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Review

Toll-like receptors as adjuvant receptors

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Abstract

The mammalian Toll-like receptors (TLRs) are expressed on macrophages and dendritic cells, which are primarily involved in innate immunity. At present, ligands for several of the TLRs, such as TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9, have been identified. Most of these ligands are derived from pathogens, but not found in the host, suggesting that the TLRs are critical to sensing invading microorganisms. Pathogen recognition by TLRs provokes rapid activation of innate immunity by inducing production of proinflammatory cytokines and upregulation of costimulatory molecules. Activated innate immunity subsequently leads to effective adaptive immunity. In this regard, the TLRs are considered to be adjuvant receptors. Distinct TLRs can exert distinct, but overlapping sets of biological effects. Accumulating evidence indicates that this can be attributed to both the common and unique aspects of the signaling mechanisms that mediate TLR family responses. For example, TLR2 and TLR9 require MyD88 as an essential signal transducer, whereas TLR4 can induce costimulatory molecule upregulation in a MyD88-independent manner. Understanding the TLR system should offer invaluable opportunity for manipulating host immune responses.

Keywords: Toll-like receptor; Adjuvant; Dendritic cell; Lipopolysaccharide; MyD88; Innate immunity

1. Introduction

Immune responses consist of two major types of immunity, i.e., innate and adaptive. Adaptive immunity, which is mediated by B and T lymphocytes, can recognize pathogens with high affinity through rearranged receptors. However, the establishment of adaptive immunity is typically not rapid enough to eradicate microorganisms. Therefore, it is the more rapidly responding innate immunity that plays a major role in host defense during the early stages of infection. Innate immunity recognition of invading pathogens is mediated by a set of germline-encoded receptors that have evolved to recognize conserved molecular patterns shared by large groups of organisms. Recent accumulating evidence has shown that this recognition can be attributed mainly to the Toll-like receptor (TLR) family. TLR signaling can induce the production of proinflammatory cytokines and upregulate expression of costimulatory molecules, thereby activating not only innate but ultimately also adaptive immunity. In this article, we describe the critical roles played by the TLR family in host defense, and the signaling mechanisms that mediate the response to TLR ligands.

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2. Host defense of *Drosophila* and mammals

Drosophila can defend against microorganisms by producing antimicrobial peptides. A type I transmembrane protein, Toll, was found to play a critical role in this antifungal defense [1]. In addition, 18-wheeler, which is structurally related to Toll, was shown to play a critical role in antibacterial defense [2]. Upon infection, Toll and 18-wheeler induce production of antimicrobial peptides such as drosomycin and attacin, respectively. Recently, sequencing of the *Drosophila* genome has revealed the existence of nine proteins that belong to Toll family [3]. Although the functions of all family members have not yet been clarified, it can be assumed that each member is involved in the defensive response against pathogens.

In the mid 1990s, the first protein that is structurally related to *Drosophila* Toll was identified and is now called as human TLR1 [4,5]. In 1997, Janeway's group characterized another mammalian Toll-like protein (human TLR4) and first implied its functional role in the immune response [6]. To date, ten human and nine murine transmembrane proteins have been shown to belong to the mammalian TLR family [7–11]. Toll and TLR family proteins are characterized by the presence of extracellular domains with a leucine-rich repeat and intracytoplasmic regions called the Toll/Interleukin-1 receptor (IL-1R) homology (TIR) domain, so designated by their similarity to IL-1R and IL-18R. The TIR domains are critical to both *Drosophila* Toll and mammalian TLR signaling, indicating that they share homologous signaling components. For example, the TLR family can activate nuclear factor (NF)-κB by inducing degradation of IκB. Toll can also lead to activation of the NF-κB family homologue, Dif, by inducing degradation of the IκB homologue, Cactus.

Thus, the Toll and TLR families are a phylogenetically conserved system for host defense.

3. How are TLRs involved in host defense?

A prerequisite for effective host defense is the recognition of pathogens. TLRs are involved in this first step. Pathogens possess several components that are not found in the host, and have been referred to as pathogen-specific molecular patterns (PAMPs) [12]. Upon infection, macrophages recognize these patterns as nonself through the TLRs. TLR signaling can stimulate macrophage activation by inducing production of proinflammatory cytokines and antimicrobial small molecules such as nitric oxide. Then, activated macrophages function to eliminate microorganisms during the early phase of infection.

However, activation of the innate immune response in peripheral tissues has a limited ability to eradicate pathogens in mammals. More effective host defense is achieved with activation of the adaptive immune response, which mainly takes place in secondary lymphoid tissues such as the lymph nodes. Dendritic cells (DCs) play an essential role in the immune response by communicating between the peripheral and lymphoid tissues [13,14]. This process mainly involves immature DCs located in the peripheral tissues, and these cells constitute a unique cell population able to activate naïve T cells. In contrast to macrophages, which mainly function to eradicate pathogens, DCs are important for sensing the invading pathogen and for instructing the adaptive immune system which is thereby recruited to fight against the infection. In order to activate T cells, DCs need to first leave the peripheral tissues and migrate to the lymph nodes [15]. This DC trafficking is mediated by chemokine receptors that are expressed on DCs following stimulation of TLRs [16–18] (Fig. 1). For example, LPS can downregulate expression of the chemokine receptors such as CCR5, but can also upregulate CCR7 expression. Concomitantly, DCs can also produce a variety of chemokines that recruit natural killer cells and naïve T cells.

TLR signaling stimulates the maturation of DCs, which migrate to the lymph nodes where they stimulate T cells by presentation of antigen–major histocompatibility (MHC) complexes. Antigen presentation alone can stimulate pathogen-specific T-cell clones, but is not sufficient to trigger efficient T-cell expansion. Clonal T-cell expansion requires an additional signal delivered by costimulatory molecules such as CD80/86. TLR signaling functions to trigger adaptive immunity by enhancing expression of not only MHC molecules but also of these costimulatory molecules in DCs (Fig. 1).

In addition to T-cell expansion, T-cell differentiation is also quite important for the establishment of

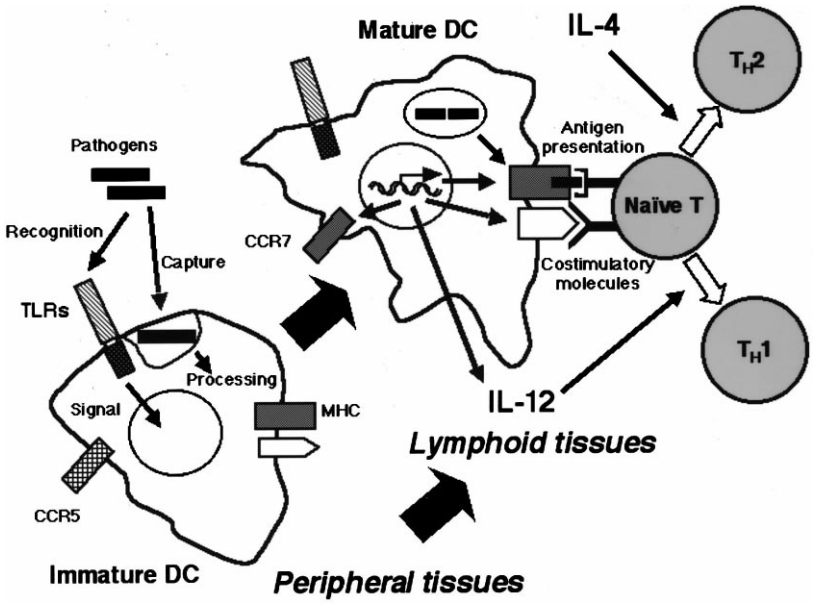

Fig. 1. Activation of adaptive immunity through TLRs. First, immature DCs in the peripheral tissues sense invading pathogens. Pathogens are recognized by TLRs or captured by endocytosis. TLR signaling can lead to DC maturation. Mature DCs exhibit low expression of CCR5 and high expression of CCR7, which can cooperatively provide DCs with the ability to exit from tissues and migrate into the draining lymphoid tissues. Concomitantly, captured pathogens are processed. Processed products are then presented to T cells as antigen–MHC complexes. TLR signaling plays critical roles also in clonal T-cell activation by augmenting expression of MHC molecules and costimulatory molecules. TLRs can also regulate T-cell differentiation status by producing proinflammatory cytokines such as IL-12. IL-12 can instruct naïve T cells to differentiate into T_H1 cells. Thus, establishment of adaptive immunity is greatly influenced by TLR-stimulated DCs.

effective adaptive immunity. T cells can differentiate into two distinct subsets, i.e., T_H1 and T_H2 [19]. T_H1 cells secrete mainly the effector cytokine IFN-γ and are involved in cellular immunity. T_H2 cells produce the effector cytokines IL-4, IL-10, and IL-13 and are involved in humoral immunity. This decision to differentiate into T_H1 or T_H2 can be directed by DCs, depending on the particular DC subset, DC maturation stage, or the DC-to-T cell ratio [20,21]. Bacterial infection activates DCs via stimulation of the TLRs and induces mainly T_H1-inducing cytokines such as IL-12. Therefore, TLR-stimulated DCs tend to direct T-cell differentiation towards the T_H1 cell type (Fig. 1). At present it remains unknown whether DCs can also be activated to instruct T_H2 cell differentiation by stimulation of certain TLRs upon infection with helminths or certain microbes.

Taken together, these observations show that TLRs are crucial not only in the early phase of infection, but also in linking innate and adaptive immunity throughout the entire course of the host defense response. Their involvement in multiple immunostimulatory activities define TLRs as the general adjuvant receptors in the body.

4. Pathogen recognition by the TLR family

In this section, we describe the relationship between TLRs and their ligands (Table 1). So far a number of ligands have been identified through in vitro systems or knockout mice. Most of these ligands can be classified as PAMPs, but non-PAMP as well as PAMP ligands seem to play important roles in eliciting not only the host defense but also various inflammatory processes.

4.1. Lipopolysaccharide (LPS) and TLR4

The best-characterized PAMP is LPS, a major component of the outer membrane of gram-negative bacteria (Fig. 2). LPS is composed of polysaccharides extending outward from the bacterial cell surface and a lipid portion, lipid A, which is embedded in the cell

Table 1
TLRs and their ligands

TLRs	Origin of ligands	Ligands
TLR2	Gram$^+$ bacteria	Lipoproteins [77,78]
		Peptidoglycan [30,79]
		(TLR2/6 [33] or TLR2/X [35])[a]
		Lipoteichoic acids [79,80]
	Staphylococcus	Modulin (TLR2/6) [81]
	Bacteria	Lipopeptides (TLR2/X) [33,35]
	Mycoplasma, *Mycobacteria*, *Spirochetes*	Lipoproteins, lipopeptides [82–85]
	Mycoplasma	MALP-2 (TLR2/6) [31,35]
	Spirochetes	Glycolipids [80]
	Listeria	Heat-killed bacteria [86]
	Mycobacteria	Lipoarabinomannan [25,87,88]
	Porphyromonas, *Spirochetes* (*Leptospira*)	LPS [28,29]
	Yeast	Zymosan (TLR2/6) [33,78]
	Trypanosoma cruzi	GPI anchors [89]
	Klebsiella	Outer membrane protein A [90]
	Neisseria meningitides	Soluble factors (TLR1/2) [34]
TLR3	Virus	dsRNA [47]
TLR4	Gram$^-$ bacteria	LPS [22–24]
	Gram$^+$ bacteria	Lipoteichoic acids [30]
	Plant	Taxol [48]
	Respiratory syncytial virus	F protein [49]
	Host	HSP60 [51,52]
		Fibronectin EDA domain [56]
TLR5	Bacteria with flagella	Flagellin [44]
TLR9	Bacteria	Unmethylated CpG DNA [10]

[a]Some TLR2 ligands are recognized by TLR2-including heterodimers. The identified dimers are shown in parentheses.

surface. LPS can provoke a variety of immunostimulatory responses, for example production of proinflammatory cytokines such as IL-12 and inflammatory effector substances such as nitric oxide. These biological activities can be ascribed to the lipid A portion of LPS. In sufficient quantity, LPS can cause a clinically life-threatening condition called endotoxin shock. A glycosylphosphatidylinositol (GPI)-anchoring protein, CD14, was identified that facilitates LPS action by binding and retaining LPS on the cell surface. However, CD14 lacks an intracytoplasmic region, suggesting that another membrane protein(s) may be essential for LPS signaling.

In 1998, genetic analysis revealed that TLR4 is a critical signal transducer for LPS [22,23]. C3H/HeJ and C57BL/10ScCr are mutant mouse strains that are defective in their responses to LPS. Both mutants carry a mutation in the TLR4 gene. The TLR4 gene isolated from C3H/HeJ mice encodes a histidine residue in place of a well-conserved proline in the intracytoplasmic region. The proline is indeed essential for TLR4 signaling [24,25]. In addition, the C57BL/10ScCr strain contains a chromosomal deletion in the TLR4 genomic locus. The idea that TLR4 is critical for LPS signaling was further confirmed by generation of TLR4-deficient mice [24]. Furthermore, in humans, TLR4 mutations are associated with impaired responsiveness to LPS [26].

Initially, experiments performed using in vitro overexpression of TLRs indicated that TLR2 is a receptor for LPS as a result of this. However, this result was found to be due to some contaminants which can function as TLR2 agonists [27]. Although certain species of LPS, derived from *Leptospira* or

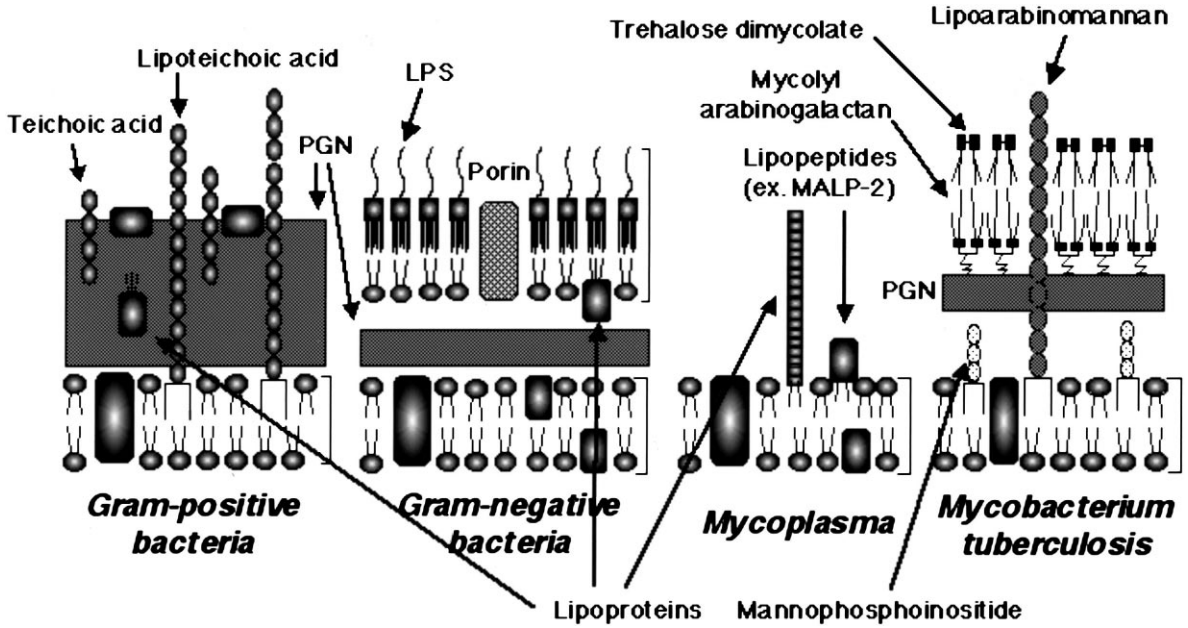

Fig. 2. Schematic representation of PAMPs. Gram-positive bacteria contain a thick layer of PGN in the cell wall. Lipoteichoic acids, teichoic acids, and lipoproteins are also embedded in this cell wall. Gram-negative bacteria have a thinner layer of PGN in the cell wall compared to Gram-positive bacteria. The cell wall of Gram-negative bacteria is further typified by the presence of LPS on the outer surface. LPS is comprised of an active component, the lipid A portion, and *O*-polysaccharide (*O*-antigen). The latter is exposed outside the cell surface. Porins are involved in forming pores through which small molecules can transverse. *Mycoplasma* lacks a cell wall, but lipoproteins and lipopeptides are embedded in its cytoplasmic membrane. *Mycobacterium tuberculosis* has a characteristic thick hydrophobic layer containing mycolyl arabinogalactan and trehalose dimycolate, in addition to a cytoplasmic membrane and a PGN layer. Lipoarabinomannan is a major cell wall-associated glycolipid. Some of these PAMPs show strong immunostimulatory activity via TLR family members.

Porphyromonas, have subsequently been shown to act through TLR2, they are structurally different from the typical *Escherichia coli* or *Salmonella* LPS [28,29]. It is generally accepted that LPS from Gram-negative bacteria stimulate inflammatory responses through TLR4.

4.2. TLR2 recognizes a variety of pathogens

Gram-positive bacteria do not produce LPS, but still can provoke immune responses similar to those generated by LPS. The cell wall of Gram-positive bacteria contains a thick layer of peptidoglycan (PGN) within which lipoproteins and lipoteichoic acids are embedded (Fig. 2). Analysis of TLR2-deficient mice demonstrated clearly that TLR2 is essential for the response to PGN [30].

Mycoplasma is a pathogen that lacks a cell wall (Fig. 2), but its cytoplasmic membrane contains various lipoproteins or lipopeptides that can also cause inflammatory responses. One of the *Mycoplasma* lipopeptides, the 2 kDa macrophage-activating lipopeptide-2 (MALP-2), was shown to utilize TLR2 as its signal transducer [31]. Furthermore, TLR2 was found to be critical for responses to a number of lipoproteins derived from a variety of pathogens (Table 1), including lipoarabinomannan, which is a major cell wall-associated glycolipid derived from *Mycobacterium tuberculosis* (Fig. 2) [32].

How can TLR2 recognize such a wide variety of PAMPs? One possibility is that TLR2 forms heterodimers with other TLRs. This possibility was demonstrated by an in vitro expression system [33]. Dominant negative forms of either TLR2 or TLR6 were found to inhibit tumor necrosis factor-α expression normally induced by zymosan, Gram-positive bacteria, or PGN. Furthermore, coexpression of TLR2 with TLR6 could confer NF-κB activation and cytokine production, while cells expressing TLR2 alone could not. Thus, TLR2 appears to function in some cases by forming heterodimers with other TLRs. Because a dominant negative form of

TLR2, but not of TLR6, can also inhibit responses to bacterial lipopeptides, it is possible that TLR2 can also heterodimerize with other TLRs. TLR2 has further been shown to form functional heterodimers with TLR1, required for recognition of certain factors released by *Neisseria meningitidis* [34]. Thus, TLR2 appears to broaden its repertoire of specificities by forming at least two distinct types of functional heterodimers with other TLRs. A similar conclusion was reached by in vivo analysis using TLR2- or TLR6-deficient mice [35]. The molecular structure of MALP-2 is only slightly different from that of bacterial lipoprotein, in that the former is diacylated, while the latter is triacylated at the N-terminal cysteine residue. Responses to both PAMPs were abolished in TLR2-deficient macrophage, but only the response to MALP-2 was abolished in TLR6-deficient macrophage [35]. We assume that TLR2/TLR6 and an as-yet undefined heterodimer, TLR2/TLRX, can recognize MALP-2 and bacterial lipopeptides, respectively. Thus, TLR2 appears to achieve its fine specificity by pairing with distinct TLRs.

4.3. CpG DNA and TLR9

In addition to cell wall components, bacterial DNAs can also function as PAMPs. This was originally demonstrated by Tokunaga et al. [36]. They found an immunostimulatory activity in extracts of bacillus Calmette Guerin (BCG) that could be attributed to the effects of DNA. The activity was eventually shown to require unmethylated CpG motifs, which are rarely detected in vertebrate DNA. Not only bacterial DNA, but oligodeoxynucleotides carrying the CpG motif can exhibit immunostimulatory activities on human and murine lymphocytes and antigen-presenting cells. This stimulation leads to the production of T_H1 cytokines and costimulatory molecule upregulation. This activity is so potent that CpG DNA has been utilized as a powerful adjuvant. CpG DNA-conjugated proteins have been shown to promote both the antigen presenting activity and maturation of DCs, thereby enhancing antigen-specific T_H1 responses [37].

Analysis of TLR9-deficient mice has further clarified the idea that the TLR family is involved in recognizing this bacterial DNA as PAMP. All CpG DNA-induced effects, including cytokine production, B-cell proliferation, DC maturation, and induction of systemic shock were completely abolished in TLR9-deficient cells and mice [10]. In humans, TLR9 is selectively expressed on plasmacytoid DCs, but not on monocyte-derived DCs [38–40]. CpG DNA can induce cytokine production from the former, but not from the latter, consistent with the fact that response to CpG DNA is dependent on TLR9 expression. Furthermore, human and mouse TLR9 confer the responsiveness via species-specific CpG motif recognition [38]. These results demonstrate that TLR9 is a critical signal transducer for CpG DNA.

Independently, Chu et al. have shown that the DNA-dependent protein kinase (DNA-PKc) is essential for the effects of immunostimulatory DNAs [41]. So far there is no evidence connecting DNA-PKc and TLR9, and further studies will be necessary to clarify whether DNA-PKc and TLR9 act sequentially or in parallel.

4.4. Flagellin and TLR5

Most bacilli including *Salmonella* possess a soluble factor, flagellin, which is the monomeric subunit of flagella. Flagellin shows potent proinflammatory activity by inducing IκB degradation, NF-κB activation, and expression of IL-8 and inducible NO synthase in intestinal epithelial cells [42,43]. Therefore, flagellin can be also regarded as another PAMPs. In vitro expression of TLR5 can confer flagellin responsiveness on Chinese hamster ovary (CHO) cells, indicating that flagellins exert its activity through TLR5 [44]. Furthermore, TLR5 is exclusively expressed on the basolateral surface of intestinal epithelia [45], which is consistent with the fact that flagellin can show proinflammatory activity through the basolateral, not through the apical, surface [46]. However, TLR5-deficient mice should be analyzed to confirm the conclusion that TLR5 is a critical signal transducer for flagellin.

4.5. Double-stranded RNA (dsRNA) and TLR3

Viral replication within infected cells results in generation of dsRNA that can provoke antiviral defense. Because host cells do not produce dsRNA,

dsRNA can also be considered as PAMPs. TLR3-deficient mice showed decreased responses to viral RNA mimic, polyinosine-polycytidylic acid (poly(I:C)), suggesting that TLR3 is involved in recognition of dsRNA [47]. TLR3-deficient mice are powerful tools to clarify the roles of TLR3 in viral infection.

4.6. Non-PAMP ligands for TLR4

TLR4 can recognize not only LPS but also other substances distantly related to LPS. For example, TLR4 can recognize plant products. A diterpene from plant extract, taxol, is widely used as an anticancer drug, but exhibits immunostimulatory activity in macrophages. This LPS-like activity depends on TLR4 signaling [48]. It is also reported that TLR4 is involved in viral recognition [49]. F protein from respiratory syncytial virus (RSV) can induce proinflammatory cytokines via its stimulation of TLR4 and CD14 [49]. They further showed that C57BL/10ScCr were impaired in their ability to eliminate RSV, arguing that TLR4 is essential for antiviral defense. However, the mutant mice possess mutation not only on TLR4 but also on IL-12Rβ2 genes [50], which may possibly be responsible for defective immunity against RSV. Therefore, further study is necessary to conclude that TLR4 is critical for antiviral immunity.

Intriguingly, the TLR family is critical for recognizing certain endogenous molecules as well as PAMPs. For example, heat sock protein 60 (HSP60) has been shown to provoke an inflammatory response in normal mice, but not in C3H/HeJ mice, suggesting that this activity is mediated by TLR4 [51]. Furthermore, Vabulas et al. found that both human and chlamydial HSP60 can activate NF-κB and mitogen-activated protein kinases (MAPKs) through TLR2 or TLR4 [52]. HSPs are released from necrotic cells in certain pathological conditions, such as injury, and induce DC maturation by activating NF-κB [53]. Such immune activation may provide a molecular basis for the danger theory of immune activation proposed by Matzinger [54]. According to this theory, the immune system does not discriminate between self and nonself per se, as has been long believed, but rather responds to antigens that are associated with danger signals released from damaged or stressed cells. CD91, which is not a TLR family member, has been identified as a receptor for HSPs [55]. It is most likely that CD91 is involved in uptake of HSPs, after which TLR delivers a signal to trigger inflammatory processes. Further studies are necessary to clarify how TLR family and CD91 coordinately function as the receptors for substances from damaged cells.

During inflammation or tissue injury, extracellular matrix components such as fibronectin or collagen, are degraded by proteases, thus accelerating the inflammatory cascades. Degraded fragments of fibronectin have been shown to exert their proinflammatory activity through TLR4 [56]. Thus, TLR4 appears to be uniquely involved in the recognition of host-derived inflammatory products, leading to the establishment of inflammatory responses.

5. TLR family signaling pathways

TLRs activate signal transduction cascades leading to expression of immune response genes following recognition of their respective ligands. The mechanism of TLR signaling is quite similar to that of the IL-1R family, because both receptor families possess TIR domains (see above). MyD88, a cytoplasmic adapter protein, associates with all members of the IL-1R and TLR families [57–60]. Activation of NF-κB and MAPK cascades involves a signaling complex that contains MyD88, IL-1R-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6) [58,61]. MyD88-deficient cells were found to lack activation of NF-κB and MAPKs and any biological responses following stimulation by IL-1 or IL-18, indicating that MyD88 is an essential adapter for signaling by these cytokines [62]. Furthermore, NF-κB activation was abrogated in MyD88-deficient cells stimulated with ligands for TLR2 and TLR9 (Fig. 3), indicating that TLR family signaling requires MyD88. However, NF-κB and MAPK activation in response to LPS was retained in MyD88-deficient cells, although with delayed kinetics, (Fig. 3 and [63]). Therefore, among IL-1R and TLR family members, TLR4 is unique in the respect that its signaling can lead to NF-κB and MAPK activation in a MyD88-independent manner.

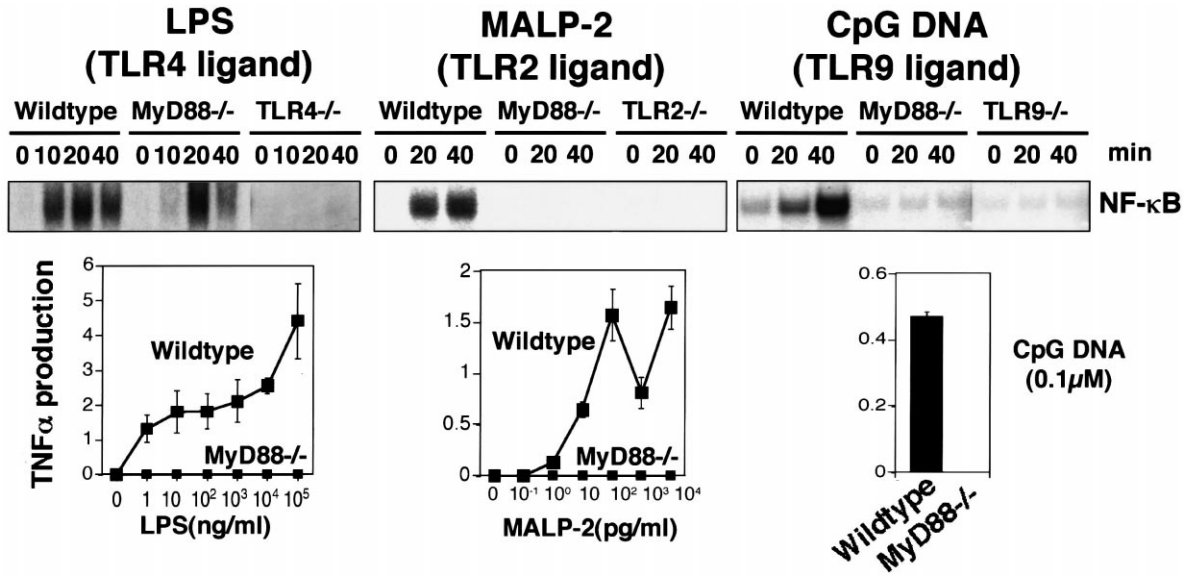

Fig. 3. LPS can induce NF-κB activity, but cannot stimulate cytokine production in MyD88-deficient macrophages. Macrophages from wild-type and mutant mice were stimulated with the ligands for TLR2, TLR4, and TLR9. In the cases of TLR2 and TLR9, ligand-induced NF-κB activity was completely dependent on MyD88 and the cognate receptor. In the case of TLR4, activation occurred in the absence of MyD88, suggesting the existence of a MyD88-independent pathway downstream of TLR4. However, cytokine induction in response to all these TLR ligands was completely abolished in MyD88-deficient cells.

6. Biological significance of the MyD88-independent pathway

Curiously, while cytokine induction in response to LPS was abolished in MyD88-deficient macrophages, activation of NF-κB was retained (Fig. 3). In addition to cytokine induction, other LPS-induced effects including NO production, B-cell blast formation and endotoxin shock were also fully abolished in MyD88-deficient mice [63]. Therefore, the question is whether stimulation via the MyD88-independent pathway has any biological consequence.

As described above, DCs express a vast repertoire of TLRs on their surface [64] and undergo maturation in response to various infectious stimuli, including LPS and CpG DNA. This process can be duplicated in vitro by culturing bone marrow (BM) cells with GM-CSF. BM DCs were generated from wild-type and various mutant mice and analyzed for their responses to LPS [65]. Cytokine production in response to LPS was abolished in both TLR4- and MyD88-deficient BM DCs, consistent with the previous results from macrophages [63]. However, upregulation of costimulatory molecules was retained in MyD88-, but not TLR4-, deficient BM DCs. This upregulation has functional consequences, since LPS could still enhance the allostimulatory activity of MyD88-deficient BM DCs. Furthermore, LPS-induced, MyD88-independent upregulation of costimulatory molecules was detected not only in vitro BM DCs, but also in vivo splenic $CD11c^{+}$ DCs [65]. Thus, two major biological effects provoked by LPS, i.e., cytokine production and costimulatory molecule upregulation, differ in their requirement for MyD88.

7. Molecular basis for the MyD88-independent pathway

At present, the molecular mechanism underlying the MyD88-independent pathway is unclear. C3H/HeJ-derived BM DCs showed impairment of both cytokine and costimulatory molecule induction in response to LPS [65]. This indicates that the conserved proline residue in TLR4 is critical for both MyD88-dependent and -independent pathways, and that both pathways presumably originate from the intracytoplasmic region of TLR4. IRAK appears to be an integral component of the MyD88-dependent pathway, as IRAK activation is abolished in MyD88-deficient DCs. It is noteworthy that IRAK-deficient

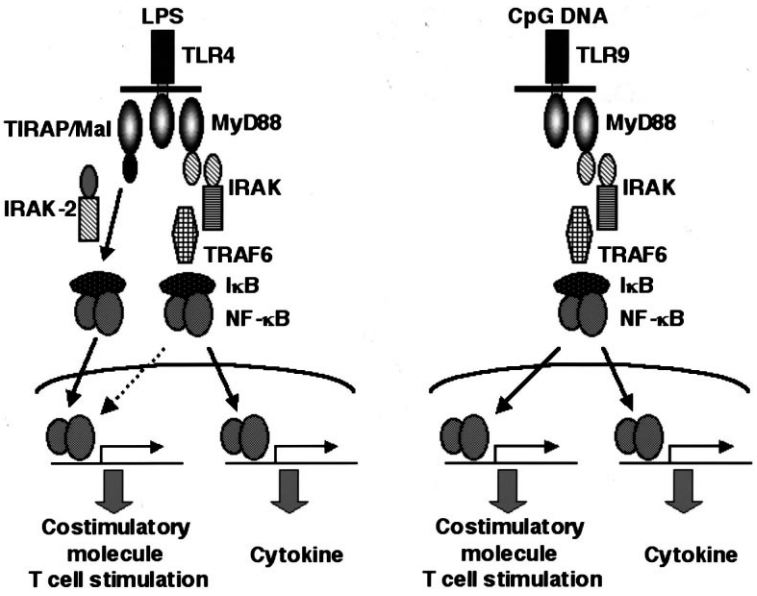

Fig. 4. TLR4 and TLR9 can exert biologically similar effects through distinct signaling pathways. Both TLR4 and TLR9 can activate signaling pathways emanating from MyD88. This pathway sequentially activates IRAK and TRAF6 and leads to activation of NF-κB and MAPKs (not shown). Notably, TLR4, but not TLR9, can activate NF-κB and MAPKs in a MyD88-independent and TIRAP/Mal-dependent manner. Cytokine induction in response to both TLR4 and TLR9 ligands is completely dependent on MyD88. Both TLR4 and TLR9 signaling can induce upregulation of costimulatory molecules and T-cell stimulatory activity. However, these effects differ in their requirement for MyD88; that is, TLR4-induced biological effects are MyD88-independent whereas TLR9-induced ones are dependent on MyD88.

macrophages showed delayed activation of NF-κB and MAPK cascades, similar to what is observed in MyD88-deficient macrophages [66]. Furthermore, in response to LPS, TRAF6-deficient embryonic fibroblasts exhibited impaired, but still detectable levels of NF-κB activation with delayed kinetics [67]. Taken together, these results suggest that the MyD88-independent pathway bifurcates at the intracytoplasmic region of TLR4, but converges again at or just downstream of TRAF6 (Fig. 4).

Subtractive hybridization analysis revealed that several interferon (IFN)-inducible genes, including a CXC chemokine, IFN-inducible protein 10 (IP-10) and IFN-regulated gene-1 (IRG-1), were induced in MyD88-deficient macrophages in response to LPS [67]. IP-10 gene induction requires IFN regulatory factor 3 (IRF-3) [68], and nuclear translocation of IRF-3 in response to LPS is detected in MyD88-deficient cells [67]. Therefore, it is likely that IRF-3 activation contributes to the MyD88-independent pathway. At present it is not known how IRF3 is activated downstream of TLR4.

Furthermore, in response to LPS, MyD88-deficient liver macrophages, i.e., Kupffer cells, can secrete active IL-18 in a caspase 1-dependent manner [69], suggesting the involvement of other factors in the MyD88-independent pathway.

It is quite intriguing to compare TLR4 with other TLR signaling. For example, TLR9 signaling exhibits similar effects to TLR4 signaling, i.e., cytokine induction and costimulatory molecule upregulation. However, in contrast to TLR4, all the effects induced by TLR9 signaling are dependent on MyD88 [65]. The results indicate that TLR4 and TLR9 can activate distinct signaling mechanisms, even while leading to similar biological effects. Thus it is quite important to elucidate differences among individual TLR signaling mechanisms.

Recently, two independent groups identified a novel adapter protein for TLR4, called as TIR domain-containing adapter protein (TIRAP) or MyD88-adapter-like (Mal) [70,71]. TIRAP/Mal associates with TLR4, but not with TLR9 and is critical for LPS-induced DC maturation [70]. TIRAP/Mal can form homodimers or heterodimers with MyD88 and also associate with IRAK-2, thereby connecting with NF-κB activation [71]. However, it still remains unknown how or whether TIRAP/Mal is involved in

IRF3 or caspase 1 activation. Furthermore, in vivo roles of TIRAP/Mal should also be clarified.

8. How TLRs recognize their ligands

Affinity between TLRs and their ligands seems lower than that between cytokines and their receptors. In spite of low affinity, TLRs themselves seem to recognize their ligands specifically. This has been demonstrated by analyzing species-specific responses to TLR ligands. Human and rodent cells differentially respond to LPS or lipid A analogues. These responses can be reconstituted in LPS-unresponsive cells by overexpressing human or rodent TLR4s [72,73]. In addition, the optimal CpG motif for activation differs between human and mice. This species-specific CpG motif recognition was also found to be mediated by TLR9 [38]. Furthermore, TLR9 and CpG DNA colocalize in the same endocytic vesicles [74]. Thus, these studies strongly suggest that at least TLR4 and TLR9 in TLR family members can directly recognize their ligands.

TLRs recognize a variety of ligands. For example, TLR4 recognizes lipids (LPS), proteins (HSP60, F protein), and diterpene (Taxol). TLR2 also recognizes a variety of products from various microorganisms. This might be partly due to their low affinity interactions. Meanwhile, as described above, TLR2 seems to discriminate fine molecular patterns depending on the heterodimerizing receptor partner. Thus, it would be quite intriguing to clarify how TLRs recognize their ligands. Crystallization experiments should contribute to the resolution of this issue.

Microbial recognition by TLRs is not determined by TLRs alone. A small secreted molecule, MD-2, was shown to associate with the extracellular domain of TLR4 and facilitate the interaction of TLR4 with ligands such as LPS and taxol [48,75]. Furthermore, a point mutation in the gene encoding MD-2 can lead to LPS-unresponsiveness, although this mutation does not abolish the ability of MD-2 to interact with TLR4 [76]. Resolving how MD-2 contributes to formation of the LPS receptor complex is another important issue for the future.

9. Concluding remarks

Antibiotics have proven to be powerful tools in the control of infectious disease. However, the use of even very powerful antibiotics has been accompanied by the emergence of pathogens with multidrug resistance. Therefore, the development of non-antibiotic agents, including vaccines, are expected to contribute to the fight against microbial pathogens. Formerly, vaccines employed live attenuated pathogens, whole inactivated organisms, and inactivated toxins, but these agents also produced undesirable side effects. Recently recombinant proteins and synthetic peptides have emerged as alternative approaches to vaccine development. However, because of their poor immunogenicity, these molecules typically must be coadministered with immunostimulatory adjuvants in order to evoke strong immune responses. The molecular mechanisms underlying adjuvant activity was poorly understood for a long time, but the discovery and functional analyses of TLRs have revealed that TLRs can function as adjuvant receptors. Therefore, we may be able to obtain novel types of immunomodulatory reagents by modulating the TLR system. For example, reagents that enhance TLR signaling pathways can be powerful adjuvants for fighting against pathogens or cancers, perhaps with fewer side effects. Furthermore, because the TLR family may be involved in the pathogenesis of inflammation via recognition of host products, reagents that inhibit TLR action may work as anti-inflammatory drugs, and possibly also as agents to treat autoimmune diseases. Thus, our increasing understanding of the TLR system could be the basis for treating a variety of pathological conditions.

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