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Invited Review

Regulation of Innate Immune Responses by Toll-Like Receptors

Kiyoshi Takeda[1,2] and Shizuo Akira[1,2]*

[1]*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University and* [2]*Solution-oriented Research for Science and Technology, Japan Science and Technology Corporation, Yamada-oka 3-1, Suita, Osaka 565-0871, Japan*

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SUMMARY: Innate immune response in *Drosophila* is mediated by signaling through Toll receptors. In mammals, Toll-like receptors (TLRs), comprising a large family, recognize a specific pattern of microbial components. So far, the roles of TLR2, TLR4, TLR5, TLR6, and TLR9 have been revealed. The recognition of microbial components by TLRs leads to activation of innate immunity, which provokes inflammatory responses and finally the development of adaptive immunity. The inflammatory response depends on a TLR-mediated MyD88-dependent cascade. However, there seems to exist additional cascades in TLR signaling. In the case of TLR4 signaling, an MyD88-independent pathway is now being characterized. In addition to the activation of innate immune responses, TLR-mediated signaling leads to suppression of the activity of innate immune cells, represented by "lipopolysaccharide (LPS) tolerance". Progress in elucidating the molecular mechanisms for LPS tolerance has been made through the analysis of TLR-mediated signaling pathways. Thus, the activity for innate immune responses is known to be finely regulated by TLRs.

1. Introduction

Host defense against invasion by pathogens relies on two types of immunity, innate and adaptive (acquired) immunity (1). Innate immunity is phylogenetically conserved and present in almost all multicellular organisms, whereas adaptive immunity is not found in invertebrates. Adaptive immunity is a system whereby a foreign antigen is recognized by antigen receptors expressed on the surface of B and T lymphocytes. In order to cope with a variety of antigens, B and T cells rearrange genes for immunoglobulin and the T cell receptor, to produce over 10^{11} types of antigen receptors. Lymphocytes bearing receptors that have suitable affinity to a specific antigen show clonal expansion when stimulated with the antigen. Thus, adaptive immunity is a highly sophisticated system to combat microorganisms. In contrast, activation of innate immunity is dependent on germline-encoded receptors to recognize microorganisms, and innate immunity seems to be a primitive system compared with adaptive immunity. However, innate immunity plays an important role not only in the first line of host defense against invasion by microorganisms but also in the instruction of adaptive immunity (2,3). T cell receptors recognize peptide antigens that are processed in and presented with major histocompatibility complex (MHC) class I and class II on antigen-presenting cells. Furthermore, activation and differentiation of naive T cells into type I helper T cells is mediated by co-stimulatory molecules expressed on the antigen-presenting cells, and cytokines such as interleukin-12 (IL-12) produced by the antigen-presenting cells (4). These antigen-presenting cells include dendritic cells (DCs) and macrophages, both of which play an important role in innate immunity by recognizing and up-taking pathogens.

In *Drosophila*, which has innate immunity but not adaptive immunity, signaling pathways via Toll receptors have been shown to play important roles in the host defense against invasion by microorganisms. Toll-like receptors (TLRs) were

*Corresponding author: Tel: +81-6-6879-8303, Fax: +81-6-6879-8305, E-mail: sakira@biken.osaka-u.ac.jp

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subsequently identified in mammals. Evidence is now accumulating that TLRs are key receptors for innate immune recognition. In this review, we will focus on recent findings on TLRs, which are responsible for the recognition of pathogens and regulation of innate immune activation.

2. Innate immune responses in *Drosophila*

In *Drosophila*, host defense is elicited by virtue of the synthesis of peptides in response to fungal invasion (Drosomycin) or bacterial invasion (Diptericin, Drosocin, Cecropin, Attacin, Defensin). The genes encoding these anti-microbial peptides possess, in the promoter regions, binding motifs analogous to those of mammalian NF-κB, the Rel family of transcription factors responsible for the gene induction of an inflammatory response (5). In *Drosophila*, three Rel types of transcription factors, Dorsal, Dorsal-type immune factor (DIF), and Relish, have been identified. Dorsal was initially identified as the morphogen defining dorso-ventral patterning during embryogenesis. Dorsal is sustained in the inactive state in the cytoplasm through interaction with the ankyrin-repeat protein, Cactus. Degradation of Cactus and translocation of Dorsal into the nucleus are triggered by an association of Spätzle with the transmembrane receptor, Toll. An adaptor Tube associates with the cytoplasmic portion of Toll and transduces the signal to activate the serine/threonine kinase Pelle in response to Spätzle. In 1996, the Toll signaling pathway was shown to be involved in the induction of an antifungal peptide, Drosomycin. Accordingly, mutant flies lacking Toll were found to be highly susceptible to fungal infection (6). Subsequently, genetic studies with mutant flies that show high susceptibility to microbial infections have established that DIF and Dorsal mediate antifungal responses in the Toll signaling pathway, whereas Relish controls antibacterial responses (7-10). Relish mutant flies do not induce the antibacterial peptide Diptericin and unlike the Toll mutant flies, show high susceptibility to bacterial infection. Toll is a large family comprising at least nine members (Toll, 18-wheeler, Toll 3-9) in *Drosophila* (11). Other Toll family members may therefore be responsible for the bacteria-induced activation of Relish. One candidate is 18-wheeler. Mutant flies lacking 18-wheeler have been shown to be sensitive to bacterial infection; however, these mutants did not exhibit a significant reduction in bacteria-induced expression of Diptericin (12). Therefore, there may be other receptors which recognize bacteria in addition to 18-wheeler, as pointed out in a recent review (13). Genetic studies have

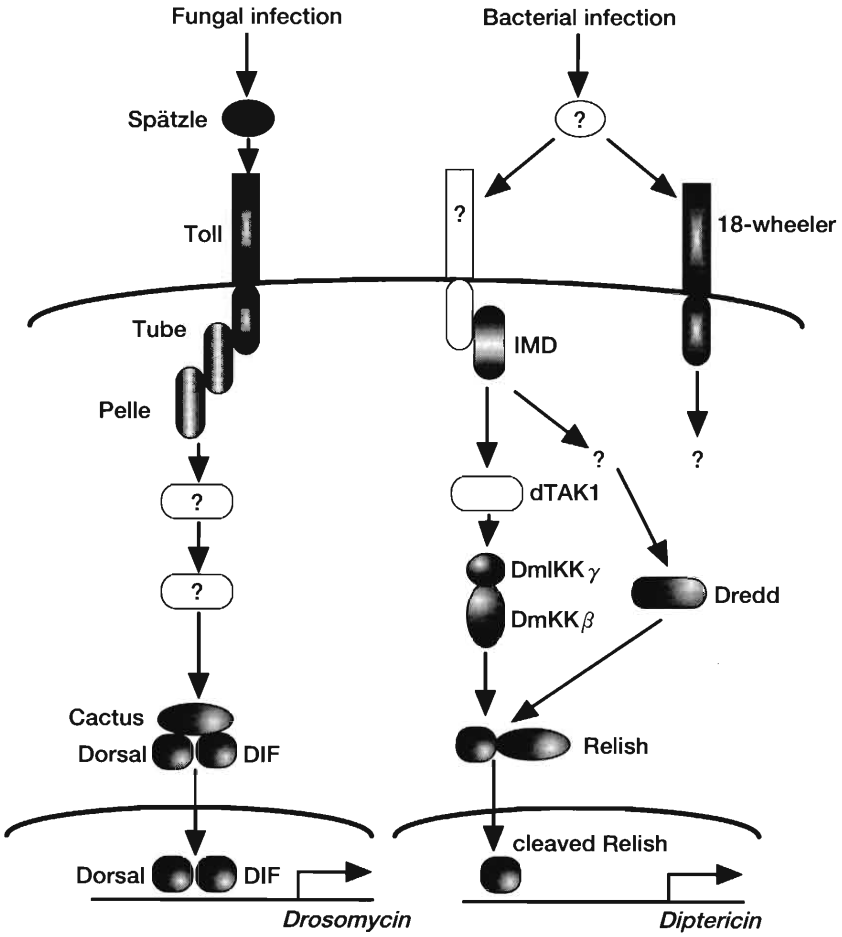

Fig. 1. Antimicrobial signaling pathways in *Drosophila*.
In *Drosophila*, fungal infection induces cleavage of pro-Spätzle. Spätzle associates with Toll and activates Pelle. This signaling pathway leads to the degradation of Cactus, then Rel-type transcription factors, Dorsal and Dorsal-type immune factor (DIF), translocate into the nucleus and induce the expression of Drosomycin. In the case of bacterial infection, the immune deficiency (IMD) pathway is activated to produce an antibacterial peptide, Diptericin. The cell surface receptor in the IMD pathway is yet to be identified. In this pathway, dTAK1 regulates the activation of an IκB kinase complex composed of DmIKK β and DmIKK γ, and finally activates Relish through cleavage. Dredd is also involved in the activation of Relish. 18-wheeler, a member of the Toll family, is also implicated in the antibacterial response; however, it does not seem to be involved in the IMD pathway.

identified several molecules involved in antibacterial responses. DmIKKβ, DmIKKγ, dTAK1 and the *immune deficiency* (*imd*) gene product (*Drosophila* homologues of mammalian IKKβ, IKKγ, TAK1 and RIP, respectively) have been shown to be involved in the expression of antibacterial peptides and resistance to Gram-negative bacteria (14-17). Other genetic screens identified Dredd, a homologue of mammalian caspase, that is involved in antibacterial peptide expression through activation of Relish (18-20). In mammals, there have been no reports of caspases involvement in the activation of NF-κB, and it remains unclear how Dredd regulates Relish activity. It has been established that *Drosophila* discriminates between pathogens and elicits a specific immune response via two signaling pathways leading to the expression of antifungal and antibacterial peptides (Fig. 1) (for a review, see references 13, 21, 22).

3. Innate immune activation by TLRs

3-1. Identification of TLRs in mammals

Following the identification of Toll as a key receptor of host defense response in *Drosophila*, a mammalian homologue of Toll was identified as hToll (now termed TLR4) by Medzhitov and colleagues. Enforced expression of TLR4 induced the activation of NF-κB and the expression of several inflammatory genes (23). Subsequent studies identified several proteins that are structurally related to TLR4. The TLR family now consists of ten members (TLR1-TLR10), and is expected to expand (24-28). The involvement of TLRs in the recognition of microorganisms has thus been well established.

3-2. TLR4 is a signaling component of the LPS receptor

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. It was first demonstrated that over-expression of TLR2 in human embryonic kidney 293 cells conferred an LPS response (29,30). It is well known that two mouse strains, C3H/HeJ and C57BL10/ScCr, are hypo-responsive to LPS. Two independent groups analyzed the gene responsible for hypo-responsiveness to LPS, and found mutations in *Tlr4* in these strains (31,32). In the C3H/HeJ mouse strain, a point mutation in the cytoplasmic region of the *Tlr4* gene resulted in an amino acid change from proline to histidine. This mutation has been shown to result in defective TLR4-mediated signaling, and to have a dominant negative effect on LPS-dependent responses (33,34). The other LPS hypo-responsive strain, C57BL10/ScCr, was shown to be null-mutated in the *Tlr4* gene (31,32). The generation of TLR4 knockout mice confirmed the essential role of TLR4 in LPS recognition (33). Subsequent studies with TLR2 knockout mice or Chinese hamster ovary (CHO) fibroblasts genetically lacking TLR2 demonstrated that TLR2 is not involved in the recognition of LPS (35,36). Thus, genetic approaches have clearly demonstrated that TLR4, not TLR2 is the LPS receptor. Overexpression of TLR2 in 293 cells seems to confer a response to the TLR2 ligand contaminating the preparation of LPS used. Indeed, re-purification of LPS demonstrated that TLR4, but not TLR2, is a receptor for LPS (37,38). However, recent studies have indicated that TLR2 recognizes the LPS extracted from *Leptospira interrogans* or *Porphyromonas gingivalis*, indicating that TLR4 recognizes LPS from enterobacteria such as *Escherichia coli* and *Salmonella*, whereas TLR2 may recognize LPS from other bacteria (39,40). Even in these studies, minor contamination with the TLR2 ligand in the LPS preparation cannot be ruled out. It will require further investigation to clarify the involvement of TLR2 in LPS recognition.

In addition to TLR4, now established as an essential receptor for the recognition of LPS, several molecules are involved in the formation of the LPS receptor complex. CD14, a glycosylphosphatidylinositol (GPI)-anchored molecule preferentially expressed in monocytes/macrophages and neutrophils, has recently been shown to physically associate with TLR4 in response to LPS (41). Miyake and colleagues identified MD-2 as a molecule that associates with the extracellular portion of TLR4 and enhances LPS responsiveness (42,43). Genetic analysis of CHO cell lines hypo-responsive to LPS led to the identification of MD-2 as a critical component in the LPS response (44). Miyake and colleagues further identified RP105, which bears leucine-rich repeats that are structurally related to TLRs in the extracellular portion (45). B cells from RP105-deficient mice showed a severely reduced response to LPS. They also showed an association between TLR4 and RP105, indicating that RP105 is closely involved in the recognition of LPS together with TLR4 in B cells (46). Thus, several components have been implicated in the recognition of LPS, indicating that the functional LPS receptor consists of a large complex of several molecules.

3-3. The TLR family is responsible for recognition of microbial components

Although it remains controversial whether or not TLR2 recognizes LPS, there is evidence that TLR2 is involved in the recognition of a variety of microbial components. These include lipoproteins from Gram-negative bacteria, Gram-positive bacteria, mycoplasma, mycobacteria and spirochetes (47-53), peptidoglycan and lipoteichoic acid (LTA) from Gram-positive bacteria (36,54-57), lipoarabinomannan from mycobacteria (58,59), and zymosan from fungi (34). In vivo roles of TLR2 have also been established in a study using TLR2 knockout mice, which show high susceptibility to infection by *Staphylococcus aureus* (60).

One of the mechanisms by which TLR2 recognizes microbial components has been elucidated. TLR2 forms heterodimers with other TLRs to discriminate among bacterial components. A study on the ectopic expression of TLR1 and TLR2 in HeLa cells revealed that TLR2 functionally cooperates with TLR1 to modulate the response to factors released from *Neisseria meningitidis* (61). Aderem and colleagues introduced the dominant negative form of TLR2 and TLR6 into the RAW264 macrophage cell line and demonstrated that TLR2 associates with TLR6 to detect the specific pattern of the peptidoglycan or modulin secreted from *S. aureus* (62,63). TLR6 knockout mice did not show any response to mycoplasma-derived lipopeptides, but showed a normal response to bacteria-derived lipopeptides (64). In contrast, TLR2 knockout mice showed no response to either type of lipopeptides. Reconstitution of the chimeric constructs of TLR2 and TLR6 in TLR2/TLR6 double knockout cells revealed that the requirement of both TLR2 and TLR6 for the recognition of mycoplasma-derived lipopeptides. Thus, TLR2 associates with other TLRs to discriminate among microbial components.

In addition to the cell wall components of pathogens, bacterial DNA is known to activate immune cells. The immunostimulatory activity of bacterial DNA is attributed to the presence of unmethylated CpG motifs. CpG motifs in vertebrate genomic DNA are observed at a reduced frequency and highly methylated, which leads to no immunostimulatory activity. Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs also activate immune cells. The involvement of TLR9 in the recognition of CpG DNA has been shown

Table 1. TLR family members and their ligands

TLR family	microbial components
TLR1	?
TLR2	peptidoglycan, lipopeptides
TLR3	?
TLR4	LPS
TLR5	flagellin
TLR6	mycoplasma-derived lipopeptides
TLR7	?
TLR8	?
TLR9	CpG DNA
TLR10	?

through the generation of TLR9 knockout mice (65).

Most recently, TLR5 has been shown to recognize flagellin, a monomeric component of bacterial flagella (66). A subsequent study indicated that TLR5 basolaterally expressed on intestinal epithelial cells confers the ability to recognize flagellin from pathogenic bacteria (67).

Thus, it has now been established that TLRs recognize a specific pattern of microbial components (Table 1). The recognition of microbial components by TLRs triggers the activation of innate immunity.

4. Signaling pathway via TLRs

4-1. Comparison of signaling pathways with *Drosophila* Toll and mammalian TLR

The signaling pathway via *Drosophila* Toll has been shown to be highly homologous to the mammalian IL-1 signaling pathway (Fig. 2). The cytoplasmic region of *Drosophila* Toll is very similar to that of the mammalian IL-1 receptor. This region is now called the Toll/IL-1 receptor (TIR) domain. In the IL-1 signaling pathway, the MyD88 adaptor molecule, a functional homologue of *Drosophila* Tube, associates with the IL-1 receptor, and recruits the Pelle-related serine/threonine kinase, IL-1 receptor associated kinase (IRAK), to the receptor upon stimulation with IL-1 (68-70). This leads to activation of IRAK through phosphorylation and the association of IRAK with TRAF6. Although *Drosophila* homologues of TRAF protein (dTRAF1-3) have been identified, it remains unclear whether dTRAF is involved in innate immune signaling. The IL-1 signaling pathway finally induces phosphorylation of inhibitory protein IκB, a homologue of Cactus, and nuclear translocation of transcription factor nuclear factor-κB (NF-κB), a homologue of Dorsal and DIF.

4-2. MyD88 is a critical component in TLR signaling

Mammalian TLRs also possess TIR domains in their cytoplasmic region and utilize MyD88 as an adaptor for signaling (71,72). The generation of MyD88 knockout mice revealed an essential role of MyD88 in the signaling pathway via the IL-1 receptor family (73). Further, it has been demonstrated that MyD88 knockout mice show no inflammatory response to LPS (74). Similarly, mice deficient in IRAK or TRAF6 exhibit impaired responses to both IL-1 and LPS, indicating that IRAK and TRAF6 are critical components of both the IL-1 receptor- and TLR4-mediated signaling pathways (75-79). In other studies, macrophages from MyD88 knockout mice were shown to produce no inflammatory cytokines in response to peptidoglycan, lipoproteins and CpG DNA (52,80-82). And MyD88 knockout mice failed to

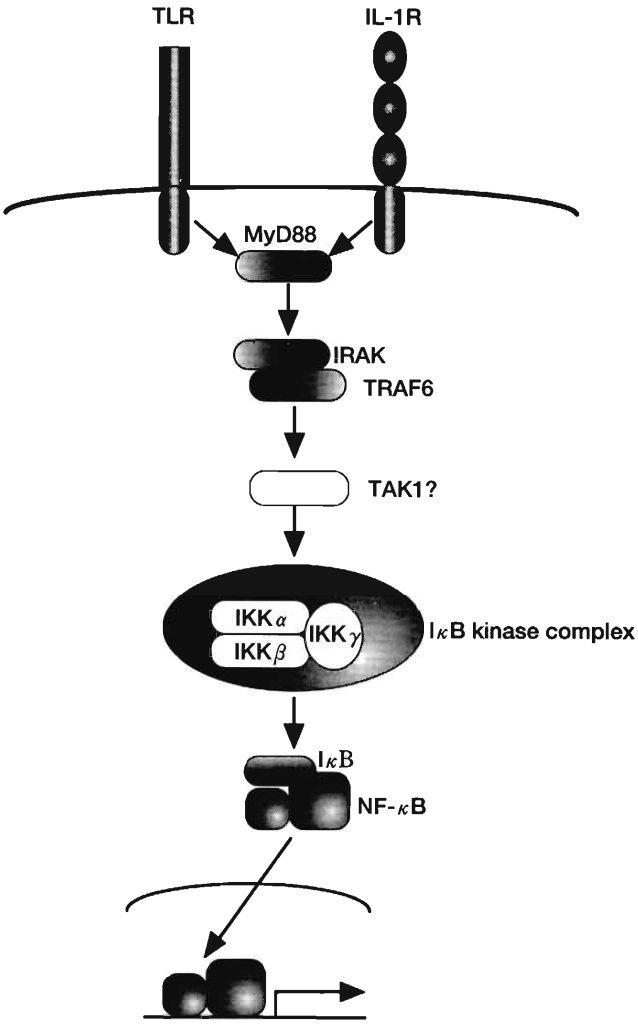

Fig. 2. Signaling pathway via the IL-1 receptor and TLRs.
Toll-like receptors (TLRs) and the interleukin-1 (IL-1) receptor utilize a common signaling pathway. An adaptor molecule, MyD88, associates with the cytoplasmic region of both receptors, and recruits IL-1 receptor associated kinase (IRAK) to the receptor upon receptor activation. IRAK then activates tumor necrosis factor receptor-associated factor 6 (TRAF6), leading to the activation of IκB kinase complex. Transforming growth factor β-activated kinase (TAK1) is implicated in the activation of IκB kinase; however, an in vivo role of TAK1 has yet to be revealed. IκB is phosphorylated by IκB kinase and degraded, leading to the nuclear localization of nuclear factor-κB (NF-κB) transcription factors.

produce a detectable level of IL-6 in response to flagellin (66). These results demonstrate that MyD88 is critical to the signaling pathway via the TLR family. Indeed, activation of NF-κB and c-Jun N-terminal kinase (JNK) in response to peptidoglycan, lipoprotein and CpG DNA was not observed in MyD88 knockout cells. However, stimulation with LPS of MyD88 knockout macrophages induced activation of NF-κB and JNK, although delayed (74). This indicates that, although MyD88 is indispensable for LPS-TLR4-mediated production of inflammatory cytokines, there exists an MyD88-independent component in the LPS signaling pathway.

4-3. MyD88-independent TLR signaling

The roles of LPS-induced activation in the MyD88-independent signaling pathway are now being clarified. The stimulation with LPS of MyD88 knockout macrophages has been shown to induce expression of IFN-inducible genes such as IP-10 and GARG16 through activation of IRF3 (83).

Similarly, the stimulation of DCs with LPS has been shown to induce the expression of distinct types of cytokines and chemokines from that with TLR2 agonist (84). Kupffer cells from MyD88 knockout mice produced IL-18 in response to LPS (85). Further, DCs from MyD88 knockout mice matured in response to LPS (86, 87). Thus, several LPS responses were demonstrated in MyD88 knockout mice.

Analysis of MyD88-independent activation of LPS signaling has recently led to identification of a novel adaptor molecule named TIR domain-containing adaptor protein (TIRAP)/ MyD88-adaptor-like (Mal) (88, 89). TIRAP/Mal possesses a TIR domain similar to MyD88, and specifically associates with TLR4. The dominant negative form of TIRAP/Mal inhibited the LPS-induced activation of NF-κB, but not TLR2 or TLR9-mediated activation. Furthermore, the blockade of TIRAP-mediated signaling by addition of a cell permeable TIRAP peptide led to impaired LPS-induced maturation of both wild-type and MyD88-deficient DCs. These findings indicate that TIRAP/Mal is an adaptor molecule involved in the LPS-induced MyD88-independent pathways (Fig. 3).

Similarly to TLR4-mediated signaling, which has a unique TIRAP/Mal-mediated component in addition to the common MyD88-dependent pathway, each TLR seems to have its own signaling pathway. In TLR2 signaling, stimulation with heat-killed *S. aureus* has been shown to cause the recruitment of active Racl and phosphatidyl-inositol-3 (PI3K) to the cytoplasmic portion of TLR2. This induced the activation of Akt followed by activation of the p65 subunit of NF-κB independently of IκBα degradation (90). In the case of CpG DNA recognition, the involvement of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) in the CpG DNA-induced immune cell activation has been demonstrated (91). DNA-PKcs is a member of the PI3K family, and was originally implicated in the repair of DNA double-stranded breaks caused by stress-induced damage from ionized radiation and by programmed DNA rearrangement (called VDJ recombination) during the development of T and B cells. Macrophages from DNA-PKcs-deficient mice were shown to be severely impaired in CpG DNA-induced production of inflammatory cytokines. Further, CpG DNA-induced activation of DNA-PKcs led to activation of NF-κB. Thus, several molecules appear to be involved in the signaling pathways of the TLR family. These molecules might be responsible for the distinct biologic responses of different TLRs.

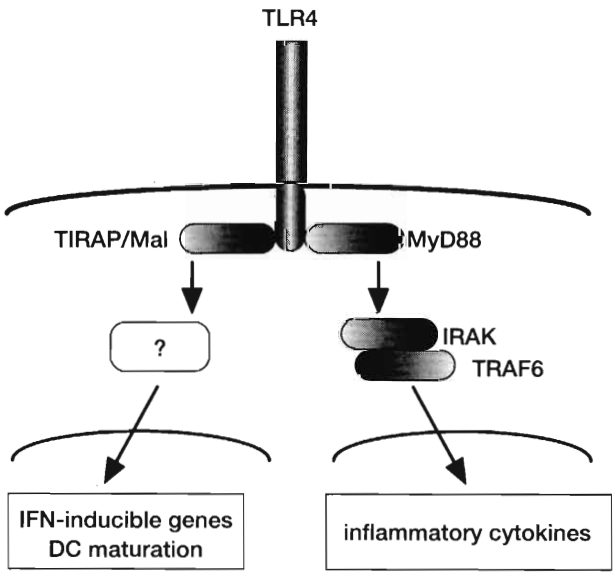

Fig. 3. Signaling pathway via TLR4.
The signaling pathway of TLR4 consists of MyD88-dependent and -independent components. The MyD88-dependent pathway is indispensable for lipopolysaccharide (LPS)-induced expression of inflammatory cytokines. In contrast, the MyD88-independent pathway is responsible for LPS-induced expression of interferon (IFN)-inducible genes and maturation of dendritic cells (DCs). An adaptor molecule that is involved in the MyD88-independent pathway, has recently been identified as Toll/IL-1 receptor domain-containing adaptor protein (TIRAP) or MyD88-adaptor-like (Mal).

5. Suppression of innate immune cell activity by microbial components

5-1. Mechanism for LPS tolerance

It has now been established that the stimulation of TLRs by microbial components activates innate immune cells such as macrophages and DCs. However, an exposure to microbial components such as LPS results in an inability among experimental animals and macrophages to respond to a second challenge of LPS. This phenomenon was first described over 50 years ago in a study in which rabbits treated with repeated injections of LPS showed a decrease in febrile response (92), and is now known as endotoxin (or LPS) tolerance. Although LPS tolerance is considered a host response to prevent the excessive production of inflammatory cytokines and shock syndrome, it is a major clinical problem in the treatment of patients with Gram-negative sepsis. In these patients, LPS from Gram-negative bacteria causes not only fatal septic shock but also LPS tolerance. Some patients that recover from septic shock become tolerant to LPS, and have a severely reduced host defense response against opportunistic infections leading to high mortality (93). Therefore, the molecular mechanism for LPS tolerance has long been a subject of investigation (94). LPS tolerance in macrophages/monocytes is characterized by a reduced production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), IL-6 and IL-12 (94-96), and alterations to the LPS-induced activation of signaling cascades, including protein kinase C, PI3K, MAP kinases, and IκB kinases (97-101). Accumulation of the p50 subunit of NF-κB, which has no transactivating activity, has also been reported (102,103). However, the precise mechanism responsible for LPS tolerance remains unclear.

The finding that TLRs are involved in the recognition of microbial components has contributed to our understanding of LPS tolerance. The utilization of monoclonal antibody that recognizes the TLR4-MD-2 complex explained one possible mechanism (104). Pre-exposure to LPS induced a time-dependent reduction in the production of inflammatory cytokines and the activation of IRAK and NF-κB in mouse peritoneal macrophages. LPS gradually reduced the surface expression of the TLR4-MD-2 complex on macrophages. This effect was well-correlated with the time-dependent onset of LPS tolerance. This study indicates that down-regulation of the TLR4-MD-2 complex (high affinity LPS receptor) is one of the major mechanisms for LPS tolerance. Internalization of the TLR4-MD-2 complex together with LPS might account for the down-regulation; however, the mechanism by which it is down-regulated remains unknown.

In THP-1 human promonocytic cell lines, it has been shown that LPS pre-treatment induces down-regulation of IRAK, and that IRAK is no longer activated in response to a second challenge of LPS (105). This is also one of the possible mechanisms which may be responsible for LPS tolerance. The

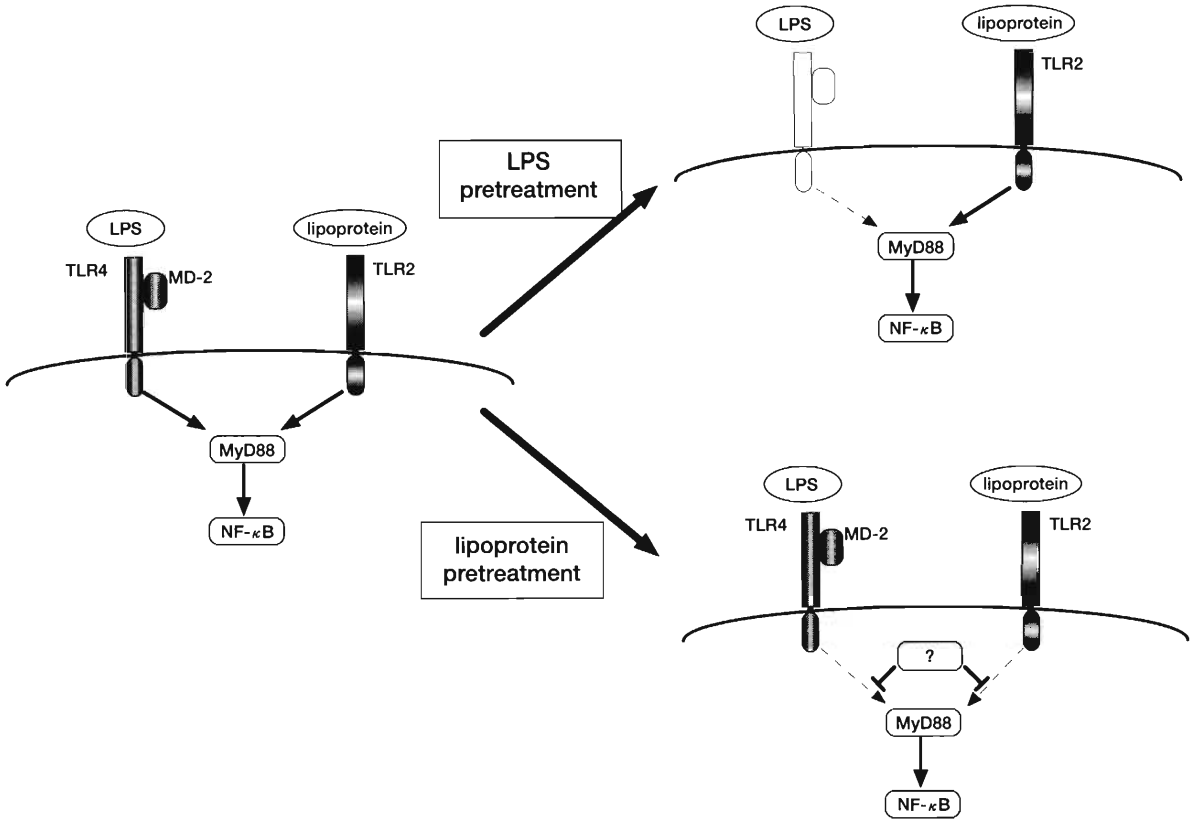

Fig. 4. Induction of LPS tolerance.
In mouse peritoneal macrophages, lipopolysaccharide (LPS) treatment induces down-regulation of the surface expression of the TLR4-MD-2 complex. This may be one of the mechanisms for LPS-induced LPS tolerance. Lipoprotein, which is recognized by TLR2, induces tolerance to lipoprotein and LPS. Lipoprotein pretreatment may affect the common signaling pathway via TLRs. Thus, LPS tolerance is induced through distinct mechanisms.

mechanism by which expression of IRAK is down-regulated in LPS-tolerant cells remains unclear.

5-2. Microbial components induce LPS tolerance

In addition to LPS, microbial components such as bacterial lipopeptides, peptidoglycan, muramyl dipeptide from Gram-positive bacteria, and CpG DNA have been shown to induce tolerance to subsequent stimulation (106-109). The mechanism for induction of tolerance in macrophages by lipopeptides or LTA, both of which are recognized by TLR2, has been investigated (57,110). Both LTA and lipopeptides were shown to induce tolerance to themselves in macrophages and mice. Further, both LTA and lipopeptides induced cross-tolerance to LPS. Unlike LPS, lipopeptides did not induce down-regulation of the TLR4-MD-2 complex in macrophages, indicating that lipopeptides-induced tolerance occurred through a mechanism distinct from LPS-induced tolerance (110). Additionally, induction of lipopeptides-induced LPS cross-tolerance was observed in IL-10-deficient mice (110). And it has been shown that LTA-induced cross-tolerance to LPS is not transferred to TLR2-deficient macrophages co-cultured with wild-type cells (57). These results indicate that soluble factors induced by microbial stimulation do not mediate induction of cross-tolerance, and that a common pathway via TLR2 and TLR4 is affected in macrophages pre-treated with TLR2 agonists. Thus, the tolerances induced by TLR4 and TLR2 agonists occur through quite different mechanisms (Fig. 4). Like lipopeptides, CpG DNA (TLR9 agonist) induces cross-tolerance to LPS in mouse peritoneal macrophages (111). Furthermore, tolerance to LPS has been shown to be induced in response to IL-1, which shares its signaling pathway with TLRs (101). These findings indicate that activation of the signaling pathway through the entire TLR/IL-1R family induces tolerance to LPS. LPS is a most potent activator of the immune system, and exposure to an excess of LPS often induces multi-organ failure with a high mortality rate. Therefore, the host may acquire a mechanism to limit inflammatory responses by inducing tolerance to LPS through several ligands which share a common signaling pathway. A recent publication described that LPS-induced tolerance was induced even in cells over-expressing TLR4 (112). One can imagine that TLR4 and other TLRs use a common signaling pathway via MyD88. Therefore, when TLR4 is constantly expressed, LPS-induced tolerance is achieved by the same mechanism as TLR2 agonist-induced tolerance. In normal macrophages, stimulation with LPS rapidly reduces the number of high affinity LPS receptors composed of the TLR4-MD-2 complex to minimize LPS-induced inflammation, as demonstrated in a study with a monoclonal antibody detecting the TLR4-MD-2 complex (104). If an antibody that detects TLR4 or MD-2 alone is used, down-regulation of both proteins might not be observed. However, the stimulation of macrophages with LPS actually induces conformational changes in the LPS receptor, leading to the destruction of the high affinity receptor. We speculate that under physiological conditions, LPS (TLR4 agonist) induces tolerance by down-regulating the high affinity LPS receptor, and other TLR agonists induce tolerance to LPS through activation of a common signaling pathway. Elucidation of the mechanism for induction of cross-tolerance to LPS by

other TLR ligands will shed further light on our understanding of LPS tolerance.

6. Future prospects

The identification of TLRs involved in innate immunity is a hot topic in immunology. A role for the TLR family in the recognition of microbial components has been elucidated. However, it remains unknown how TLRs recognize invading pathogens under physiological conditions. Phagocytosis plays a major role in innate immunity. TLR9 is known to be co-localized with CpG DNA in endosomes (113). TLR2 and TLR6 have been shown to be recruited to phagosomes from the cell surface after stimulation (34,62). From these findings, we speculate that TLRs recognize microbial components in the phago-lysosome, where pathogens are phagocytosed, digested and degraded, and microbial components are exposed. However, flow cytometric analyses with monoclonal antibodies have demonstrated that some TLRs, such as TLR1, 2, and 4, are actually expressed on the cell surface (42,43,114, 115). In this respect, we suspect that some TLRs recognize microbial components that are secreted from pathogens on the cell surface in addition to those in phago-lysosomes. Visualization of the interaction between TLRs and pathogens will be important to understand how innate immune responses are triggered. Elucidation of the pathway of signaling via each TLR will also be of interest. This will reveal why activation of each TLR evolves a distinct response, and how innate immune responses are positively (activation of innate immunity leading to development of adaptive immunity) and negatively (LPS tolerance) regulated. Analysis of the molecular mechanism behind innate immunity has only just restarted with the identification of TLRs, and many questions and mysteries remain.

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