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Do you see what I see: Recognition of protozoan parasites by Toll-like receptors

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Abstract

Toll-like receptors (TLRs) are important for recognizing a variety of pathogens, including protozoan parasites, and initiating innate immune responses against them. TLRs are localized on the cell surface as well as in the endosome, and are implicated in innate sensing of these parasites. In this review, we will discuss recent findings on the identification of parasite-derived pathogen associated molecular patterns and the TLRs that bind them. The role of these TLRs in initiating the immune response against protozoan parasitic infections *in vivo* will be presented in the context of murine models of infection utilizing TLR-deficient mice. Additionally, we will explore evidence that TLRs and genetic variants of TLRs may impact the outcome of these parasitic infections in humans.

Keywords

glycophosphatidylinositol; innate immune response; MyD88; parasite; protozoan; Toll-like receptor

Introduction

Protozoan parasites continue to be an extensive global health concern. According to recent WHO estimates, protozoan infections cause the second largest number of infectious disease related deaths after only HIV infection. They account for approximately 720,000 deaths annually worldwide [1]. Protozoan parasitic infections include fatal diseases such as malaria, visceral leishmaniasis, toxoplasmic encephalitis, and trypanosomiasis. The lack of vaccination strategies and an increase in drug-resistant parasites means these infections are still and will remain a major socio-economic issue in developing countries. These infections are often associated with immune-mediated pathologies. Hence, to design better immunotherapies against these parasitic infections, it is crucial that we recognize the underlying intricacy of the immune system and the parasite derived factors that trigger it in order to understand the resultant disease.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

The immune response against protozoan infections involves a strong innate immune response followed by predominantly a T helper type 1 (T_H1) response. The innate immune system is comprised of a number of cell types, including dendritic cells (DCs), macrophages (Mφ), granulocytes (neutrophils, basophils and eosinophils) and natural killer (NK) cells. Recognition of pathogens by these cell types leads to phagocytosis in some cases (DCs, Mφs & neutrophils), and also the production of pro-inflammatory cytokines, which assist in shaping the subsequent adaptive immune response. Hence, it is important to understand the mechanisms by which the innate immune system gets activated in response to infection with protozoan pathogens.

Toll-like receptors (TLRs) are type-I integral trans-membrane receptors, crucial for recognition of different pathogen associated molecular patterns (PAMPs). These PAMPs play a critical role in activating the innate immune system [2, 3]. Currently thirteen members of the TLR superfamily have been identified in mammals, of which humans express ten and twelve are found in mice. Expression of TLR-1, -2, -4, -5, and -6 are found on the cell surface, while TLR-3, -7, -8, and -9 are expressed in the endosomal compartment [4, 5]. While TLR-12 is expressed in the endosomal compartment, the cellular localization of TLR-11 is unclear. There is evidence showing that this TLR is expressed on the cell surface and/or in the endosome [6–10]. The adapter protein myeloid differentiation primary response 88 (MyD88) is utilized by all TLRs, except TLR-3, for signal transduction, making MyD88 a master regulator of TLR mediated activation of the immune system. Another adapter protein toll-like receptor adaptor molecule 1 (TRIF) is shared by TLR-3 and TLR-4 for signal transduction. The TLRs localized on the cell surface share the responsibility of recognizing microbial surface associated PAMPs. On the other hand, the endosomally localized TLRs (TLR-3, TLR-7, TLR-8, and TLR-9) detect primarily nucleic acids. The activation of TLRs triggers the stimulation of interferon regulatory factors (IRFs) and the canonical NF-κB pathway, leading to the production of pro-inflammatory cytokines, type I interferons, anti-microbial proteins and chemotactic factors.

In this review, we will discuss the role of TLRs in host resistance to protozoan infections with a particular emphasis on the protozoan-specific PAMPs that have been identified, and the corresponding TLRs that they bind. Differential stimulation of TLRs by these parasite-specific PAMPs dictates the immune response elicited against these pathogens. For instance, one PAMP common amongst protozoans is glycophosphatidylinositol (GPI), which is a glycolipid that can be attached to the C-terminus of proteins in the cell membrane. GPIs are the dominant glycolipids that coat the surface of protozoan parasites. Several studies over the last decade suggest GPI anchors from the protozoa *Leishmania, Toxoplasma, Trypanosoma,* and *Plasmodium* can stimulate the innate immune response through TLR mediated pathways, leading to the production of pro-inflammatory cytokines [11–21]. Although mammalian cells carry GPI anchors, parasite-derived GPI anchors have a longer glycan core and are expressed 100-fold more compared to mammalian cells [22]. The GPI anchors from different parasites vary due to differences in glycosylation patterns, including number, length, and degree of saturation of the hydrocarbon chains. Even within the same parasite species the expression of GPI anchors varies depending on the life cycle stage. For example, the hyperglycosylated GPI (Lipophosphoglycan or LPG) of *Leishmania* spp. is

prevalent on the surface of the infective promastigote stage, while it is down-regulated considerably in the intracellular amastigote stage [23]. Even though GPIs are prevalent on the surface of protozoan parasites they are not the sole PAMPs that stimulate the activation of the innate immune response. There is increasing evidence that infection with protozoan parasites results in the activation of multiple TLR and PRR pathways. Whether these PRRs are stimulated simultaneously after infection or at different stages of the parasite's life cycle within the host is currently under investigation.

TLR activation in Plasmodium infection

Plasmodium infection causes the disease malaria, which is marked by high parasitemia followed by fever, rigors, malaise and severe anemia during acute stage infection. These symptoms are related to the production of pro-inflammatory cytokines [24]. While $CD8^+$ effector T-cells are required for protection against the hepatic stage of infection [25, 26], a $CD4^+$ T-cell biased immune response characterized by the production of interferon-γ (IFN-γ) is absolutely necessary for the resolution of peak parasitemia during blood-stage infection [27–30]. Additionally, the presence of parasite specific antibodies correlates with clearance of infection in mice [31–35] and protection in humans [36, 37]. Hence, a varied range of immune activation is required for proper control and resolution of infection. MyD88, TLR-2, TLR-9, and to a small extent TLR-4, have been implicated to play a significant role in the activation of the immune response against *Plasmodium* [19, 21, 38–42]. However, most of this data is based on *in vitro* stimulation of isolated cell populations with parasite-derived material. The role of MyD88 and the TLRs *in vivo* is debatable, but they may contribute to the pathogenesis associated with *P. falciparum* infection in humans [43, 44]. Upregulation in TLR expression has been noted in patients infected with *P. falciparum* [45]. Additionally, genetic polymorphisms of TLR-2, TLR-4, TLR-9, and MyD88 adapter like (MAL) proteins in malaria endemic areas have been found to increase or reduce disease severity [43, 44, 46–48]. Nonetheless, selective pressure by malaria does not seem to be a contributing factor for development of these polymorphisms amongst malaria endemic populations [48].

GPI anchors associated with *P. falciparum* proteins contribute to the pathology of infection, and they have long been considered a malaria toxin. Administration of *Plasmodium* GPIs to mice can be sufficient to cause the symptoms of acute malaria, such as fever, hypoglycemia and TNF-α–mediated sepsis [49]. GPI anchors of *P. falciparum* act as strong antigenic epitopes, and are found to activate the innate immune system via a TLR-2/MyD88 dependent pathway triggering the production of pro-inflammatory cytokines (IL-1 and TNF-α) from macrophages and endothelial cells [19–21, 49]. TLR-2 heterodimerizing with TLR-1, or TLR-6 serves as the major receptor for GPI, but it is also recognized to a lesser extent by TLR-4 [19, 21]. The functional association of TLR-2 with either TLR-1 or TLR-6 allows it to discriminate between microbes with triacylated or diacylated lipoproteins respectfully [50–52]. The GPIs of protozoan parasites contain glycolipids with three or two fatty acid substituents [15, 17, 39, 49, 53, 54], which is similar to bacterial lipoproteins. Hence, TLR-2/TLR-1 would be capable of recognizing protozoan glycolipids with three fatty acid chains, while TLR-2/TLR-6 would bind glycolipids with two fatty acid chains. Supporting this hypothesis, the triacylated GPIs derived from *P. falciparum* are

preferentially recognized by TLR-2/TLR-1 heterodimers on human macrophages, whereas GPI derivatives containing two-fatty acid chains are bound by the TLR-2/TLR-6 complex [19].

There is compelling *in vivo* evidence for and against a role for TLR-2 in the recognition of malarial PAMPs following infection of mice with rodent *Plasmodium* spp. Infection of C57BL/6 mice with *P. berghei* strain ANKA is commonly used as a model to study cerebral malaria (CM), one of the pathologies commonly seen in children infected with *P. falciparum*. TLR-2 deficient mice infected with *P. berghei* ANKA are protected from CM (Table I) [55], while in other studies its absence had no effect on the outcome of infection [56, 57]. Also, no difference in parasitemia or survival was noted between wild-type and TLR-2$^{-/-}$ mice infected with non-lethal blood-stage *P. yoelii* 17×, which causes anemia in mice [58]. Although infection of C57BL/6 mice with *P. chabaudi* AS also results in a non-lethal infection, in contrast to *P. yoelii* 17× infection this parasite species induces chronic infection in mice. While there was a partial decrease in TNF-α and IFN-γ production observed after *P. chabaudi* AS infection of TLR-2$^{-/-}$ mice this reduction did not effect the outcome of infection, as TLR-2$^{-/-}$ mice had a similar peak parasitemia and weight loss as wild-type mice over the course of the infection [59].

TLR-9, which is expressed on the inner surface of the endosome, is an important sensor of unmethylated bacterial CpG DNA motifs [60]. Hemozoin, the byproduct of hemoglobin digestion by *Plasmodium*, was initially described to be the parasite-derived ligand for stimulating TLR-9 [38], making it the first identified non-DNA ligand for TLR-9. Interestingly, hemozoin has been reported to have immunomodulatory effects with evidence that it can induce [38, 42], or inhibit [61–63] DC maturation, or induce the production of pro-inflammatory cytokines such as IL-12 and TNF-α [64, 65], as well as IL-10 [66]. In support of hemozoin as a ligand for TLR-9, *Plasmodium falciparum* crude extract and CpG DNA were shown to bind certain peptides derived from the extracellular domain of TLR-9, and this was dependent on a CXXC motif that was present in the bound peptides [42]. However, results using synthetic heme as a ligand for TLR-9 are mixed as in one study synthetic heme was found to have no inflammatory activity [41], while in another it was found to have adjuvant activity and was capable of binding to TLR-9, but it's ability to act as an adjuvant was shown to be independent of its ability to bind TLR-9 [42]. Differences in the source of heme and preparation of synthetic hemozoin offer one possible explanation for variations seen between native and synthetic hemozoin, as well as differences seen between different synthetic forms of hemozoin. Another potential explanation for these differences between native and synthetic hemozoin is that something other than hemozoin can interact with TLR-9. Evidence for this idea comes from a study by Parroche et al., in which hemozoin was found in a complex with parasite-derived DNA, and was capable of targeting malarial DNA to endosomal compartments to facilitate TLR-9 activation [41]. Binding and stimulation of TLR-9 was abolished with nuclease treatment of hemozoin. This finding was rather surprising since, the malarial genome is almost 80% AT-rich [67, 68] and TLR-9 conventionally recognizes unmethylated CpG-containing DNA. Other studies have indicated that histone-DNA complexes (nucleosomes) of *P. falciparum* are capable of activating DCs to produce pro-inflammatory cytokines in a TLR-9 dependent fashion [69, 70].

Although the exact mechanism of TLR-9 activation is uncertain, in its absence a marked impairment of pro-inflammatory cytokine and T_H1 driven antibody production was observed, which led to higher recorded parasitemia and increased susceptibility of mice to infection with *P. yoelii* 17× [58]. This demonstrates the importance of TLR-9 in mediating protective cellular and humoral immunity to malaria infection. However, additional studies of TLR-$9^{-/-}$ mice with *P. yoelii* 17× infection showed no difference in the course of infection between TLR-$9^{-/-}$ and wild-type C57BL/6 mice [71]. Furthermore, TLR-$9^{-/-}$ mice showed no difference in parasitemia compared to wild-type C57BL/6 mice after *P. chabaudi* AS infection [59], although one study did note a decrease in TNF-α and IFN-γ production in TLR-$9^{-/-}$ mice following *P. chabaudi* AS infection [72]. Similar to results seen with TLR-$2^{-/-}$ mice, one group reported that TLR-$9^{-/-}$ mice infected with *P. berghei* ANKA show increased protection against CM [55], whereas other groups have indicated that the absence of TLR-9 does not afford increased protection against CM [56, 57].

MyD88 as mentioned previously is a critical adaptor protein downstream of all TLR signaling except TLR-3. It is also activated after IL-1R and IL-18R activation. The absence of MyD88 leads to impaired pro-inflammatory cytokine production in response to hemozoin, nucleosomes and GPI mediated stimulation of DCs and Mφs [19, 38, 41, 70], implicating its role in induction of the innate immune response after TLR signaling. Mice deficient in MyD88 exhibit defective immune responses after infection with a number of different rodent species of malaria [55–59, 71–73]. For instance, following *P. chabaudi* AS infection wild-type and MyD$88^{-/-}$ mice display a comparable ability to control and clear parasitemia, yet MyD$88^{-/-}$ mice display a reduced capacity to produce TNF-α and IFN-γ [59, 72], which resulted in no changes in body weight and temperature during the infection [59]. Infection of MyD$88^{-/-}$ mice with *P. yoelii* 17× resulted in an increased susceptibility of these mice to infection compared to wild-type mice due to impairments in the cell-mediated and humoral immune response [58, 71]. Interestingly in the latter study, TLR-2/4/9 triple knockout mice (all of which are MyD88 dependent TLRs) had no impact on parasitemia or survival, indicating that MyD88 signaling downstream of another receptor(s) is important for the control of parasitemia in this model [71]. In the case of lethal *P. berghei* NK65 infection in mice, in which liver pathology caused by the immune response to the parasite contributes to their susceptibility, MyD$88^{-/-}$ mice displayed decreased amounts of serum IL-12 and reduced liver pathology; however, MyD88 was found to not be involved in the control of parasite replication [73]. In studies using *P. berghei* ANKA, MyD88 was shown to be involved in the development of CM, as a reduction in mortality was noted in the absence of MyD88 [55, 74]. In contrast to this finding, Togbe et al. indicated that MyD88 does not contribute to development of CM [57]. In support of the latter finding, infection of TLR-2/4/9 triple-knockout mice with *P. berghei* ANKA resulted in no difference in survival between wild-type and triple-knockout mice [56]. Unfortunately, reasons for all of the observed discrepancies seen in the *in vivo* models of *Plasmodium* infection using various TLR and MyD88 deficient mice is unknown, as mice used in these studies were of the same genetic background, and similar strains and inoculums of the parasite species were used for the experiments.

Comparison of the phenotype between TLR-9$^{-/-}$ and MyD88$^{-/-}$ mice after *P. yoelii* 17× infection indicates that MyD88$^{-/-}$ mice display more pronounced defects, indicating that additional MyD88-dependent TLRs and/or IL-1R/IL-18R signaling may be important for induction of pro-inflammatory cytokines and immune activation [56–59, 71]. As mentioned TLR-4 has a limited capacity in recognizing GPIs, but it has also been found to recognize heme, hemozoin and microparticles released from parasitized erythrocytes [75–77]. Yet, TLR-4 deficient mice do not display an increase in parasitemia or susceptibility to infection with malarial parasites [58, 59, 71]. Whereas, the absence of TLR-4 by itself does not prevent mice from developing CM after *P. berghei* ANKA infection [56, 57], TLR-2/4$^{-/-}$ mice show considerable protection from CM [74]. Interestingly, simultaneous blockade of the endosomal nucleic acid sensing TLRs by chemical and genetic means resulted in a more dramatic increase in protection against CM than that seen in just TLR-9$^{-/-}$ mice [78]. This suggests that TLR-3 or TLR-7 may also participate in the recognition of *Plasmodium*. While infection of TLR-7$^{-/-}$ mice with *P. chabaudi* AS did not result in a difference in parasitemia or clearance of the parasite compared to wild-type mice, signaling through TLR-7 was shown to contribute to an early burst of type I interferon production within the first 24 hours after infection [72]. The authors of this study suggest that TLR-7 is important for early recognition of the parasite, but that as the infection progresses the contribution of TLR-9 to the host response increases. This may be due to changes in availability of TLR-7 and TLR-9 ligands during the course of infection. IL-1β and IL-18, which are downstream products of inflammasome activation and whose receptors utilize MyD88 for signaling, may also contribute to the development of protective immunity against *Plasmodium*. Peak parasitemia was slightly increased in IL-1R$^{-/-}$ mice and significantly increased in IL-18$^{-/-}$ mice after *P. yoelii* 17× infection [71]. Higher amounts of IL-1β and IL-18 were also detected in the serum of TLR-9$^{-/-}$ compared to MyD88$^{-/-}$ mice after *P. yoelii* 17× infection [58]. However, while infection of MyD88$^{-/-}$ mice results in a lethal phenotype, IL-1R$^{-/-}$ and IL-18$^{-/-}$ mice clear their parasitemia at the same time as wild-type mice [58, 71]. This indicates that their individual contribution to the development of protective immunity is minimal, and redundant with that of various TLRs that are activated during *Plasmodium* infection.

A recent study indicated that the high AT-rich motifs present in *Plasmodium* genomic DNA can be sensed by an unknown cytosolic DNA sensor that interacts with the adaptor protein stimulator of interferon genes (STING) in order to induce production of pro-inflammatory cytokines, such as TNF-α and type-I interferons [79]. However, the involvement of STING and a cytosolic DNA sensor in the *in vivo* recognition of malarial parasites is currently unknown, as it is unclear as to how parasite DNA could gain access to the cytosol of DCs during a blood-stage infection. Thus, while a number of different TLRs are capable of recognizing *Plasmodium*-derived PAMPs *in vitro*, the *in vivo* studies to date indicate a potential redundant role for TLRs, and other PRRs in the recognition of malarial parasites.

TLR activation in Toxoplasma infection

Toxoplasma gondii is another important apicomplexan parasite that causes toxoplasmic encephalitis. Although, almost 70% of the infected population is largely asymptomatic, immunocompromised individuals with this infection show a high rate of morbidity and mortality [80]. IFN-γ is absolutely necessary for control of this infection [81]. TLR-

mediated activation of the NF-κB pathway contributes to production of IL-12 by DCs, monocytes and Mφs, which in turn drives IFN-γ production from NK cells, $CD4^+$ and $CD8^+$ T-cells. Evidence for the role of TLRs in activation of the immune response after *T. gondii* infection comes from $MyD88^{-/-}$ mice, as these mice are highly susceptible to this infection (Table I) [82–84]. An increase in susceptibility is the result of impairment in production of the T_H1 cytokines IL-12 and IFN-γ, leading to an inability to control parasite replication. While there is a clear role for MyD88 in innate sensing of *T. gondii* and production of IL-12, administration of IL-12 to $MyD88^{-/-}$ mice only partially restores the T_H1 response [85]. Interestingly, T cells also express MyD88, and chimeric mice in which T cells are deficient for expression of MyD88 succumb by 30 days post-infection due to development of severe toxoplasmic encephalitis. Additionally, it was determined that the MyD88 dependent signal occurred downstream of TLRs as the susceptibility of these chimeric mice was independent of IL-1R and IL-18R signaling [85]. Thus, in this case MyD88 expression by T cells as well as innate cells is required for resistance to this parasitic infection.

While involvement of MyD88 in the immune response against *T. gondii* is clear, identification of the upstream receptor that signals through MyD88 in response to this pathogen has been less straightforward. TLR-1, TLR-2, TLR-4, TLR-6, TLR-9, IL-1 and IL-18, which all require MyD88 signaling, are not individually required for control of *T. gondii* infection [16, 83, 86–88]. However, following oral *T. gondii* infection two studies indicated that TLR-9 was partly responsible for promoting a T_H1 response and ileitis in the gut [89, 90]. It was suggested that the DNA from the microflora and not *T. gondii* was responsible for activating TLR-9, and thus serving as a natural adjuvant for the T_H1 response [89]. Although GPIs of *T. gondii* are recognized by TLR-2 and TLR-4 [16] a profilin-like protein of the parasite was shown to induce IL-12 production by mouse DCs in a TLR-11–dependent manner [91]. However, unlike infection of $MyD88^{-/-}$ mice with *T. gondii* $TLR\text{-}11^{-/-}$ mice survive acute infection [91], indicating that this may not be the sole TLR responsible for recognizing *T. gondii*. The protein UNC-93 homolog B1 (UNC93B1) associates with the endosomal TLRs such as TLR-3, TLR-7 and TLR-9 mediating their transport from the endoplasmic reticulum (ER) to the endosome [92, 93]. Triple-deficient '3d' mice, with a mutation in UNC93B1 are susceptible to intraperitoneal (i.p.) infection with *T. gondii* [6, 7, 86], indicating that these TLRs may have a redundant function in recognizing *T. gondii*-derived nucleic acids, and thus potentially explaining the lack of a phenotype in single gene deficient mice [7, 90, 91]. However, triple mutant mice lacking TLR-3/7/9 do not fully recapitulate the phenotype seen in $MyD88^{-/-}$ and 3d mice after i.p. *T. gondii* infection [7]. It was later established that TLR-11 is found in the endosome, it interacts with UNC93B1, and 3d mice fail to activate TLR-11 after infection with *T. gondii* [6]. Furthermore, based on these findings Andrade et al. decided to generate a quadruple knockout mouse deficient in the genes for TLR-3/7/9/11. Infection of quadruple knockout mice with *T. gondii* recapitulated the 3d phenotype, resulting in impaired IL-12 and IFN-γ production leading to an inability to control parasite replication [7].

In addition to TLR-11 the structural homologue TLR-12 is capable of binding *T. gondii* profilin [7, 8, 10]. TLR-12 localizes to the endosome, it can heterodimerize with TLR-11, and it interacts with UNC93B1 as well. In contrast to the phenotype observed in $TLR\text{-}11^{-/-}$

mice, TLR-12$^{-/-}$ mice were found to rapidly succumb to infection with *T. gondii* [8]. The difference in phenotype was attributed to a variance in TLR-12 expression, while TLR-11 and TLR-12 are expressed in conventional DCs and Mφs, plasmacytoid DCs only express TLR-12. Furthermore, plasmacytoid DC derived IL-12 and IFN-α was shown to promote IFN-γ production by NK cells, which is essential for early control of parasite replication as depletion of NK cells or plasmacytoid DCs in TLR-11$^{-/-}$ mice increased their susceptibility to infection [8]

Although TLR-11 and TLR-12 are essential for recognition of profilin and induction of IL-12 production in mice these TLR proteins are not expressed in humans. However, the other resident endosomal TLRs in humans, TLR-7, -8 and -9, may be important for triggering the innate immune response upon *T. gondii* infection. Stimulation of human PBMCs resulted in production of pro-inflammatory cytokines, including IL-12, in response to *T. gondii* DNA and RNA, but not profilin [7]. Lastly, there has been a recent report linking single-nucleotide polymorphisms in TLR-9 with susceptibility to ocular toxoplasmosis in humans [94]; therefore, these nucleic-acid sensing TLRs and the signaling pathways they trigger may be essential for resistance to this parasitic infection and prevention of clinical disease in humans.

TLR activation in Leishmania infection

Similar to other protozoan parasites a protective T_H1 response characterized by IL-12 and IFN-γ production is necessary to limit parasite growth of the intracellular parasite *Leishmania* [95]. This T_H1 response is absolutely essential for resolution of cutaneous lesions caused by *L. major* or *L. brasiliensis* infection in man or mice. However, the protective T_H1 response can also lead to immune-mediated pathology in the liver and spleen, which is associated with visceral leishmaniasis caused by *L. donovani* and *L. infantum* infection in humans and mice [96, 97]. Evidence of the involvement of TLR signaling in the development of a protective T_H1 response against *Leishmania* comes from studies in MyD88$^{-/-}$ mice. As was the case with *T. gondii* infection, MyD88$^{-/-}$ mice are unable to control parasite growth, and resolve cutaneous lesions after infection with *L. major* or *L. brasiliensis* (Table I) [98–102]. This susceptibility of MyD88$^{-/-}$ mice to *L. major* infection was attributed to a lack of IL-12 production as administration of IL-12 to MyD88$^{-/-}$ mice rescued these mice by restoring a functional T_H1 response [100]. As IL-1R and IL-18 deficient mice show no exacerbation of disease after *Leishmania* infection [103, 104], this indicates that TLR signaling through MyD88, and not IL-1R or IL-18R signaling induces the protective IL-12 response.

Studies of *Leishmania* glycolipids indicated that GPI-anchored LPG, and not other GPI-linked proteins, could serve as a TLR ligand for TLR-2 [98, 105]. LPG is a virulence factor that is prominently expressed on the surface of this parasite, and it can stimulate production of pro-inflammatory cytokines by Mφs and NK cells [98, 105]. However, TLR-2$^{-/-}$, and TLR-2/4$^{-/-}$ mice show no deficiency in their immune response or their ability to resolve their infections [99, 102, 106–109]. Surprisingly, parasite clearance occurred faster in the absence of TLR-2 compared to wild-type mice [102, 107–109]. Furthermore, TLR-4$^{-/-}$ or TLR-4$^{0/0}$ C57/BL10ScN mice, which carry a homozygous null mutation in the TLR-4 gene,

show an early defect in their ability to control parasite growth, but they ultimately resolve their infections in a TLR-4 independent manner [108, 110, 111]. However, another study utilizing TLR-4$^{-/-}$ mice observed no phenotypic changes compared to wild-type mice after *L. major* infection [106].

Additional studies have been conducted in order to determine the ability of endosomal TLRs to contribute to recognition of nucleic-acid structures from *Leishmania* parasites. There are several studies that support a role for TLR-9 in sensing *L. major* infection [106, 112–114]. TLR-9 deficiency in mice results in an enhanced T_H2 response, and only transiently inhibits the development of a T_H1 response and parasite clearance, as these mice are eventually able to heal their cutaneous lesions after *L. major* and *L. brasiliensis* infection [106, 112, 113, 115, 116]. Also, similar to TLR-9$^{-/-}$ mice TLR-7$^{-/-}$ mice are slightly more susceptible to *L. major* infection, but TLR-3$^{-/-}$ mice are resistant to infection [115]. Infection of TLR-7/9 and TLR-3/7/9 knockout mice with *L. major* indicated that these mice were more susceptible to infection then single-gene deficient mice. Furthermore, triple TLR-3/7/9 knockout mice are more susceptible then TLR-7/9 knockout mice and display a phenotype similar to that seen in 3d mice after *L. major* infection. Treatment of 3d mice with IL-12 restored IFN-γ production, reduced parasite load and promoted resolution of lesions, thus rescuing these mice from high susceptibility to this parasitic infection. Moreover, triple knockout mice and 3d mice developed more severe lesions than MyD88$^{-/-}$ mice implying that TLR-3 and its signaling adaptor TRIF contribute to host resistance to *Leishmania* infection [115]. Indeed there is evidence that *Leishmania* can activate TLR-3 [117]. Thus, these studies indicate a functional redundancy for TLRs, particularly nucleic-acid sensing TLRs, in recognition of *Leishmania*, and induction of protective T_H1 immunity.

A far as a role for TLRs in sensing *Leishmania* infection in humans, expression of TLR-2, TLR-4 and TLR-9 have been shown within lesions of patients infected with *Leishmania brasiliensis* [118, 119]. Also, Mφs isolated from the blood of patients with non-healing cutaneous *L. major* lesions were shown to express reduced amounts of TLR-2 and -4 compared to Mφs from patients who were capable of healing their lesions [120], suggesting a role for TLR-2 and -4 in the outcome of cutaneous leishmaniasis. Additionally, although TLR-4 gene polymorphisms have been found in patients with visceral leishmaniasis these polymorphisms do not contribute to an increase in susceptibility to visceral leishmaniasis [121]. Yet, TLR-4 polymorphisms may lead to increased susceptibility and severity of cutaneous leishmaniasis, as patients with chronic cutaneous leishmaniasis had an increased frequency of polymorphisms in TLR-4 compared to non-infected individuals [122].

TLR activation in Trypanosoma infection

Trypanosoma cruzi is a trypansomatid protozoan parasite, known for causing Chagas disease. Resolution of this infection is dependent on activation of a strong innate immune response that promotes T_H1 driven IFN-γ production [123]. IFN-γ subsequently promotes production of reactive nitrogen intermediates, which have a microbicidal effect on the parasite [124]. As seen with other protozoan parasites recognition of parasite-derived PAMPs by host cell TLRs are critical for host resistance against *T. cruzi*. This is illustrated in studies performed utilizing MyD88$^{-/-}$ mice in which *T. cruzi* infection resulted in

increased susceptibility to this infection characterized by higher parasitemia, and diminished pro-inflammatory cytokine and nitric oxide (NO) production (Table I) [125]. Similar conclusions were reached with another trypanosome species *T. brucei* [126], although *T. brucei* is an extracellular rather than an intracellular parasite.

Among immunostimulatory PAMPs present in *T. cruzi* the most prominent one is the GPI anchors linked to mucin-like glycoproteins on the cell surface of trypomastigotes [11, 13, 127, 128]. The GPIs from *T. cruzi* are known to be recognized by TLR-2, resulting in activation of MAPK and NF-κB pathways, leading to expression of NO and pro-inflammatory cytokines (IL-12, TNF-α) [13, 14, 127, 129]. Additionally, CD14 along with TLR-4 aide in recognition of a free GPI anchor containing a ceramide as a lipid tail (GIPL-ceramide) from *T. cruzi* epimastigotes [130]. Also, human and murine DCs were found to recognize a protein released by *T. cruzi* known as Tc52, which is related to proteins of the thioredoxin and glutaredoxin families through a TLR-2-mediated mechanism [131]. However, as with other protozoan parasites, infection of TLR-$2^{-/-}$ or TLR-$4^{-/-}$ mice with *T. cruzi* or *T. brucei* resulted in no significant phenotype in terms of cytokine production or parasitemia, when compared to wild-type mice [125, 132–134]. Unexpectly, in the case of TLR-$2^{-/-}$ mice the lack of this TLR resulted in a heightened T_H1 response after *T. cruzi* infection [134].

Genomic DNA from *T. cruzi* has a high GC content [135], and unmethylated CpG motifs can activate Mφs to stimulate IL-12, TNF-α, and NO production in a TLR-9 dependent manner [12, 135]. This suggested an *in vivo* role for TLR-9 in activation of a T_H1 response against *T. cruzi*. Indeed, in the absence of TLR-9, mice were highly susceptible to *T. cruzi* or *T. brucei* infection, resulting in increased parasitemia, impaired cytokine production and macrophage function [12, 133]. Additionally, TLR-2/9 deficient mice displayed an even more pronounced susceptibility phenotype after *T. cruzi* infection. However, while TLR-2/9 knockout mice had similar quantities of parasites in their blood compared to MyD88$^{-/-}$ mice they survived longer than MyD88$^{-/-}$ mice after infection [12], indicating involvement of additional TLRs that signal through MyD88 that contribute to resistance to *T. cruzi*. Strikingly, the $CD8^+$ T-cell response in TLR-$2^{-/-}$, TLR-$4^{-/-}$, TLR-$9^{-/-}$ or MyD88$^{-/-}$ mice has been found to be normal after *T. cruzi* infection [132].

Recent studies conducted with 3d mice that contain a mutation in UNC93B1, showed that these mice are extremely susceptible to *T. cruzi* infection with severely impaired IL-12 and IFN-γ production [136], indicating that nucleic-acid sensing TLRs are important for promoting the immune response against this parasite. Although TLR-9 had already been shown to be involved in host resistance to infection with *T. cruzi* the phenotype observed in 3d mice more closely resembled that of MyD88$^{-/-}$ mice, and not TLR-$9^{-/-}$ mice, which display a less severe phenotype in response to *T. cruzi* infection. As UNC93B1 also mediates translocation of TLR-3 and TLR-7 to the endosome involvement of these TLRs in sensing *T. cruzi* was evaluated. TLR-$7^{-/-}$ mice are susceptible to infection with *T. cruzi* with a phenotype that resembles that of TLR-$9^{-/-}$ mice, while TLR-$3^{-/-}$ mice are able to control parasite replication and survive infection [136]. Whereas TLR-$3^{-/-}$ mice produced normal amounts of IL-12 and IFN-γ, production of these cytokines was greatly diminished in 3d and TLR-$7^{-/-}$ mice. Mφs from wild-type, but not TLR-$7^{-/-}$ mice were responsive to parasite

derived RNA, as noted by induction of TNF-α by these cells [136]. As UNC93B1 has also been shown to be involved in activation of $CD8^+$ T cells [93, 137], possibly by controlling translocation of cross-presentation machinery, susceptibility of 3d mice to infection could be explained by a defect in antigen presentation by DCs. Therefore, the phenotype of TLR-3/7/9 triple knockout mice was evaluated after *T. cruzi* infection. Unlike the phenotype observed after *T. gondii* infection, TLR-3/7/9 triple knockout mice display a phenotype similar to 3d mice after *T. cruzi* infection in terms of survival, parasitemia, and decreased IL-12 and IFN-γ production [136]. Furthermore, $CD11c^+$ DCs from triple knockout mice and 3d mice display no defect in presentation of parasite-derived antigen to syngeneic $CD8^+$ T cells [136]. Thus, the phenotype observed in 3d mice after *T. cruzi* infection is associated with a combined deficiency of TLR-7 and TLR-9 signaling rather than a defect in cross-presentation. As TLR-7 conventionally recognizes ssRNA in the endosome, an *in silico* analysis was performed to identify potential TLR-7 agonists in the *T. cruzi* transcriptome, particularly guanosine- and uridine-rich single stranded RNA. This analysis identified 344 GU motifs in genes found to be expressed on the surface of the parasite [136]. However, further studies are required to identify the *Trypanosoma* specific PAMP that stimulates TLR-7 activation. Lastly, the phenotype of 3d mice does not completely resemble the phenotype observed in $MyD88^{-/-}$ mice after *T. cruzi* infection, indicating that additional TLRs (TLR-2 and/or TLR-4) or cytokine receptors (IL-1 and/or IL-18) utilize MyD88 for their signaling in response to this infection. However, IL-1R and IL-18 deficient mice are not more susceptible to infection with *T. cruzi* than wild-type mice [136, 138], indicating that these cytokines are not essential for controlling this infection.

There is also evidence that TLRs are involved in the immune response against *T. cruzi* in humans as asymptomatic individuals infected with *T. cruzi* that are heterozygous carriers of the MAL/TIRAP variant S180L have decreased signaling through TLR-2 and TLR-4 upon ligand binding, and thus may have a lower risk of developing chronic Chagas cardiomyopathy (CCC) [139]. Furthermore, an additional study in Chile indicated that a heterozygous TLR-4 deficiency genotype D299G/T399I occurred more often in asymptomatic patients then individuals with CCC, indicating that an impaired TLR-4 response might be beneficial in preventing CCC [140].

Concluding Remarks

In this review, we discussed how different TLRs in the host recognize various parasite-specific PAMPs and thereby activate immune cells such as DCs and Mφs to elicit a protective immune response that controls these parasitic infections. In the context of protozoan infections, TLR activation leads to production of IL-12 that will serve to promote the development of a T_H1 response characterized by production of IFN-γ. Early research on the role of TLRs during parasitic infections primarily focused on TLRs expressed on the cell surface (TLR-1, -2, -4 and -6) and their ability to recognize GPIs, which are highly expressed on the surface of protozoan parasites. However, most of the current literature indicates a limited role for these TLRs in sensing protozoan parasites *in vivo*, and instead nucleic-acid sensing TLRs associated with the endosome play a predominant role in initiating the innate response against these parasites *in vivo*. Additionally, the implication of STING and a DNA sensor in the recognition of *Plasmodium* DNA [79] indicates that the

contribution of cytosolic PRRs in promoting the innate response against protozoan parasites needs to be considered and warrants future investigation. Moreover, evidence indicates that multiple TLRs are capable of recognizing several parasite-specific PAMPs, and insight into how various cells can simultaneously interpret signals associated with activation of multiple TLRs over the course of infection is currently lacking. While activation of NF-κB is thought to be the primary MyD88 dependent signaling pathway activated by the majority of TLRs to induce pro-inflammatory cytokine production, recent evidence indicates that other signaling pathways downstream of MyD88 may contribute to the regulation of cytokine expression during parasitic infections. For instance, TLR-11/-12 and TLR-9 activation in murine $CD8^+$ DCs after stimulation with *T. gondii* profilin induced IL-12 production preferentially via activation of IRF-8 rather than NF-κB [10].

Although mouse models of protozoan infections have been useful for initial identification of parasite-related PAMPs and evaluating TLRs that are responsible for initiating the immune response against these pathogens, they also have their limitations. For instance, TLR-11 and TLR-12 which are essential for recognizing a profilin protein expressed by *T. gondii* in mice are not expressed in humans, indicating other TLRs, such as TLR-9 and TLR-7, may play a more important role in recognizing this intracellular parasite in humans, as suggested by Andrade et al., [7]. Unfortunately, there is limited *in vitro* and *in vivo* evidence linking activation of human TLRs to generation of protective immunity against these parasites. One of the best associations between TLRs and protection comes from studies that illustrated an increased correlation between single nucleotide polymorphisms in TLR-9 and IRAK4 with an increased risk of ocular and congenital toxoplasmosis respectively [94, 141]. Thus, additional human studies are needed to determine the contribution of TLRs to the protective outcome from infection with protozoan species in order to gain further insight into how the immune system operates to control these infections. Identifying polymorphisms within TLR genes, or other genes that participate in TLR signaling, will improve our understanding of how they may contribute to increased pathology and disease after infection.

Thus, although a definitive role for TLR signaling in protective immunity to parasitic protozoans remains unclear, the manipulation of TLR signaling remains an attractive method to promote immunity to infection, or in some cases to prevent excessive activation of the immune response. Future studies examining whether TLR modulation can improve vaccination, pathology and memory responses are critical for delineating the molecular mechanisms of protozoan parasite infections.

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List of Abbreviations

TLR	Toll-like receptor
PRR	Pattern recognition receptor

PAMP	Pathogen associated molecular pattern
MyD88	Myeloid differentiation primary response 88
TRIF	Toll-like receptor adaptor molecule 1
IRF	Interferon regulatory factor
STING	Stimulator of interferon genes
UNC93B1	UNC-93 homolog B1
GPI	glycophosphatidylinositol
CM	cerebral malaria
LPG	lipophosphoglycan
NO	Nitric oxide

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Table I

Phenotype of TLR and MyD88 deficient mice with protozoan infections

Parasite	Knockout mouse	Immune response	Outcome	References
P. yoelii 17×	MyD88$^{-/-}$; TLR-9$^{-/-}$	Impaired IL-12 and IFN-γ; Lower IgG2a and IgG2b, higher IgG1 antibody; Increased IL-10 (TLR-9$^{-/-}$ only)	Higher parasitemia and susceptibility	[58, 71][a]
	TLR-2$^{-/-}$; TLR-4$^{-/-}$; TLR-2/4/9$^{-/-}$;TRIF$^{-/-}$	No difference	No difference in parasitemia or survival	[58, 71]
	IL-1R$^{-/-}$; IL-18$^{-/-}$	Decreased serum IL-10 and IFN-γ in IL-18$^{-/-}$	Higher peak parasitemia; no difference in survival	[71]
P. berghei ANKA	MyD88$^{-/-}$	Decreased infiltration of immune cells in brain; Impaired IL-12, TNF-α and IFN-γ	Increased protection from cerebral malaria (CM)	[55, 74]
	TLR-9$^{-/-}$	Impaired TNF-α, but not IL-12 or IFN-γ		[55]
	TLR-2$^{-/-}$	No difference in cytokine production		[55]
	TLR-2/4$^{-/-}$	Increased serum IL-10 and IFN-γ		[74]
	3d	Decreased serum IL-12, IFN-γ,TNF-α, IL-10 and MCP1		[78]
P. berghei ANKA	MyD88$^{-/-}$; TLR-9$^{-/-}$; TLR-2$^{-/-}$; TLR-2/4$^{-/-}$	No difference	Dispensable for development of CM	[57]
	TLR-1$^{-/-}$; TLR-3$^{-/-}$; TLR-4$^{-/-}$; TLR-5$^{-/-}$; TLR-7$^{-/-}$; TRIF$^{-/-}$; TIRAP$^{-/-}$; CD14$^{-/-}$			[55, 57]
	IL-18$^{-/-}$; IL-1β$^{-/-}$			[74]
	TLR-2/-4/-9$^{-/-}$			[56]
P. berghei NK65	MyD88$^{-/-}$	Decreased serum IL-12; Impaired T_H1 cell development	Decreased liver pathology; No effect on parasitemia or survival	[73]
P. chabaudi AS	MyD88$^{-/-}$	Reduced TNF-α and IFN-γ in serum and by splenocytes; Increased IgG1 antibody	No difference in parasitemia or survival	[59, 72]
	TLR-2$^{-/-}$	Reduced TNF-α by splenocytes		[59]
	TLR-4$^{-/-}$	No difference		[59]
	TLR-7$^{-/-}$	Reduced Type I IFNs, TNF-α and IFN-γ within first 24 h post-infection		[72]
	TLR-9$^{-/-}$	Reduced IFN-γ by splenocytes		[59, 72]
T. gondii	TLR-1$^{-/-}$; TLR-2$^{-/-}$ TLR-3$^{-/-}$; TLR-4$^{-/-}$; TLR-6$^{-/-}$; TLR-7$^{-/-}$; TLR-9$^{-/-}$; ICE$^{-/-}$; IL-18$^{-/-}$; TRIF$^{-/-}$; CD14$^{-/-}$	No defect in IL-12 or IFN-γ production	No difference in parasitemia or survival	[16, 83, 86–88]
	TLR-7/9$^{-/-}$; TLR-3/7$^{-/-}$; TLR-7/8$^{-/-}$; TLR-2/4$^{-/-}$			[7, 16]

Parasite	Knockout mouse	Immune response	Outcome	References
	TLR-11$^{-/-}$	Early impairment in IL-12, but no impairment of IFN-γ production	Increased cyst burden, but no increase in susceptibility	[7, 91]
	TLR-3/7/9$^{-/-}$	No defect in IL-12 or IFN-γ production	Increased parasitemia, marginal increase in susceptibility	[7]
	MyD88$^{-/-}$	Impaired IL-12 and IFN-γ production	Increased parasitemia and susceptibility	[82–84]
	TLR-12$^{-/-}$; TLR-11/12$^{-/-}$	Decreased IL-12 production; Decreased IFN-γ production by NK cells		[8]
	3d	Early impairment of MCP-1, IL-6, IL-12 and IFN-γ production		[6, 7, 86]
	TLR-3/7/9/11$^{-/-}$; TLR-7/9/11$^{-/-}$	Impaired IL-12, IL-6 and MCP-1 production		[7]
L. major L. brasiliensis	MyD88$^{-/-}$	Impaired IL-12 and IFN-γ production; Increased IgE and decreased IgG2a antibody	Increased parasitemia and lesion size; non-healing lesions	[98–102]
L. brasiliensis L. infantum L. donovani L. amazonensis	TLR-2$^{-/-}$	Enhanced T_H1 response	Accelerated parasite clearance	[102, 107–109]
L. donovani	TLR-4$^{-/-}$	Decreased TNF-α, IFN-γ and iNOS expression	Delayed parasite clearance	[108]
L. major	TLR-4$^{0/0}$	Increased IL-4, IL-10 and IFN-γ production	Increased parasitemia; Delay in clearance, but eventually resolve lesions	[110, 111]
	TLR-2$^{-/-}$; TLR-3$^{-/-}$; TLR-4$^{-/-}$; TLR-2/4$^{-/-}$	No difference	No difference	[99, 106, 115]
	IL-1R$^{-/-}$; IL-18$^{-/-}$	Increased IL-4 production	Increased parasitemia, but no delay in lesion resolution	[103, 104]
L. brasiliensis	TLR-9$^{-/-}$	Increased IFN-γ production in dLN	Increased parasitemia and lesion size; no delay in lesion resolution	[116]
L. major	TLR-9$^{-/-}$	DecreasedIL-12 and IFN-γ production, and enhanced T_H2 response	Increased parasitemia and lesion size; Moderate to no delay in resolution	[106, 112, 113, 115]
L. major	TLR-7$^{-/-}$; TLR-7/9$^{-/-}$; TLR-3/7$^{-/-}$; TLR-3/9$^{-/-}$	No difference in IFN-γ production	Increased parasitemia and moderate increase in lesion size	[106]
	TLR-3/7/9$^{-/-}$; 3d	DecreasedIL-12 and IFN-γ, and enhanced IL-10; Decreased IgG2c antibody	Increased parasitemia and lesion size	
T. brucei	MyD88$^{-/-}$	Impaired T_H1 response	Higher parasitemia and susceptibility	[126, 133]
	TLR-1$^{-/-}$; TLR-2$^{-/-}$; TLR-4$^{-/-}$; IL-1R$^{-/-}$; IL-18R$^{-/-}$ CD14$^{-/-}$; TLR-2/4$^{-/-}$	No difference	No difference	

Parasite	Knockout mouse	Immune response	Outcome	References
	TLR-9$^{-/-}$; TLR-2/9$^{-/-}$	Decreased T cells in brain; Decreased IFN-γ production	Higher parasitemia and susceptibility with non-clonal parasites	
T. cruzi	MyD88$^{-/-}$	Impaired T_H1 response	Higher parasitemia and susceptibility	[125]
	TLR-2$^{-/-}$; TLR-4$^{-/-}$	Heightened T_H1 response in TLR-2$^{-/-}$ but not in TLR-4$^{-/-}$	Higher peak parasitemia, but no difference in control	[125, 132, 134]
	TLR-3$^{-/-}$	No difference	No difference	[136]
	TLR-7$^{-/-}$; TLR-9$^{-/-}$; TLR-2/9$^{-/-}$; 3d; TLR-3/7/9$^{-/-}$	Impaired T_H1 response	Higher parasitemia and susceptibility	[12, 136]
	IL-1R$^{-/-}$; IL-18$^{-/-}$	Decreased IFN-γ production in IL-18$^{-/-}$	No difference	[136, 138]

[a]The bracketed numbers in the reference column refer to the numerical order in which the references were cited within the body of the text.