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Activation and regulation of Toll-Like Receptors (TLRs) by helminth parasites

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Abstract Helminth (worm) infections are major public health problems that have important socioeconomic consequences for the more than 2 billion infected individuals. Chronicity (their hallmark) can lead to anemia (in hookworm infection), river blindness (onchcerciasis), cirrhosis (schistosomiasis), and elephantiasis (lymphatic filariasis). Although there have been many studies examining innate immune responses (including TLR expression and function) in response to intracellular pathogens, fewer have examined the interaction of the multicellular helminth parasites and the innate immune system. This review will focus on two "systemic" helminth parasitic infections (lymphatic filariasis and schistosomiasis) and the regulation of TLRs that may contribute to infection outcome.

Keywords APC · Lymphatic filariasis · Schistosomiasis · Dendritic cells

Introduction

Helminth infections are a major public health problem resulting in many physical disabilities and having important socioeconomic impact. Helminth parasites have developed mechanisms to evade host responses allowing them to survive in hostile environments such as the gastrointestinal tract, the lymphatics, and the bloodstream [1].

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Almost 130 million people are estimated to be infected by one of three parasites causing lymphatic filariasis in humans: *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* [2]. The infection is initiated by third-stage larvae (L3) deposited in the skin after a mosquito bite. With development of L3 to L4 and then to adult worms, the parasite evades the primary line of defense at the skin site and migrates to the lymphatics, where adult parasites reside and, when mature, release microfilariae (MF), a stage felt to mediate some of the immunologic 'defects' associated with chronic lymphatic filariasis. While the clinical manifestations vary slightly depending on the specific lymph-dwelling filariae, the most consequential manifestations of lymphatic filariasis include lymphedema, elephantiasis, and hydrocele each related to dilatation of or inflammatory damage to the afferent and efferent lymphatics where the adult worms are typically localized [3].

The schistosomes are the causative agents of schistosomiasis that affects ~300 million people worldwide [4]. This parasite infects its human host through the skin when individuals come in contact with the cercarial-contaminated fresh water. Common symptoms are largely related to the granulomatous response to the schistosome eggs [5]. Schistosomes, like the filariae, can survive within the host without inducing serious disease symptoms, and adult worms are estimated to be able to survive for up to 40 years, with each worm producing 300–3,500 eggs per day. Its chronic nature is explained by the pro- and anti-inflammatory responses that are vital to the containment of immune-mediated damage to tissue [6, 7].

The chronicity, disability, social impact, and overall burden of these worm infections have led to much research on the immune responses and of pathogenesis of these infections. Specifically, studying the roles of both innate and adaptive branches of the immune response has focused on the mechanism of pathogen recognition, and studies in endemic areas suggest both innate and adaptive immune systems play a role in host defense.

Antigen presenting cells (APCs) play a major role in the innate immune responses in that they are capable of recognizing a wide range of molecular patterns expressed on pathogens, commonly known as pathogen-associated molecular patterns (PAMPs). In recent years, it has been shown that APCs recognize these PAMPS through Toll-like Receptors (TLRs) and NOD-like receptors (NLRs) leading to signaling [8, 9] through pathways that induce production of inflammatory cytokines. Understanding how recognition of these helminth parasites through the TLR pathway is paramount if the host-parasite interface is to be elucidated.

TLR structure

The function of TLRs is to recognize non-self molecules through recognition of PAMPs found on a variety of microorganisms including bacteria, fungi, and viruses. TLRs are type-1 transmembrane proteins that are pattern recognition receptors (PRRs) that function as sensors for innate immune responses that, in turn, direct the responses of the adaptive immune system. This innate immune response can be thought of as an early defense system that can recognize conserved motifs among molecules found in both animals and plants. The TLRs are evolutionarily conserved molecules and were identified by their homology to Toll, a molecule in *Drosophila melanogaster* that induces production of an antimicrobial protein [10].

TLRs are expressed on many cells of the immune system, in different combinations, at cell surface and endosome membrane of cells such as dendritic cells (DCs), macrophages, neutrophils, endothelial cells, and lymphocytes. This cell-specific but differential pattern of

expression is one mechanism to ensure a more diverse response to different types of pathogens.

Mammalian species typically have 10 to 13 distinct TLRs that recognize conserved PAMPS, 10 of which are found in humans [11]. Mammalian TLRs have been characterized based on stimulation patterns by different ligands in vitro (reviewed in [12]). Due to the heterogeneity of the extracellular domains of TLRs, a variety of ligands are recognized by specific TLRs (Table 1). The extracellular domains of TLRs contain variations of 18–31 leucine-rich repeats [11]. Commonly TLRs associate into homodimers with the exception of TLR2, which preferentially forms a heterodimer with either TLR1 or TLR6 (reviewed in [13]). Pathogen-encoded TLR ligands are divided into three categories: lipids and lipopeptide (TLR2/TLR1; TLR2/TLR6; TLR4), nucleic acids (TLR3, TLR7, TLR8, TLR9), and proteins (TLR5 and, in mice, TLR11). The ligand for TLR10 has not been yet identified (see Table 1; reviewed in ref. [14]).

TLR signaling

The binding of TLRs triggers a series of signaling events that lead to the activation of the nuclear factor-κB (NF-κB) pathway and, as a result, induction of inflammatory responses. TLRs have a common conserved domain (TIR) that is located intracellularly. Once this Toll/IL-1R (TIR) domain is activated, it initiates a signal through five different adaptor molecules that eventually leads to activation of the NF-κB-dependent pathway along with the interferon regulatory factor (IRF) pathway (reviewed in [13]).

After interaction with a specific ligand, the TLR recruits an adaptor protein to its TIR domain. The first discovered adaptor molecule was myeloid differentiation primary response gene 88 (MyD88), a molecule involved in the signaling pathway for all TLRs except TLR3. MyD88 contains a death domain at its N-terminus and a TIR domain at its C-terminus. It often couples with MyD88 adaptor-like (MAL) protein for certain TLR signaling. Another adaptor protein, TIR-related adaptor protein inducing interferon (TRIF), is the sole adaptor molecule for TLR3 that signals through a MyD88-independent pathway.

Table 1 TLR and ligands

TLR	Ligand	Cellular location	Adaptor molecule
1	Triacyl lipoproteins	Cell surface	MAL/MyD88
2	Peptidoglycan, fungi, virus glycoproteins, lipoproteins, lipoteichoic acids	Cell surface	MAL/MyD88
3	ds RNA, poly (I:C)	Endosome	TRIF
4	Lipopolysaccharide (LPS)	Cell surface	MAL/MyD88 and TRAM/TRIF
5	Flagellin	Cell surface	MyD88
6	Diacyl lipoproteins	Cell surface	MAL/MyD88
7	ssRNA, small synthetic compounds	Endosome	MyD88
8	ssRNA	Endosome	MyD88
9	Unmethylated CpG DNA	Endosome	MyD88
10	Unknown	Unknown	Unknown
11	Profilin-like protein in *Toxoplasma gondii*	Innate immune cells, particularly DCs	MyD88

TRIF interacts with adaptor molecule TRIF-related adaptor molecule (TRAM) for signaling through TLR4, which also can signal through the MAL/MyD88 adaptor protein complex (reviewed in ref. [13]). Activation through TLR4 by LPS, for example, induces production of proinflammatory cytokines IL-6, TNF-α, and IL-12 [15]. Through the MyD88-independent pathway, TLR4 can induce activation of IRF3 and induce production of IFN-α/β through the TRAM/TRIF complex [16–18].

NF-κB and MAPK are involved in the downstream affects of TLR signaling pathways. NF-κB is a major regulator of gene transcription made up of five subunits: p50, p65, p52, RelB, and c-Rel (reviewed in [19]). Two of these subunits dimerize to allow translocation into the nucleus, and NF-κB binds to DNA. Once in the nucleus, NF-κB regulates the production of more than 150 genes coding for cytokines, Ag receptors, apoptosis, and host defense. Both sensory and effector functions of TLRs are involved during immune response to pathogens. The production of proinflammatory cytokines and increased APC costimulatory potential are perhaps the immediate response of the host to pathogens by the host via TLR recognition of that particular pathogen [20].

TLR activation by helminth parasites

Downregulation of an Ag-specific T cell proliferative response is a hallmark of several different parasitic infections [21–23] and may reflect a mechanism by which parasite survival is promoted in the host. Factors such as regulatory cytokines [24], altered function of APC [25–30], T cell apoptosis [31], and inducible NO synthase [22, 32] have each been implicated in mediating this downregulated response. We have previously shown that microfilarial antigen (MF Ag) as well as live microfilaria (MF) exhibit a suppressive effect on DCs. For example, they impair production of both IL-12 and IL-10 by DCs [27, 28]. Furthermore, the infective stage (L3) of *B. malayi* also downregulates the function of human Langerhans' cells (LCs), leading to a decreased proliferation of $CD4^+$ T cells that encounter these parasite-exposed LCs [29].

Although there have been many studies examining TLR signaling in response to intracellular pathogens (including the parasitic protozoa) [33–35], fewer studies have examined interaction of the multicellular helminth parasites and the TLR system. The mechanisms underlying inflammation induced by filarial infection are not fully understood.

The observation that *W. bancrofti*, *B. malayi*, and *Onchocerca volvulus* harbor an obligate intracellular rickettsia-like Wolbachia bacteria [36–38] has raised the possibility that filarial-infected individuals may respond to this endosymbiont in a manner that promotes or initiates an inflammatory response [39–43]. In fact, the evidence that filarial parasites elicit immune response through TLRs originally came from a study by Taylor et al. [44] indicating that Wolbachia extracts derived from a mosquito cell line induced similar LPS-dependent response in murine macrophages, perhaps through TLR4. Furthermore, in 2004, Brattig et al. [45] advanced these studies by showing that the major surface protein of Wolbachia (wsp) in filarial nematodes can indeed elicit immune response in human embryonic kidney 293 (HEK293) cell line through both TLR2 and TLR4. In the same study, these investigators also showed that wsp induced an inflammatory response measured by proinflammatory cytokines in murine macrophages and DCs again through a TLR2- and TLR4-dependent mechanism, as mice deficient in either of these TLRs failed to elicit the same response. These studies were further pursued by Hise et al. [46] using human TLR-transfected HEK cell line as well as murine macrophages from TLR and adaptor molecule gene knockout to show that the inflammatory

response to Wolbachia is mediated primarily by engagement of TLR2 and TLR6 and is indeed dependent on MyD88 and the TIR domain-containing adaptor protein (TIRAP)/MAL.

Major secreted products (ES) also contribute to the immunomodulatory response seen in helminth infections. In the rodent filarial nematode *Acanthocheilonema viteae*, the phosphorylcholine-containing glycoprotein ES-62 was found to inhibit the activation of B and T lymphocytes through TLR recognition. Using mice deficient in TLR4 or MyD88, Goodridge et al. were able to show that the effect of ES-62 on IL-12 and TNF-α production was mediated by an MyD88-dependent TLR4 pathway [47]. Furthermore, utilizing human embryonic kidney 293 (HEK293) cells, we have shown that live mf of *B. malayi* can activate TLR2 directly, but not TLR4 or TLR3 [48].

Immune deviation during *S. mansoni* infection is characterized by an alteration in the number and activation state of many cell types including macrophages and DC [4]. During this infection, the immune response is initially Th1-like but, following the onset of egg production, this response becomes highly Th2 polarized. After the release of eggs by this parasite, a profound granulomatous remodeling response driving the production of IL-4 and IL-13 is observed [49]. The primary inducer of this Th2 response that characterizes schistosomiasis [50, 51] is known to be both complex carbohydrates ([52] ,Yazdanbaksh, M (published and unpublished)) expressed on the soluble egg Ag (SEA) secreted by *S. mansoni* ova and parasite egg secreted ribonuclease (Jankovic, D, unpublished). Tissue-resident macrophages play an important role during the course of this infection by producing cytokines, chemotactic factors, and free radicals [53–55]. Furthermore, with chronic Th2-like conditions seen in schistosome infections, a subset of macrophages undergoes an alternative activation [56] that may mediate the tissue-destructive fibrotic response seen most commonly in the liver. Indeed, it has been documented that bone marrow-derived macrophages from mice with *S. mansoni* egg-induced pulmonary granulomas have an augmented response to TLR2 and TLR3 activation compared to control mice [57]. Furthermore, when live schistosome larvae of different maturation stages or soluble preparations from whole larvae were used to stimulate cytokine production by thioglycollate-elicited macrophages (tMφ), the parasite-derived molecules released from the schistosome larvae were shown to partly act through TLR4, MyD88-dependent pathway [58].

In addition to macrophages, signals delivered by DC can greatly influence Th polarization in the course of *S. mansoni* infection [59, 60] in that DCs, in general, use PRRs to identify and respond to the pathogens and produce a series of positive and negative signals involved in effector cell differentiation of naïve T cells [61, 62]. Although the schistosomal PAMPs, lysophosphatidylserine, and schistosomal glycolipids [63, 64] have been shown incapable of transducing a signal through TLRs on HEK-transfected cell lines, lysophosphatidylserine was found to activate DC through TLR2 in such a way that resulted in skewing toward a Th2 response and toward the development of Tregs [63]. Studies by Layland et al. [65] extended the link between triggering of TLR2 and the induction of Tregs, a finding that suggested that pathology induced in schistosomiasis could be mediated by TLR2-activated cells that resulted in Treg expansion.

Other studies have also indicated the activation of TLRs by this parasite, as they have shown that the eggs of *S. mansoni* can activate transcription of genes including cell surface markers CD40 and CD86, and cytokines IFN-β, TNF-α, and IL-12-p40 in mouse myeloid DCs [66]. Of interest, schistosome eggs can activate TLR2 (but not TLR4), and the dsRNAs from these eggs activate TLR3 in transfection assays [67].

TLR regulation by helminth parasites

Once activated by microbial PAMP, TLRs transduce signals through two pathways involving distinct adaptor proteins containing TIR domains discussed above. The end result of TLR signaling is activation of NF-κB, triggering induction of proinflammatory cytokines or IRF-dependent induction of type I interferons. TLR-dependent proinflammatory cascades triggered by infections with protozoan parasites and other microbial agents must be tightly regulated to avoid severe pathology or even mortality. Furthermore, this TLR regulation can be at the level of expression, function, or combination of the two, resulting in tight control of the immune response. We review below the regulation of expression and function of TLRs by these parasitic helminths (also see Table 2).

TLR expression

Although bacterial (and non-bacterial intracellular) pathogens typically cause increases in TLR expression, downregulation of TLR expression appears to be an important evasion strategy utilized successfully by some bacterial pathogens (reviewed in [68]). For example, a mechanism of bacteria-induced immune suppression has been suggested based on TLR4 downregulation and tolerance by LPS and TLR2 downregulation by bacterial lipoprotein [69, 70]. Similarly, protozoan parasites such as *Entamoeba histolytica* and *Trypanosoma spp*. have been shown to inhibit immune responses by suppressing TLR-mediated signaling [71, 72], particularly by downregulating TLR2 expression [73].

Direct evidence that multicellular helminths can downregulate the gene or protein expression of TLRs in human came from studies [48] that demonstrated that exposure of monocyte-derived DC to live MF of *B. malayi* significantly downregulated mRNA expression of TLR3, TLR4, TLR5, and TLR7. Furthermore, using immunoblot analysis TLR3 protein expression induced by Brugian parasites was shown to be unaltered, while the protein expression of TLR4 was markedly diminished.

In clinical settings, Babu et al. [74] have shown that filaria-infected individuals indeed have decreased expression of TLR1, TLR2, TLR4, and TLR9 on B cells based both on mRNA expression and protein (surface or intracellular expression) levels. They also showed that filarial infected individuals had a diminished ability to upregulate TLR expression upon parasite Ag stimulation in both B cells and monocytes. Other studies have

Table 2 Activation and regulation of TLRs by helminth parasites

	Filarial infections				Schistosome infections			
	Murine models		Human infections		Murine models		Human infections	
	in vitro	in vivo	in vitro	ex vivo	in vitro	in vivo	in vitro	ex vivo
Direct Activation of TLR by parasite products	+	+	+	ND	+	+	+	ND
Alteration of TLR expression on APCs	+	ND	+	+	ND	ND	ND	ND
Inhibition of signaling through TLR	+	+	+	+	+	+	+	+

ND = Not Determined

also shown diminished surface expression of TLR after exposure of murine macrophages to *B. malayi* female worm extracts (BMFE) [75].

One characteristic of lymphatic filarial infection is a modulated Ag-driven Th1 response [3] and the major adaptive immune response critical for elimination of the most pathogens. Previous studies have shown that exposure to MF results in a diminished ability of APCs to stimulate $CD4^+$ T cells [27, 28]. Diminished T cell activation would naturally have a profound effect on the ability of the adaptive immune system to fulfill its role. It has also been reported that T cells express many of the TLRs at the mRNA gene level and therefore may play an additional role in TLR signaling. T cells from patients with lymphatic filariasis, in the presence of B cells and monocytes, expressed significantly lower levels of TLR1, 2, and 4 than did T cells from filarial-uninfected individuals [76] suggesting that filarial parasites (and presumably other helminth parasites) can affect T cell development.

TLR function

Regulation of TLRs can be manipulated by helminth parasites at the level of TLR expression and function. We have recently shown that not only do live MF of *B. malayi* downregulate the mRNA expression of TLR3 and TLR4 in human monocyte-derived DC, they also diminish the response of these cells to TLR3 and TLR4 ligands. DCs that were exposed to live MF for 48 h had a significant decrease in production of IL-12p40 following activation with poly I:C or LPS, and IFN-α, IL-12 p40, IL-12 p70, and MIP-1α following activation with poly I:C. Moreover, the mRNA and protein expression of MyD88 was significantly decreased in DCs that were exposed to live MF. Notably, mRNA expression of inhibitory molecules such as SOCS1 and SOCS3 were upregulated in these MF-exposed cells. Finally, live MF downregulated the binding ability of p50 and p65 (of the NF-κB complex) in DC following activation with either LPS or poly I:C, which may, in turn, explain the diminished cytokine production by these cells [48]. The possibility that parasitic helminths may bind particular TLRs but not signal has been given credence in murine studies by Goodridge et al. [47] in which ES-62 of the filarial nematode *Acanthocheilonema vitiae*, induces by itself low production of IL-12 and TNF-α in a TLR4- and MyD88-dependent manner but also leads to subsequent inhibition of cytokines (IL-12, TNF-α, and IL-6) production induced by the TLR4 ligand LPS.

In a study by Babu et al. [74], in which the expression of several TLRs was shown to be lower in filaria-infected compared to filarial-uninfected individuals, the response to specific TLR ligands was also diminished; the diminished cytokine production appeared to reflect chronic exposure to filarial parasites causing diminished ability to signals through TLRs [74].

In studies with the obligate intracellular endosymbiont Wolbachia of *Brugia malayi*, Turner et al. [75] suggested a role for Wolbachia in promoting macrophage tolerance to TLR through a TLR2/MyD88 pathway. This study revealed that pre-exposure of murine macrophages to BMFE inhibited production of TNF-α and IL-12 in response to secondary stimulation by the worm, ligands for TLR2, TLR4, and TLR9 and through a TLR3-mediated but MyD88-independent process. Interestingly, this BMFE-mediated heterotolerance of macrophages was abrogated in the absence of MyD88 and TLR2 but not TLR4, suggesting a direct role of TLR2 in this system [75].

The negative regulatory effect of helminth parasites also applies to SEA. For example, it has become apparent that SEA exerts an inhibitory effect on DC maturation induced by TLR ligands in that DCs pulsed with both SEA and LPS produce less IL-12 than DCs pulsed only with the TLR ligand alone [77, 78]. Furthermore, SEA has been shown to

inhibit the ability of CpG, poly I:C, hyaluronic acid, and LPS to induce IL-12 production or upregulate CD80, CD86, and MHC class II surface expression on DCs [79]. Also, SEA has been shown to prevent LPS-induced downregulation of other genes based on microarray analysis [79]. This suppressive effect extends to human DCs; SEA has been shown to suppress maturation of human DCs induced by both poly I:C and LPS as indicated by a decrease in both cytokine production (IL-12, IL-6, and TNF-α) and by costimulatory molecule (CD80/86) surface expression [80]. In addition, SEA suppressed Th1 responses following coculture of poly I:C-pulsed DCs and T cells, and shaped the LPS-induced responses toward a Th2 response.

Interestingly, cells from *Schistosoma haematobium (Sh)*-infected children in Gabon had a lower level of cytokine responses to a schistosomal phosphatidylserine fraction containing a TLR2 ligand as well as to a TLR4 ligand as compared with *Sh*-uninfected children [81]. In contrast, uninfected children had a higher response to schistosomal adult worm glycolipids (none being TLR ligands) than did infected children. These data suggest that chronic and presumably continuous exposure to helminth antigens can negatively regulate the response of cells to PAMPs that are derived from these parasites and change the immune response in helminth-infected individuals.

Concluding remarks

TLRs have many modes of action that allow them to play an important role in the immune response to pathogens. Each TLR clearly has a unique role in generating that response. The chronic nature of the two systemic helminth infections described in this review provides a window into mechanisms used by the parasite to subvert the host immune system, most notably by interfering with both the expression and function of TLRs.

The fact that helminth parasites can both activate (to a small degree) and negatively regulate TLRs (to a much larger degree) suggests that the immune response to invasive helminths is under tight control. Perhaps the timing of this early activation and subsequent regulation is important for the individuals infected with these parasites. However, because exposure to helminth parasites (and their antigens) is prolonged, the function of the cells involved in innate immune responses is dampened. This negative regulation of TLRs results in diminished pro-inflammatory cytokine production that may be protective to the host by preventing pathology. Compromised TLR expression and function, however, can have bystander effects such that response to non-helminth pathogens (e.g., bacteria or viruses) is also dampened. Thus, exploring the interface between tissue-invasive helminth parasites and the innate immune system should shed additional light onto the role played by TLR dysregulation and provide new areas of study for therapy and vaccine development that may involve alterations in TLR expression and function.

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