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Review Article

Toll-Like Receptor Initiated Host Defense against *Toxoplasma gondii*

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Toxoplasma gondii is an intracellular pathogen notable for its ability to establish a stable host-parasite relationship amongst a wide range of host species and in a large percentage of the human population. Toll-like receptor signaling through MyD88 is a critical pathway in initiating defense against this opportunistic protozoan and may also be a mediator of pathology during immune dysfunction. Other MyD88 independent signaling pathways are also involved in the host-parasite interaction. These responses can be triggered by the parasite itself, but interactions with the intestinal microbiota add additional complexity during enteric infection.

1. Introduction

Host defense against infection relies upon effective mechanisms of pathogen detection coupled with the ability to discriminate between infectious and noninfectious material. For the case of *Toxoplasma gondii*, an intracellular protozoan with worldwide distribution in humans and animals [1], recognition of infection elicits a rapid and strong Th1-polarized immune response that is necessary for host survival and long-term parasite persistence [2]. In the context of chronic infection, characterized by presence of parasite cysts in tissues of the central nervous system and skeletal muscle, IFN-γ-producing $CD4^+$ and $CD8^+$ T lymphocytes are required to maintain quiescent infection [3, 4]. The most dramatic evidence underlining this concept is found during AIDS progression, where cyst reactivation may occur as T cell numbers decline, often with devastating consequences [5].

Studies spanning the last two decades have established the importance of IL-12 as an early cytokine steering immunity to a polarized Th1 response that allows control of *Toxoplasma* [6–8]. In the absence of IL-12, mice rapidly succumb to infection due to overwhelming parasite replication and tissue destruction. The need for IL-12 to allow host survival extends into chronic infection [9]. During *T. gondii* infection, this cytokine is produced most prominently by dendritic cells, but also macrophages and neutrophils [7, 10–12]. Each of these cell types is targeted for invasion during in vivo infection, but whether infected cells themselves are triggered to produce IL-12 or whether the cytokine is made by noninfected cells responding to parasite products is less clear. Regardless, it is also apparent that production of IL-12 must be tightly controlled to prevent proinflammatory pathology. The cytokine IL-10 is a key player in downregulating the response to *Toxoplasma*. Thus, IL-10 knockout mice succumb to infection with kinetics remarkably similar to IL-12 and IFN-γ gene deleted animals [13]. However, death in animals lacking IL-10 is associated with low parasite numbers and overwhelmingly high levels of IL-12, TNF-α, and IFN-γ [13, 14]. While it was originally thought that IL-10 was produced mainly by cells of the macrophage lineage, more recent data indicate that Th1 cells themselves are an important source of this cytokine during *Toxoplasma* infection [15].

How these complex and essential responses are initiated during infection with *T. gondii* and other microbes has been of long-standing interest to investigators in the field. The concept that cells of innate immunity use pattern recognition receptors (PRRs) to respond to pathogen-associated molecular patterns (PAMPs) was introduced by Janeway in the late 1980's [26]. Ten years later, Janeway and Medzhitov discovered Toll-like receptors (TLR), a major family of PRR [27]. Even though TLRs are structurally similar, they are

Table 1: Summary of findings in TLR/MyD88 knockout mice.

Mouse strain	Susceptibility	Phenotype	References
MyD88$^{-/-}$	Highly susceptible	Uncontrolled parasite growth; defective IL-12 production; delayed emergence of Th1 response	[16, 17]
ICE$^{-/-}$	Normal resistance	Normal	[18]
TLR11$^{-/-}$	Intermediate resistance	Higher cyst burden; mice are resistant to ileitis during enteric infection	[19, 20]
TLR2$^{-/-}$	Intermediate resistance	Mice are susceptible under high infectious dose conditions	[21]
TLR4$^{-/-}$	Unclear	Absence of TLR4 confers resistance to gut pathology; absence of TLR4 results in increased susceptibility to peroral infection	[22, 23]
TLR2x4$^{-/-}$	Intermediate resistance	Higher cyst burden in the absence of TLR4 and TLR2; decreased intestinal pathology during enteric infection	[20, 24]
TLR9$^{-/-}$	Intermediate resistance	Deceased gut pathology; increased parasite burden	[25]

remarkable in that they recognize an extremely diverse array of microbial molecules, including proteins, lipids, DNA, and RNA [28]. Triggering of TLR by microbial ligands plays an important role in initiating immunity to many pathogens, including *T. gondii*.

2. Importance of MyD88-Dependent Toll-Like Receptor Signaling in Defense against *T. gondii*

Initial studies on TLR and their ligands focused on recognition of bacterial and viral molecules such as lipopolysaccharide and nucleic acids, but it soon became clear that TLRs were also central players in recognition of protozoan products and in triggering the immune response to this group of pathogens [29]. Studies in MyD88 gene knockout mice first suggested the importance of TLR in resistance to *T. gondii* [16]. The MyD88 molecule is referred to as the common adaptor of TLR signaling. This is because almost all TLRs use this molecule to relay signals in the host cell [30]. Accordingly, genetic inactivation of MyD88 results in loss of almost all TLR signaling. The exceptions are TLR3 and TLR4, which use another adaptor molecule called Toll-IL-1 receptor domain containing adaptor-inducing interferon (TRIF). In an intraperitoneal mouse injection model absence of MyD88 led to rapid death during infection with the normally low virulence ME49 parasite strain [16]. Mortality was associated with high parasite numbers and low levels of IL-12 and IFN-γ. In addition, neutrophil recruitment was found to be defective in the absence of MyD88, an effect that has been reported in other infectious disease models using this mouse strain [31, 32].

More recently, increased susceptibility in the absence of MyD88 was confirmed in animals undergoing *Toxoplasma* infection initiated through the oral route [17]. MyD88 is most often associated with signaling in innate immune cells, but there is evidence that cells of adaptive immunity also use this common adaptor molecule. Thus, studies in bone marrow chimera mice showed that T cell expression of MyD88 is necessary to prevent emergence of toxoplasmic encephalitis during chronic infection [33]. Although the susceptibility of MyD88 knockout mice to *T. gondii* implicates TLR in recognition of the parasite, this adaptor molecule is also involved in signaling through receptors for IL-1 and IL-18. However, mice lacking IL-1β-converting enzyme (ICE), a molecule that is required to produce bioactive IL-1 and IL-18, display normal resistance to infection [18]. This is an important result because it provides strong evidence that TLR signaling per se is required for resistance to *T. gondii* infection.

While results from studies using *MyD88*$^{-/-}$ mice argue for their importance, there is as yet no evidence for a master TLR that controls the immune response to *Toxoplasma*. As described further in what follows, a parasite ligand for mouse TLR11 has been identified. Yet, mice lacking this TLR are only modestly increased in susceptibility relative to MyD88 knockout animals [19]. Along similar lines, *TLR2*$^{-/-}$ mice have been reported to display increased susceptibility to *T. gondii*, but this effect is only observed using high inocula [21]. In a study using *TLR2x4*$^{-/-}$ mice, the animals survived infection but were reported to harbor an increase in cyst number in the brain [24]. Another study suggested that lack of TLR4 increased susceptibility to enteric infection [22], although differing results were obtained by others [23]. Given the molecular complexity of this eukaryotic pathogen, it is reasonable to propose that *Toxoplasma* possesses multiple TLR ligands, such that knockout of any single TLR has minor effects on host resistance (Table 1). Similar conclusions have been reached in resistance to *Trypanosoma cruzi* and *Mycobacterium tuberculosis*, where TLR2 and TLR9 cooperate to provide optimal immunity to infection [34, 35].

3. Parasite TLR Ligands

Two *Toxoplasma* molecular structures serving as TLR ligands have been identified to date. The tachyzoite surface is uniformly covered with glycosylphosphatidyl inositol-(GPI-)anchored proteins [36]. Purification of parasite GPI anchors as well as core glycans and lipid moieties followed by stimulation of cells cotransfected with plasmids encoding TLR and NFκB reporter genes revealed that GPI moieties triggered TLR2 and TLR4 [24, 37]. In macrophages, this leads to production of TNF-α and upregulation of major histocompatibility complex class II molecules [38]. Interestingly, fatty acids isolated from the parasite block TNF-α inducing properties of GPI moieties [39]. This could possibly be related to the observation that live parasites not

only do not induce TNF-α but actively suppress its synthesis during lipopolysaccharide-TLR4 triggering [40].

To date, GPI moieties come closest to serve as bona fide protozoan PAMP molecules [29]. Thus, in addition to those from *Toxoplasma*, GPI anchors isolated from *Trypanosoma cruzi*, *T. brucei*, *Leishmania major*, *L. donovani*, and *Plasmodium falciparum* all possess TLR2- or TLR4-activating properties [41–44]. In some cases, this promotes protective responses (*L. major*, *T. cruzi*), but in others this may be an underlying cause of pathology (*Plasmodium*) [45]. Why parasite GPI would activate TLR whereas (as far as is known) mammalian GPI do not trigger autoimmune reactions may relate to the fact that GPI molecules are expressed in great abundance on the surface of these protozoan parasites as well as the fact that the fine specificity of these structures varies amongst species [46].

A soluble parasite lysate (STAg) prepared by sonication of tachyzoites followed by high speed centrifugation is well known for its ability to stimulate dendritic cell IL-12 production in a manner dependent upon MyD88 [16]. Straightforward biochemical fractionation of the lysate resulted in identification of a parasite profilin as the MyD88-dependent IL-12-inducing molecule [19]. Interestingly, a profilin molecule with IL-12-inducing properties was simultaneously identified from the related apicomplexan protozoan *Eimeria* [47]. Nevertheless, not all apicomplexan profilins possess the capacity to trigger IL-12 production, because *P. falciparum* profilin has at most only weak IL-12-inducing activity [48]. For *Toxoplasma*, TLR11 was identified as the host cell receptor for profilin [19]. The TLR11 molecule, expressed in mice but not humans, was originally implicated in responses to uropathogenic bacteria [49], but to date *T. gondii* profilin is the only molecule identified as a ligand for this receptor. In addition to triggering host cell IL-12 synthesis, profilin is required for invasion, revealing a dual role in the host-parasite interaction [48, 50]. Profilin is not a secreted molecule but rather is expressed in the tachyzoite cytoplasm. This suggests that triggering of IL-12 synthesis may be mediated by cells responding to parasite degradation products rather than infection itself.

The TLR/MyD88 pathway is believed to play a role in controlling T cell immunodominance [51]. This is because professional antigen presenting cells such as dendritic cells can simultaneously process and present antigen as well as upregulate T cell costimulatory molecules in an MyD88-dependent manner. In support of this model, it was shown that intraperitoneal STAg injection triggered an immunodominant $CD4^+$ T cell response to profilin that was dependent upon TLR11 expression and major histocompatability class II recognition within the same dendritic cell population [52].

4. Role of TLR/MyD88 in the Gut—Who Is Doing the Driving?

It is well established that during high dose *Toxoplasma* infection of certain inbred mice, such as the C57BL/6 strain, animals develop severe pathology in the small intestine mediated by proinflammatory cytokines [53]. A key role for IL-10 in preventing this pathology under low dose infection conditions has been shown using *IL*-$10^{-/-}$ mice [14]. Similar intestinal pathology has been reported in other species infected with *T. gondii*, although whether this occurs in humans is not clear [54]. In several respects, pathology in the C57BL/6 gut resembles Crohn's disease in humans, which is now believed to involve dysregulated Th1 and Th17 responses in the intestinal mucosa [55–57]. As in inflammatory bowel disease in humans, pathology is associated with changes in the microbiota in the gut. Whether these changes are the result or the trigger of pathology is a current area of active interest.

During high dose *Toxoplasma* infection, gram-negative bacteria accumulate at sites of intestinal damage, and bacteria translocate into subepithelial regions of the intestine [58]. Interestingly, both TLR4 and TLR9 knockout mice are resistant to parasite-induced damage to the intestinal mucosa, and this is associated with decreased levels of proinflammatory cytokines [23, 25]. In addition, mice depleted of intestinal flora by treatment with broad-spectrum antibiotics are resistant to ileal damage triggered by *T. gondii* [58]. These combined results suggest that TLR-based recognition of bacteria, rather than sensing of the parasite itself, causes proinflammatory pathology in the intestinal mucosa.

Recent evidence also suggests that under low dose *Toxoplasma* infection conditions, the endogenous gut microbiota have an adjuvant effect on immunity to the parasite [20]. Thus, TLR11 knockout mice develop a robust Th1 response during enteric infection, despite being unable to respond to the IL-12-inducing TGPRF molecule. However, emergence of Th1 cells associated with protection is defective in *TLR*2*x*4$^{-/-}$ and *TLR*9$^{-/-}$ mice. Reinforcing these results, *Toxoplasma* infection of germ-free mice resulted in defective IL-12 responses in the intestinal mucosa but this response was restored by feeding mice with the TLR4 ligand lipopolysaccharide. Thus, it is possible that during orally initiated *Toxoplasma* infection, TLR ligands expressed by the parasite are less important than TLR ligands expressed by the intestinal microbiota [20, 59]. In this view, *T. gondii* infection would cause localized damage to the intestine, possibly as a result of the invasive and cell-lytic nature of the parasite. This would allow translocation of bacteria which would, under low dose conditions, promote differentiation of protective Th1 cells but that, under high dose conditions, and would lead to fulminant pathology. Nevertheless, it is interesting to note that *TLR*11$^{-/-}$ mice were reported to be resistant to parasite-induced intestinal pathology, a result suggesting a role for *T. gondii* TGPRF in addition to the contribution of TLR4 and TLR9 to intestinal inflammation [20, 23, 25] (Table 1).

5. Immune Recognition and Protective Immunity without MyD88

While TLR and MyD88-dependent signaling is important in the host response to *T. gondii*, it is not the only pathway for recognition of the parasite. During in vitro infection

of bone marrow-derived macrophages, cells produce IL-12 independently of MyD88 signaling [60]. Interestingly, there is a parasite strain specificity to this effect because high virulence Type I parasites trigger low level IL-12 production that does not depend upon MyD88, whereas low virulence Type II strains induce higher amounts of this cytokine in partial dependence upon MyD88 [60, 61]. Control of IL-12 production has been linked to ROP16, a polymorphic rhoptry kinase that mediates its effects through activation of signal transducer and activator of transcription (STAT)-3 [62, 63].

During enteric infection, we found that *MyD*88$^{-/-}$ animals display uniform mortality that is associated with uncontrolled parasite replication and dissemination [17]. Nevertheless, while there is a delay in emergence of Th1 cells, IFN-γ-producing Th1 cell responses reach normal levels by one week after infection. We hypothesize that although MyD88 is required for controlling the parasite, it is not essential to trigger an adaptive response to the parasite in this situation. It is possible that in the absence of MyD88, immunity is triggered by bacterial recognition systems such as NOD2 that do not involve TLR/MyD88 signaling, or that non-TLR-based recognition of the parasite itself is sufficient to induce a delayed response.

In an effort to get at these issues and to test whether MyD88 knockout mice could generate a functionally protective response, we immunized mice with an avirulent uracil auxotrophic parasite strain called *cps*1-1 and challenged with the virulent RH strain [17, 64]. In this case, immunization triggered emergence of parasite-specific Th1 cells and as predicted the animals were resistant to lethal challenge. Thus, this appears to be a case where MyD88-independent recognition of *T. gondii* results in fully functional adaptive immunity.

The molecular basis for recognition of *Toxoplasma* that does not rely on TLR/MyD88 is currently not clear. However, the parasite triggers G_i-protein coupled receptors (G_iPCR) leading to release of several chemokines including CCL17 and CCL22 [65, 66]. One particular chemokine receptor, CCR5, has been linked to IL-12 production [67]. As described above, the parasite activates STAT3 as well as STAT6 during invasion. Precisely how this occurs is not clear, but phosphorylation of these transcription factors does not require MyD88 (Butcher and Denkers, unpublished observation).

6. Importance of Human TLR in Resistance to Toxoplasma

Extensive studies in mice have shown the importance of the TLR/MyD88 pathway in resistance to a broad range of viral, bacterial, fungal, and protozoan infections. Nevertheless, the relevance of this pathway in human host defense is less clear. A cohort of 9 pediatric patients with inherited MyD88 deficiency was found to be susceptible to pyogenic bacterial infections but was unexpectedly resistant to other infections, including *Toxoplasma* [68]. Thus, TLR/MyD88 may play a more narrow role in human host defense relative to the major effect of this signaling pathway mouse resistance to infection. However, another interpretation is that MyD88 deficiency results in early lethal susceptibility to infection, so that patients are therefore simply not available for study. For the specific case of *Toxoplasma*, lack of TLR11 expression argues that parasite profilin is not a major IL-12-inducing molecule in humans. Nevertheless, after transfection with human TLR2, CHO cells respond to derivatives of tachyzoite GPI molecules, arguing for a possible role of TLR2 in human infection [24]. Thus, the importance of TLR/MyD88 in humans for resistance to *Toxoplasma* and other infections is still an open question.

7. Future Directions

The mouse model has been extremely valuable in determining how the innate and adaptive immune system is coordinated to provide protection against *T. gondii*, and how dysfunction in the immune system may lead to immunopathology and death during infection with this opportunistic protozoan. The TLR/MyD88 pathway is clearly important in early recognition of infection. Yet, molecular details of how this occurs, and how, in some circumstances, the parasite evades detection, are still lacking. By applying genetic tools that are available to study this parasite, we can expect significant progress in this area in the future. It will also be important in the future to clarify the basis of *Toxoplasma* immune recognition in humans. Working towards these goals will permit a better understanding of this important human pathogen allowing us to develop more effective strategies to prevent and treat disease.

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