Toll-like receptors and their signaling mechanism in innate immunity

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In *Drosophila* the Toll family, a group of transmembrane proteins, plays crucial roles in the host defense against invading pathogens. Mammalian species also conserve this system as the Toll-like receptor (TLR) family, which includes more than 10 members that have been identified so far. Both the Toll and TLR families recognize various kinds of microorganisms through pathogen-associated molecular patterns. Mammalian TLRs are expressed on macrophages and dendritic cells and mediate the signal for cytokine release or upregulation of costimulatory molecules. These activities cooperatively generate host defense mechanisms. Recently, gene targeting experiments, including ours, have contributed much to clarifying not only the function but also the signaling mechanism of TLRs. TLR2 is essential for recognizing lipopeptides and lipoproteins from several microorganisms and also peptidoglycans derived from gram-positive bacteria. TLR4 recognizes lipopolysaccharides and lipoteichoic acids from gram-negative and -positive bacteria, respectively. Furthermore, TLR9 is critical for recognizing bacterial DNAs. Thus, TLRs distinguish various immunostimulatory molecular patterns. Although TLRs can produce similar biological responses, studies with mutant mice lacking a TLR-associating protein, MyD88, showed that TLR signaling is differentially regulated among TLR family members. Here, we describe recent progress in elucidating the function and signaling mechanisms of the TLR family. □ *Dendritic cells; lipopolysaccharide; MyD88; Toll-like receptor; unmethylated cytosine-guanosine DNA*

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Immune responses comprise innate and adaptive immunity (Table I). Adaptive immunity is phylogenetically established in the jawed vertebrates and mediated by B and T lymphocytes, which carry immunoglobulins and T-cell receptors, respectively. These receptors, created by somatic gene rearrangement, recognize fine molecular determinants based on the amino acid structures of pathogens. Innate immunity is mediated mainly by macrophages and dendritic cells (DCs). This immune system is an ancient mechanism but plays a central role in eradicating pathogens also in vertebrates. This system is evident especially in the early phase of infection because it usually takes several days for hosts to establish effective adaptive immunity. A group of surface molecules belonging to the Toll-like receptor (TLR) family was recently found to be critical in innate immunity. The family recognizes pathogen-associated molecular patterns (PAMPs) as nonself. PAMP recognition by TLRs then leads to cytokine production and expression of costimulatory molecules such as CD40, which can shape up protective innate and adaptive immunity.

The *Drosophila* Toll family

Insects can cope with microorganism infection despite their lack of adaptive immunity. They rapidly synthesize antimicrobial peptides in response to microbial invasion and secrete them into the hemolymph. Expression of the genes encoding antifungal and antibacterial peptides is differentially regulated through the Toll family signaling. *Drosophila* Toll, an originally identified Toll family member, is involved in induction of the antifungal peptide, drosomycin (1). Another Toll family member, 18W, is critical for induction of antibacterial genes such as attacin and cecropin (2). Thus, *Drosophila* immune responses against microorganisms are regulated by pathogen-specific Toll family members. The Toll family, a battery of type-I transmembrane proteins, is characterized by the extracellular domain, with the leucine-rich repeat (LRR), and the intracytoplasmic domain, which shows homology with that of the mammalian interleukin-1 receptor (IL-1R) family. The intracytoplasmic region is now known as the Toll/IL-1R homology (TIR) domain. Whole genome sequencing of *Drosophila* showed that the Toll family consists of nine members (3).

The outline of Toll family signaling is depicted in Fig. 1. Toll associates with an adaptor protein, Tube. Subsequently, a serine-threonine kinase, Pelle, is activated, and Cactus is degraded. Cactus degradation can make Dif competent for nuclear translocation. Then, the Drosomycin gene is induced by activated Dif. Cactus and Dif are *Drosophila* homologues of vertebrate IκB and NF-κB, respectively. In addition to Cactus and Dif, other molecular components involved in this pathway are also functionally and structurally similar to mammalian IL-1R or TLR signaling components (Fig. 1).

Table 1. Innate and adaptive immunity

	Innate immunity	Adaptive immunity
Cells involved	Macrophages Dendritic cells	T and B cells
Receptors	Not rearranged	Rearranged
Recognition	Pathogen-associated molecular patterns (for example, LPS, PGN)	Fine structures (for example, peptides)

LPS = lipopolysaccharide; PEN = peptidoglycan.

Pathogen recognition by the mammalian TLR family

TLR4

The most characterized PAMP is lipopolysaccharide (LPS), which is embedded in the outer membrane of gram-negative bacteria (4). LPS can activate macrophages to produce not only proinflammatory cytokines, including tumor necrosis factor (TNF)-α and IL-12, but also inflammatory substances such as NO. In addition, LPS can cause life-threatening endotoxin shock.

LPS binds to LPS-binding protein (LBP) in serum and then interacts with CD14 on the surface of target cells. However, CD14 alone is not sufficient for LPS signaling. First, CD14 is a glycophosphatidylinositol (GPI)-anchored protein lacking transmembrane and intracytoplasmic domains. In addition, CD14-deficient mice can respond to high doses of LPS (5). These findings indicate that other transmembrane protein(s) are essential for LPS signaling.

A mammalian TLR family member, TLR4, has been identified as an essential signal transducer for LPS, on the basis of several lines of evidence. First, genetic approaches showed that an LPS-hyporesponsive strain of mice, C3H/HeJ, carries a missense point mutation in the intracytoplasmic region of TLR4 (6, 7). A histidine was substituted for a proline, which is a highly conserved amino acid residue among the TLR family. TLR4 carrying this mutation was defective for LPS signaling (8, 9). Furthermore, TLR4-deficient mice were also hyporesponsive to LPS (8). These results clearly suggested that TLR4 was a long-sought receptor for LPS.

Initial studies also showed that TLR2 mediates LPS signaling. This is based on transfection experiments utilizing the 293 cell line and overexpression of TLR2 (10, 11). However, gene targeting experiments showed that TLR2 deficiency does not disturb any responses to LPS (12). Furthermore, Chinese hamster-derived cells, which lack functional TLR2, can respond to LPS (13). These findings suggest that TLR2 is dispensable for LPS signaling. The discrepancy can be explained by the fact that commercial preparations of LPS often contain some TLR2 agonistic activity (14). This residual TLR2 agonist can stimulate 293 cell lines overexpressing TLR2. It is now generally accepted that TLR2 is not involved in LPS signaling.

TLR4 also functions as a signal transducer for ligands

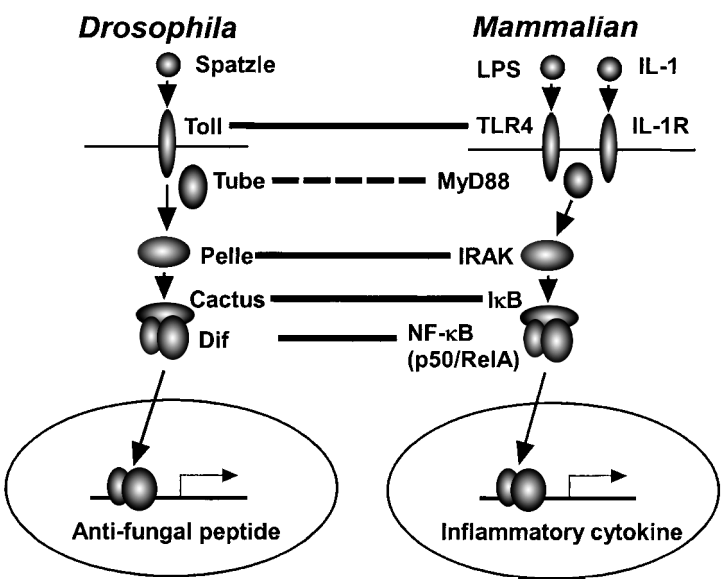

Fig. 1. Comparison of *Drosophilla* Toll and mammalian interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) signaling pathways. Spatzle is generated by serine protease activation and functions as a ligand for Toll. After ligand binding, both pathways sequentially activate phylogenetically conserved molecular components. Corresponding molecules are indicated with thick solid lines. Tube and MyD88 are connected with broken lines, on the basis of their less conserved molecular structures when compared with other components (see text).

Table 2. Toll-like receptor (TLR) family and pathogen-associated molecular patterns (PAMPs)

TLRs	Origin	PAMPs
TLR2	Mycoplasma Mycobacteria Spirochetes	Lipopeptides Lipoproteins
	Gram-positive bacteria	Lipoproteins Peptidoglycan
	Yeast	Zymosan
TLR4	Gram-negative bacteria	LPS
	Gram-positive bacteria	Lipoteichoic acids
	Plant	Taxol
	Virus (RS virus)	F protein
	Host	HSP60 Fibronectin
TLR9	Bacteria	CpG DNA

LPS = lipopolysaccharide; HSP = heat shock protein; CpG = unmethylated cytidine-phosphate-guanosine.

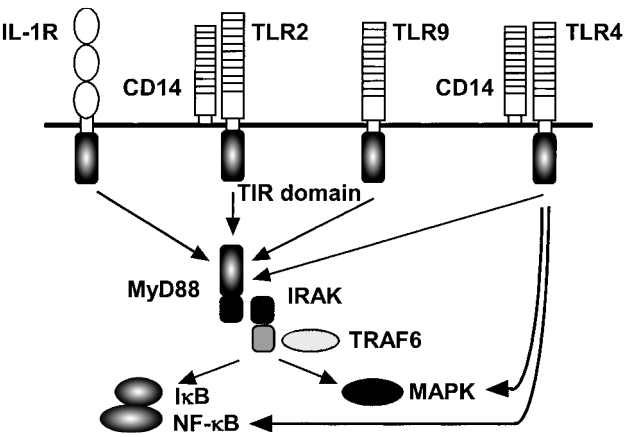

Fig. 2. Schematic representation of mammalian interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) family signaling. White ovals indicate Ig-like domains of IL-1R. CD14 facilitates the ligand–TLR interaction by retaining the ligands. Horizontal bars in the extracellular domains of CD14 and TLRs represent leucine-rich repeat (LRR). Intracytoplasmic Toll/IL-1R (TIR) domains of TLRs initiate the signaling cascades, which can lead to activation of necrosis factor (NF)-κB and MAPK. Note that only TLR4 signaling can activate the MyD88-independent pathway.

other than LPS (Table 2). For example, gram-positive bacteria-derived lipoteichoic acids and the plant-derived anticancer agent, taxol, can also activate macrophages through TLR4 (12, 15; our unpublished data). Furthermore, TLR4 also recognizes the fusion protein derived from respiratory syncytial (RS) virus, indicating the involvement of TLR4 in antiviral immunity (16). In addition to these exogenous ligands, an endogenous ligand, heat shock protein (HSP) 60, is a potential ligand for TLR4 (17). HSP proteins are released from necrotic cells that have encountered danger signals such as bacterial infection or some stress. They can be potent stimulators for macrophages and DCs (18, 19). Finally, fragments of fibronectin, produced on tissue injury, can provoke inflammatory responses through TLR4 (20). Thus, interestingly, both host-derived proteins and microbial products can coordinately activate innate immunity through TLR4.

TLR2

To clarify how TLR2 is involved in innate immunity, TLR2-deficient mice were analyzed for their responses to various PAMPs (12). TLR2-deficient macrophages were shown to be hyporesponsive to gram-positive cell wall preparations from *Staphylococcus aureus*, *Corynebacterium diphtheriae*, and *Nocardia coeliaca*. The cell wall of gram-positive bacteria contains a thick layer of peptidoglycan (PGN). PGN can also elicit cytokine production by macrophages, and we have found that TLR2 is essential for the response to *S. aureus* PGN. In addition, TLR2-deficient macrophages did not respond to the 2-kDa macrophage-activating lipopeptide-2 (MALP-2), which is a lipopeptide from the wall-less bacterium, *Mycoplasma* (21). In addition to our findings, several lines of evidence suggest that TLR2 is essential for the immune response to a wide variety of pathogens, including mycobacteria, spirochetes, and yeast (22–27) (Table 2).

TLR9

In the 1980s Tokunaga et al. (28) showed that bacterial DNA is responsible for the immunostimulatory and antitumor activity of Bacillus Calmette-Guérin (BCG). This immunostimulatory effect depends on an unmethylated cytidine-phosphate-guanosine (CpG) motif (29, 30). This motif is underrepresented and mostly methylated in eukaryotic DNAs lacking immunostimulatory activity. Therefore, bacterial DNAs can also behave as PAMPs. Cytokine release induced by bacterial DNAs containing the CpG motif (CpG DNA) is dependent on MyD88, suggesting that the receptor for CpG also belongs to the TLR family (31). We have cloned a novel TLR family member, TLR9, and established TLR9-deficient mice (32). The mutant mice retained the responsiveness to LPS but were completely refractory to CpG DNA, showing that TLR9 is a critical signal transducer for CpG DNA (Table 2).

Chu et al. (33) also showed that the DNA-dependent protein kinase (DNA-PK) is essential for the effects of immunostimulatory DNA on innate immunity. It is unclear at present whether the DNA-PK locates downstream of TLR9 or parallel to the TLR9 signaling pathway.

Critical roles of MyD88 in TLR family signaling

After microbial recognition TLRs activate the signaling pathway that can lead to inflammatory responses. As described above, the pathway is first triggered by the TIR domain. The TIR domain associates with an adaptor protein, MyD88, through homophilic interaction of their TIR domains. MyD88 associates not only with IL-1R but also with TLR family members (Fig. 2). To clarify the critical roles of MyD88, MyD88-deficient mice were generated (34). IL-1 family cytokines such as IL-1 and IL-18 cannot activate either NF-κB or MAPK in MyD88-deficient cells, suggesting that MyD88 is essential for IL-1/IL-18 signaling (34). In addition, MALP-2-induced, TLR2-mediated activation of these factors was abolished in MyD88-deficient macrophages (21), suggesting that MyD88 is also essential for TLR2 signaling. Furthermore, CpG DNA-induced activation of NF-κB was abolished not only in TLR9- but also in MyD88-deficient cells (32; our unpublished data). These results suggest that signaling through TLR2 and TLR9 and through the IL-1R family is dependent on MyD88.

The MyD88-independent pathway in TLR4 signaling

TLR4-mediated signaling stimulated by LPS can induce NF-κB and MAPK activity in MyD88-deficient cells, even though the induction is delayed (35). Thus, TLR4 signaling is unique with regard to its causing the activation of NF-κB and MAPK cascades in an MyD88-independent manner.

MyD88 associates with a serine threonine kinase, IL-1R-associated kinase (IRAK) (Fig. 2), through the homophilic interaction of their death domains. When IRAK is activated, it subsequently leads to activation of NF-κB and MAPK cascades via the TNF receptor-associated factor (TRAF) 6. How is the MyD88-independent pathway integrated in TLR4 signaling?

LPS-induced IRAK activation is abolished in MyD88-deficient cells (35), suggesting that IRAK is activated in a MyD88-dependent manner. Interestingly, LPS can induce the activation of NF-κB and MAPK cascades with delayed kinetics in IRAK-deficient macrophages as in MyD88-deficient macrophages (36). Furthermore, delayed activation of NF-κB in response to LPS was also observed in TRAF6-deficient embryonic fibroblasts (our unpublished data). Collectively, it can be assumed that neither IRAK nor TRAF6 is involved in the MyD88-independent pathway (Fig. 2).

Biological significance of the TLR4-induced, MyD88-independent pathway

Although MyD88-deficient cells retain biochemical responses to LPS, their biological responses have never been reported. First, LPS cannot provoke MyD88-deficient macrophages to produce inflammatory cytokines. Second, LPS does not induce blast formation of MyD88-deficient B cells. Third, MyD88-deficient mice are completely resistant to LPS-induced septic shock.

Thus, the question is whether the MyD88-independent pathway can lead to any biological responses. So far, TLR

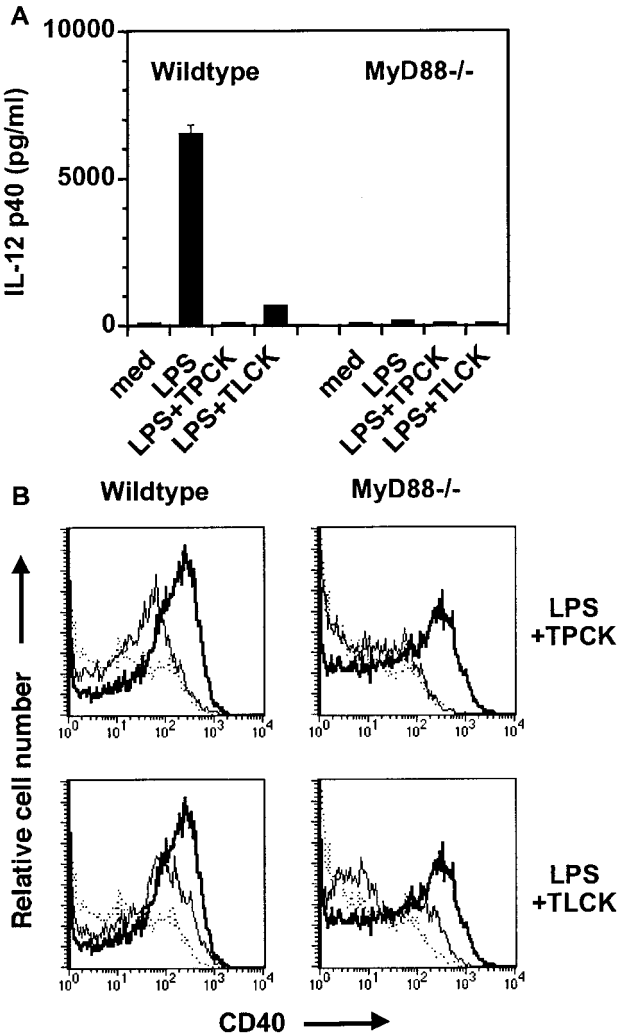

Fig. 3. The serine protease inhibitors can block both cytokine production and CD40 expression in response to lipopolysaccharide (LPS). BM cells were cultured with GM-CSF for 6 days and used for BM DCs. BM DCs were pretreated with or without *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) or *N*-alpha-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) for 1 h and cultured for a further 8 h in the absence or presence of LPS. 3A. IL-12 p40 production from wildtype and MyD88-deficient BM DCs. The levels of IL-12 p40 were measured by means of enzyme-linked immunosorbent assay. 3B. Surface expression of CD40 on DCs stimulated with (solid thick and thin lines) or without (dotted lines) LPS. Solid thin lines represent data from LPS-stimulated DCs treated with TPCK or TLCK.

signaling has mainly been investigated in macrophages and B cells. In addition to these cells, DCs are also important as innate immunity players. DCs reside in the peripheral tissues, such as skin, in an immature state, and they mature in response to microbial infection (37, 38). PAMPs, including LPS, are critically involved in this process. Mature DCs leave the tissues and migrate to the regional lymph nodes. There, DCs stimulate naive T cells by offering cytokines and costimulatory molecules such as CD40 and CD86. Thus, DC function is critical for a link between innate and adaptive immunity. To clarify the biological significance of the MyD88-independent pathway, we have focused on the effects of LPS on DCs (39).

The DC maturation process can be reproduced in vitro. Bone marrow (BM) cells stimulated with GM-CSF can give rise to immature DCs. LPS can induce cytokine production from wildtype immature BM DCs. However, LPS-induced cytokine release was completely abolished in not only TLR4- but also MyD88-deficient BM DCs (40) (Fig. 3A). This is consistent with previous observations in macrophages and indicates that LPS stimulates cytokine production from DCs in a MyD88-dependent manner.

LPS can also enhance surface expression of costimulatory molecules including CD40, CD80, and CD86 on wildtype DCs. This phenotypic modulation is a typical feature of DC maturation. As expected, LPS-induced maturation was abolished in TLR4-deficient DCs. However, on LPS stimulation, surface expression of costimulatory molecules was significantly upregulated in MyD88-deficient DCs (40) (Fig. 3B). Thus, the MyD88-independent pathway can lead to another important biological function, costimulatory molecule upregulation.

Rescigno et al. (41) found that LPS-induced DC maturation, as measured by upregulation of costimulatory molecules such as MHC and CD86, is dependent on NF-κB activation. They utilized serine protease inhibitors to block nuclear translocation of NF-κB activation. We have also tested whether the inhibitors block the effects of LPS on BM DCs. The reagents could inhibit LPS-induced cytokine production from wildtype BM DCs (Fig. 3A). Futhermore, they could also decrease upregulation of CD40 expression in response to LPS in both wildtype and MyD88-deficient BM DCs (Fig. 3B). Their results indicate that residual NF-κB activation detected in MyD88-deficient cells is critical for costimulatory molecule upregulation in response to LPS.

Differential signaling mechanism in TLR family signaling

Similar to LPS, CpG DNA, a TLR9 ligand, can induce not only cytokine release but also CD40 upregulation of DCs (42). CpG DNA-induced cytokine production was dependent on MyD88 (31). Therefore, we have also investigated whether TLR9 signaling can cause upregulation of costimulatory molecules. Both cytokine production and CD40 upregulation were completely abolished in the absence of MyD88, indicating that TLR9 signaling is completely dependent on MyD88 (40). This is also supported by the fact that no biochemical responses such as NF-κB activation were induced in CpG DNA-stimulated, MyD88-deficient cells (data not shown). The results suggest that different TLRs activate distinct signaling pathways despite their similar biological consequences. Ozinsky et al. (43) have also shown that TIR domains among the TLR family are functionally heterogeneous. According to them, dimerization of the intracytoplasmic region of TLR4, but not TLR2, can induce cytokine production. In addition, TLR2 can associate with TLR1 or TLR6 and lead to cytokine production as heterologous TLR complexes. These findings also suggest that the TIR domains are not functionally equivalent among the TLR family.

Concluding remarks

Many issues still remain unresolved in the TLR research field. Currently, public databases and our unpublished results have shown that the mammalian TLR family consists of more than 10 members. Do other TLRs, apart from TLR2, TLR4, and TLR9, also function as signal transducers for certain PAMPs?

Another question concerns the molecular basis of the MyD88-independent pathway. Mammalian MyD88 may not be a real homologue of *Drosophila* Tube because MyD88, but not Tube, carries a TIR domain. *Drosophila* carries both MyD88 (GenBank Accession No. AAF58953) and Tube. It is therefore probable that TLR4 interacts with other adaptor(s) such as a mammalian homologue of Tube. At present, however, other TIR domain-associated molecule(s) apart from MyD88 remain unknown.

We should also keep in mind that there is more to be clarified about the MyD88-dependent pathway. Considering that the MyD88-independent NF-κB and MAPK cascades are not sufficient to induce cytokine production, some unknown pathway(s) must be critical in MyD88-dependent cytokine production.

Clarifying the molecular mechanisms of TLRs in innate immunity may help to identify potential targets for future therapeutic intervention to regulate host defense. This can also contribute to the development of effective adjuvants.

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Awards

Excellent contribution to dental research

Professor Dowen Birkhed from Göteborg University was selected by the Prize Committee as the 2001 recipient of the Acta Odontologica Scandinavica Award for his excellent contributions to dental research. The award, NOK 50,000, will be presented to Professor Birkhed at the 84th Annual Meeting of NOF in Copenhagen, Denmark, on 23–25 August 2001. The Prize Committee appointed by the Board of the Acta Odontologica Scandinavica Foundation consisted of Drs. Stefán Finnbogason, Anne Bjørg Tveit, Marianne Lenander-Lumikari, Palle Holmstrup, and Folke Lagerlöf.

The best article

As the best article published in Acta Odontologica Scandinavica in 2000 the Prize Committee selected 'Growth hormone and cortisol in serum and saliva' by Panu J. F. Rantonen, Ilkka Penttilä, Jukka H. Meurman, Kari Savolainen, Sakari Närvänen, and Tuula Helenius (Acta Odontol Scand 2000;58:299–303). The award constitutes NOK 10,000. The Prize Committee appointed by the Board of the Acta Odontologica Scandinavica Foundation consisted of Drs. Stefán Finnbogason, Karin Heyeraas, Peter Holbrook, Palle Holmstrup, Pentti Kirveskari, Thomas Modéer, and Folke Lagerlöf.