GST	glutathione-S-transferase
HAART	highly active antiretroviral therapy
HASPB	hydrophilic acylated surface protein, B family
HAT	human African trypanosomiasis
HAV	hepatitis A virus
hBD-1	human β-defensin-1
HBsAg	Hepatitis B surface antigen
HDL	high density lipoprotein
HEP17	hepatocyte erythrocyte protein 17
HHVI	human hookworm vaccine initiative
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IAG	immunological activity group
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IDO	indoleamine 2,3 dioxygenase
IEC	intestinal epithelial cells
iE-DAP	g-D-glutamyl-mesodiaminopimelic acid
IFN	interferon
IgA	immunoglobulin subtype A
IgE	immunoglobulin subtype E
IgG	immunoglobulin subtype G
IgM	immunoglobulin subtype M
IKK	IκB kinase
IL	interleukin

INF	infected individuals
iNOS	inducible nitric oxide synthase
IP	intra-peritoneal
IPSE/α1	IL-4-inducing principle of *Schistosoma mansoni* eggs / alpha-1
IPTp	intermittent preventative treatment of malaria in pregnancy
IRAK	IL-1 receptor-associated kinase
IRG	interferon inducible p47 GTPases
IRIS	immune reconstitution inflammatory syndrome
ISA	Immune stimulating adjuvant
ISCOM	Immune stimulating complexes
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
iTreg	inducible T regulatory cell
ITS	internal transcribed spacer
IV	intra-venous
Jak	janus kinase
J_HD	heavy-chain knockout mice
JNK	c-Jun N-terminal kinase
kDNA	kinetoplastid DNA
KIR	killer inhibitory receptor
KMP11	kinetoplastid membrane protein 11
KSAC	polyprotein vaccine antigen from *Leishmania* composed of KMP11, SMT, A2 and CPB
L_3	infective third-stage larvae of filarial nematodes
L_4	fourth-stage larvae of filarial nematodes
LacDiNAc	glycan structures GalNAcb1-4GlcNAc
LACK	*Leishmania* homologue of receptors for activated C kinase
LEF	large exoerythrocytic form
LeIF	*Leishmania* elongation initiation factor
LF	lymphatic filariasis
LFA	lymphocyte Function-Associated
LL	laminated layer
LmSTI1	*Leishmania major* homologue to eukaryotic stress-inducible protein
LPG	lipophosphoglycan
LPS	lipopolysaccharide
LSA	liver-stage antigen
LTR	long terminal repeat region
Lyso-PS	lysophosphatidylserine
mAb	monoclonal antibody
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MASPS	mucin-associated proteins
MBL	mannose-binding lectin
MD2	myeloid differentiation factor-2
MDA	mass drug administration
MDA5	melanoma differentiation-associated gene-5
MDP	muramyl dipeptide
MEP	metalloendopeptidase
Mf	microfilariae
MHC	major histocompatibility complex

MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
mL_3	moulting third-stage larvae of filarial nematodes
MLN	mesenteric lymph nodes
MM7	matrix metalloproteinase 7 (also known as matrilysin)
mMCP	mouse mast cell protease
MPL	monophosphoryl lipid A
MPL-SE	monophosphoryl lipid A in stable emulsion
MS	multiple sclerosis
MSP-1	merozoite surface protein-1
MTCT	mother-to-child HIV transmission
mtDNA	mitochondrial DNA
MTP	metalloprotease
MVA	modified *Vaccinia* virus Ankara
NADPH	reduced form of nicotineamide adenine dinucleotide phosphate$^+$
MyD88	myeloid differentiation primary response gene-88
NBL	new born larvae
NCC	neurocyticercosis
NET	neutrophil extracellular trap
NF-κB	nuclear factor κ-light-chain enhancer of activated B cells
NIF	neutrophil inhibitory factor
NK	natural killer cell
NKT	natural killer T cell
NKBP	natural killer cell-binding protein
NLR	nucleotide-binding oligomerisation domain (NOD)-like receptor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NO	nitric oxide
NOD	non-obese diabetic mice
NOD	nucleotide-binding oligomerisation domain-containing proteins
NTD	neglected tropical disease
nTreg	natural T regulatory cell
NTS	non-typhoid *Salmonella*
Ov-RAL	*Onchocerca volvulus*-reactive with antiserum against larvae
Ov-RBD	*Onchocerca volvulus*-retinol binding protein
TMY	tropomyosin
PAM	pregnancy associated malaria
PAM- 3-Cys	a synthetic lipopeptide: (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride
PAMP	pathogen-associated molecular pattern
PAS-1	protein from *Ascaris suum*-1
PBMC	peripheral blood mononuclear cells
PC	phosphorylcholine moiety
PCA	passive cutaneous anaphylaxis
PD-1	programmed death-1
PDL-1	ligand for the PD-1molecule
PEXEL	*Plasmodium* export elements

*Pf*EMP-1	*Plasmodium falciparum* erythrocyte membrane protein-1
*Pf*s	*Plasmodium falciparum* schizont antigen preparation
*Pf*s25	25kDa surface protein from *Plasmodium falciparum*
*Pf*s28	28kDa surface protein from *Plasmodium falciparum*
*Pf*s48/45	48kDa/45kDa surface protein from *Plasmodium falciparum*
*Pf*s230	230kDa surface protein from *Plasmodium falciparum*
PGE2	prostaglandin E2
PI	protease inhibitor
PI	putatively immune
PI3K	phosphoinositide-3-kinase
pIgR	poly immunoglobulin receptor
PKC	protein kinase-C
PKDL	post Kala-azar dermal leishmaniasis
pMHC	peptide-loaded MHC
PMN	polymorphonuclear
PRR	pattern recognition receptor
PSA	polysaccharide antigen of *Bacteroides fragilis*
PSG	promastigote secretory gel
PV	parasitophorous vacuole
PZQ	praziquantel
QS-21	saponin derivative from the soap-bark tree *Quillaja saponaria*
Quil-A	purified saponin fraction obtained from the soap-bark tree *Quillaja saponaria*
RA	radiation attenuated
RA	retinoic acid
RAG	recombination activating gene deficient mouse (with no adaptive immune cells)
RANTES	regulated upon activation, normal T cell expressed and secreted
RBC	red blood cell
RBP	retinol binding protein
rDNA	ribosomal DNA
RELM	resistin-like molecule
RESA	ring-infected erythrocyte surface antigen
RhA	rheumatoid arthritis
RIG-I	retinoic acid inducible gene-I
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rNAPc2	recombinant anticoagulant protein c2 from *Ancylostoma duodenale*
ROI	reactive oxygen intermediates
PRR	pattern recognition receptor
RTS,S	candidate anti-malarial vaccine composed of the repeat region of CSP (R), the carboxy terminal region of CSP containing T cell epitopes (T) fused to hepatitis B surface antigen (S) and surface subunits (S)
SAA	surface-associated antigen
SAR	stramenopiles, alveolates and *Rhizeria*
Sc	sub-cutaneous
SCFA	short chain fatty acids

SCID	severe combined immunodeficiency (no functional adaptive cells)
SEA	Schistosome egg antigen
SEM	scanning electron microscopy
SERA	serine-repeat antigen
Siat4c	sialyltransferase 4C
SMA	severe malarial anaemia
SmLRR	*Schistosoma mansoni* leucine-rich repeat protein
SMT	sterol 24-c-methyltransferase
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
S*Pf*66	candidate anti-malarial vaccine composed of peptides from erythrocytic stage antigens of *Plasmodium falciparum*
SRA	serum resistance-associated
SSU	small subunit
STAT	signal transducer and activator of transcription
sTNF-RII	soluble tumour necrosis factor receptor II
SWA	Schistosome soluble worm antigen
T1D	type 1 diabetes
TAK	TGF-β-activated kinase
TAP	transporters associated with antigen processing
TARC	thymus and activation-regulated chemokine
Tat	transcriptional transactivator
T-bet	T-box expressed in T cells
TBI	transmission blocking immunity
TBK	TANK-binding kinase
TBV	transmission blocking vaccine
Tc	cytotoxic T cell (CD8+ T cell)
Tc52	*Trypanosoma* cruzi protein of 52kDa
TCR	T cell receptor
TCS	tricarboxylic acid cycle
TE	toxoplasmic encephalitis
TEM	transmission electron microscopy
TES	*Taenia* excretory / secretory soluble antigens
TFF	trefoil factor
TGF-β	transforming growth factor-β
Th	T helper cell
TIMP	tissue matrix metalloproteinase inhibitor
TIR	Toll / IL-1 receptor
TLF	Trypanosome lytic factor
TLR	Toll-like receptor
TMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor-α
Tpl2	tumour progression locus-2
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRAP	thrombospondin-related adhesive protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
Treg	T regulatory cell
ts	trans-sialidase

TSA	thiol-specific antioxidant
TSLP	thymic stromal lymphopoietin
TSOL-18	*Taenia solium* antigen of 18kDa
TSOL-45-1A	*Taenia solium* antigen of 45kDa, homologous to *To*45W
UC	ulcerative colitis
UIS	up-regulated in sporozoites
UNC93B1	12-membrane spanning protein found in the endoplasmic reticulum
VAH	venom allergen antigen homologue
VAL	VAH/ASP-like
VAT	variant antigen type
VCAM	vascular cell adhesion molecule
VL	visceral leishmaniasis
VSA	variant surface antigen
VSG	variant surface glycoprotein
VSP	variant-specific surface protein
WoLP	*Wolbachia* lipoprotein
WSP	*Wolbachia* surface protein
XID	X-linked immunodeficiency
xL_3	X-ray attenuated third-stage larvae of filarial nematodes
XLAAD	X-linked autoimmunity-allergic dysregulation syndrome
ZYA	zymosan-induced arthritis

Glossary

Antibody-dependent cellular cytotoxicity: immunological effector mechanism, mediated by antibodies, which acts to inhibit the survival of pathogens by inducing NK cell-mediated cell lysis.

Antibody-dependent cellular inhibition: immunological effector, mechanism mediated by antibodies, which acts to inhibit the survival of pathogens by inducing phagocytosis.

Achromatic zone: a histological term used to describe an area in the body of protozoan parasites that does not stain with chromatin dye.

Acute phase response: an innate immune response, initiated by macrophages, involving the production of the cytokines IL-1, IL-6 and TNF and the secretion of acute phase proteins from the liver. This reaction is associated with the induction of fever.

Adjuvant: a substance used in vaccines to stimulate or aid an immune response to purified vaccine antigens. (Latin: *adjuvare*)

Agglutinate: a clump of pathogens in a complex with antibodies.

Agonist: a molecule or particle that triggers the activation of a receptor upon binding.

Allergen: an innocuous antigen that can cause an allergic response by triggering a type 1 hypersensitivity reaction.

Allergic airway hyper-responsiveness: an allergic reaction that occurs in the airways due to inhalation of an allergen; leads to breathing difficulties.

Alloreactivity: reaction of immune components such as antibodies or immune cells towards molecules that are shared by some other individuals, but not by others (e.g. MHC molecules from different alleles).

Altruistic vaccine: vaccines that do not protect the vaccinated individual but prevent transmission of the infection to other individuals.

Alum: an adjuvant composed of aluminium hydroxide that can precipitate antigen and release it slowly in the body.

Amastigotes: an intracellular life cycle stage of some protozoan parasites of the *Kinetoplastida* class that do not possess a flagellum.

Amphidial glands: secretory glands containing anti-coagulant proteins found in hookworms.

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Anaemia: a decrease in the amount of circulating haemoglobin in the blood as a result of reduced number of red blood cells or a haemoglobin deficiency. This can result in a hypoxic environment in the organs.

Anaphylaxis: a systemic allergic reaction leading to the systemic release of inflammatory mediators throughout the body and a swelling of the airways leading to difficulty breathing.

Anaphylotoxin: small fragments of components of the complement cascade pathway (primarily C3a and C5a) that can bind to specific receptors and induce an inflammatory response.

Anergy: a state of immune unresponsiveness.

Aneurysm: a weakness in a blood vessel wall causing a balloon-like bulge in the vessel.

Angioedema: an inflammatory reaction in the dermis of the skin, leading to swelling and oedema such as that found around the eyes and lips during an allergic reaction. This is a similar to hives, in which the reaction occurs in the in the upper dermis.

Angiogenesis: the growth of new blood vessels from pre-existing vessels.

Anorexia: inability to consume food due to loss of appetite.

Antagonist: a molecule or particle that inhibits the activation of a receptor upon binding.

Anthropophilic: a preference for humans over other animals.

Anthroponotic transmission: transmission from humans to animals (opposite of zoonotic transmission) or transmission between humans.

Anti-haemostatic: an agent that causes bleeding.

Apicoplast: a non-photosynthetic plastid found in Apicomplexan protozoan parasites and derived from an ancient symbiotic relationship with an algae.

Apoptosis: a process in which cells are induced to die in a controlled manner via programmed cell death; apoptotic cells are cleared by phagocytic cells without inducing inflammation.

Arrhythmias: conditions that cause an abnormal heartbeat that can be too fast, too slow or dysregulated.

Asthenia: muscle weakness.

Attenuated: reduction in the ability of an infectious organism to undergo replication or cause disease.

Autoantibodies: antibodies that react with 'self' proteins.

Autoimmune: immune responses generated against 'self' proteins causing damage to the body.

Autoinfection: the infection of a primary host whereby the complete life cycle of the parasite occurs in a single host without the requirement for another host.

Autologous: in reference to transplantation, a transplant in which the donor and the recipient are the same person.

Autophagy: a process of 'autophagocytosis' whereby a cell degrades its own components by using lysosomal machinery present in the cell.

Axostyle: a flexible sheet of microtubules found at the base of the flagella of flagellated protozoan parasites. This structure generally contributes to the movement of the parasite.

Balancing selection: the balancing of selective processes that maintain the number of alleles for a gene in a population at frequencies above the rate of mutation, for example when heterozygotes have a reproductive or survival advantage over homozygotes. This process conserves genetic polymorphism.

Binary fission: the division of a cell in half during asexual reproduction in protozoan parasites.

Bothria: weak muscular structures found on cestodes that can form suckers for attachment.

Bradyzoite: the dormant encysted stage of *Toxoplasma* parasites found in the tissue of infected individuals.

Calabar swelling: a transient subcutaneous swelling, usually on the extremities, caused by angioedema and characterised by swollen lumps of subcutaneous tissue.

Cell line: a continuously dividing culture of cells, normally derived from tumours, in which cell division is uncontrolled. Examples include Caco2, a human cell line derived epithelial colorectal adenocarcinoma (see Chapter 6), or HELA cells derived from cervical cancer of Henrietta Lacks.

Chancre: a localised ulcerated skin reaction resulting from the bite of a Tsetse fly.

Chorioretinitis: inflammation of the choroid (coating) and retina (inner surface) of the eye.

Chromatoid bodies: structures of accumulated RNA in the cytoplasm of some amoebae.

Chronic: a persistent or long-lasting infection, normally in excess of three months.

Chemokine: chemoattractant proteins that induce the migration of immune cells.

Chylocele: a cyst-like lesion caused by the effusion of chyle (a cloudy pale liquid containing emulsified fats from the digestive tract) to the genitals (in males infected with filarial nematodes, normally the sheath of the testis).

Clade: a phylogenetic grouping containing all descendants of a common ancestor.

Clonal: genetically identical cells (single-celled organisms or immune cells) descended from a common ancestor.

Cognate antigen: an antigen that is related in origin; commonly used to describe antigen that is recognised by both B cells and CD4+ T cells, allowing the acquisition of T cell help by B cells during activation.

Commensal: a relationship between two organisms, in which one organism derives benefit while the other is unaffected.

Complement pathway: a set of sequentially activated plasma proteins that leads to the formation of pores in the membrane of an invading pathogen.

Concomitant immunity: see 'premunition'.

Congenital: a condition that already exists at birth.

Conoid: a cone-shaped structure in Apicomplexan parasites that is used for cell invasion.

Coprological: anything that is faecal in origin.

Co-stimulation: the second signal required for expansion and activation of adaptive immune cells (CD4+ T cells, CD8+ T cells and B cells).

Cristae: compartments formed from the inner membrane of the mitochondrion.

Cross-presentation: the presentation of exogenous antigen on MHC-I molecules of antigen-presenting cells.

Cross-priming: the priming of CD8+ T cells to exogenous antigen by dendritic cells.

Cruzipain: a cysteine protease secreted by *Trypanosoma cruzi*.

Cryptic antigens: epitopes on proteins that cannot be recognised by the receptor of an immune cell unless the protein is broken down and processed first.

Cysticercosis: tissue infection with cysticerci of tapeworms.

Cytolytic: destruction of cells by lysis.

Cytophilic antibody: antibodies that have an affinity for cells. The cytophilic subclasses IgG1 and IgG3 are effective opsonins and mediate a number of immune effector mechanisms.

Cytostome: a feeding organelle used to ingest material by protozoan parasites of the class *Euglenozoa*.

Danger Hypothesis: a hypothesis proposed by Polly Matzinger in 1994 to explain why immune responses occur to some antigens and not others. This hypothesis superseded the 'self /non-self' hypothesis, which stated that the immune system reacts to anything foreign (non-self) but not to molecules making up the human body ('self'). Rather than focusing on immune reactions to foreign non-self antigens, the danger hypothesis focuses on immunoreactivity to anything which is 'dangerous' to the body, whether it be infection or molecules released upon necrosis of tissue.

Dauer: a developmental stage of nematodes in which the larvae enter a dormant stage, enabling survival through harsh environmental conditions.

Defensins: anti-microbial pore-forming peptides that are capable of causing death of microorganisms via pore formation.

Definitive host: the host in which a parasite reaches sexual maturity and reproduces.

Dense granules: secretory organelles found in Apicomplexan parasites.

Dermatitis: inflammation of the skin, normally caused by an allergic reaction.

Digenetic: parasites that can alternate between sexual and asexual reproduction.

Dioecious: parasitic species that have separate males and females.

Disability adjusted life years: a measure of overall health burden of disease, as measured by the number of years lost due to ill-health or disability caused by a particular disease.

Distal: in reference to the GI tract, a point situated down the tract away from the mouth; opposite to proximal.

Diurnal periodicity: a circadian rhythm that favours the release or formation of transmissible stages during the day in some parasitic infections, to coincide with the biting habits of the relevant vector, in turn maximising the chance of transmission.

Duffy antigen: an antigen located on the surface of red blood cells that is a non-specific receptor for some chemokines and a receptor for the malaria parasite *Plasmodium vivax*.

Dysbiosis: an imbalance of microbial infection in the body.

Ectoparasite: a parasite that lives on the surface of the body.

Embryonated ova: eggs of *Ascaris* nematodes that contain a larval embryo.

Endocytosis: the process of uptake of molecules or microorganisms by phagocytic cells.

Endoparasite: a parasite that lives inside the body.

Endosymbiont: an organism that lives within the body of another, normally providing essential nutrients to the host (an obligate endosymbiont).

Enteropathy: a term that refers to pathology of the intestine.

Eosinophilia: increased numbers of eosinophils in the bloodstream.

Epitope: a portion of an antigen that is recognised by an antigen receptor on immune cells.

Erythema: a redness of the skin caused by an inflammatory reaction and increased blood flow to the area.

Erythrophagocytosis: endocytosis of red blood cells, including healthy and undamaged red blood cells, by phagocytes.

Erythropoiesis: the production of red blood cells from stem cell progenitors.

Extravasation: the traversal of immune cells across endothelial layers from the bloodstream into the tissue.

Fc receptors: receptors that bind to the crystallisable fragment of antibodies containing the constant region.

Fe-S cluster assembly: iron-sulphur clusters required for the functioning of several biochemical pathways in microorganisms.

Fibril: thread-like structures in muscle tissue.

Filaricele: an accumulation of straw-coloured lymph fluid resulting in enlargement of the scrotal sac in males infected with filarial nematodes.

Flagellate: protozoan parasites that have one or more flagella for movement.

Freund's adjuvant: an adjuvant composed of paraffin mineral oil and mannide monooleate which is emulsified with antigen to allow slow release of the antigen into the body; complete Freund's adjuvant contains the addition of inactivated and dried *Mycobacterium tuberculosis* for potent immunostimulatory activity.

Gametogenesis: the differentiation of precursor cells into mature gametes.

Gamma globulin: antibodies of the IgG isotype.

Glycocalyx: the carbohydrate-rich extracellular coating on the surface of some helminth species.

Glycoproteins: proteins containing covalently-attached oligosaccharide chains.

Glycosome: a membrane-bound organelle that contains glycolytic enzymes and is found in some protozoan parasites of the *Kinetoplastida*.

Gonochoristic: see 'dioecious'.

Granuloma: the formation of a cage-like structure of immune cells initiated by macrophages to contain pathogens.

Haematophagous: blood-feeding; an example of a hematophagous insect is a mosquito.

Haemolymph: fluid found in the circulation of insects that contains components analogous to those normally found in both blood and lymph of mammals.

Haematopoiesis: the process of differentiation and development of blood cells from stem cell precursors.

Haptenisation: the combination of a small antigenic component onto a carrier protein to increase the molecular weight for immune stimulation.

Hepatosplenomegaly: simultaneous enlargement of the liver and spleen.

Hepta-laminate: structure of seven layers.

Heterogonic: a life cycle that has both parasitic and free-living stages.

Heterotrophic: an organism that cannot fix carbon and requires consumption of organic carbon to generate carbohydrates, fats and proteins.

Heterologous antigen: an antigen that can cross-react with antibodies or T cells activated by other antigens.

Holoendemic: a disease in which most of the members of a population are infected.

Homeostasis: the regulation of immune cells in the body to maintain a stable number.

Homologues: the same, or similar, in relation to structure.

Hyaline: a substance that appears pink and glassy upon staining with haemotoxylin and eosin.

Hydatid disease: a disease caused by infection with the cestode *Echinococcus* in which cysts form in the liver and lungs.

Hydrocele: the accumulation of fluid in a body cavity, such as the scrotal sack in the case of some males infected with lymphatic filariasis.

Hydrogenosomes: organelles present in Trichomonads that contain a number of enzymes facilitating the production of hydrogen, carbon dioxide and ATP.

Hyperplasia: an increased number of cells, resulting in enlargement of an organ.

Hypnozoites: a dormant stage of pre-erythrocytic malaria parasites in hepatocytes; specific to *Plasmodium vivax* and *Plasmodium ovale* species.

Hypobiotic: an organism in an arrested stage of development.

Immune compromised: individuals who are unable to mount an effective immune response because of a deficiency in one or more facets of the immune system.

Immunological memory: the ability of activated antigen-specific adaptive immune cells to persist in the body after a primary immune response, providing a fast and efficient immune response to the same antigen upon a secondary challenge (e.g. upon reinfection or a vaccination booster).

Immunopathology: pathology directly related to the immune response, rather than to the invading pathogen.

Inflammasomes: molecular platforms containing caspases which act to mature the inflammatory cytokines IL-1-β and IL-18 and initiate an inflammatory innate immune response.

Inflammation: an immune response which leads to vasodilation and the accumulation of fluid and white blood cells, resulting in redness and swelling.

Intermediate host: a host that harbours a parasite for a short period of time, normally during one or more developmental stages of the life cycle.

***in utero*:** within the womb.

***in vitro*:** within the test-tube; commonly used to refer to experiments carried out in tissue culture.

***in vivo*:** within the living organism.

Inactivation by irradiation: exposure to ionising radiation that leads to irreparable (and terminal) damage to the DNA of the exposed organisms.

Katayama fever: a fever associated with acute schistosomiasis, in particular the migration of larvae to their final destination in the portal vein and the initial production of schistosome eggs.

Keratitis: inflammation of the cornea of the eye.

Kinetosome: an organelle composed of a centriole and an array of microtubules which forms a point of attachment for flagella.

Kinins: polypeptides that cause contraction of the smooth muscle and vasodilation by inducing the release of prostacyclins. Kinins can reduce blood pressure.

Kupffer cell: macrophages found in the lining of the sinusoids of the liver that form part of the reticuloendothelial system.

Latency: the ability of a pathogen to remain dormant; can also refer to the time between exposure to a pathogen and the appearance of symptoms of a disease.

Leishmaniasation: method of vaccination against *Leishmania* by deliberate inoculation of live viable *Leishmania* parasites.

Leukotrienes: lipid mediators of inflammation synthesised from arachidonic acid and produced by several immune cell types including macrophages and granulocytes.

Life history: another term used to describe the life cycle of an organism.

Lymph nodes: secondary lymphoid organs found throughout the body, in which local immune responses are generated.

Lymphadenopathy: abnormal lymph nodes, most commonly in relation to enlarged lymph nodes during infection.

Lymphadenitis: inflammation of the lymph nodes involving a cellular infiltrate.

Lymphangiogenesis: the formation of new lymphatic vessels from pre-existing lymphatic vessels; similar to angiogenesis in the development of new blood vessels.

Lymphangitis: an infection of the lymphatic vessels.

Lymphocele: accumulation of lymph fluid in a body cavity – in the case of males, individuals infected with lymphatic filariasis in the scrotal sack.

Lymphoedema: localised swelling due to lymph accumulation, normally due to an obstruction in the lymphatic vessel.

Lymphoid organ: organs of the body pertaining to the immune system.

Lysosome: vesicular organelles containing acid hydrolases for break down of engulfed materials upon fusion with phagosomes.

Macropinocytosis: the process by which small molecules enter cells by the invagination of the membrane and formation of small vesicles; primarily used for absorption of extracellular fluids into a cell ('cell-drinking').

Maculae: skin lesion, commonly caused by entry of schistosome cercariae, in which the colour of the skin is altered but the lesion is flat and undetectable by touch.

Maurer's clefts: disc-like structures tethered both to the red blood cell membrane and to the parasitophorous vacuole membrane in malaria-infected red blood cells; slender membrane extensions connect neighbouring Maurer's clefts on the same red blood cell.

Merozoite: a life cycle stage of Apicomplexan parasites, normally the first stage in an intracellular part of the life cycle of the parasite.

Meningoencephalitis: infection and inflammation of the brain and the meninges (a system of membranes making up the central nervous system).

MHC haplotype: the set of particular MHC alleles found in an individual.

MHC-II restriction: the limitations placed on T cell receptor recognition by particular MHC alleles, which themselves influence binding of the peptide-loaded MHC complex to the T cell receptor.

Micronemes: electron-dense secretory organelles found in Apicomplexan parasites near the apical end of the parasite; involved in cell invasion.

Mitogen: molecules that can induce cell proliferation of a cell upon binding by triggering signalling pathways that induce mitosis.

Mixotrophic: an organism that obtains energy from different sources, e.g. by direct fixation of carbon as well as from consumption of organic carbon sources.

Monoclonal: a population of T or B cells that have identical clonotypical receptors because they have arisen from a single clone. In reference to antibodies, monospecific antibodies made by identical plasma cells that have arisen from a single clone; the opposite of polyclonal.

Monoecious: having male and female sex organs on the same individual.

Monophyletic: a group of organisms that form a clade, containing all the descendants of the same ancestor.

Mucosal hyperplasia: an increase in the number of cells in the mucosa.

Multivalent antigen: an antigen that containing two or more antibody-binding sites allowing the formation of antibody-antigen complexes and cross-linking of Fc receptors.

Multivariate model: a statistical model that incorporates and simultaneously analyses multiple variables that could contribute to a given observation.

μMT: a genetically modified mouse strain in which the heavy chain of IgM antibodies (μ-chain) has been deleted, preventing the successful development of B cells and resulting in B cell deficiency.

Myalgia: muscle pain.

Myeloid: in reference to white blood cells, any cell that is not from the lymphoid lineage.

Ngana: a wasting disease of cattle caused by infection with *Trypanosoma brucei*.

Naïve: an individual or laboratory animal that has not been exposed to the infection under study.

Natural antibody: low-affinity antibodies with a broad specificity; normally of the IgM isotype found in the serum in the absence of any apparent infection.

Nemepsins: nematode-specific hydrolases.

Neutrophilia: increased numbers of neutrophils in the bloodstream.

Neurocyticercosis: a disease of the central nervous system caused by infection with the larval stages of *Taenia solium* tapeworm and the formation of cysts in the brain; causes seizures.

New permeation pathways: channels that become activated in red blood cells infected with malaria parasites, increasing the permeability of the red blood cells to solutes such as amino acids, ionically charged molecules and carbohydrates.

New world: a term of reference to the Americas and Australasia.

Nude mice: a laboratory mouse strain which has a mutation that causes the thymus not to develop, in turn leading to an absence of mature T cells capable of participating in an immune response.

Oedema: an accumulation of fluid beneath the skin or in the body cavities, leading to swelling.

Old world: a term of reference to Europe, Asia and Africa.

Oocyst: a thick-walled structure containing the developing zygote of Apicomplexan parasites.

Ookinete: the motile diploid zygote stage of malarial parasites formed in the mosquito stomach by fertilisation of a macrogamete by a microgamete.

Opsonic: any molecule of the immune system that has the capability of coating particulate matter promoting engulfment by phagocytosis. Examples include antibodies or acute phase proteins.

Orthologues: homologous gene sequences found in different species that were separated by a speciation event in a common ancestor.

Ovalbumin: a protein antigen found in the white of chicken eggs; used to model antigen-specific immune responses.

Ovalbumin hypersensitivity model: a model of hypersensitivity in which an immune response is generated to ovalbumin protein, and a subsequent immune challenge with ovalbumin protein leads to a delayed-type hypersensitivity reaction.

Overdispersed frequency distribution: an epidemiological term which describes the frequency of the distribution of an infection among a population which is more variable than expected. For example, within an endemic population, a few individuals harbour heavy burdens of *Ascaris* parasites ('wormy individuals'), whereas most individuals have light or no infections.

Oviparous: species that lay eggs (as opposed to giving birth to live offspring).

Oxazolone: a chemical allergen that gives rise to a delayed-type hypersensitivity reaction.

Papulae: skin lesions commonly caused by entry of schistosome cercariae, in which the skin is red and elevated but with no visible fluid underneath.

Parabasal bodies: cytoplasmic bodies found in flagellate parasites modified from the Golgi and associated with the kinetostomes at the base of flagella.

Parasitophorous vacuole: a cellular compartment enclosing an engulfed parasite, often formed by invaginated cell membrane during phagocytosis.

Paratenic host: a host which is dispensable for the development of a particular parasite species, but which serves to maintain the life cycle.

Parenteral: a route of administration that does not involve the alimentary tract; normally one that involves piercing of the skin.

Parthogenetic: arising from asexual reproduction.

Patent infection: the appearance of detectable parasites in the periphery or faeces.

Pattern recognition receptors: receptors of the innate immune system that recognise common patterns on pathogen molecules.

Pedunculate: growing on, or from, a stalk.

Peri-bronchial inflammation: inflammation surrounding the bronchi of the respiratory tract.

Peri-urban: the area between the suburbs of a city and the countryside.

Peroxisomes: organelles involved in the synthesis of metabolites such as fatty acids and polyamines.

Phagocytosis: the process of 'cell-eating', in which phagocytes (cells capable of phagocytosis) internalise particular matter.

Phasmids: sensory structures in nematodes.

Phosphorylation: the addition of a phosphate group to a molecule; a common method of activating proteins involved in intracellular signalling pathways.

Phytohaemagglutinin: a lectin found in plants which is an effective T cell mitogen.

Piperazine: an anti-helminthic drug that activates the inhibitory γ-aminobutyric acid receptor on helminths, inducing paralysis and expulsion.

Placebo: a medically inactive substance given as a control treatment during clinical trials; controls for the effects of patient-perceived benefits of the treatment under trial.

Platelet: anucleate cellular structures that bud from megakaryocyte precursors and play a role in blood clotting.

Pleomorphic: the existence of multiple structural forms.

Plerocercoids: the elongated infective larval stage of some tapeworm species.

Polyclonal: a population of T or B cells that have different clonotypical receptors because they have arisen from many clones. In reference to antibodies, made by plasma cells with different specificities; the opposite of monoclonal.

Premunition: resistance to infection provided by an existing immune response generated to a chronic infection of the same, or a closely-related, species.

Prevalence of infection: an epidemiological measurement calculated from the number of infected individuals in a population divided by the total number of

individuals in the population. It provides an estimate of how common a particular infection is within the population under study.

Promastigote: a developmental stage of some Kinetoplastid parasites in which the parasite has developed a flagellum attached to the kinetoplast in front of the nucleus of the parasite (compare with 'trypomastigote').

Prostaglandins: lipid compounds derived from arachidonic acid that can induce smooth muscle contraction and inflammation in a similar way to leukotrienes; prostaglandin E2 is able to induce fever via actions on the hypothalmus.

Proximal: in reference to the GI tract, a point situated up the tract closest to the mouth; opposite to distal.

Pruritis: itching of the skin.

Pseudocoele: a hollow space or cavity between the body wall and internal organs.

Pseudocysts: cyst-like pockets of parasites in larger cells, such as those formed by *Trypanosoma cruzi* in muscle cells.

Pyrogen: a molecule that can cause fever.

Recombinant: in reference to DNA, artificial DNA constructed by molecular methods in the laboratory. In reference to proteins, preparations that have been produced by microorganisms (such as bacteria or yeast) after transfection with a vector encoding the protein's sequence.

Respiratory burst: the transient increase in the consumption of oxygen by a cell such as a macrophage or neutrophil during the production of toxic oxygen radicals.

Rete: an interwoven network of blood vessels.

Reticulocytes: immature anucleate red blood cells that contain a network of ribosomal RNA.

Retroinfection: infection that occurs contrary to the usual course, such as mother-to-child transmission.

Rhoptries: secretory organelles of some Apicomplexan parasites, containing enzymes involved in cell invasion.

Rosetting: the formation of red blood cell clusters by the erythrocytic stages of malaria, whereby naïve red blood cells stick onto an infected red blood cell.

Scavenger receptors: cell receptors that bind to low-density lipoproteins.

Schizogony: the process by which malaria merozoites develop and burst from schizonts to begin a new erythorcytic cycle.

Schizont: a multinucleate stage of the erythrocytic cycle of malaria, in which daughter merozoites form.

Scolex: the head-like part of a tapeworm, normally bearing hooks and suckers for attachment to host tissue.

Senescent: aged.

Sepsis: a life-threatening systemic inflammatory immune response, normally caused by a systemic bacterial infection.

Sickle cell anaemia: a condition arising from a mutation in the β-chain of haemoglobin leading to the formation of sickle-shaped red blood cells which are refractory to the development of malaria parasites.

Solenophagic: blood vessel-feeding arthropods such as mosquitoes.

Sowda: a skin disease associated with infections of *Onchocerca volvulus* filarial nematodes characterised by the formation of papulae and darkening of the skin.

Splenomegaly: enlargement of the spleen.

Spleen: a secondary lymphoid organ that filters the blood; located in the upper left quadrant of the abdomen.

Sporogenesis: reproduction via the release of spores.

Sporozoite: transmissable stage of Apicomplexan parasites arising from sporogeny.

Stercorarian trypanosome: a trypanosome of faecal origin, specifically a reference to the *Trypanosoma cruzi* stages transmitted via the faeces of Triatomine bugs.

Strobila: the segments of the body of an adult tapeworm.

Stichocytes: secretory cells found in the intestine of *Trichinella spiralis* nematodes.

Sub-conjunctival: under the conjunctiva of the eye.

Sub-patent infection: an infection that is not detectable in the periphery nor the faeces.

Surra: a disease of domestic and wild animals in tropical and sub-tropical regions, primarily caused by *Trypanosoma evansi*, leading to weakness, lethargy and anaemia. It can be fatal.

Syncytia: multi-nucleated cells that arise from the fusion of many individual cells.

Tachyzoite: a developmental stage of some Apicomplexan parasites, associated with fast replication and rapid growth.

Tetramers: complexes of four biotinylated peptide-loaded MHC-molecules specific for an epitope of interest bound together by tetravalent streptavidin; used to identify antigen-specific T cells in studies of T cell responses during infection.

Thallassaemia: an inherited blood disorder that results from the formation of mutant haemoglobin and leads to anaemia.

Thylakoid: a membrane-bound compartment of chloroplasts where photosynthesis occurs.

Transcription factor: a DNA-binding factor that influences the transcription of that DNA sequence to messenger RNA.

Transcytosis: the transport of dimeric IgA across the epithelial cells of the mucosal membranes to the luminal surface by the poly-Ig receptor.

Translocon: a complex of proteins associated with the transport of nascent protein (protein that has not yet folded) across membranes.

Transgenic: organisms that have been genetically modified.

Tri-laminate: structure of three layers.

Trophic: related to food and nutrition.

Trophozoite: a developmental growing stage of Apicomplexan parasites with high absorption of nutrients from the host.

Tropism: growth of an organism in response to an environmental stimulus.

Triatomine: a subfamily of the reduviid bugs that carry parasites of the species *Trypanosoma cruzi* and are the vectors for Chagas disease.

Typomastigotes: a free-swimming developmental stage of trypanosomes that contains a flagellum arising from the kinetoplast at the anterior of the parasite behind the nucleus (compare with 'promastigote').

Ungulates: animals that are hoofed.

Urticaria: also called hives; a skin rash commonly caused by activation of mast cells in the skin, leading to red raised itchy bumps.

UV-inactivated: exposure to ultraviolet electromagnetic radiation that leads to sterilisation and prevents successful development because of indirect damage to the DNA of the exposed organisms by free radicals and oxidative stress.

Vitelline: the outer surface of the plasma membrane of an egg, composed of protein fibres which bind sperm.

Viviparous: species that give birth to live offspring (as opposed to releasing eggs).

Vasculitis: inflammation of the blood vessels.

Vasodilator: a substance that can cause relaxation of the smooth muscle cells within the blood vessel walls, leading to widening of the blood vessel.

Vector: in reference to a life cycle, an insect host of a parasite (either intermediate or definitive) that transmits infective parasites to new hosts.

Virulence: the degree of pathogenicity of an infection which arises as a genetic property of a particular host-parasite pairing, rather than just from the genetic makeup of the pathogen alone.

Western blotting: an immunological technique that uses antibodies to detect specific proteins separated by gel electrophoresis and transferred onto a membrane.

Wild-type: a term used to refer to parasites or hosts which have a phenotype typical of the species (e.g. those that are not genetically modified).

Zoonotic transmission: transmission from animals to humans (opposite of anthroponotic transmission).

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Note: **Bold** type indicates pages with figures or tables.

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