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# Toll-Like Receptors (TLRs) and Innate Immunity

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## Preface

In the second half of the 20th century innate immune responses of cellular or humoral type were treated like stepchildren by many immunologists: that is, somewhat neglected. This disregard turned into an exciting research field over the past several years and led to the identification of receptor families involved in the recognition of microbes. This has resulted in a remarkable refinement of our understanding of innate immunity as it relates to host defense. Additionally, it has led to the appreciation of the role of the innate immunity in “guiding” the development of the adaptive immune system.

The Toll-like receptors represent an important family within the innate immune system. Originally identified in *Drosophila* as Toll receptors, various homologous Toll-like receptors (TLRs) have been identified in vertebrates. These receptors recognize chemically diverse ligands such as lipoproteins, proteins, lipopolysaccharides, nucleic acid, and nucleoside analogues and lead to the activation of immune cells. The mechanisms of TLR-driven cellular activation are quite well understood; however, information on the ligand-receptor interaction of the TLRs are scarce. A great body of evidence suggests that TLR ligands, especially nucleic acid and its analogues, have great pharmacological potential in the treatment of infectious diseases and cancer.

This book reviews and highlights our recent understanding on the function and ligands of TLRs, as well as their role in autoimmunity, dendritic cell activation and target structures for therapeutic intervention.

Overall, recent research on TLRs has led to tremendous increase in our understanding of early steps in pathogen recognition and will presumably lead to potent TLR-targeting therapeutics in the future.

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# Toll-Like Receptors (TLRs) and Their Ligands

Satoshi Uematsu and Shizuo Akira(✉)

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**Abstract** The innate immune system is an evolutionally conserved host defense mechanism against pathogens. Innate immune responses are initiated by *pattern recognition receptors* (PRRs), which recognize microbial components that are essential for the survival of the microorganism. PRRs are germline-encoded, non-clonal, and expressed constitutively in the host. Different PRRs react with specific ligands and lead to distinct antipathogen responses. Among them, *Toll-like receptors* (TLRs) are capable of sensing organisms ranging from bacteria to fungi, protozoa, and viruses, and they play a major role in innate immunity. Here, we review the mechanism of pathogen recognition by TLRs.

## 1 Introduction

In mammals, host defenses sense pathogen invasion through PRRs. Toll-like receptors are evolutionally conserved transmembrane proteins and play crucial roles as PRRs. Recent molecular biological studies have clarified the function of TLRs

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in microbial infection. TLRs recognize specific components of microorganisms, including fungi, protozoa, and viruses, and they induce innate immune responses. Here, we summarize the current knowledge regarding TLR family members and their ligands.

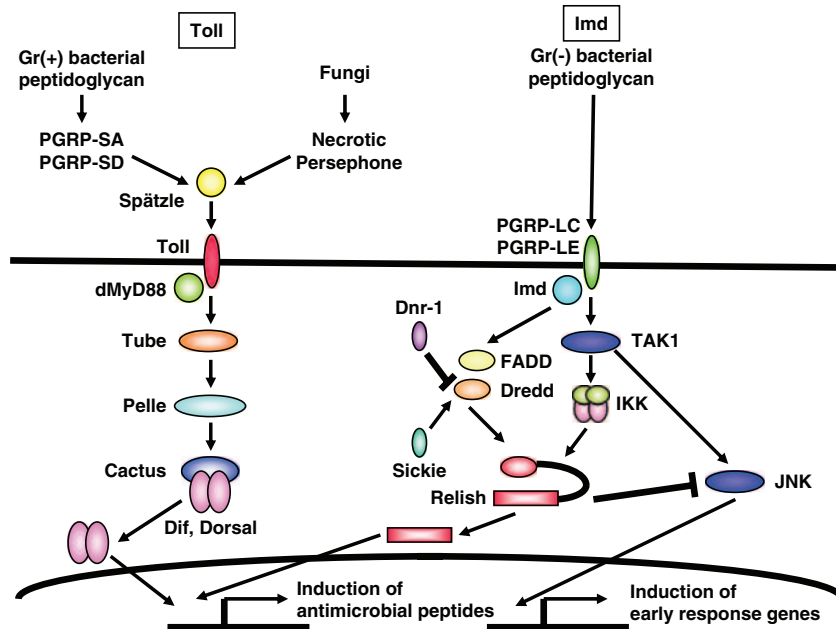
## 2 Innate Immunity

The mammalian immune system is divided into two types of immunity: *innate* and *adaptive*. Adaptive immunity is characterized by specificity and develops by clonal selection from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated by gene rearrangement. This mechanism allows the host to generate immunological memory. However, it takes time for specific clones to expand and differentiate into effector cells before they can serve for host defense. Therefore, the primary adaptive immune system cannot induce immediate responses to invasive pathogens. To induce immediate responses when it encounters a pathogen, a host is equipped with innate, nonadaptive defenses that form preemptive barriers against infectious diseases. Although the innate immune system was first described by Elie Metchnikoff over a century ago, it has long been ignored: viewed as merely a nonspecific response to simple phagocytose pathogens and as something that presents antigens to the cells involved in acquired immunity (Brown, 2001). However, in 1996, Hoffmann and colleagues demonstrated that the *Drosophila* protein Toll is required for flies to induce effective immune responses to *Aspergillus fumigatus* (Lemaitre et al., 1996). This study made us aware that the innate immune system functions as a pathogen detector. The targets of innate immune recognition are conserved molecular patterns of microorganisms. Therefore, the receptors involved in innate immunity are called pattern-recognition receptors (Medzhitov and Janeway, 1997). These molecular structures were originally called pathogen-associated molecular patterns (PAMPs). However, it is more appropriate to designate them as microorganism-associated molecular patterns (MAMPs) since they are found not only in pathogenic but also in nonpathogenic microorganisms. MAMPs are generated by microbes and not by the host, suggesting that MAMPs are good targets for innate immunity to discriminate between self and non-self. Furthermore, MAMPs are essential for microbial survival and are conserved structures among a given class, which allows innate immunity to respond to microorganisms with limited numbers of PRRs. There are many PRRs associated with opsonization, phagocytosis, complement and coagulation cascades, proinflammatory signaling pathways, apoptosis, and so on. Among them, Toll receptors and the associated signaling pathways represent the most ancient host defense mechanism found in insects, plants, and mammals (Akira, 2004). Studies of the fruit fly have shown that the Toll family is one of the most crucial signaling receptors in innate immunity.

## 2.1 Immune Responses in *Drosophila*

Insects do not have counterparts of mammalian B and T cells and cannot induce acquired immune responses based on producing antibodies to pathogenic organisms. Nonetheless, insects can recognize the invasion of various microorganisms and induce antimicrobial responses. Recent studies using a model organism, *Drosophila melanogaster*, have shown that the induction of antimicrobial peptides, which are important for survival after infection, depends on Toll and immune deficiency (Imd) signaling pathways (Tanji and Ip, 2005). A transmembrane protein, Toll, originally identified as an essential component in dorsal-ventral embryonic development (Wu and Anderson, 1997), is also involved in innate immune responses (Lemaitre et al., 1996). Gram-positive bacterial peptidoglycan might bind directly to extracellular peptidoglycan recognition protein (PGRP)-SD (Michel et al., 2001) and SD (Bischoff et al., 2004), which then stimulate the Toll pathway. Another pattern recognition protein, Gram-negative binding protein-1 (GNBP-1), is also involved in the recognition of Gram-positive bacteria (Gobert et al., 2003; Pili-Floury et al., 2004). Not only Gram-positive bacteria but also fungi stimulate the Toll pathway. Fungi are recognized by a serine protease, Persephone, and a protease inhibitor, Necrotic (Levashina et al., 1999; Ligoxygakis et al., 2002). All upstream cascades lead to the cleavage of pro-Spätzle to Spätzle, and the binding of proteolytically processed Spätzle to Toll induces the dimerization of Toll (Hu et al., 2004; Weber et al., 2003). After activation of Toll, the adapter proteins MyD88 and Tube, and a serine-threonine kinase, Pelle, are recruited to Toll (Sun et al., 2004). Then, activated Pelle acts on the Cactus, a *Drosophila* I $\kappa$ B. Dif and Dorsal are transcription factors of the Rel protein family and are retained in the cytoplasm by Cactus. By the stimulation of the Toll pathway, Cactus is degraded and Dorsal and Dif translocate into the nucleus, leading to the induction of antimicropeptides (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003).

The Imd pathway is responsible for the induction of antimicrobial peptides in response to Gram-negative bacteria (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003; Lemaitre, 2004). Imd is an adapter protein for this pathway (Georgel et al., 2001). Recent reports show that PGRP-LC (Choe et al., 2002; Gottar et al., 2002) and PGRP-LE (Takehana et al., 2004), which have putative transmembrane domains, are the pattern recognition receptors in this pathway. There are at least three branches downstream of Imd (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003; Lemaitre, 2004). First is TAK1, which induces the proteolytic cleavage of IKK, followed by activation of the transcription factor, Relish (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2003; Silverman et al., 2000; Stoven et al., 2003; Vidal et al., 2001). Second is the FADD-Dredd pathway that also activates Relish (Balachandran et al., 2004; Chen et al., 1998; Elrod-Erickson et al., 2000; Georgel et al., 2001; Hu and Yang, 2000; Leulier et al., 2000; Leulier et al., 2002). Two new components, Sickie and Dnr-1, have been identified; whereas Sickie positively regulates the Relish activation by Dredd, Dnr-1 inhibits



**Fig. 1** Toll and Imd pathways in the *Drosophila* innate immune response. The Toll pathway mediates the response to fungal and Gr(+) bacterial infection, whereas the Imd pathway mediates the response to Gr(-) bacterial infection. These pathways are similar to the signaling pathway of the mammalian Toll-like receptor, and are essential for *Drosophila* to survive infection

this pathway (Foley and O'Farrell, 2004; Khush et al., 2002). Third is the JNK pathway that is activated through TAK1. The JNK pathway induces immediate early genes after septic shock, which is negatively regulated by Relish (Boutros et al., 2002; Park et al., 2004).

As stated above, recent genetic and genomic analyses of *D. melanogaster* have shown that insects have an evolutionally primitive recognition and signaling system (Figure 1). Collectively, these analyses' results provide important insights into the mechanism of pathogen recognition and host responses in mammalian systems.

## 2.2 Toll-Like Receptors

A mammalian homologue of Toll receptor (now termed TLR4) was identified through database searches and shown to induce expression of the genes involved in inflammatory responses (Medzhitov et al., 1997). Subsequently, a mutation in the *tlr4* gene was identified in C3H/HeJ mice that were hyporesponsive to lipopolysaccharide (LPS) (Poltorak et al., 1998). TLR4-deficient mice confirmed LPS's essential role in the LPS recognition (Hoshino et al., 1999). So far, 13 mammalian members of the TLR family have been identified (Akira, 2004). TLRs

are type I integral member glycoproteins characterized by a cytoplasmic signaling domain and extracellular domains. As the cytoplasmic domain of TLRs is similar to that of the interleukin (IL)-1 receptor family, it is called the Toll/IL-1 receptor (TIR) domain. However, the extracellular region of TLRs and IL-1R are markedly different: Whereas IL-1R possesses an Ig-like domain, TLRs contain leucine-rich repeats (LRR) (Akira, 2004). The LRR domains are composed of 19–25 tandem LRR motifs, each of which is 24–29 amino acids in length, containing the motif XLXXLXLXX as well as other conserved amino acid residues (XΦXXΦXXXXFXXLX; Φ = hydrophobic residue). Each LRR consists of a β-strand and an α-helix connected by loops. The LRR domain of TLRs was supposed to form a horseshoe structure with the ligand binding to the concave surface. However, the three-dimensional structure of the human TLR3 LRR motifs suggested that negatively charged dsRNA is more likely to bind to the outside convex surface of TLR3. It is uncertain whether this model fits the other TLR family members. Future crystallographic analysis of other TLRs will be necessary for elucidating the ligand/receptor binding mechanism.

The TLR family is an important group of receptors through which innate immunity recognizes invasive microorganisms. TLRs are key molecules for microbial elimination, such as the recruitment of phagocytes to infected tissues and subsequent microbial killing. Recent gene targeting studies have revealed that TLRs sense organisms ranging from bacteria to fungi, protozoa, and viruses (Tables 1–4).

**Table 1** TLRs and bacterial ligands

Bacterial component	Species	TLR usage
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	<i>Mycoplasma</i>	TLR2/TLR6
Triacyl lipopeptides	Bacteria	TLR2/TLR1
Peptidoglycans	Gram-positive bacteria	TLR2(?)
Lipoteichoic acid	Gram-positive bacteria	TLR2/TLR6
Phenol-soluble modulin	<i>Staphylococcus aureus</i>	TLR2
Glycolipids	<i>Treponema maltophilum</i>	TLR2
Atypical LPS	Non-entero bacteria	TLR2(?)
Flagellin	Flagellated bacteria	TLR5
CpG DNA	Bacteria	TLR9
Not determined	Uropathogenic bacteria	TLR11

**Table 2** TLRs and fungal ligands

Fungal component	Species	TLR usage
Zymosan	<i>Saccharomyces cerevisiae</i>	TLR2/TLR6
Mannan	<i>Saccharomyces cerevisiae</i>	TLR4
	<i>Candida albicans</i>	
Phospholipomannan	<i>Candida albicans</i>	TLR2
Glucuronoxylomannan	<i>Cryptococcus neoformans</i>	TLR4

**Table 3** TLRs and protozoan ligands

Protozoan component	Species	TLR usage
(GPI anchors)		
GPI anchor	<i>Trypanosoma cruzi</i>	TLR2/TLR6
Glycoinositolphospholipids	<i>T. cruzi</i>	TLR4
LPG	<i>Leishmania major</i>	TLR2
Galbeta1, 4Manalpha-Po(4)-containing phosphoglycans	<i>Leishmania donovani</i>	TLR2
GPI anchor	<i>Plasmodium falciparum</i>	TLR2, TLR4
Native GPI anchors	<i>Toxoplasma gondii</i>	TLR2, TLR4
(Non-GPI anchors)		
Tc52	<i>Trypanosoma cruzi</i>	TLR2
Genomic DNA	<i>Babesia bovis</i> , <i>T. cruzi</i> and <i>T. brucei</i>	TLR9
Hemozoin	<i>P. falciparum</i>	TLR9
Profilin-like protein	<i>T. gondii</i>	TLR11

**Table 4** TLRs and viral ligands

TLRs (localization)	Virus and components
(cell surface)	
TLR2	envelope proteins of Measles virus, human cytomegalovirus and herpes simplex virus type I
TLR4	F protein of respiratory syncytial virus (RSV) Envelope protein of mouse mammary tumor virus (MMTV)
(endosome)	
TLR3	Viral dsRNA, synthetic dsRNA (Poly(I:C))
TLR7/TLR8	ssRNA, synthetic imidazoquinoline derivatives (anti-viral drugs)
TLR9	CpG DNA

### 3 Pathogen Recognition by TLR

#### 3.1 Bacteria

Lipopolysaccharide is a cell wall component of Gram-negative bacteria and a strong immunostimulant. As described above, TLR4 is essential for recognition of LPS, which is composed of lipid A (endotoxin), core oligosaccharide, and O-antigen. TLR4 recognizes lipid A of LPS. For LPS recognition, a complex formation of TLR4, MD2, and CD14 on various cells, such as macrophages and dendritic cells, is necessary (Shimazu et al., 1999). LPS is associated with an accessory protein, LPS-binding protein (LBP) in serum, which converts oligomeric micelles of LPS to monomers for delivery to CD14, which is a glycosyl phosphatidylinositol

(GPI)-anchored, high-affinity membrane protein. CD14 concentrates LPS for binding to the TLR4/MD2 complex (Takeda et al., 2003).

TLR2 recognizes various bacterial components, such as lipoproteins/lipopeptides and peptidoglycans from Gram-positive and Gram-negative bacteria, and lipoteichoic acid from Gram-positive bacteria, a phenol-soluble modulins from *Staphylococcus aureus*, and glycolipids from *Treponema maltophilum* (Takeda et al., 2003; Takeuchi et al., 1999a). TLR2 is also reported to be involved in the recognition of LPS from non-enterobacteria, including *Leptospira interrogans*, *Porphyromonas gingivalis*, and *Helicobacter pylori* (Takeda and Akira, 2005). These are atypical LPSs whose structures are different from typical LPSs of Gram-negative bacteria (Netea et al., 2002b). However, a recent report has indicated that lipoproteins contaminated in LPS preparation from *P. gingivalis* stimulated TLR2 and that LPS from *P. gingivalis* itself had poor TLR4 stimulation activity (Hashimoto et al., 2004). There are also controversial reports regarding peptidoglycan recognition by TLR2. Careful analyses are needed to ensure the exclusion of any possible contaminants.

TLR1 and TLR6 are structural relatives of TLR2 (Takeuchi et al., 1999b). TLR2 and TLR1 or TLR6 form a heterodimer that is involved in the discrimination of subtle changes in the lipid portion of lipoproteins. TLR6-deficient macrophages do not produce inflammatory cytokines in response to diacyl lipopeptides from mycoplasma; however, they normally produce inflammatory cytokines in response to triacyl lipopeptides derived from a variety of bacteria (Takeuchi et al., 2001). Contrarily, TLR1-deficient macrophages show normal responses to triacyl lipopeptides but not to diacyl lipopeptides (Alexopoulou et al., 2002; Takeuchi et al., 2002). These results suggest that TLR2 interacts not only physically but also functionally with TLR1 and TLR6.

CD36 is a member of the class II scavenger family of proteins. A recent report has shown that CD36 serves as a facilitator or co-receptor for diacyl lipopeptide recognition through the TLR2/6 complex (Hoebe et al., 2005).

Bacterial flagellin is a structural protein that forms the major portion of flagella that contribute to virulence through chemotaxis, adhesion to, and invasion of host surfaces. TLR5 is responsible for the recognition of flagellin (Hayashi et al., 2001; Uematsu et al., 2006). Unlike other TLRs, TLR5 is not expressed on conventional dendritic cells or macrophages in mice (Uematsu et al., 2006). Gewirtz et al. reported that TLR5 is expressed on the basolateral surface, but not the apical side of intestinal epithelial cells, suggesting that flagellin is detected when bacteria invade across the epithelium (Gewirtz et al., 2001). However, the expression of TLR5 in mouse intestinal epithelial cells is not high (Uematsu et al., 2006). By contrast, TLR5 is expressed mainly on intestinal CD11c<sup>+</sup> lamina propria cells (LPCs). CD11c<sup>+</sup> LPCs detected pathogenic bacteria and secreted proinflammatory cytokines in a TLR5-dependent way (Uematsu et al., 2006). A common stop codon polymorphism in the ligand-binding domain of TLR5 (TLR5 392STOP SNP) is unable to mediate flagellin signaling and is associated with susceptibility to pneumonia caused by *Legionella pneumophila* (Hawn et al., 2003). However, researchers in Vietnam reported that TLR5 392STOP SNP is not associated with susceptibility to typhoid fever (Dunstan et al., 2005). Although TLR5 initially induced host defenses

against flagellated bacteria, TLR5-deficient mice were resistant to oral *Salmonella typhimurium* infection. The transport of *S. typhimurium* from the intestinal tract to the mesenteric lymph nodes (MLNs) was impaired in TLR5-deficient mice. These results suggest that *S. typhimurium* utilizes TLR5 on CD11c<sup>+</sup> LPCs for systemic infection (Uematsu et al., 2006).  $\alpha$  and  $\epsilon$  *Proteobacteria*, including *Helicobacter pylori* and *Campylobacter jejuni*, change the TLR5 recognition site of flagellin without losing flagellar motility (Andersen-Nissen et al., 2005). This modification may contribute to the persistence of these bacteria on mucosal surfaces.

Bacterial DNA is a potent stimulator of the host immune response. This immune stimulation is mediated by unmethylated CpG motifs. In vertebrates, the frequency of CpG motifs is severely reduced and the cytosine residues of CpG motifs are highly methylated, which leads to abrogation of the immunostimulatory activity. Analysis of TLR9-deficient mice showed that CpG DNA recognition is mediated by TLR9 (Hemmi et al., 2000).

Mouse TLR11, a relative of TLR5, is expressed abundantly in the kidney and bladder. TLR11-deficient mice are susceptible to uropathogenic bacterial infections, indicating that TLR11 senses the component of uropathogenic bacteria. However, the human *Tlr11* gene appears to contain a stop codon that would prevent expression of the protein (Zhang et al., 2004) (Table 1).

### 3.2 Fungi

TLRs have been implicated in the recognition of the fungal pathogens such as *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Pneumocystis carinii* (Netea et al., 2004; Takeda et al., 2003). Several components located in the cell wall or cell surface of fungi have been identified as potential ligands. Yeast zymosan, derived from *Saccharomyces cerevisiae*, activates TLR2/TLR6 heterodimers, whereas mannan, derived from *S. cerevisiae* and *C. albicans*, are detected by TLR4. TLR4-deficient mice show increased susceptibility to disseminated candidiasis due to the decreased release of chemokines and the impaired recruitment of neutrophils to infected sites (Netea et al., 2002a).

Phospholipomannan, present on the cell surface of *C. albicans*, is also recognized by TLR2, while TLR4 mainly interacts with glucuronoxylomannan, the major capsular polysaccharide of *C. neoformans* (Netea et al., 2004).

Dectin-1 is a lectin family receptor for the fungal cell wall component,  $\beta$ -glucan, which is a major component of zymosan (Brown et al., 2002). Dectin-1 has been reported to functionally collaborate with TLR2 in response to yeast (Netea et al., 2004). The Dectin-1-mediated signaling pathway uses spleen tyrosine kinase (Syk), and interactions with Syk directly induce cellular responses such as the respiratory burst and IL-10 production. Dectin-1 is also reported to collaborate with TLR2 and to induce proinflammatory responses such as the induction of TNF- $\alpha$  and IL-12 (Gantner et al., 2003; Rogers et al., 2005; Underhill et al., 2005). Gross et al. reported that Card9 is required to link Dectin-1/Syk activation to Bcl10-Malt1-dependent NF- $\kappa$ B activation by zymosan (Gross et al., 2006). Recently, two groups



generated Dectin-1-deficient mice (Saijo et al., 2007; Torok et al., 2004). When stimulated with zymosan, IL-10 production was completely dependent on Dectin-1. However, TNF- $\alpha$  production was not impaired in Dectin-1-deficient mice but was dependent on TLR-mediated signaling pathway. When stimulated with purified  $\beta$ -glucan, both IL-10 and TNF- $\alpha$  production was dependent on Dectin-1, though the total amount of TNF- $\alpha$  production markedly decreased compared with zymosan stimulation. Thus, Dectin-1 is the sole receptor for  $\beta$ -glucan, and most inflammatory cytokine production by zymosan might be the result of TLR recognition of their ligands, except for  $\beta$ -glucan contained in zymosan (Saijo et al., 2007). Experimental infection models of disseminated candidiasis in Dectin-1-deficient mice showed different phenotypes between the two research groups. Taylor et al. (2007) reported that Dectin-1-deficient mice were more susceptible to *C. albicans* infection than wild-type mice. These results were inconsistent with the study of Saijo et al. (2007), who found Dectin-1-deficient mice and wild-type mice equally susceptible to candida infection. Interestingly, Dectin-1-deficient mice were more susceptible than wild-type mice to pneumocystis infection (Saijo et al., 2007). Further study is obviously necessary to clarify the *in vivo* function of Dectin-1.

### 3.3 Protozoa

#### 3.3.1 Protozoan GPI Anchors

Several studies have shown that glycosylphosphatidylinositol (GPI) anchors (or their fragments) from protozoan parasites activate cells of both lymphoid and myeloid lineages (Camargo et al., 1997; de Veer et al., 2003; Debierre-Grockiego et al., 2003; Magez et al., 1998; Schofield and Hackett, 1993). GPI moieties are abundantly expressed by many protozoan parasites and function as anchors to the surface of eukaryotic cells. GPI anchors consist of a glycan core and a lipid component. GPI anchors are featured with variations in the carbohydrate branches, the lipid inositol portion (glycerol versus ceramide), and the number, length, and degree of saturation in the hydrocarbon chains (Gazzinelli and Denkers, 2006). Although GPI anchors are expressed on mammalian cells, they do not initiate host immune responses. The expression levels of GPI anchors on mammalian cells are much lower than those of protozoan parasites. Moreover, the structure of protozoan-derived GPI anchors is different from mammalian-derived ones in the length of the glycan core and lipid component. All these differences may determine the activation of host immunity (Gazzinelli and Denkers, 2006).

TLRs sense GPI anchors of protozoa including *Trypanosoma cruzi*, *Leishmania* spp., *Toxoplasma gondii* and *Plasmodium falciparum*. *T. cruzi*-derived GPI anchors were shown to activate host cells through TLR2 (Campos et al., 2001). Also, recognition of the GPI anchors requires a host cell surface molecule, CD14, which is involved in the recognition of LPS by TLR4 (Campos et al., 2001). As mentioned earlier, TLR2 functionally associates with either TLR2 or TLR6. TLR6-deficient macrophages failed to respond to *T. cruzi* GPI anchors, suggesting that a complex

of TLR2/TLR6/CD14 is involved in the recognition of these molecules (Ropert and Gazzinelli, 2004). Glycoinositolphospholipids, a subset of free GPI anchors of *T. cruzi*, also activated CHO cells transfected with TLRs. These molecules are recognized by TLR4 and CD14 but not TLR2 (Oliveira et al., 2004). Thus, *T. cruzi* contains two types of ligands that are recognized by TLR2/TLR6 or TLR4.

*Leishmania major* is an obligate intracellular eukaryotic pathogen of mononuclear phagocytes. Promastigotes invade target cells by receptor-mediated phagocytosis, transform into nonmotile amastigotes, and establish in the phagolysosome. Glycosylphosphatidylinositol-anchored lipophosphoglycan (LPG) is a virulence factor and a major parasite molecule involved in this internalization process. LPG from *L. major* has been shown to activate natural killer cells and macrophages through TLR2 (Becker et al., 2003; de Veer et al., 2003). In addition, *in vivo* studies in mice revealed an important role for TLR4 in the control of *L. major* infection, possibly through the regulation of inducible NO synthase expression. However, it is unclear which molecule of *L. major* is a ligand for TLR4 (Kropf et al., 2004). A recent study of RNA interference showed that TLR3 and TLR2 are involved in the secretion of NO and TNF- $\alpha$  induced by *L. donovani* promastigotes. TLR2-mediated responses are dependent on Galbeta1,4Manalpha-PO(4)-containing phosphoglycans, whereas TLR3-mediated responses are independent of these glycoconjugates. TLR2 and TLR3 participated in the phagocytosis of *L. donovani* promastigotes and TLR3 plays a role in the leishmanicidal activity of the IFN- $\gamma$ -primed macrophages.

GPI anchors of *P. falciparum* are the major factors that contribute to malaria pathogenesis, doing so through their ability to induce proinflammatory responses. *P. falciparum* GPIs are structurally distinct from those of *T. cruzi*; the former contain a diacylated glycerol moiety and fatty acid acylation at C-2 of inositol, whereas the latter have sn-1-alkyl-sn-2-acylglycerol and lack inositol acylation (Channe Gowda, 2002; Gerold et al., 1994; Naik et al., 2000). The proinflammatory responses to *P. falciparum* GPIs by macrophages are mediated mainly through TLR2 and to a lesser but still significant extent also through TLR4. Interestingly, *P. falciparum* GPIs are degraded by macrophage surface phospholipase A2 and phospholipase D; in addition, intact GPIs and sn-2-lyso-GPIs are differentially recognized by TLR2/TLR1 and TLR2/TLR6 heterodimers (Krishnegowda et al., 2005).

Native GPI anchors purified from *Toxoplasma gondii* tachyzoites, as well as synthetic fragments of the proposed structure of these GPI anchors, activate NF- $\kappa$ B and induced TNF- $\alpha$  in a mouse macrophage cell line, and these responses also appeared mediated through TLR2 and TLR4 (Gazzinelli and Denkers, 2006).

### 3.3.2 Other Protozoan TLR Ligand

Other protozoan molecules also serve as important mediators of proinflammatory responses except for GPI anchors and their related molecules. The *T. cruzi*-released protein Tc52 contains a tandemly repeated structure characteristic of GSTs, notably of the  $\theta$  group, and a set of small heat shock proteins, and it is a crucial factor for parasite survival and virulence. Tc52 also plays a central role in innate and adaptive

immunity through TLR2 on human and murine dendritic cells (DCs) (Ouaissi et al., 2002).

TLR9, a receptor for unmethylated bacterial CpG DNA motifs, is also important for resistance to protozoan parasite infections. DNA from the protozoan parasites *Babesia bovis*, *T. cruzi*, and *T. brucei* activate macrophages and DCs, leading to the induction of inflammatory responses (Brown and Corral, 2002; Harris et al., 2006; Shoda et al., 2001). A recent study demonstrates that DNA from *T. cruzi* stimulates cytokine production by APCs in a TLR9-dependent manner. *T. cruzi*-infected TLR9-deficient mice show elevated parasitemia and decreased survival, suggesting that TLR9 plays a crucial role for the protection to *T. cruzi* infection (Bafica et al., 2006).

*Plasmodium* parasites within erythrocytes digest host hemoglobin into a hydrophobic heme polymer, known as hemozoin (HZ) (Arese and Schwarzer, 1997; Sullivan, 2002). Intracellular HZ is released into the bloodstream during schizont rupture and is phagocytosed by myeloid cells resulting in the concentration of HZ in the reticulo-endothelial system (Arese and Schwarzer, 1997). Several studies have shown that HZ purified from *P. falciparum* activates macrophages to produce proinflammatory cytokines, chemokines, and nitric oxide, and it enhances human myeloid DC maturation (Coban et al., 2002; Sherry et al., 1995). A recent report demonstrated that HZ purified from *P. falciparum* is a novel ligand for TLR9 (Coban et al., 2005). Synthetic HZ, which is free of the other contaminants, also activated innate immune responses *in vivo* in a TLR9-dependent manner (Coban et al., 2005). This work is interesting as it provides the first evidence of a non-DNA ligand as recognized by TLR9.

Cerebral malaria is a lethal complication of malaria caused by *P. falciparum* in humans. Besides the high mortality rates, persistent neurocognitive deficits after recovery have become an increasing concern (Aikawa, 1988; Idro et al., 2005; Miller et al., 2002). *Plasmodium berghei* ANKA (PbA) infection in mice is a good experimental model of cerebral malaria (CM) (Engwerda et al., 2005; Good et al., 2005; Schofield and Grau, 2005). Recently, the role of TLRs in the pathogenesis of cerebral malaria was investigated by using this PbA infection model. A significant number of MyD88 (myeloid differentiation primary response gene 88; adapter molecule of TLRs)-deficient mice compared with wild-type mice survived CM caused by PbA infection. Although systemic parasitemia was comparable, sequestration of parasite and HZ load in blood vessels in the brain was significantly lower in MyD88-deficient mice than wild-type mice. Furthermore, brain-specific pathological changes were associated with MyD88-dependent infiltration of CD8<sup>+</sup>, CCR5<sup>+</sup> T cells, and CD11c<sup>+</sup> dendritic cells, including CD11c<sup>+</sup>, NK1.1<sup>+</sup>, and B220<sup>+</sup> cells, and up-regulation of genes such as Granzyme B, Lipocalin 2, Ccl3 and Ccr5. TLR2- and TLR9-deficient mice, but not TLR4-, TLR5-, and TLR7-deficient mice, have decreased susceptibility to cerebral malaria, suggesting that TLR2- and/or TLR9-mediated brain pathogenesis may play a critical role in CM, a lethal complication during PbA infection (Coban et al., 2007).

A profilin-like protein of *T. gondii* (PFTG) is a relatively conserved molecule in a number of apicomplexans. Profilins are small actin-binding proteins that in other eukaryotic cells regulate actin polymerization. Mammalian cell profilins also

interact with a number of different proteins and regulate a variety of biological processes, such as membrane trafficking, receptor clustering, as well as small GTPase and phosphoinositide signaling pathways. Thus, profilins are thought to play critical roles in governing a number of motility-related functions for eukaryotic cells. Although PFTG is a phylogenetical relative of profilin, the profilins in apicomplexan parasites are quite distinct from those present in mammals, plants, and other microorganisms, including other protozoan species (Yarovinsky and Sher, 2006). The exact function of PFTG is unclear but it seems to be involved in parasite motility and invasion of host cells (Gazzinelli and Denkers, 2006). Murine TLR11 senses PFTG (Yarovinsky et al., 2005). TLR11-deficient mice showed increased susceptibility to infection of *T. gondii*, whose phenotype is associated with decreased IL-12 production *in vivo* (Yarovinsky et al., 2005). In addition, PFTG is an immunodominant protein in the CD4<sup>+</sup> T cell response to a soluble extract of the tachyzoite stage of the parasite as well as to live *T. gondii* infection. The immunodominance of PFTG depends on TLR11 both *in vivo* and *in vitro* (Yarovinsky et al., 2006). As TLR11 is non-functional in humans, it would seem that PFTG does not activate human DCs (Gazzinelli and Denkers, 2006).

### **3.4 Virus**

#### **3.4.1 Viral Protein**

TLR4 recognizes not only bacterial components but also viral envelope proteins. The fusion (F) protein from respiratory syncytial virus (RSV) is sensed by TLR4 (Kurt-Jones et al., 2000). C3H/HeJ mice were sensitive to RSV infection (Haynes et al., 2001). The envelope protein of mouse mammary tumor virus (MMTV) directly activates B cells via TLR4 (Rassa et al., 2002).

TLR2 has also been reported to be involved in the recognition of envelope proteins of measles virus, human cytomegalovirus, and HSV-1 (Bieback et al., 2002; Compton et al., 2003; Kurt-Jones et al., 2004).

#### **3.4.2 Viral Nucleic Acid**

Double-stranded (ds) RNA is generated during viral replication. TLR3 is involved in the recognition of a synthetic analog of dsRNA, polyinosine-deoxycytidylic acid (poly I:C), a potent inducer of type I interferons (IFNs) (Alexopoulou et al., 2001; Yamamoto et al., 2003). Consistent with this result, TLR3-deficient mice were hyper susceptible to mouse cytomegalovirus (Tabeta et al., 2004). Contrarily, TLR3-deficient mice showed more resistance to West Nile virus (WNV) infection. WNV triggers inflammatory responses via TLR3, which results in a disruption of the blood brain barrier, followed by enhanced brain infection (Wang et al., 2004). These findings suggested that WNV utilizes TLR3 to efficiently enter the brain.

Mouse splenic DCs are divided into CD11c high B220- and CD11c dull B220+ cells. The latter contain plasmacytoid DCs (pDCs), which induce large amounts of IFN- $\alpha$  during viral infection. CpG DNA motifs are also found in genomes of DNA viruses, such as Herpes simplex virus type 1 (HSV-1), HSV-2, and murine cytomegalovirus (MCMV). Mouse pDCs produce IFN- $\alpha$  by recognizing CpG DNA of HSV-2 via TLR9 (Lund et al., 2003). TLR9-deficient mice were also shown to be susceptible to MCMV infection, suggesting that TLR9 induces anti-viral responses by sensing CpG DNA of DNA virus (Krug et al., 2004a; Krug et al., 2004b; Tabeta et al., 2004). However, in the case of macrophages, HSV-2-induced IFN- $\alpha$  production is not dependent on TLRs. Mice lacking TLR9 or the adapter molecule MyD88 can still control HSV-1 infection (Hochrein et al., 2004). Thus, TLR9-mediated IFN- $\alpha$  response to DNA virus is limited to pDCs, and the TLR-independent system plays an important role in DNA viral infection.

TLR7 and TLR8 are structurally highly conserved proteins (Akira, 2004). The synthetic imidazoquinoline-like molecules imiquimod (R-837) and resiquimod (R-848) have potent antiviral activities and are used clinically for treatment of viral infections. Analysis of TLR7-deficient mice showed that TLR7 recognizes these synthetic compounds (Hemmi et al., 2002). Human TLR7 and TLR8, but not murine TLR8, recognize imidazoquinoline compounds (Ito et al., 2002). Murine TLR7 has also been shown to recognize guanosine analogs such as loxoribine, which has antiviral and antitumor activities (Akira and Hemmi, 2003). Since all these compounds are structurally similar to ribonucleic acids, TLR7 and human TLR8 are predicted to recognize a nucleic acid-like structure of a virus. TLR7 and human TLR8 have been shown to recognize guanosine- or uridine-rich single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), and influenza virus (Diebold et al., 2004; Heil et al., 2004). Although ssRNA is abundant in hosts, host-derived ssRNA is not usually detected by TLR7 or TLR8. As TLR7 and TLR8 are expressed in the endosome, host-derived ssRNA is not delivered to the endosome and so is not recognized by TLR7 and TLR8.

Besides TLR7 and TLR8, TLR3 and TLR9 are exclusively expressed in endosomal compartments not on cell surfaces (Latz et al., 2004). After phagocytes internalize viruses or virus-infected apoptotic cells, viral nucleic acids are released in phagolysosomes and are recognized by TLRs. However, intracellular localization of TLR9 is not required for ligand recognition but prevents recognition of self DNA. Localization of nucleic acid-sensing TLRs is critical for discriminating between self and non-self nucleic acids. Clarification of these mechanisms should lead to a comprehensive understanding of the immune system and so contribute to the development of new therapies for infection and immune disorders.

## 4 Conclusion

The function of TLRs has been extensively clarified in recent decades. TLRs are critically involved in bacterial infection as well as fungal, protozoan, and viral infections. Some TLR ligands, especially nucleic acids, are synthesized *in vitro* and

have already been applied to treatment of viral infections and allergic diseases. Also, TLR-independent pathogen recognition has been demonstrated.

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