Toll-Like Receptor Signaling and Regulation of Cytokine Gene Expression in the Immune System

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ABSTRACT

Dendritic cells, macrophages, and granulocytes are derived from hematopoietic stem cells and provide a first line of defense against infectious pathogens. Toll-like receptors (TLRs) expressed on these cells recognize molecular structures present in the pathogens. Upon binding of a pathogen ligand, TLRs trigger a cascade of signaling pathways that is conserved from insect to plants to humans, which ultimately activates NF κ B. In mammalian cells, this leads to the induction of cytokine genes and the establishment of innate immunity. For example, TLR signals induce type I interferons (IFN α/β) in dendritic cells conferring an antiviral state upon host cells. Moreover, TLR signals stimulate not only pro-inflammatory cytokines such as IFNs, IL-1, TNF α , and IL-12 but also anti-inflammatory cytokines such as IL-10 and IL-6. IL-12 and IL-10 are cytokines that bridge early innate responses and the ensuing specific immune responses. TLR signals also enhance an antigen presentation capacity in dendritic cells and macrophages. Recent studies with mouse and human cells indicate that TLRs activate multiple signaling cascades that involve chromatin structure alterations as well as activation of many transcription factors (e.g., IRF-3, IRF-8/ICSBP, and PU.1). Together, although the basic backbone is conserved throughout evolution, the TLR signaling system in mammalian species has an added complexity to accommodate a mechanism that links innate and adaptive immunity.

THE TOLL-LIKE RECEPTOR SYSTEM

Toll-like receptors (TLRs) are transmembrane molecules that contain a leucine-rich repeat motif in the external domain. In mammalian species, there are 10 TLRs, which are structurally similar to the IL-1 receptor and IL-18 receptor in the TIR domain, although the external domain is divergent (28,31,57). The TLR system is conserved throughout evolution, and similar receptors and signaling pathways are present in insects and plants (37,57). The conservation of the TLRs is likely to be attributed to their critical role in host defense, although TLRs in *Drosophila* play an important role in early development as well (37).

Table 1 lists the ligands identified for each TLR, showing that TLRs recognize a diverse set of ligands with a high level of specificity. TLR4, the best-studied TLR, recognizes bacterial lipopolysaccharides (LPSs) whose active moiety is lipid A. The

TLR4 gene is mutated in the mouse strains C3H/HeJ and C57B/10ScCr that fail to respond to LPS [i.e., they are resistant to LPS-mediated endotoxin shock and are susceptible to infection by Gram-negative bacteria (61)]. A later study showed that TLR4 $^{-/-}$ mice exhibit basically the same phenotype as C3H/HeJ (27). TLR4 $^{-/-}$ and C3H/HeJ mice do not express TNF α , IL-6, and IL-12 in response to LPS, although these mice are capable of producing the cytokines in response to the TLR2 ligands.

Interestingly, TLRs can recognize not only membrane components of pathogens but also nucleic acids. TLR3 and TLR9 recognize dsRNA and unmethylated CpG DNA, respectively. It is known that CpG DNA from bacterial sources are largely unmethylated and elicit strong inflammatory responses that induce many cytokines, such as IL-12 and IL-10 (38). On the other hand, CpG DNA in the mammalian genomes are highly methylated, evading TLR recognition. TLR9 recognition of unmethylated CpG DNA was verified by the analysis of TLR9 $^{-/-}$ mice and "gain of function" experiments (6,23). While TLR9 $^{-/-}$ cells fail to induce cytokines in response to CpG (but not to LPS or PGN), transfection of TLR9 confers responsiveness to CpG. Analysis of TLR3 $^{-/-}$ mice (2) and a study with cells transfected with TLR3 (50) established that this receptor recognizes dsRNA. Illustrating specificity for dsRNA, neither DNA nor ssRNA is recognized by TLR3.

In the event of infection, multiple TLRs simultaneously engage in ligand recognition, potentially generating complex downstream pathways. How different TLRs affect each other's activity upon ligand engagement is not fully understood. Simplistically, a certain degree of synergy may be expected among different TLRs. Indeed TLR2 and TLR4 are shown to synergize when two respective ligands are added together (68). However, the mechanism by which TLRs can recognize diverse molecular structures is currently unknown.

REGULATION OF TLR EXPRESSION

Among the major cell types that express TLRs, granulocytes and macrophages are derived from a common myeloid progenitor. The two cell types develop through an intrinsic mechanism

Table 1. Mammalian TLRs

TLR	Ligand	Pathogen	Reference
TLR 1	Lipopeptides	Mycobacteria	74
TLR 2	Lipoproteins	Gram-positive Bacteria	73,84
	Zymosan Lipoarabino-mannan	Yeast Mycobacteria	
TLR3	Ds RNA	RNA and DNA Viruses	2
TLR4	LPS	Gram-negative Bacteria	27,61,63
TLR5	Flagellin	Bacteria	21
TLR6	Peptidoglycan Lipopeptides	Mycoplasma	59
TLR7	Imidazoquinolin		22
TLR8	Imidazoquinolin	?	29
TLR9	Unmethylated CpG DNA	Bacteria	6,23
TLR10	?	?	

involving the activity of specific transcription factors including IRF-8/ICSBP, PU.1, and C/EBP, although cytokines such as M-CSF and G-CSF influence the course of their development (75,76). In the adult bone marrow, the pro-inflammatory cytokine IFN γ influences myeloid development, favoring macrophage differentiation in a IRF-8/ICSBP-dependent manner (81). Dendritic cells are the most efficient cells that produce

cytokines in response to TLR signaling (5). Recent evidence indicates that IRF-8/ICSBP plays an important role in dendritic cell development (82).

At present, developmental regulation of the TLR genes (and genes for TLR signaling) is not well delineated, although it is likely to be an important part of the growth and differentiation of dendritic cells and macrophages. Nevertheless, in light of the observations that TLR-deficient mice are born normal and fertile in pathogen-free conditions, mammalian TLRs are apparently not critical for early development. TLR4 expression is shown to be regulated by IRF-8/ICSBP and PU.1 (64), both transcription factors critical for myeloid and dendritic cell development. TLR ligand binding is central to the maturation of dendritic cells. Upon ligand binding, immature dendritic cells express costimulatory molecules at higher levels and gain a higher antigen presentation capacity, which coincides with the induction of a series of cytokines. In humans, different dendritic cell subsets express TLRs at different levels and respond to different microbes. Accordingly, they produce cytokines at different levels (26,85). While “plasmacytoid” dendritic cells (pDCs) produce a large amount of IFN α/β , “monocyte” (or myeloid) dendritic cells (mDCs) predominantly manufacture IL-12 (13,30).

TLR expression by dendritic cells is transiently increased following ligand addition both in humans and mice (85). In monocytes and macrophages, LPS enhances the expression of TLR4 (55) and TLR9 (2). Thus, TLR expression is stimulated by their respective ligands in these cells. However, prolonged ligand engagement results in ligand-specific negative regulation of receptor expression, as reported for CpG and LPS (26,51). Thus, ligand binding may cause different modes of regulation in TLR expression, depending on the stage of cell maturation. TLR expression in macrophages is markedly enhanced by IFN γ , as documented for TLR4 and other TLRs (8,22). This up-regulation provides an explanation for the long-acknowledged “priming effect” of IFN γ on macrophages (43,72).

Figure 1. TLR signaling pathway. Ligand-bound TLRs recruit the adaptor MyD88 and transmit signals through the activation of a series of kinases. In addition to MyD88, TLR4 utilizes another adaptor, TIRAP. Both TIRAP and MyD88 pathways result in the activation of NF κ B. TLR4 also activates IRF-3 through a MyD88-independent mechanism.

TLR SIGNALING PATHWAYS

A rough outline of the TLR signaling pathway has been described in both mammals and *Drosophila*. It is clear that the backbone of the pathway is conserved throughout evolution (28,31,37,57). Fundamental to this process is the activation of NF κ B, the endpoint of TLR signaling (Figure 1). However, mammalian species clearly have added complexities at various steps of signaling (see below).

Upon ligand binding, TLR4 complexes with the cytoplasmic adaptor protein MyD88. Discovered as a gene expressed during myeloid cell differentiation (41), MyD88 has a TIR domain similar to that of TLRs in the C-terminal domain, but it does not have the external domain present in TLRs. The TLR-MyD88 complex recruits IRAK (90) and then TRAF6 (12). It is then complexed with the signalsome, a multi-protein complex that activates IKK (56) and p38 MAPK plus MEKK (57). This results in the activation of NF κ B (and presumably AP-1) (31,57).

The importance of MyD88 in TLR signaling was confirmed by the finding that MyD88 $^{-/-}$ mice fail to induce cytokines in response to LPS and CpG (34). MyD88 is required for signaling not only by TLRs but also by IL-1R and IL-18R, as

MyD88^{-/-} cells do not respond to IL-1 and IL-18 (1). When IKK is activated, it phosphorylates IκB, a cytoplasmic molecule that sequesters NFκB. This prompts IκB degradation, leading to the activation of NFκB and the transcription of numerous genes involved in inflammatory responses. It is notable that MyD88 is not indispensable for LPS-mediated signaling, as evidenced by the activation, albeit delayed, of NFκB and MAPK in MyD88^{-/-} mice (32,35).

TIRAP (also called Mal) is another adaptor recruited to TLR4 and that takes part in LPS signaling (17,25). TIRAP has a nonredundant function, as LPS induction of IFNβ is dependent on TIRAP, but not on MyD88 (79). TIRAP also activates PKR, a kinase with antiviral activities. Another adaptor protein (Tollip) that also interacts with TLRs was recently reported (93). Tollip appears to negatively affect TLR signaling, possibly by limiting inflammatory responses.

By screening LPS-inducible genes in MyD88^{-/-} macrophages, Kawai et al. (35) identified a MyD88-independent pathway downstream of TLR4. This pathway activates IRF-3, a latent cytoplasmic factor, which is phosphorylated and complexed with p300/CBP to stimulate IFNα/β genes (40,87,92). The mechanism by which TLR signaling activates IRF-3 is not completely understood. It has been reported that the DNA-dependent kinase contributes to the activation of IRF-3 (33), although other kinases may also play a role in the process.

CYTOKINE GENE EXPRESSION AFTER TLR SIGNALING

Following ligand-TLR binding, numerous genes are induced in both macrophages and dendritic cells, including many cytokines. Other genes involved in antimicrobial activities, including chemokines, proteolytic enzymes, and extracellular matrix, are also induced, along with genes important for antigen presentation, such as major histocompatibility complex class II and co-stimulatory molecules. This is well illustrated in a recent genome-wide microarray study (19) in which global gene expression patterns were investigated in a dendritic cell line following exposure to *E. coli*. Within 4–6 h, cytokines that regulate inflammatory responses (including IL-1α, IL-1RA, IL-2, IL-6, IL-10, IL-12p35, IL-12 p40, IL-15, and TNFα) were induced. Other cytokines that affect cell growth/survival, such as VEGF, M-CSF, and GM-CSF, were also induced. It is likely that most if not all of these cytokines are downstream targets of TLR signaling, although formal proof is lacking. Other ligands such as CpG are known to induce a large array of cytokines (38,91).

From these studies, it is clear that TLR signaling results in the expression of not only pro-inflammatory cytokines but also anti-inflammatory cytokines such as IL-10 and IL-6, suggesting that balancing pro- and anti-inflammatory responses is part of TLR signaling and that this activity begins at an early stage of TLR response. Another aspect of note is the induction of IL-12 and IL-10. These are cytokines that do not have antimicrobial activities by themselves. Their main activity is to regulate expression of effector cytokines, including IFNγ and IL-4, thereby coordinating the innate responses with subsequent adaptive immune responses (54,80). Thus, in mammals, the linking of the innate and adaptive immune responses is an integral part of TLR signaling. Al-

though the basic TLR system is conserved from insects to vertebrates (37), this aspect may well represent a point of divergence.

TYPE I IFNs (IFNα/β)

IFNα/β are a major mechanism of innate defense against viral infection, as these cytokines activate PKR, Mx, and RNaseL. IFNα/β are expressed through TLR signaling, involving TLR3, TLR9, and probably other TLRs. In humans, all 15 IFN genes are encoded in the IFN cluster in chromosome 9 (14). Although IFNβ can be expressed broadly in many types of cells, IFNα is produced predominantly by “leukocytes”. Recently, dendritic cells have been identified as a single major cell type that produces IFNα in humans (13,70). Plasmacytoid dendritic cells produce greater than 200–1000 times higher amounts of IFNα than other blood cells in response to viruses, dsRNA, and CpG DNA (30). Mouse pDCs also express high levels of IFNα (3,18).

IRF-3, activated by dsRNA and viruses, plays an essential role in the transcription of IFNα/β genes (40,67,87,92). IRF-3 is activated by LPS through TLR4 (35). Thus, TLR3 and TLR9 are anticipated to activate IRF-3. In line with the role for TLR3, expression of IFNα and IFNβ gene is diminished in TLR3^{-/-} macrophages stimulated by dsRNA (2). The dependence of IFNα/β on TLR signaling is also supported by the observation that imidazoquinolin (TLR7)-induction of IFNα requires MyD88 (22).

Among the many type I IFN genes, only IFNβ and IFNα4 are immediate-early genes that are directly activated by viruses (45,48). Other IFN genes are induced secondarily by IFNβ and IFNα4 in a STAT1-dependent manner (48). It is interesting that while the IFNβ promoter has a κB site (45), IFNα genes seem to lack a canonical κB element (44), suggesting that NFκB is dispensable for IFNα expression. IFNα genes contain a PRDI like element and “TG element”, both resembling the ISRE, a binding site for IRF proteins (9). Induction of IFNβ genes requires assembly of many factors, including NFκB and IRFs, as well as histone acetylases, and is associated with alterations of local chromatin structure (49,53).

IL-1 AND TNFα, TYPICAL PRO-INFLAMMATORY CYTOKINES

IL-1 elicits inflammatory responses upon infection and injuries (15) by signaling through the TLR-MyD88 pathway (Figure 1) (34). Alternatively, IL-1β gene expression itself is under control of TLR signaling. The IL-1β promoter has a κB site that is important for LPS induction of the gene (24). However, this site alone is not sufficient for the full activation of promoter activity. It requires an additional site, the IRF/PU.1 composite element on which IRF-8/ICSBP, IRF-4, and PU.1 assemble, leading to cooperative activation of IL-1β transcription (47). The mechanism by which TLR signaling triggers the activity of the composite element is not fully understood. Interestingly, similar elements assembling IRF-8/ICSBP and PU.1 are present in other genes activated by TLR signaling (see below).

TNFα is another potent pro-inflammatory cytokine induced by the TLR signaling. TNFα's powerful biological effects are at-

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tributed to its ability to activate NF κ B. LPS, CpG, and imidazoquinolin activate TNF α in a TLR- and MyD88-dependent manner (22,23,34). The TNF α promoter has three NF κ B binding sites, which function in a sequence-context and cell type-dependent manner. Unlike other cytokine promoters that require additional elements and other transcription factors, TNF α may require only NF κ B for activation. Recent studies suggest that while the p65-p50 κ B heterodimer activates transcription, the p50 homodimer inhibits TNF α transcription (4). Interestingly, a naturally occurring variant of TNF α promoter has a κ B site that preferentially binds to the p50 homodimer, attenuating TNF α expression (83).

IL-10, A Th2 CYTOKINE

IL-10 is an immunosuppressive cytokine produced in many cell types in response to LPS, mycobacteria, viruses, and TNF α (54). IL-10 down-regulates expression of IL-12 and IFN γ , there-

by inhibiting Th1 responses. The IL-10 promoter binds to Sp1 and Sp3, both of which regulate constitutive transcription (78). Sp1 is also shown to be involved in LPS activation of IL-10 promoter (11). In macrophages, LPS induction of IL-10 transcription requires new protein synthesis, indicating that induction depends on some other cofactors or modification of Sp1. IL-10 transcription may also be regulated at the level of mRNA stability (62). The IL-10 promoter is unusual in that it does not have a canonical κ B site. Details of the pathways by which TLR signals activate IL-10 gene expression await further investigation.

IL-12, A Th1 CYTOKINE

This cytokine is a key mediator of Th1 responses, as it induces expression of IFN γ in NK and T cells (80). IL-12 is composed of a heterodimer of p40 and p35. p40 is a highly inducible subunit and regulates the overall production of biologically active IL-12p70. Although previously thought to be constitutively expressed, the p35 subunit is also regulated by external stimuli (71). IL-12 is induced by bacteria, viruses, parasites in macrophages, and dendritic cells. Induction by CpG, dsRNA, and imidazoquinolin is dependent on TLR3, TLR4, TLR7, and TLR9, respectively, all of which require MyD88 (2,22,23,32).

Because of its central role in bridging innate and adaptive immune responses, IL-12 transcription has been extensively studied. IL-12 p40 is synergistically induced in macrophages by LPS and IFN γ , which requires new protein synthesis (43). IFN γ also synergizes with CpG and imidazoquinolin to induce IL-12 p40 expression (22). We found that the mode of IL-12 induction in dendritic cells differs from that in macrophages, in that IL-12 p40 induction in dendritic cells does not require a new protein synthesis and does not depend on IFN γ for full expression (Tsumimura et al., unpublished observations).

Contrary to IFN γ 's stimulatory activity, relatively high doses of IFN α/β are shown to inhibit IL-12 induction in a variety of systems in both mouse and human (7). For example, IL-12 production in vivo is markedly reduced following infection with lymphocytic choriomeningitis virus. In addition, pretreatment of monocytes with IFN α or IFN β also reduces IL-12 p40 expression in vitro. However, the mechanism by which IFN α/β inhibit IL-12 expression is not well understood. Interestingly, LPS induction of IL-12 p40 in macrophages is dependent on p38 MAPK, as evidenced by the absence of IL-12 in Mkk3 $^{-/-}$ mice, although induction of IL-1, TNF α , and IL-6 is normal in these mice (42).

Activity of the IL-12 p40 promoter has been studied in mouse (60,89) as well as human (20,43,86) and involves multiple regulatory elements, including the κ B, C/EBP, and Ets sites. Consistent with the importance of the κ B site, disruption of c-Rel, a member of the NF κ B family, selectively diminishes IL-12 p40 expression in macrophages, while p65 $^{-/-}$ and p50 $^{-/-}$ mice express the gene (66). However, the lack of c-Rel alone did not show a defect in expressing IL-12 p40 in dendritic cells (58). Thus, NF κ B may play a differential role in macrophages and dendritic cells.

Although current information suggests that NF(B plays a critical role, there are other factors that regulate IL-12 p40 expression. We have obtained evidence that IRF-8/ICSBP is essential for the expression of IL-12 p40 (69,75,82,86). IRF-8/ICSBP is synergistically induced by LPS and IFN γ in

Figure 2. Gene activation after TLR signaling. (A) Complexity of TLR signaling in vertebrates. TLR signaling prompts chromatin remodeling and activation of multiple transcription factors (in addition to NF κ B), initiating a mechanism to link innate and adaptive immunity. (B) TLR signaling stimulates IRF-8/ICSBP and PU.1 that cooperatively activate downstream genes with specific binding motifs.

macrophages, but its expression is constitutive in dendritic cells (76,82). IRF-8/ICSBP^{-/-} macrophages and dendritic cells fail to express IL-12 p40 in response to IFN γ + LPS and all other stimuli tested, although they express TLR4, TLR9, and MyD88 at normal levels (82). We showed that transfection of IRF-8/ICSBP into ^{-/-} cells restores expression of endogenous IL-12 p40 mRNA along with co-transfected mouse and human IL-12 p40 promoters (86). In addition, myeloid progenitor cells derived from IRF-8/ICSBP^{-/-} cells, when IRF-8/ICSBP was reintroduced, acquired the ability to induce IL-12 p40 in response to LPS and IFN γ , along with the ability to differentiate into macrophages (75). Further analysis indicated that IRF-8/ICSBP is assembled into a multi-protein complex containing IRF-1, which binds to the IL-12 p40 promoter (86). A similar complex that contains PU.1, IRF-8, and c-Rel is shown to assemble on the human IL-12 promoter (20).

In vivo footprinting analysis of the mouse IL-12 p40 promoter by Weinmann et al. (89) demonstrated that upon LPS and IFN γ stimulation the promoter undergoes a dramatic chromatin remodeling, most prominently in nucleosome 1 (adjacent to the transcription start site). Concomitant with the remodeling was a marked increase in restriction site accessibility in and around the nucleosome. This remodeling event was dependent on de novo protein synthesis. A more recent study (88) shows that the increased restriction site accessibility depends on TLR4, but not on NF κ B, as c-Rel^{-/-} macrophages undergo nucleosomal remodeling. These studies indicate that TLR-MyD88 signaling activates NF κ B as well as another protein/pathway(s) important for IL-12 p40 transcription.

TRANSCRIPTIONAL MECHANISMS BY WHICH TLRs ACTIVATE GENE EXPRESSION

A major downstream target of TLR signaling is NF κ B, a feature conserved throughout evolution. However, in mammalian species, transcription factors other than NF κ B are activated by TLR signaling and probably play a major role in gene expression. These transcription factors may be particularly indispensable for regulating genes that coordinate innate and adaptive immune responses. In this context, it is interesting that expression of major histocompatibility complex class II and co-stimulatory molecules, as well as elicitation of allogeneic mixed lymphocyte reaction, is not impaired in cells lacking both NF κ B p50 and c-Rel (58). This supports the hypothesis that NF κ B is not required for certain TLR pathways, such as those linking innate and adaptive immune responses.

Other transcription factors that are expected to take part in TLR signaling in mammalian cells are proteins of the IRF family (77). Besides IRF-3, IRF-8/ICSBP is likely to have a significant role in TLR signaling. IRF-8/ICSBP is a key factor for induction of IL-12 p40, IL-1 β , gp91^{phox}, and IL-18R (16,36,46, 47,64,86). In each of these promoters, IRF-8 acts by interacting with PU.1, a member of the Ets family whose expression pattern parallels that of IRF-8/ICSBP. The two factors bind to the Ets-IRF composite element (EICE) to stimulate transcription (Figure 2). The canonical EICE contains the Est consequence site (A)GGAA and a half IRF site GAAA (10). Although a canonical EICE is not present, the IL-12 p40 promoter is shown to assem-

ble a complex containing PU.1 and IRF-8/ICSBP (20). Moreover, EICE-like elements (designated EIRE) are found to be present in some IFN-inducible genes (Figure 2). IRF-8/ICSBP and PU.1 are reported to stimulate transcription through this element (52). We have shown that IRF-8/ICSBP interacts with TEL, another Ets family protein, representing an additional possible mechanism of regulation (39).

Finally, it is likely that TLR signaling not only activates individual transcription factors but also mediates a large-scale alteration in chromatin structure, affecting global gene expression patterns. Indicative of a chromatin change, Saccani et al. (65) reported that many TLR ligands activate p38 MAP in human primary dendritic cells, which results in promoter-specific phosphorylation and acetylation of histone H3. This change is seen within 10 min after stimulation and probably involves recruitment of histone acetylases and other chromatin modifiers, leading to increased transcriptional activation of a series of genes. However, the specific nature of chromatin changes and mechanisms causing these changes are still largely unknown.

In conclusion, TLR signaling governs the expression of many cytokine genes in macrophages and dendritic cells, establishing innate immunity and opening a road to adaptive immune responses. Although NF κ B is a factor critical for the conserved function of TLR signaling, additional transcription factors are expected to participate in the TLR signaling in vertebrates to link innate and adaptive immunity.

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