Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism

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Abstract | Toll-like receptors (TLRs) have emerged as a major receptor family involved in non-self recognition. They have a vital role in triggering innate immunity and orchestrate the acquired immune response during bacterial and viral infection. However, the role of TLRs during infection with protozoan pathogens is less clear. Nevertheless, our understanding of how these parasitic microorganisms engage the host TLR signalling system has now entered a phase of rapid expansion. This Review describes recent insights into how parasitic protozoans are sensed by TLR molecules, and how the TLR system itself can be targeted by these microbial pathogens for their own survival.

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Infection with protozoan parasites is a major human health issue, causing vast morbidity and mortality that, particularly in developing countries, contributes to political, social and economic instability. There are currently no vaccines available to prevent these devastating infections, and the development of drug resistance in these pathogens is a growing problem (TDR Diseases web site). Therefore, there is an urgent need for the development of new prophylactic strategies and therapies for patients infected with this class of pathogen. Understanding the complexity of the immunological mechanisms involved in resistance to infection, as well as in pathogenesis caused by protozoan parasites, is essential for the development of effective prophylactic and therapeutic vaccines.

During the early stages of infection, the host innate immune system must rapidly detect and respond to protozoan parasite infection, and this is achieved through innate immune receptors. Furthermore, innate immunity and TLRs orchestrate the development of an acquired immune response, which is necessary for protection against re-infection. Therefore, in the absence of recognition by innate immune receptors, the host will quickly become overwhelmed by the parasitic pathogen, resulting in disease and possibly death. Conversely, if activation of innate immune receptors is excessive, high levels of pro-inflammatory mediators such as interferon-γ (IFNγ), tumour-necrosis factor (TNF) and reactive nitrogen intermediates (RNIs) can be detrimental to the host. Therefore, how the innate immune system detects and responds to protozoan parasites is crucial to understanding how infection is controlled, as well as how excessive immune responses are avoided.

In mammalian cells, the Toll-like receptor (TLR) family is an important group of receptors through which the innate immune system recognizes invasive microbes[1,2]. TLRs are crucial for many aspects of microbial elimination, including recruitment of phagocytes to infected tissue, and subsequent microbial killing. TLRs expressed by macrophages and dendritic cells (DCs) also have a role in shaping long-term acquired immunity. However, when activated to excess, TLRs mediate pathology, as is the case in septic shock induced by infection with Gram-negative bacteria and by lipopolysaccharide (LPS)[3,4].

Although the importance of innate immunity in resistance to parasitic infections, and in the pathogenesis of protozoan infections, is well recognized, the molecular mechanisms underlying recognition of parasitic protozoans by innate immune cells are only now beginning to be understood. Major advances have identified bacterial and viral molecules that act as TLR agonists and have identified how these pathogens can manipulate TLR-induced signalling cascades to prolong their own survival. Now, this area of research is emerging with new and exciting insights into how the TLR signalling system responds to infection by protozoans, including *Leishmania* spp., *Trypanosoma cruzi*, *Trypanosoma brucei*, *Plasmodium* spp. and *Toxoplasma gondii* — pathogens that every year account for immense human suffering and death worldwide (BOX 1). In this Review, we present recent studies describing protozoan molecules

Box 1 | **Protozoan parasites and human diseases**

Trypanosomatids

Cutaneous leishmaniasis. This is an immunologically silent infection that leads to cutaneous lesions. Development of parasite-specific T helper 1 (T_H1)-cell responses and interferon-γ (IFNγ) are protective and associated with lesion healing. T_H2-cell responses are associated with severe diffuse leishmaniasis. Worldwide, 12 million people are infected. The aetiological agents are *Leishmania major*, *Leishmania mexicana*, *Leishmania brasiliensis* and *Leishmania amazonensis*.

Visceral leishmaniasis. This is an immunologically silent infection that leads to marked parasitism in the bone marrow and spleen. Severe disease is associated with parasite-specific T-cell anergy. Protective immunity is mediated by T_H1-cell-associated IFNγ. Approximately 0.5 million people acquire visceral leishmaniasis each year. The aetiological agents are *Leishmania donovani* and *Leishmania chagasi*.

Sleeping sickness. Sleeping sickness is characterized by marked parasitaemia, cytokinaemia and associated symptoms, including fever, headache and lethargy. Protective immunity is mediated by T_H1-cell responses, IFNγ and antibodies. Approximately 1 million people are infected with African trypanosome species.

Chagas disease. Chagas disease has the same general characteristics as sleeping sickness, but also involves myocarditis. Protective immunity is mediated by T_H1 and $CD8^+$ T cells, IFNγ and antibodies. Chronic pathology is associated with scarce parasitaemia in the blood, but with marked T-cell infiltration in the heart that coincides with tissue parasites. Approximately 18 million people in Latin America are infected with *Trypanosoma cruzi*.

Apicomplexans

Malaria. Malaria is characterized by marked parasitaemia associated with cytokinaemia and related symptoms. Protective immunity is mediated by a T_H1-cell response and $CD8^+$ T cells, IFNγ and antibodies. Later stages of infection are associated with immune tolerance. Acquired immunity is non-persistent, unlike most other protozoan infections. Approximately 300 million individuals become infected and 1 million children die each year. The aetiological agents are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malarae*.

Toxoplasmosis. Toxoplasmosis results in marked immune activation as indicated by lymphoadenopathy, splenomegaly, fever and headache. Protective immunity is mediated by T_H1 cells, $CD8^+$ T cells and IFNγ. It is an extremely common infection (30–50% of the human population are seropositive) that is benign, except in immunodeficient individuals. The aetiological agent is *Toxoplasma gondii*.

that act as TLR agonists and describe a role for TLRs and Toll/interleukin-1 receptor (TIR)-domain-containing adaptor molecules in resistance to these parasites. Also, emerging examples of the novel mechanisms by which protozoans can manipulate TLR-dependent signal-transduction cascades to establish host parasitism are discussed. Together, these studies argue for the vital role TLR signalling pathways have in innate immune recognition of protozoans, in the emergence of acquired immunity, and conversely in certain cases in the development of immunopathology during protozoan infection.

Protozoan PAMPs

The term pathogen-associated molecular pattern (PAMP) was coined to describe infectious, non-self targets of the innate immune system. Three main features characterize molecules containing PAMPs: they are usually expressed by microbes and not host cells, they are conserved among microorganisms of a given class and their expression is essential for microbial survival. Whereas the first two characteristics allow recognition of microbes and not host cells, the third prevents the development of mutants that escape recognition by the host immune system[5]. Although the identification of protozoan PAMPs is at an early stage compared with the identification of PAMPs contained within bacterial and viral molecules (BOX 2), several TLR agonists derived from protozoans have been identified in recent years[6].

Several studies have shown that glycosylphosphatidylinositol (GPI) anchors (or their fragments) from *Leishmania major*, *T. brucei*, *T. cruzi*, *Plasmodium falciparum* and *T. gondii* activate cells of both lymphoid and myeloid lineages[7–11] (TABLE 1). GPI moieties function to anchor proteins to the surface of eukaryotic cells, and they are abundantly expressed by many protozoan parasites. GPI anchors are composed of a glycan core and a lipid component. Specificity is conferred through variations in the carbohydrate branches, the lipid inositol portion (glycerol versus ceramide), and the number, length and degree of saturation in the hydrocarbon chains. For *T. cruzi* trypomastigotes derived from mammalian cells, the pro-inflammatory activity of GPI anchors covalently linked to mucin-like glycoproteins (GPI mucin) expressed on the surface of the parasite depends on the GPI anchor's fine structure. Non-saturated, fatty acyl chains and periodate-sensitive components from the GPI anchor of *T. cruzi* have been shown to be required to trigger the production of cytokines by macrophages[7,12,13].

Because parasite GPI anchors can initiate host immune responses, this raises the question as to why mammalian GPI anchors do not themselves induce unrestrained autoimmune disease. Mammalian cells typically express 10^5 copies of GPI anchors per cell, whereas parasitic protozoans express up to 10^7 copies (and related structures) per cell[14]. Furthermore, protozoan-derived GPI anchors contain a longer glycan core and a lipid component (which is not present in mammalian GPI anchors)[15]. Consequently, both the amount and the fine structure of at least some protozoan-derived GPI anchors are important aspects in determining activation of innate defence mechanisms, as well as inflammation in the vertebrate host.

TLR activation by protozoan GPI anchors. The *T. cruzi*-derived GPI anchors trigger the phosphorylation of mitogen-activated protein kinase (MAPK) and inhibitor of nuclear factor-κB (IκB) family members. Activation of both of these pro-inflammatory signalling pathways is triggered by activation of TLRs. By using Chinese hamster ovary (CHO) cells transfected with genes encoding different TLR molecules, *T. cruzi*-derived GPI anchors were shown to trigger nuclear factor-κB (NF-κB) activation through TLR2. In addition, recognition of the GPI anchors required CD14, a host cell-surface molecule involved in the recognition of bacterial LPS by TLR4 (REF. 16). Furthermore, the induction of pro-inflammatory cytokines by GPI anchors was ablated in macrophages derived from TLR2-deficient mice[16]. Indeed, live tissue-culture-derived trypomastigotes have been shown to activate CHO cells transfected

Trypomastigote
The extracellular non-replicative stage of *Trypanosoma cruzi* or the replicative stage of *Trypanosoma brucei*.

Box 2 | **Parasites — unique pathogenic organisms**

Unlike bacteria and viruses, protozoans often differentiate within the host into discrete forms that are morphologically and molecularly distinct. This reflects the unique ability of this class of organism to adapt to the host environment and establish a biological niche within the constraints of the host immune system.

Recognition of protozoan pathogens by the host innate immune system presents further challenges, because, like their hosts, they are eukaryotic organisms. Therefore, the immune system might not have as many targets to use in the recognition of protozoan parasites as it does for viruses and bacteria. For example, nucleic acids (single-stranded RNA, double-stranded RNA and DNA that contains CpG motifs) are the main viral molecular targets that are recognized by Toll-like receptor 7 (TLR7) and TLR8, TLR3, and TLR9, in corresponding order[2]. In bacteria, the range of molecules recognized is even more diverse. Cell-wall components such as lipopolysaccharides and lipopeptides, and structural proteins such as flagellin and DNA that contains CpG motifs are the targets for recognition by TLR4, TLR2, TLR5 and TLR9, in corresponding order[2]. Nevertheless, protozoan-associated molecular patterns exist that are recognized by TLRs. These include dominant surface glycolipids (glycosylphosphatidylinositol anchors that are recognized by TLR2 and TLR4), structural proteins (profilin-like proteins that are recognized by TLR11) and genomic DNA that activates TLR9 (REF. 6).

For some of the described protozoan molecules, the concentrations that are necessary to activate TLRs are high compared with viral and bacterial TLR agonists. Consistently, the host can sustain relatively high parasitism. By contrast, tolerance to bacteria and viruses is much lower, and acute episodes with bacterial or viral infections generally require a much smaller pathogen load to result in dramatic activation of the innate immune system. Nevertheless, myeloid differentiation primary-response gene 88 (MyD88) and/or TLRs have been found to be crucial for initiating pro-inflammatory cytokine synthesis and controlling protozoan replication[29,30,36,47–49], and for cytokine-mediated pathology during rodent malaria[51].

with human *TLR2* (REF. 16) and promote production of interleukin-1 (IL-1) and cardiomyocyte hypertrophy through TLR2 (REF. 17). It is well established that TLR2 functions as a heterodimer with either TLR1 or TLR6. Because macrophages that lack TLR6 expression fail to respond to *T. cruzi* GPI anchors[18], the data indicate that a complex of TLR2–TLR6 and CD14 is involved in the recognition of these parasite molecules (FIG. 1).

In addition to these *T. cruzi* TLR2–TLR6 ligands, a subset of free GPI anchors from *T. cruzi*, known as glycoinositolphospholipids, that contain ceramide activate CHO cells transfected with TLRs. This response is dependent on TLR4 and CD14 but not TLR2 (REF. 19). *In vivo*, glycoinositolphospholipids that contain ceramide trigger the production of chemokines, such as CXC-chemokine ligand 2 (CXCL2) in wild-type mice, but not in animals that express a non-functional TLR4 (REF. 19). Therefore, *T. cruzi* contains PAMPs that are recognized by both TLR2–TLR6 complexes and TLR4.

Similar to *T. cruzi*, other kinetoplastids such as *Leishmania* spp. have GPI-linked molecules that trigger TLR activation. Infectious promastigotes are decorated at their surface with GPI-linked molecules. At this particular stage of development, the main GPI-linked molecules are lipophosphoglycans (LPGs), which contain long carbohydrate branches with repeating phosphoglycan units[14,20]. LPGs from *L. major* have been shown to stimulate mouse macrophages and human natural killer (NK) cells through TLR2 (REFS 8,21). Furthermore, use of RNA interference to knock down the expression of various TLRs revealed that activation of macrophages by *Leishmania donovani* is also, at least in part, dependent on TLR2 (REF. 22).

There is evidence that GPI-related molecules from apicomplexan parasites also trigger TLR2 and TLR4 activation. For example, GPI anchors derived from *P. falciparum* merozoites have been shown to induce TNF synthesis through the interaction of the three fatty acyl chains of the GPI anchor with the TLR2–TLR1 complex, which involves a minor contribution from TLR4 (REFS 23,24). Native GPI anchors purified from *T. gondii* tachyzoites, as well as synthetic fragments of the proposed structure of these GPI anchors, were shown to promote NF-κB activation and stimulate TNF synthesis by a mouse macrophage cell line[9], and these responses

Kinetoplastids
A group of flagellate protozoans that are distinguished by the presence of a kinetoplast, a DNA-containing organelle that is closely associated with the flagellum base. Members of this group include *Trypanosoma* spp. and *Leishmania* spp.

Promastigote
The form of the *Leishmania* parasite that is inoculated into the vertebrate host by the bite of an insect vector. The promastigote enters cells such as macrophages through receptor-mediated uptake and then differentiates into an amastigote.

RNA interference
The use of double-stranded RNAs with sequences that precisely match a given gene, to 'knock down' the expression of that gene by directing RNA-degrading enzymes to destroy the encoded mRNA transcript.

Table 1 | **Protozoan pathogen-associated molecular patterns (PAMPs)**

PAMPs	Parasite	Expression stage	Structure	TLR	References
GPI anchors	*L. major*	Promastigotes	LPG	TLR2	8
	L. donovani				20
	T. cruzi	Trypomastigotes	Contains unsaturated alkylacylglycerol	TLR2	16
		Epimastigotes	GIPLs containing ceramide	TLR4	19
	T. brucei	Trypomastigotes	GPI anchors of VSGs	ND	10
	P. falciparum	Merozoites	GIPLs and GPI anchors of the MSP	TLR2 TLR4	23
	T. gondii	Tachyzoites	GIPLs and GPI anchors	TLR2 TLR4	9
Genomic DNA	*T. brucei*	All stages	Contains unmethylated CpG motifs	TLR9	27,30
	T. cruzi	All stages	Contains unmethylated CpG motifs	TLR9	27,29
Haemozoin	*P. falciparum*	Merozoites	β-Haematin crystal made from haemin	TLR9	33,35
PFTG	*T. gondii*	Tachyzoites	Profilin-like protein	TLR11	36

GIPL, glycoinositolphospholipid; GPI, glycosylphosphatidylinositol; *L. donovani*, *Leishmania donovani*; *L. major*, *Leishmania major*; LPG, lipophosphoglycan; MSP, merozoite surface antigen; ND, not determined; *P. falciparum*, *Plasmodium falciparum*; PFTG, profilin-like protein; *T. brucei*, *Trypanosoma brucei*; *T. cruzi*, *Trypanosoma cruzi*; *T. gondii*, *Toxoplasma gondii*; TLR, Toll-like receptor; VSGs, variant surface glycoproteins.

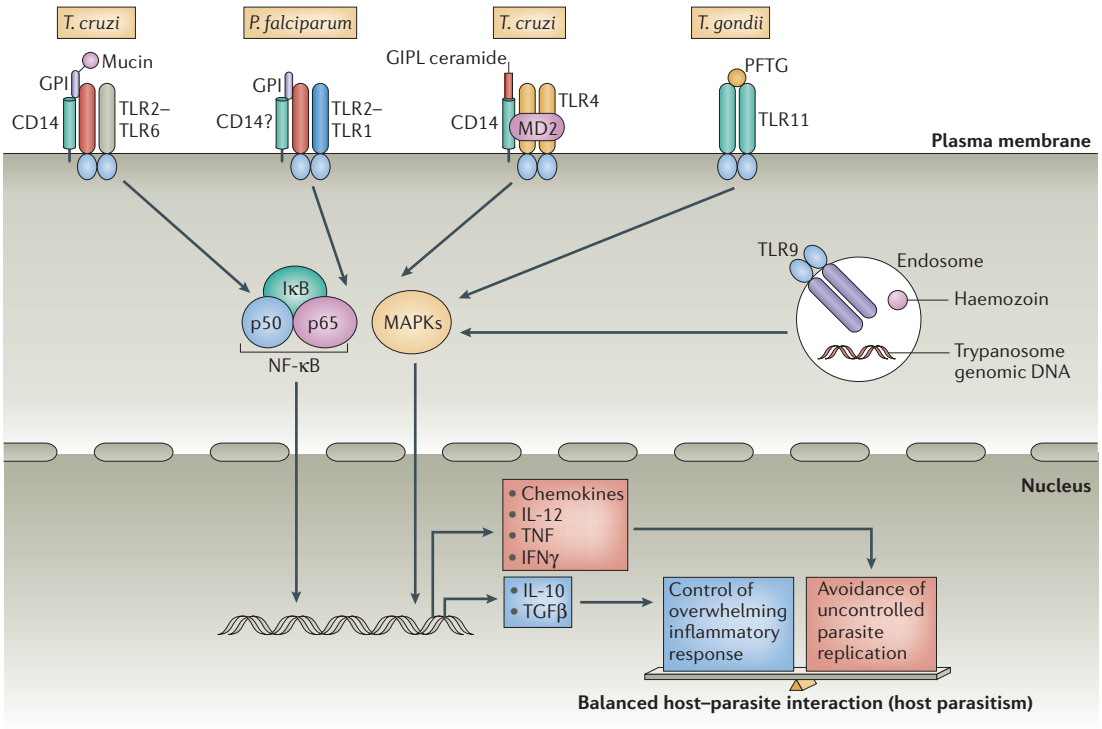

Figure 1 | **Activation of Toll-like receptors by protozoan pathogen-associated molecular patterns.** *Trypanosoma cruzi* glycosylphosphatidylinositol (GPI) covalently linked to mucin-like glycoproteins (GPI mucin) are ligands for the Toll-like receptor 2 (TLR2)–TLR6 heterodimer, and an additional role for CD14 has been implicated. In the case of *Plasmodium falciparum* GPI anchors, three fatty acyl chains preferentially activate the TLR2–TLR1 heterodimer, but involvement of CD14 has not been determined. The TLR4–MD2 complex recognizes free *T. cruzi* GPI anchors, known as glycoinositolphospholipids containing ceramide (GIPL ceramide). The TLR11 molecule, which is expressed by mouse but not human cells, recognizes a profilin-like protein (PFTG) from *Toxoplasma gondii* and possibly other related apicomplexan parasites. TLR9, which is localized in endosomes and the endoplasmic reticulum, recognizes *T. cruzi* and *Trypanosoma brucei* genomic DNA, as well as haemozoin derived from red blood cells that are infected with *P. falciparum*. TLR activation by parasite molecules triggers nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways to induce the expression of pro-inflammatory cytokine genes and genes that directly control parasite replication. At the same time, genes that encode anti-inflammatory cytokines are induced. These control an excessive and, therefore, deleterious immune response. An appropriate balance between the pro-inflammatory and anti-inflammatory response facilitates host parasitism and persistent infection. IκB, inhibitor of NF-κB; IL, interleukin; IFNγ, interferon-γ; TGFβ, transforming growth factor-β; TNF, tumour-necrosis factor.

Apicomplexan
A member of the phylum Apicomplexa. These intracellular protozoans are distinguished by a complex of apical organelles that discharge during the process of invasion. *Toxoplasma gondii* and *Plasmodium* spp. are prominent members of this phylum.

Merozoite
The intra-erythrocytic replicative stage of the malaria parasite that is associated with pathogenesis.

Tachyzoite
The immunostimulatory replicative form of *Toxoplasma gondii* found inside vertebrate host cells that is associated with pathogenesis. This contrasts with the quiescent bradyzoite form that is contained within cysts located in skeletal muscle and central nervous tissue.

Haemozoin
The crystalline product resulting from digestion of haemoglobin by intra-erythrocytic *Plasmodium* merozoites.

also seem to be mediated through TLR2 and TLR4 (F. Debierre–Grokiego *et al.*, manuscript in preparation). So, parasite GPI anchors and related molecules seem to fit the definition of a PAMP. Collectively, they serve as ligands for the TLR2–TLR6 heterodimer, the TLR2–TLR1 heterodimer and TLR4, and CD14 is involved in at least some of these cases.

Non-GPI-related protozoan TLR ligands. Although most of the limited literature on innate immune activators from parasites has focused on GPI anchors, other protozoan molecules also serve as important orchestrators of pro-inflammatory responses. One example is a *T. cruzi*-derived protein, Tc52, which induces the synthesis of pro-inflammatory cytokines by host cells through TLR2 (REF. 25). In addition, it is becoming clear that TLR9, well known as a receptor for unmethylated bacterial CpG DNA motifs, is important for the induction of pro-inflammatory cytokines during infection with protozoans. DNA from protozoan parasites such as *T. cruzi*, *T. brucei* and *Babesia bovis* stimulates both macrophage and DC activation[26,27] probably through unmethylated CpG motifs[28]. Recently, the pro-inflammatory activity of *T. cruzi* and *T. brucei* genomic DNA has been shown to be mediated by TLR9 (REFS 29,30) (FIG. 1).

The genomic DNA from *Plasmodium* spp. has a high AT (70–80%) and low GC (20–30%) content, and its role in TLR9 activation has not been determined. In fact, several studies have shown that non-DNA components of *P. falciparum* can activate innate immune cells through TLR9. Therefore, a non-DNA heat-labile component and haemozoin, which had previously been shown to stimulate the production of pro-inflammatory cytokines by macrophages[32,33], were shown to activate human and mouse DCs through TLR9 (REFS 34,35). An important question that remains to be solved is whether fragments of parasite genomic DNA that are associated with these *P. falciparum* preparations are involved in TLR9 activation.

Profilin
The actin-binding protein from eukaryotes that has a role in regulating actin polymerization.

A profilin-like protein from *T. gondii* (PFTG) has been found to activate TLR11 in mouse cells[36]. PFTG is present as a relatively conserved molecule in a number of apicomplexans, indicating that these proteins might serve as another broad class of protozoan PAMPs. Although its exact function in the parasite is unknown, PFTG is predicted to bind to actin and, like flagellin, might be involved in parasite motility and invasion of the host cell. The induction of IL-12 in DCs exposed to PFTG is mediated by TLR11, as the response was abolished in DCs from TLR11-deficient mice. In addition, mice lacking TLR11 show increased susceptibility to infection with *T. gondii*, and this is associated with decreased IL-12 production *in vivo*[36]. However, the *TLR11* gene in humans has a premature stop codon and therefore encodes a non-functional form of TLR11. Accordingly, it would seem that PFTG does not activate human DCs to produce IL-12. Finally, other *T. gondii* molecules, such as tachyzoite heat-shock proteins and other partially purified tachyzoite preparations, have been shown to activate TLR4 and TLR2, respectively[37,38]. From these collective studies, the pattern that emerges is that for any given protozoan, multiple TLR-binding molecules are likely to be expressed (FIG. 1).

Role of TLR signalling in resistance to protozoans

It is now well established that TLRs are important for defence against every known category of human microbial pathogen. Once activated by microbial PAMPs, TLRs interact with adaptor proteins. TLR molecules have a cytoplasmic domain that is homologous to the IL-1 receptor, and is known as the TIR domain[39]. The best-studied TIR-domain-containing adaptor protein is myeloid differentiation primary-response gene 88 (MyD88), which transduces signals for all TLRs except TLR3, as well as the signals for IL-1 and the IL-18 receptors[40,41]. Other TIR-domain-containing adaptors are MyD88-adaptor-like protein (MAL; also known as TIRAP), TIR-domain-containing adaptor protein inducing interferon-β (TRIF; also known as TICAM1), and TRIF-related adaptor molecule (TRAM)[42–44]. MAL is required for TLR2 and TLR4 signalling, whereas TRIF is used by TLR3 and TLR4 (REFS 44–46). TRAM has been shown to associate only with TLR4 (REF. 43).

The most convincing data indicating the importance of the TIR signalling pathway in host resistance and pathogenesis during parasitic diseases are those obtained from infections of MyD88-deficient mice with various protozoan parasites[30,47–49] (TABLE 2). MyD88-deficient mice are highly susceptible to infection with *T. gondii*,

Table 2 | **TLR signalling pathways in host resistance to and pathogenesis of parasitic infection**

Parasite	Knockout	Phenotype	References
L. major	*Myd88*$^{-/-}$	T_H2 phenotype and increased susceptibility	8,48
	Tlr4 mutant	No major effects on immune response	53
T. brucei	*Myd88*$^{-/-}$	Decreased pro-inflammatory cytokines and increased susceptibility	30
	Tlr1$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr6*$^{-/-}$ or *Cd14*$^{-/-}$	Normal cytokine responses, parasitaemia and survival	
	Tlr2$^{-/-}$ and *Tlr4*$^{-/-}$ or *Tlr9*$^{-/-}$	Impaired IL-12 and IFNγ production and increased susceptibility	
T. cruzi	*Myd88*$^{-/-}$	Impaired pro-inflammatory cytokines and increased susceptibility	47
	Tlr2$^{-/-}$	Increased IL-12 and IFNγ, and unaffected parasitaemia and survival	18
	Tlr4$^{-/-}$, *Tlr6*$^{-/-}$ or *Cd14*$^{-/-}$	Normal cytokine responses, parasitaemia and survival	29
	Tlr9$^{-/-}$	Impaired IL-12 and IFNγ production, increased parasitaemia and accelerated mortality	
	Tlr2$^{-/-}$ and *Tlr9*$^{-/-}$	More pronounced effects than just *Tlr9*$^{-/-}$ mice	
P. berghei	*Myd88*$^{-/-}$	Impaired pro-inflammatory cytokines and decreased pathology	51
	Tlr2$^{-/-}$, *Tlr4*$^{-/-}$ or *Tlr6*$^{-/-}$	Normal cytokine responses, parasitaemia and pathology	
T. gondii	*Myd88*$^{-/-}$	Impaired IL-12 and IFNγ production, and increased susceptibility	49
	Tlr2$^{-/-}$	High-dose inocula — increased susceptibility to infection; low or intermediate dose — normal cytokine responses, parasitaemia and survival	54
	Tlr4$^{-/-}$	Normal cytokine responses, parasitaemia and survival	54
	Tlr11$^{-/-}$	Impaired production of IL-12 and IFNγ, and increased susceptibility	36

IFN, interferon; IL, interleukin; *L. major*, *Leishmania major*; *Myd88*, myeloid differentiation primary-response gene 88; *P. berghei*, *Plasmodium berghei*; *T. brucei*, *Trypanosoma brucei*; *T. cruzi*, *Trypanosoma cruzi*; *T. gondii*, *Toxoplasma gondii*; T_H, T helper; *Tlr*, Toll-like receptor.

such that all animals die within 10 days of infection. Increased susceptibility is associated with impaired production of the T helper 1 (T_H1)-associated cytokines IFNγ and IL-12 and marked parasitaemia[49]. Similar results were obtained from MyD88-deficient mice infected with either *T. cruzi*[47] or *T. brucei*[30]. Knockout of *Myd88* in otherwise resistant C57BL/6 mice confers susceptibility to infection with *L. major*. This susceptibility is characterized by large non-healing lesions, marked parasitism and the development of a T_H2-type immune response, as opposed to the normal IL-12-dependent T_H1-type protective immune response that develops in the wild-type C57BL/6 mice[48].

A distinct scenario was observed in the case of rodent malaria. Similar to infection with other protozoans, innate immunity and activation of MyD88 in particular, have an important role in the production of pro-inflammatory cytokines, such as IL-12, TNF and IFNγ[50]. However, infection of MyD88-deficient mice with *Plasmodium berghei* (the causative agent of rodent malaria) resulted in impaired cytokine production but showed less pathology and an improved outcome[51]. Therefore, although signalling through MyD88 in innate immune cells has a protective role in most cases of protozoan infection by activating a T_H1-associated immune response, in other situations decreased pro-inflammatory responses resulting from a lack of MyD88 signalling might be beneficial to the host.

Despite studies indicating an important role for TLR signalling in resistance to protozoan infection as is observed in *Myd88*$^{-/-}$ mice, mice deficient in a single TLR do not show marked increases in susceptibility to infection in most cases[19,30,47,49,51–54]. For example, lack of expression of TLR2, which recognizes protozoan GPI anchors, did not affect the susceptibility of mice to infection with *T. cruzi*. By contrast, mice that have an inactivating mutation in *Tlr4* (C3H/HeJ mice) show a small but significant enhancement in susceptibility to infection with *T. cruzi* compared with wild-type C3H/HeN mice, which have a functional TLR4 molecule. However, recent data indicate that susceptibility to infection was not significantly altered in *Tlr4*-knockout mice on the C57BL/6 genetic background (R.T.G., unpublished observations), which might point to a role for the genetic background of the mouse in their susceptibility to infection in the absence of TLR4. No major phenotype is observed in terms of parasitism or immune responses when either TLR2-deficient or TLR4-deficient mice are infected with *T. brucei*[30], *T. gondii*[52,54], *P. berghei*[51] or *L. major*[53]. By contrast, although not as susceptible as the *Myd88*$^{-/-}$ mice, TLR9-deficient mice show increased parasitaemia, as well as accelerated mortality, following infection with either *T. cruzi*[29] or *T. brucei*[30]. Furthermore, TLR11-deficient mice are more susceptible to infection with *T. gondii*, showing an increase in cyst numbers in the central nervous system, and a decrease in IL-12 and IFNγ production, compared with wild-type mice[36]. However, these mice are not as susceptible as *Myd88*$^{-/-}$ mice infected with *T. gondii*. Therefore, these results indicate that protozoan parasites are recognized by multiple TLRs, and the lack of a specific functional TLR might not be sufficient to result in the dramatic enhancement of host susceptibility to infection that is seen with MyD88 deficiency[19].

We propose that host defence against pathogens is orchestrated by multiple TLRs that, in addition to having a redundant role, might lead to distinct responses by different host cells. In support of this hypothesis, it has been shown *in vitro* that GPI mucin and DNA from *T. cruzi* can provide synergistic signals to induce cytokine synthesis by macrophages and DCs. In addition, mice that are deficient in both TLR2 and TLR9 are more susceptible to acute infection with *T. cruzi* than mice that are deficient in one of the receptors[29]. The inability to control parasite growth was found to correlate with deficient T_H1-cell responses *in vivo* after infection with *T. cruzi*. However, mice that are deficient in both TLR2 and TLR9 are not as susceptible to *T. cruzi* as *Myd88*$^{-/-}$ mice, indicating that another TLR, possibly TLR4, might contribute to early host resistance to infection with this parasite. Nevertheless, this study clearly indicates a dominant role for TLR9 in the induction of IL-12 and IFNγ expression during infection with *T. cruzi*. In addition, the B2 receptor for bradykinin was shown to cooperate with TLR2 in inducing T_H1-cell responses during infection with *T. cruzi*[55]. Furthermore, emerging evidence indicates that other innate immune receptors, which are different from TLRs, might be involved in the initial recognition of protozoan parasites (BOX 3). Therefore, future studies with mice that are deficient in multiple TLRs and possibly other host receptors are clearly required to fully understand how TLRs cooperate

Box 3 | **Beyond TLRs: evidence for other innate immune receptor families**

Although the Toll-like receptor (TLR) family is an important class of sensors for protozoan infection, there are hints of the existence of other innate recognition systems that fulfil the same function. A role for non-TLR, non-MyD88 (myeloid differentiation primary-response gene 88)-based recognition during protozoan infection is indicated by studies in mice that are deficient for MyD88. For example, although *Myd88*$^{-/-}$ mice infected with *Trypanosoma cruzi* show increased susceptibility to infection, host resistance is nonetheless greater than in interferon-γ (IFNγ)-deficient mice[47]. Furthermore, *T. cruzi* infection continues to elicit low levels of interleukin-12 (IL-12) and IFNγ in the absence of MyD88. And although *Myd88*$^{-/-}$ mice are susceptible to *Toxoplasma gondii* infection, a residual IL-12 response persists[49]. Moreover, infection with low-virulence *T. gondii* is non-lethal to *Myd88*$^{-/-}$ mice, but causes death in IL-12- or IFNγ-deficient mice[118]. Therefore, protozoans might also be recognized by TLRs that use other adaptor molecules or other innate immune receptors that are not related to TLRs. Whereas the nature of MyD88-independent mechanisms for protozoan recognition is a largely unexplored area, examples emerging from investigations with other microbial pathogens might prove useful.

Recent studies indicate the existence of non-TLR pattern-recognition systems that function as sensors of viral and bacterial components. For example, the peptidoglycan component of bacterial cell walls is recognized by a family of peptidoglycan-recognition proteins (PGRPs)[119]. Also, NOD1 (nucleotide-binding oligomerization domain 1) and NOD2 proteins act as cytosolic sensors of peptidoglycan-derived bacterial components. Activation of NOD proteins, as with TLRs, triggers host NF-κB (nuclear factor-κB) and MAPK (mitogen-activated protein kinase) pathways. In addition to the well known RNA-binding capability of PKR (IFN-inducible double-stranded (ds)RNA-dependent protein kinase), the RNA helicase RIG-I (retinoic-acid-inducible gene I; also known as DDX58) recognizes dsRNA[120]. Likewise, the intracellular FADD (FAS-associated via death domain) molecule is involved in recognition of viral dsRNA[121]. Whether these or other yet-to-be discovered non-self sensing systems contribute to protozoan recognition and how they cooperate with TLR signalling pathways are exciting areas awaiting future discovery.

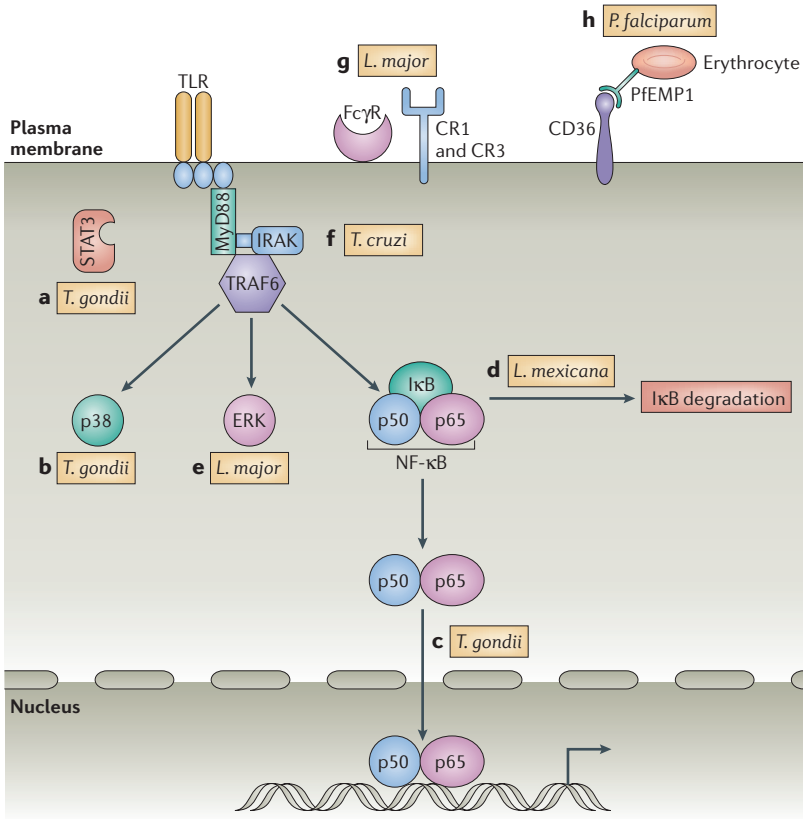

Figure 2 | **Manipulation of Toll-like receptor signalling pathways by protozoans.** **a** | Direct activation of signal transducer and activator of transcription 3 (STAT3) by *Toxoplasma gondii* downregulates interleukin-12 (IL-12) and tumour-necrosis factor (TNF) production. **b**,**c** | The parasite also prevents p38 mitogen-activated protein kinase (MAPK) phosphorylation (**b**) and blocks accumulation of nuclear factor-κB (NF-κB) (**c**) in the host cell nucleus following the triggering of Toll-like receptor 4 (TLR4). **d** | A *Leishmania mexicana* cysteine protease has been identified that is involved in the degradation of inhibitor of NF-κB (IκB) and NF-κB molecules. **e** | The activation of the extracellular-signal-regulated kinase 1 (ERK1)/ERK2 MAPK pathway has been implicated in the ability of *Leishmania major* to downregulate IL-12 production by macrophages. **f** | *Trypanosoma cruzi* induces tolerance to secondary stimulation, and this is characterized by the induction of type 2A protein phosphatase, which deactivates IL-1-receptor-associated kinase (IRAK), MAPK and NF-κB molecules. **g** | Antibody- or complement-coated *L. major* interacts with host receptors for antibodies (such as FcγR), complement receptor 1 (CR1) and CR3, resulting in downregulation of TLR-driven IL-12 production. **h** | *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed on the surface of erythrocytes infected with *P. falciparum*, interacts with CD36 expressed by the host cell, resulting in dendritic-cell deactivation, characterized by unresponsiveness to TLR ligands. MyD88, myeloid differentiation primary-response gene 88; TRAF6, TNF receptor-associated factor 6.

among themselves and/or with other endogenous host components to trigger pro-inflammatory responses and initiate host defence against protozoan infection.

Protozoan regulation of TLR activation

TLR-dependent pro-inflammatory cascades that are triggered by infections with protozoan parasites and other microbial pathogens must be tightly controlled to avoid immunopathology or possibly death. Also, an over-exuberant immune response is a negative outcome for the parasite, which seeks to keep the host alive long enough to allow its transmission to another host. Accordingly, a common aspect of protozoan infection is the induction of endogenous anti-inflammatory mediators. Not only do these mediators avert an over-exuberant immune response, but, at least in some cases, can directly facilitate parasite persistence. In addition, it is becoming increasingly apparent that protozoan parasites directly interfere with signalling cascades that emanate from cognate recognition receptors such as the TLR family.

Manipulation of TLR signalling pathways by endogenous host mediators. The anti-inflammatory cytokine IL-10, which is induced during infection with protozoan parasites, and is well known for its ability to downregulate pro-inflammatory mediators such as IL-12, TNF and RNIs that are triggered through the TLR signalling pathway[56]. Infection of IL-10-deficient mice with normally avirulent *Toxoplasma* strains results in increased mortality, which is associated with hyperproduction of pro-inflammatory cytokines[57,58]. A similar lethal pro-inflammatory phenotype occurs during infection of IL-10-deficient mice with *T. cruzi*[59], and the severity of both cerebral malaria associated with infection with *P. falciparum* in humans and malaria in rodents is also associated with a lack of IL-10 production[60–62]. By contrast, lack of IL-10 during infection with *L. major* is not lethal to the host, but does result in total elimination of the parasite[63]. This is presumably a consequence of increased microbicidal activity mediated by pro-inflammatory cytokines. So, IL-10 controls pro-inflammatory responses and effector mechanisms during infection, and allows long-term persistence of the parasite within the host.

In addition to IL-10, some parasites induce the production of transforming growth factor-β (TGFβ). This cytokine also has anti-inflammatory properties that promote the survival of protozoans, including *Plasmodium chabaudi*, *Leishmania chagasi*, *T. cruzi* and *T. gondii*[64–68]. The activity of TGFβ that favours protozoan infections might be due to suppression of innate cells that produce IFNγ and thereby trigger T_H1-cell differentiation[69], and might be due to modulation of the effector mechanisms of macrophages[70,71]. Evidence also indicates that parasitic induction of host lipoxin A_4, an anti-inflammatory eicosanoid, might contribute to the downregulation of IL-12 and the avoidance of immunopathology during infection with *T. gondii*[72,73].

Manipulation of TLR signalling pathways by protozoan parasites. In addition to the activation of TLR signalling pathways, there are several examples of interference with pro-inflammatory signal-transduction pathways by protozoans (FIG. 2). Although *T. gondii* seems to express multiple TLR ligands, macrophages, neutrophils and DCs become unresponsive to LPS-induced activation following infection with this parasite, as measured by the production of IL-12 and TNF, the upregulation of co-stimulatory molecules, and the ability to stimulate T cells[74–77]. These effects are mediated, at least in part, through activation of signal transducer and activator of transcription 3 (STAT3; FIG. 2a), a signalling intermediate through which IL-10 exerts its anti-inflammatory effects[78]. In STAT3-deficient macrophages the ability of

Eicosanoids
Biologically active compounds that are primarily derived from arachidonic acid, in part through cyclooxygenases and lipoxygenases, including prostaglandins, prostacyclins, thromboxanes, leukotrienes and lipoxins.

T. gondii to block LPS-induced cytokine production is greatly reduced[79]. Rather than inducing IL-10 directly, *T. gondii* triggers STAT3 phosphorylation during infection[79]. Whether this is due to parasite-induced activation of upstream Janus kinase (JAK) molecules, which phosphorylate STATs, or direct STAT3 phosphorylation is not yet clear. Nor is it clear how STAT3 functions during infection with *T. gondii* or even during IL-10 stimulation. Nevertheless, the implication of these studies is that this parasite can directly hijack the IL-10 signalling pathway to downregulate pro-inflammatory responses.

Toxoplasma gondii also regulates LPS-induced MAPK activation[80]; in particular, the activation of p38 MAPK, which is required for IL-12 production (FIG. 2b). The effect of *T. gondii* on p38 MAPK signalling, however, seems to be distinct from the phenomenon of LPS tolerance, which is also characterized by MAPK inactivation[3,81,82]. This is because tolerance induced by LPS targets signalling molecules that are found immediately proximal to the TLR. By contrast, stimulation of *T. gondii*-infected cells with LPS results in the activation of MAPK kinase 3 (MKK3; also known as MAP2K3) and MKK6 (also known as MAP2K6), which are upstream activators of p38 MAPK; degradation of IκB is also observed[80]. This indicates either that the target for inactivation by *T. gondii* is another p38-activating kinase (such as MKK4; also known as MAP2K4) or that *T. gondii* induces a p38 MAPK phosphatase, which prevents the phosphorylation and therefore activation of this kinase.

Activation of NF-κB is also targeted by *T. gondii*. Although infection induces rapid IκB phosphorylation and degradation, the NF-κB molecule fails to translocate to the nucleus[83,84]. Similarly, NF-κB fails to translocate when infected cells are stimulated with LPS (FIG. 2c), although this effect is lost within 6 hours of infection[74]. Recent studies indicate that the apparent lack of translocation might in fact be the result of increased nuclear export rather than decreased nuclear import of NF-κB[85]. There are also indications that the defect in NF-κB activation might be a characteristic of high-virulence parasite strains, not low-virulence strains[86].

Amastigotes of *Leishmania mexicana*, which are largely devoid of LPGs, specifically suppress IL-12 production by macrophages[87]. This seems to be due to the expression of amastigote-specific cysteine peptidases[88]. These molecules, previously identified as virulence factors, modulate the NF-κB signalling pathway by proteolytic breakdown of IκB and NF-κB (FIG. 2d). How these cysteine peptidases exit the vacuole that contains *L. mexicana* and enter the cytoplasm of the host cell has not yet been determined. Related to these studies, *L. major* promastigotes also specifically suppress IL-12 production by macrophages during LPS stimulation. In part, this might be due to the stimulation of an extracellular-signal-regulated kinase 1 (ERK1)/ERK2 MAPK pathway by promastigote LPGs, which results in the negative regulation of IL-12 production[89] (FIG. 2e). It is of interest that LPG itself is a TLR2 ligand, because other TLR2 ligands, such as Pam3Cys (tripalmitoyl-*S*-glyceryl cysteine), have also been reported to inhibit IL-12 production through the induction of the ERK1/ERK2 MAPK pathway[90]. Such cross-regulation in TLR pathways is likely to be an important aspect of the innate response to infection.

Nevertheless, it is clear that not all TLR2 ligands act to downregulate IL-12 production directly. GPI mucin from *T. cruzi* trypomastigotes induces potent IL-12 responses. Similar to LPS, GPI mucin also induces tolerance to secondary stimulation[91]. Stimulation with either LPS or GPI mucin induces protein phosphatase 2A (PP2A) activity that acts to dismantle the TLR signalling cascade, modulating macrophage pro-inflammatory responses to TLR agonists. This occurs through the deactivation of interleukin-1 receptor-associated kinase 1 (IRAK1), MAPKs and IκB molecules, thereby inducing tolerance[92] (FIG. 2f). Control of PP2A occurs through an autoregulatory loop, as induction of its phosphatase activity is dependent on signalling through both p38 MAPK and NF-κB.

IL-12 suppression during infection with *Leishmania* spp. can also be induced by receptor-mediated uptake of opsonized parasites (FIG. 2g). Signalling through complement receptor 1 (CR1; also known as CD35) and CR3 negatively regulates IL-12 production through impaired tyrosine phosphorylation of STAT1 during activation with LPS and IFNγ[93]. For infection with *L. major*, this might be due to the induction of protein tyrosine phosphatases, such as SHP1 (SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 1) (REFS 94,95). Related to these findings, ligation of the macrophage FcγR (a high-affinity receptor for IgG) by opsonized parasites leads to the suppression of IL-12 transcription[96]. Although this might be a direct inhibitory effect on the IL-12p40 promoter, it was also found that FcγR-mediated uptake of *L. major* amastigotes stimulated IL-10 release, inhibiting IL-12p40 transcription and IL-12 synthesis. As a consequence, infected macrophages are refractory to the activating effects of IFNγ, enhancing intracellular survival of the parasite[97].

DCs that phagocytose red blood cells infected with *P. falciparum* or *Plasmodium yoelii* become unresponsive to LPS-induced activation, resulting in defects in T-cell activation[98–100]. In the case of *P. falciparum*, these effects are mediated by the interaction of the scavenger receptor CD36, which is expressed by DCs, and PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), which is expressed on infected erythrocytes[101] (FIG. 2h), although the link between CD36 and TLR signalling pathways is not clear. On another level, phagocytosis of malaria haemozoin, which is a TLR9 agonist, during *P. chabaudi* infection results in the non-responsiveness of DCs to LPS and an inability to activate T cells[102].

So, protozoans target TLR signalling pathways from within cells (*Leishmania* spp., *T. gondii* and *T. cruzi*) to influence host immune responses and might also exploit host cell-surface receptors (for example, *Plasmodium* spp. and *Leishmania* spp.) to suppress these pathways. The diverse mechanisms used by protozoans to downregulate TLR signalling are likely to reflect the need for these organisms to establish long-term residence in their hosts.

LPS tolerance
A transient, non-responsive cell state induced by Toll-like receptor (TLR) ligands such as liposaccharide (LPS). Cells such as macrophages and dendritic cells that have been made tolerant fail to respond to secondary stimulation with LPS or other TLR ligands. This could be a mechanism to avoid pathology that is associated with overproduction of pro-inflammatory cytokine.

Amastigote
The intracellular replicative stage of *Leishmania* spp. that is found in the acidified endosomes of macrophages or the replicative stage of *Trypanosoma cruzi* that is found in the cytoplasm of any nucleated host cells.

Table 3 | **Areas of potential for the use of TLR-based strategies to prevent or treat diseases caused by protozoan parasites**

Disease	Therapeutic agent	TLR	TLR-based strategy	References
TLR agonists as adjuvants				
Cutaneous leishmaniasis and malaria	MPL	TLR4	Prophylactic vaccines — MPL with protozoan antigens; advanced stages of development	104,105
Toxoplasmosis	Flagellin — agonist	TLR5	Elicits *T. gondii*-specific mucosal immunity	(Hypothetical)
	CpG ODN — agonist	TLR9	Effective in experimental models of all diseases listed	106–110
TLR agonists alone as direct inducers of protection				
Cutaneous leishmaniasis	Imiquimod — agonist	TLR7 and TLR8	Prevents or cures disease in mice and humans	114,115
	CpG ODN — agonist	TLR9	Prevents or cures disease in primates	116
Malaria, Chagas disease and leishmaniasis	TLR agonist	To be defined	Eradicates specific infections when combined with chemotherapy	(Hypothetical)
TLR agonists as anti-pathology vaccines				
Malaria	Anti-protozoan PAMP	TLR2 or TLR4	Neutralizes pro-inflammatory activity and pathogenesis elicited by GPI anchors	117
	TLR antagonist	TLR2 or TLR4	Prevents cytokinaemia and pathologies during acute episodes	(Hypothetical)
Chagas disease	TLR antagonist	TLR2, TLR4 and/or TLR9	Prevents local stimulation of innate immunity, recruitment of T cells and consequent damage to heart tissue during chronic *T. cruzi* infection	(Hypothetical)

MPL, monophosphoryl lipid A; CpG ODNs; CpG-containing oligodeoxynucleotides; GPI, glycosylphosphatidylinositol; PAMP, pathogen-associated molecular pattern; *T. cruzi*, *Trypanosoma cruzi*; *T. gondii*, *Trypanosoma gondii*; TLR, Toll-like receptor.

TLR-based strategies against protozoan infection

Protozoan infections exert a devastating toll on human health worldwide, and there is an urgent need for new strategies to treat and prevent disease. Exploitation of chemically defined microbial-derived TLR ligands as therapeutic and prophylactic tools holds promise in this regard. As discussed earlier, activation of TLRs leads to expression of co-stimulatory molecules and pro-inflammatory cytokines (for example, IL-12) that promote differentiation of T_H1 cells. These cells mediate antigen-specific, cell-mediated immunity and produce IFNγ, which also drive protective antibody production by B cells, and therefore these cells are the basis of protective immunity against microbial infections[1]. In fact, many of the chemically defined microbial products being used as adjuvants or immunostimulants are TLR agonists (these include polyinosinic–polycytidylic acid (poly I:C) (TLR3 agonist), lipid A (TLR4 agonist), flagellin (TLR5 agonist), imiquimod (TLR7 and TLR8 agonist) and CpG DNA (TLR9 agonist))[2]. However, a crucial issue is to dissociate the beneficial effects (potentiation of immune responses) from the detrimental effects (oedema and pain) caused by these adjuvants[103]. In the case of infection with protozoan parasites, monophosphoryl lipid A (MPL) is in advanced stages of development for use in vaccine formulations using recombinant antigens of *P. falciparum*[104] and *Leishmania* spp.[105] to protect against these infections (TABLE 3). In addition, CpG-containing oligodeoxynucleotides have been successful in vaccine formulations that induce effective protective immunity against challenge from different protozoan parasites (such as *Leishmania* spp., *Plasmodium* spp., *T. gondii* and *T. cruzi*) in experimental models[106–110]. Bacteria-derived flagellin has been shown to be highly effective in stimulating mucosal immunity[111]. This might be of potential value for vaccine development against the subset of protozoan parasites, such as *T. gondii*, that infect through the intestinal tract.

A second area of interest with regard to clinical use of immunostimulatory TLR agonists is in the treatment of persistent chronic infections that are often refractory to conventional chemotherapy. Many chemotherapeutic drugs currently in use, especially those used for leishmaniasis and Chagas disease, are more effective when used in combination with vaccines or immunostimulants such as IL-12 or *Mycobacterium bovis* bacillus Calmette–Guérin[112,113]. Therefore, it might be valuable to use therapeutic vaccines in combination with TLR agonists, or simply to use TLR agonists in combination with commercially available anti-parasite drugs. In fact, studies using imiquimod[114,115] or CpG-containing oligodeoxynucleotides[116] are apparently effective in the treatment of cutaneous leishmaniasis in experimental models.

Another attractive area of immunological intervention in parasite disease is the use of TLR antagonists in cases in which activation of a TLR pathway is involved in pathology. MyD88 seems to be involved in the pathology associated with malaria[51]. So, use of a TLR antagonist during acute malaria episodes might potentially be of clinical effectiveness, as MyD88 and TLRs might not be crucial for protective immunity and parasite clearance during infection with *Plasmodium* spp. As *P. falciparum* GPI anchors are recognized by TLRs[23], it is of interest that synthetic GPI anchors mimicking those of the parasite are effective as an anti-toxic vaccine, blocking the pathological effects of malaria[117]. However, an important issue is whether blocking TLR–TLR ligand interactions might interfere with the development of protective immunity during infection with other protozoans. This

Adjuvant
An agent that is mixed with an antigen and increases the immune response to that antigen following immunization.

might be the case during infection with trypanosomatids or *T. gondii* that seem to depend largely on activation of MyD88 for resistance[30,47–49].

The beneficial effect of the TLR-based interventions discussed here are at present largely hypothetical, and their potential as therapeutic agents needs to be further tested in experimental models and in the field to help define their efficacy. Nevertheless, the information accumulated in recent years regarding the cellular and molecular biology of TLRs, and their importance in the immunology to infectious diseases, brings high expectations in terms of the development of new strategies for prophylaxis of infections with protozoan parasites.

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Competing interests statement
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