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Toll-like receptor signal transduction

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Abbreviations: CpG-ODN, synthetic oligodeoxyribonuclotides containing CpG motifs; CRP, C reactive protein; DC, dendritic cells; DOK1, downstream of kinase 1; GARG 16, glucocorticoid- attenuated response gene 16 product; IAQ, imidazoquinolines; IKK, IκB kinase; IKKε, IκB kinase epsilon; IP-10, interferon activated gene 10; IRAK4, interleukin 1 receptor-associated kinase 4; IRF, interferon regulatory factor; LBP, lipopolysacchride binding protein; LRR, leucine rich repeat; LTA, lipoteichoic acid; MAL, MyD88 adapter like; MALP2, macrophage-activating lipopeptide 2; MBL, mannose binding lectin; MDA5, melanoma differentiation-associated gene 5; MMTV, mouse mammary tumor virus; MyD88, myeloid differentiation primary response gene 88; NALP, NACHT, leucine rich repeat and PYD containing; NOD1, nucleotide-binding oligomerization domain protein 1; OAS, 2,5-oligoadenylate synthetase; PAM2CSK4, synthetic diacylated lipopeptide Pam_2Cys-SKKKK × 3 TFA; PAM3CSK4, synthetic triacylated lipopeptide Pam_3Cys-SKKKK × 3 HCl; PAMP, pathogen associated molecular pattern; pDC, plasmacytoid dendritic cells; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PMN, polymorphonuclear phagocytes; Poly-IC, polyinosinic-polycytidylic acid; PRR, pattern-recognition receptor; PTX3, pentraxin 3; RANTES, regulated upon activation, normal T-cell expressed, and secreted; RIG1, retinoic acid inducible gene 1; SAP, serum amyloid protein; sE-selectin, soluble form of E-selectin; SHIP, SH2-containing inositol phosphatase; sICAM, soluble intercellular adhesion molecule; SOCS1, suppressor of cytokine signaling 1; SR, scavenger receptor; ssRNA, single stranded RNA; ST2, suppression of tumorigenicity 2; TBK1, TANK-binding kinase 1; TIR, Toll/interleukin-1 receptor; TLRs, Toll-like receptors; TRAF6, tumor necrosis factor receptor-associated factor 6; TRAM, TRIF-related adapter molecule; TRIF, TIR domain-containing adapter protein inducing IFN-β; VSV-G, vesicular somatitis virus G protein; ZYM, zymosan

Abstract

Toll-like receptors (TLRs) are the archetypal pattern recognition receptors in sensing exogenous pathogens. Activation of TLRs is a first line of defense of the immune system, leading to the activation and recruitment of neutrophils and macrophages to sites of infection and enhances antimicrobial activity. The TLR signaling through different intracellular molecules, such as MAP kinases and IκB kinases which are conserved signaling elements for many receptors, leads to a distinct set of proinflammatory gene expressions. However, how these pathways differentially and precisely control the transcription of identical genes remains largely unknown. Our review focuses on the details of up-to-date signaling molecules including negative regulators and their role in controlling innate immune response. We also stress the importance of developing systemic approaches for the global understanding of TLR signaling so that appropriate drug therapeutic targets can be identified for regulating inflammatory diseases.

Keywords: immunity, natural; ligands; signal transduction; systems biology; Toll-like receptors

Introduction

Toll-like receptors (TLRs) are closely related type I transmembrane proteins, which form the major pattern-recognition receptors (PRRs) to transduce the signals in response to microbial intruders such as protozoa, bacteria, fungi and viruses (Takeda and Akira, 2005). The signal transduction pathways that are activated downstream of TLRs have been the subject of intensive analysis by numerous laboratories (Fitzgerald *et al.,* 2003; Kawai *et al.,* 2004; McDonald *et al.,* 2006). The continued focus of research on TLR signaling has provided a lot more information on pathways activated by TLRs, and new layers of complexity and regulation have been revealed. Stimulation of TLRs underpins the activation of several aspects of innate and adaptive immune responses *via* cytokines, type I interferons, chemokines, cell surface molecules and chemokine receptors, increases effector functions such as phagocytosis, and enhances capacity to present antigen to T cells (Sioud, 2005). Although TLRs are crucial for innate immunity, they are also prerequisite for the induction of adaptive immune responses in combating many infections. The pur-

pose of this review is to discuss TLR signal transduction, including adapter molecules, negative regulators, and transcription factors.

TLR structure, location and ligands

TLRs are *bona fide* 'pattern recognition' receptors

The conserved patterns unique to microbial surface are called pathogen associated molecular patterns (PAMPs), that allow the cell to recognize dangerous non-self molecules from self-molecules. Cells contain unique PRRs like TLRs to fight against the foreign intruders. PRRs handle the microbial infection and they are broadly distributed in various cells of the immune system, which include polymorphonuclear phagocytes (PMN), monocytes/macropahges, dendritic cells, natural killer cells as well as mucosal epithelial and endothelial cells (Becker *et al*., 2000; Cario *et al*., 2000; Muzio *et al*., 2000; Faure *et al*., 2001; Visintin *et al.,* 2001). Recognition of PAMPs results in activation of macrophages, leading to a plethora of biological responses required for eliciting both the innate and adaptive arms of the immune response, and they are distributed all over the body (Table 1). TLRs are highly conserved through evolution and its presence is reported as low as the phylum from Porifera which includes sponges (Wiens *et al*., 2005). Toll was first discovered in *Drosophila*. Researches working in the mechanisms mediating embryonic development in *Drosophila* found a remarkable sequence similarity between Toll, a transmembrane protein involved in embryogenesis, and the human interleukin-1 receptor (IL-1R) (Hashimoto *et al*., 1988; Gay and Keith, 1991) which is discussed in the following paragraph.

Structural similarity with interleukin-1 receptor (IL-1R)

TLRs belong to a superfamily called the Toll/IL-1 receptor (TIR) family and all members of which contain cytoplasmic TIR domains. The endodomain of all TLRs differs from IL-1R ectodomain in which TLR has leucine rich repeats (LRRs) whereas IL-1R posses Ig-like domains. The cytoplasmic region of TLRs shares a stretch of TIR domain, which mediates homo- and heterophilic interactions between TLRs and TIR-containing adapters (Akira *et al*., 2001). The TIR domain is approximately 160 amino acids long and is essential for cellular signaling, and contains three regions of particular importance, termed boxes 1, 2 and 3, although regions outside these boxes also show conservation. Box 1 is the signature sequence of the TIR domain. Box 2 forms an important loop in the structure, which probably engages with downstream elements (or with

Table 1. Representation of pattern recognition receptors (PRRs) in body fluids, cell membrane and cytoplasm.

Body fluids	Cell membrane	Cytoplasm	Endogenous
LBP	Plasma membrane	Antibacterial	SR
Pentraxin family members	TLR1	NOD-like receptor	CD36
SAP, CRP, PTX3	TLR2	Antiviral	NALP3
MBL, C1q, C3	TLR4	RIG1	
	TLR5	MDA5	
	TLR6	PKR	
	Endosomes	OAS	
	TLR3		
	TLR7		
	TLR8		
	TLR9		

PAMPS are opsonized by the PRRs present in the body fluids. LPS is captured by LPS binding protein (LBP) in blood (Ulevitch and Tobias, 1995). Complement pathways are activated by mannose binding lectin (MBL), pentraxin family members serum amyloid protein (SAP), C reactive protein (CRP) and pentraxin 3 (PTX3) (Gasque, 2004, Garlanda *et al*., 2005) and also by complement components C3 and C1q (Gasque, 2004). TLR1, 2, 4, 5, and 6 are in plasma membrane and TLR3, 7, 8, and 9 are in endosomes, respectively. The cytoplasmic PRRs can be classified into antibacterial, antiviral and endogenous ligand recognizers. NOD1 (Nucleotide-binding oligomerization domain protein 1) and NOD2 recognize peptidoglycan derived peptides, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Chamaillard *et al*., 2003) and muramyl dipeptide (MDP) (Girardin *et al*., 2003; Inohara *et al*., 2003), respectively. Antiviral detectors include retinoic acid inducible gene 1 (RIG1), melanoma differentiation-associated gene 5 (MDA5, also called as Helicard) and 2-5-oligoadenylate synthetase 1A (OAS) (Stark *et al*., 1998). Scavenger receptor (SR) and CD36 can mediate phagocytosis of apoptotic cells, whereas NALP3 (NACHT, leucine-rich repeat and PYD containing 3) can recognize endogenous danger signals such as extracellular ATP (Taylor *et al*., 2005; Mariathasan *et al*., 2006).

another TLR because TLRs appear to dimerize during signaling). Most TIR Box 2 sequences have a conserved proline, which when mutated to histidine renders the protein unable to signal. The function of Box 3 is not fully understood, although it contains residues important in signaling (based on mutational analysis of the type I IL-1 receptor) (Slack *et al*., 2000) (Figure 1).

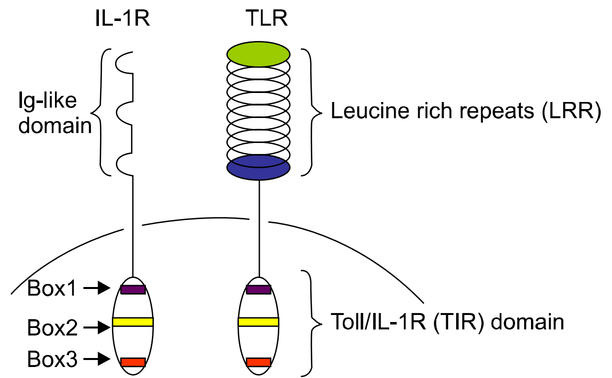

Figure 1. High level structural similarity between IL-1R and TLR. Both receptors differ in their extracellular domain, in which IL-1R possesses Ig-like domain, whereas TLR has leucine rich repeats (LRR). The intracellular region is the same, which contains Toll/IL-1R (TIR) domain indicating the high level signaling similarity between the receptors.

Types of TLRs and their location in the cell

In mammals, to date, 13 TLRs (Roach *et al*., 2005) with distinct specificities to recognize highly conserved structural motifs of microbial pathogens as well as several host-derived molecules (Akira and Takeda, 2004) have been identified. The specific locations of TLRs remain unclear. For example, the first group (TLRs 1, 2, 4, 5 and 6) are found at the plasma membrane. The second group (TLRs 3, 7, 8 and 9) are intracellular and likely signal from acidic endosomes (Figure 2). According to Barton

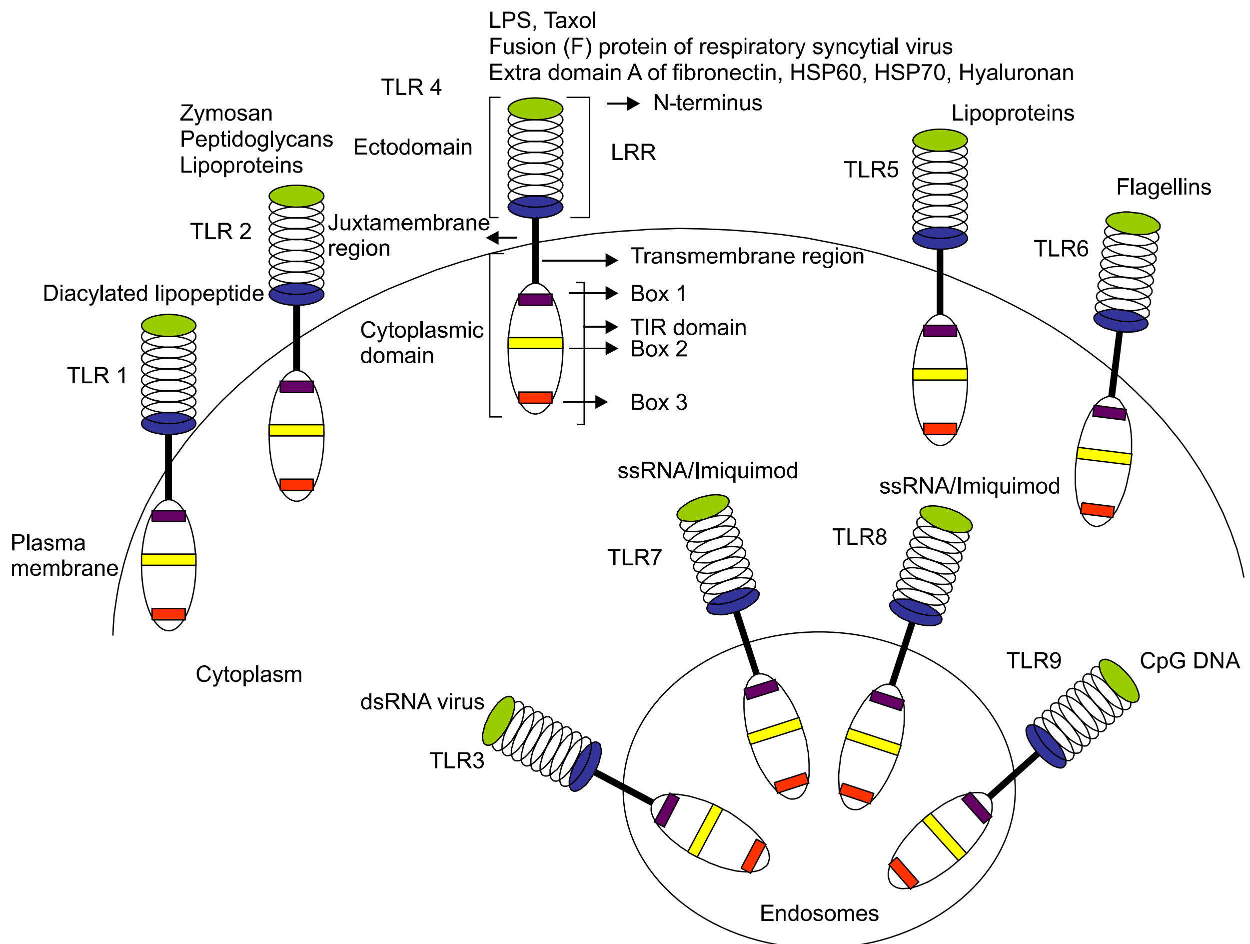

Figure 2. The localization of TLRs in the cell. TLRs on the plasma membrane include TLR 1, 2, 4, 5 and 6. TLRs which are present on the endosomal membrane include TLR 3, 7, 8 and 9.

et al. (2006), the subcellular localization is of great importance for the discrimination of viral nucleic acids from self nucleic acids. In a chimeric experiment of TLR9 ectodomain and TLR4 endo- and transmembrane domain they showed that these hybrids responded to self nucleic acids when present at plasma membrane but TLR9 in the endosomes responded only to foreign nucleic acids. They concluded that the localization of the nucleic acid-sensing TLRs is critical in discriminating between self- and nonself nucleic acid.

Ligands of TLRs

We classified the known mammalian Toll-like receptors with the adapters recruited, location, species and the ligands which stimulate TLRs (Table 2). TLR4 has been genetically identified as a signaling molecule essential for the recognition of LPS, a component of Gram-negative bacteria (Poltorek *et al*., 1998; Hoshino *et al*., 1999). Individual TLRs can recognize several structurally unrelated ligands. For example, TLR4 recognizes not only LPS but also taxol (Kawasaki *et al*., 2000), fusion (F) protein of respiratory syncytial virus (Kurt-Jones *et al*., 2000), extra domain A of fibronectin (Okamura *et al*., 2001), heat shock protein (HSP) 60 (Ohashi *et al*., 2000), HSP 70 (Habich *et al*., 2002), and hyaluronan (Taylor *et al.,* 2004, 2007). It is important to understand how TLR4 recognizes these structurally unrelated ligands. TLR2 responds to mycobacteria, yeast cell wall component zymosan, and Gram-positive bacteria (Takuechi *et al*., 1999; Underhill *et al*., 1999a, b; Takuechi *et al*., 2000,). TLR6 associates with TLR2 and recognizes lipoproteins from mycoplasma (Ozinsky *et al*., 2000). TLR5 mediates the induction of the immune response by bacterial flagellins (Hayashi *et al*., 2001).

Recent studies showed that ssRNA is the natural ligand for TLR7/8 (Hemmi *et al*., 2000; Diebold *et al*., 2004; Heil *et al*., 2004). Activation of innate immunity by siRNAs is sequence dependent and occurs mainly in immune cells that express functional TLR7/8 (Hornung *et al*., 2005; Judge *et al*., 2005; Sioud, 2005, 2006). Consistent with the role of endosomal TLRs in siRNA sensing, TLR7 knockout mice did not mount immune activation in response to siRNAs (Hornung *et al*., 2005). A synthetic compound (imidazoquinoline compound R848) with antiviral activity has been described as a ligand for TLR7 and TLR8 (Thomassen *et al.,* 1999; Hemmi *et al*., 2002; Jurk *et al*., 2002). TLR9 has been shown to recognize unmethylated bacterial CpG DNA (Hemmi *et al*., 2000) and TLR3 recognizes double stranded RNA (dsRNA) (Alexopoulou *et al*., 2001). TLR11 is involved in the recognition of uropathogenic bacteria (Zhang *et al*., 2004). The natural ligands for TLRs 10, 12 and 13 are still not known.

Immunostimulatory siRNA can also induce the maturation and differentiation of monocytes into

Table 2. TLRs and their ligands.

TLRs	Adapters	Location	Species	Ligands
TLR1	MyD88/Mal	Cell surface	Human/Mouse	PAM3CSK4
TLR2	MyD88/Mal	Cell surface	Human/Mouse	PAM2CSK4, MALP2, LTA, ZYM
TLR3	TRIF	Cell compartment	Human/Mouse	dsRNA, Poly-IC, viral RNA, siRNA, endogenous mRNA
TLR4	MyD88/Mal/TRIP/TRAM	Cell surface	Human/Mouse	LPS, MMTV, VSV-G, Taxol, F protein, Fibronectin, HSP60, HSP70, Hyaluronan
TLR5	MyD88	Cell surface	Human/Mouse	Flagellins
TLR6	MyD88/Mal	Cell surface	Human/Mouse	MALP2, LTA, Zym
TLR7	MyD88	Cell compartment	Human/Mouse	ssRNA, IAQ (R848)
TLR8	MyD88	Cell compartment	Human/Mouse	ssRNA, IAQ (R848)
TLR9	MyD88	Cell compartment	Human/Mouse	CpG-ODN
TLR10	Unknown	Cell surface	Human	Unknown
TLR11	MyD88	Cell surface	Mouse	Profilin
TLR12	Unknown	Unknown	Mouse	Unknown
TLR13	Unknown	Unknown	Mouse	Unknown

CpG-ODN, synthetic oligodeoxyribonuclotides containing CpG motifs; IAQ, imidazoquinolines, including resiquimod and imiquimod; LTA, lipoteichoic acid; MAL, MyD88 adapter-like; MALP2, macrophage-activating lipopeptide 2; MMTV, mouse mammary tumor virus; PAM3CSK4, synthetic triacylated lipopeptide Pam_3Cys-SKKKK $\times$ 3 HCl; PAM2CSK4, synthetic diacylated lipopeptide Pam_2Cys-SKKKK $\times$ 3 TFA; Poly-IC, polyinosinic-polycytidylic acid; ssRNA, single stranded RNA; VSV-G, vesicular somatitis virus G protein; ZYM, zymosan.

macrophages, dendritic cells, and human bone marrow CD34$^+$ progenitor cells, in addition to the induction of cytokines and interferons (Sioud *et al.,* 2006). Thus, signaling *via* TLRs not only induces cytokine production against the invader but it can also induce immune cell maturation for effective immune responses. TLRs contribute to both the war against the invader as well as the maturation of important immune cells. The identification of endogenous compounds like HSPs and fibronectin have also been recognized by TLRs, leading to the suggestion that TLRs might also act as danger sensing receptors rather than just being involved in microbial recognition as the aforementioned proteins can also be autoantigens. Many types of cells also have TLR-independent, intracellular detection systems to sense viral invasion and initiate innate immune responses.

Adapter proteins recruited by TLRs

TLR mediated MyD88-dependent and independent cellular signaling

For the last one decade, how PRRs convert the message gleaned from recognition of a pathogen into an appropriate cellular response has been the subject of intensive investigation. The signaling pathways activated by TLRs are broadly classified into MyD88-dependent and independent pathways (Takeda and Akira, 2005) as MyD88 is the universal adapter protein recruited by all TLRs except TLR3. The major pathways activated by TLR engagement are passed through IκB kinase (IKK), MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. These pathways regulate the balance between cell viability and inflammation.

The signaling pathways activated by a specific TLR are largely dictated by the adapter proteins

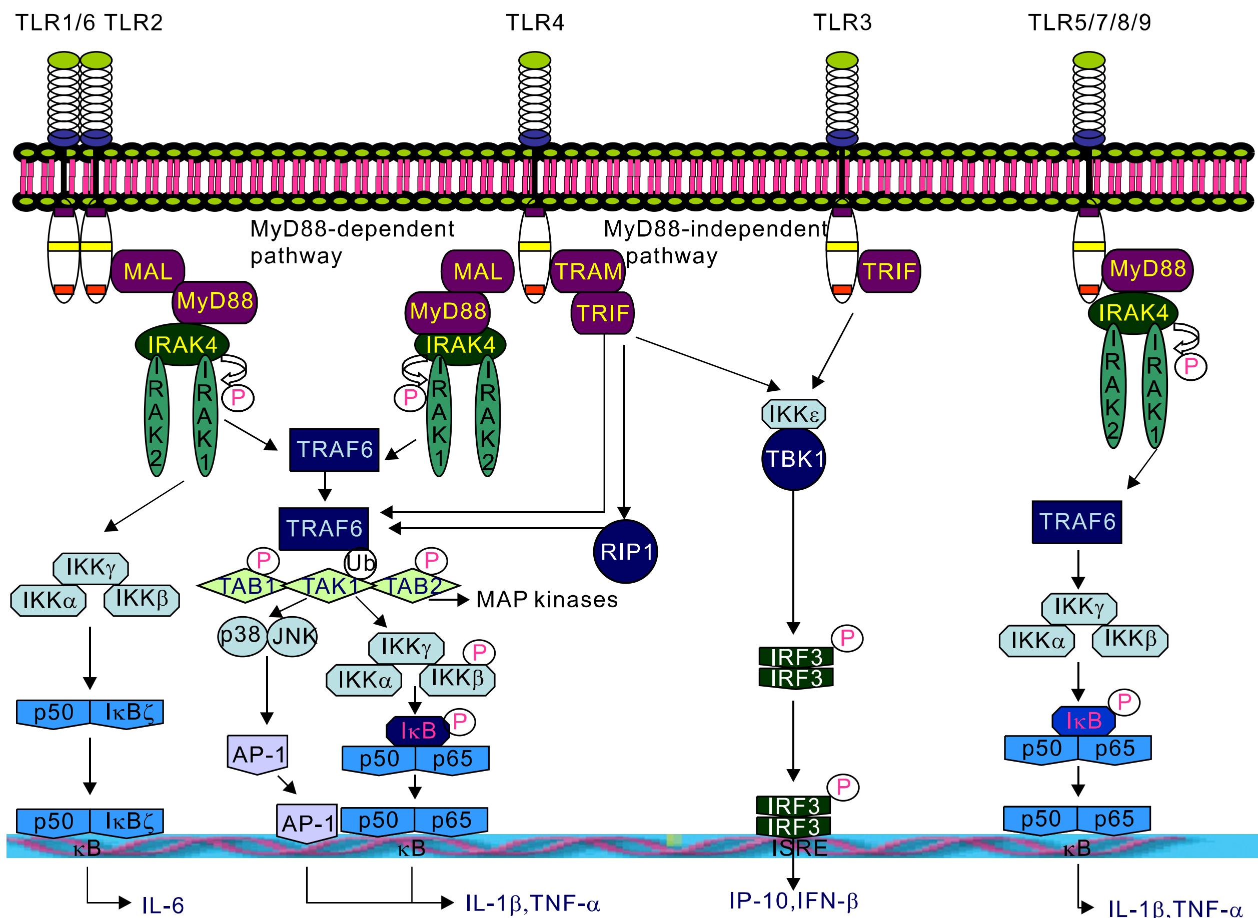

Figure 3. Signaling mediated by TLR is broadly classified as MyD88-dependent and independent pathways. All TLRs utilize MyD88 with the exception of TLR 3. TLR 7, 8 and 9 pathways are predominant in pDCs. MyD88 binds with the TIR domain of the receptor and phosphorylates IRAK4 which in turn phosphorylates IRAK1. IRAK1 phosphorylates TRAF6 leading to the ubiquitination of TAK complex. Activation of IKK, JNK and p38 pathways leads inflammatory and antiviral responses. ISRE, interferon stimulatory response element; κB, κB site; p, phosphorylation; ub, ubiquitination.

recruited to the intracellular domain of the TLR upon ligand binding (Akira and Takeda, 2004). There are currently four cytosolic adaptor proteins that are thought to play a crucial role in specificity of individual TLR-mediated signaling pathways. Amongst them, TLR4 signaling involves all four adapter proteins, MyD88 (myeloid differentiation primary response gene 88), MyD88 adapter like [MAL; also known as TIRAP (TIR domain-containing adapter protein)], TIR domain-containing adapter protein inducing IFN-β [TRIF; also known as TICAM1 (TIR domain-containing adapter molecule 1)], and TRIF-related adapter molecule [TRAM; also known as TICAM2 (TIR domain-containing adapter molecule 2)] (McGettrick and O'Neill, 2004). The differential recruitment of these adapter proteins by different TLRs form the basis for the specificity in the signaling process activated by them. Our current understanding about TLR signaling is illustrated in Figure 3.

MyD88 is the primary adapter for microbial signaling

Every TLR member differentially utilizes adapters, but MyD88 (296 amino acid protein) seems to be the widely used adapter molecule. MyD88 harbors a TIR domain as well as a death domain. The carboxy terminal of TIR domain interacts with the cognate domains in the cytoplasmic tails of the TLRs, and the amino terminal death domain mediates the interaction with the corresponding domain of interleukin 1 receptor-associated kinase 4 (IRAK4) (Wesche *et al.*, 1997; Li *et al.*, 2002). MyD88 was originally isolated as a myeloid differentiation primary response gene that is rapidly induced upon IL-6 stimulated differentiation of M1 myloleukemic cells into macrophages (Lord *et al.*, 1990). MyD88$^{-/-}$ mice were resistant to LPS-induced shock compared to that of wild type (Kawai *et al.*, 1999) that provided an invaluable tool for analyzing the critical role of MyD88 in TLR signaling. However, LPS is still capable of activating NF-κB and MAPK in MyD88-deficient mice, and this activation in MyD88-deficient cells is delayed in reaching a peak in comparison with wild-type cells (Kawai *et al.*, 1999). Importantly, LPS can induce IFN-β production by macrophages and DCs (dendritic cells) in a manner independent of MyD88 (Kawai *et al.*, 2001). Further studies pointed out that MyD88$^{-/-}$ mice still can produce type I interferon inducible gene products such as IP-10 and GARG 16 (Kawai *et al.*, 2001). Together, these observations suggested other adapters mediate late activation of NF-κB and MAPK which is associated with type I IFN induction in TLR4 signaling.

MAL specificity in TLR signaling

MAL has been identified as an adapter molecule that mediates responses to TLR2 and TLR4 ligands (Equils *et al.*, 2004). Although MAL has similarities to MyD88, it differs in N-terminal portion in which MAL is 75 amino acids shorter and lacks a death domain (Fitzgerald *et al.*, 2001). MAL is utilized by a subset of TLRs that signal from the plasma membrane but not by TLRs that signal from endosomes. Recently, Kagan and Medzhitov (2006) found that MAL resides on membranes that shuttle between the plasma membrane and endosomes by an ADP ribosylation factor 6 (ARF6) dependent processes. They further demonstrated that MAL contains a phosphatidylinositol 4, 5-bisphosphate (PIP2) binding domain that mediates MAL recruitment to membranes and is required for TLR4 signaling. Therefore, the primary function of MAL in TLR signaling seems to control the recruitment of MyD88 to TLR4. They proposed that the unique phosphoinositide composition of cellular membranes dictates differential adapter recruitment to TLRs residing in different compartments. MAL and MyD88 together may be required for a rapid and optimal response. Surprisingly, MAL$^{-/-}$ mice also displayed impairment in TLR2-mediated responses, suggesting MAL as an important component of TLR2-mediated innate host defense (Horng *et al.*, 2002). Studies with MAL gene targeted mice revealed that MAL functions in the MyD88-dependent NF-κB activation pathway shared by TLR2 and TLR4 (Horng *et al.*, 2002; Yamamoto *et al.*, 2002).

Adapters mediating MyD88-independent signaling

Most of the TLRs seem to be absolutely dependent on the expression of MyD88 for all of their functions whereas TLR3 and TLR4 are unique in their ability to activate MyD88-independent responses. TLR3 and TLR4 appear to have evolutionarily diverged from other TLRs to activate gene expression programs and trigger antiviral responses by a mechanism involving the activation of IRF3. MyD88-independent signaling events are controlled by TRIF (for TLR3) or TRIF/TRAM (for TLR4) and induce IRF3-dependent type I interferon production (Fitzgerald *et al.*, 2003; Hoebe *et al.*, 2003; Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2003a, b) (Figure 3).

TIR domain containing adapter inducing interferon-β (TRIF) and TRIF related adapter molecule (TRAM)

TRIF is an adapter for TLR3 and TLR4, and is associated with the MyD88-independent cascade.

TRIF was identified through database searches and has 712 amino acids (Yamamoto *et al.,* 2002, 2003a). TRIF was found to be primarily associated with virus infections owing to the induction of IFN-α and IFN-β (Yamamoto *et al.,* 2002). However, there are studies which show the role of TRIF in bacterial infection. $TRIF^{-/-}$ mice infected with *E. coli* display attenuated neutrophil migration and attenuated production of NF-κB, TNF-α and IL-6, and LPS induced C-X-C chemokines in the lungs (Jeyaseelan *et al.,* 2007). Another *in vitro* study using TLR3 ligands suggests that TRIF regulates three distinct signaling pathways leading to IRF3 activation, NF-κB activation, and apoptosis *via* a RIP/FADD/caspase 8-dependent pathway (Han *et al*., 2004). Two noncanonical IκB kinases (IKKs), TBK1 (TANK-binding kinase 1) and IKKε (IκB kinase ε), interact with TRIF, activate IRF3/IRF7 and finally lead to IFN-β production (Fitzgerald *et al*., 2003).

TRAM (253 amino acids) was identified as a small TIR domain containing protein (Bin *et al.,* 2003). TRAM is placed upstream of TRIF in the induction of IFN-β through MyD88-independent pathway. The role of TRAM in LPS signaling appears to act as a bridging adapter connecting TLR4 and TRIF. In TRAM deficient mice, normal inflammatory cytokine production induced by TLR2, TLR7 and TLR9 ligands was observed (Yamamoto *et al.,* 2003b). TLR4 mediated phosphorylation of IRAK was induced normally, indicating that activation of the MyD88-dependent pathway was unaffected.

Kinases involved in signaling from adapters to transcription factors

Downstream of TLR signaling by adapters are mediated by IRAK family

The next component of downstream TLR signaling is the IRAK family members. IRAKs are important mediators in the signal transduction of the TLR family as they may act to potentiate the downstream signaling. So far, four IRAKs have been identified, such as IRAK1, IRAK2, IRAK4 and IRAKM. IRAK1 and IRAK4 possess intrinsic serine/threonine protein kinase activities, whereas IRAK2 and IRAKM lack this activity, that may negatively regulate TLR mediated signaling. IRAKM deficient cells show hyperproduction of inflammatory cytokines in response to various TLR ligands (Suzuki *et al*., 2002). IRAK4 deficient mice also show no response to a variety of bacterial components suggesting the critical involvement of IRAK4 in TLR signaling (Suzuki *et al*., 2002). IRAK1 has three TRAF6 (tumor necrosis factor receptor-associated factor 6) binding motifs to mediate the interaction with TRAF6 (Ye *et al*., 2002) and undergoes autophosphorylation. LPS responses were not diminished in IRAK2 deficient mice, indicating the kinase activity of IRAK2 is not required for downstream signaling events (Li *et al*., 2002). Upon stimulation, IRAK4 and IRAK1 are sequentially phosphorylated and dissociated from MyD88, which results in activation of TRAF6 (Figure 3).

TRAF6 is the central activator of MAPK during microbial infection

TRAF6 belongs to an E3 ubiquitin ligase family, which facilitates the synthesis of lysine 63 linked polyubiquitin chains (Chen, 2005). TRAF6 is the activator of canonical NF-κB pathway (Hayden and Ghosh, 2004). TRAF6 is ubiquitinated at K63 chains and this K63 polyubiquitinated TRAF6 mediates activation of the next component in the pathway, which is most likely to be TGF-β activated kinase-1 (TAK1) (Sun *et al*., 2004). In fact, the TAK1 associated proteins, TAB2 and TAB3, contain a domain that interacts specifically with K63-ubiquitin chains. This model for TLR signaling predicts that the TAK1-TAB complex associates with K63-ubiquitinated TRAF6 to activate TAK1 kinase, which then activates the IKK complex as well as the JNK kinases. Sato *et al*. (2003b) reported that TRAF6 is involved in TRIF mediated IRF3 activation and NF-κB activation during TLR signaling. However, a recent paper delineated the involvement of TRAF6 in TLR signaling, where TRAF6 is involved in MyD88 mediated NF-κB activation but not TRIF mediated NF-κB activation (Gohda *et al*., 2004).

Transcription factors activated by TLR engagement

PAMPs stimulation through TLR-dependent and independent pathways converges at the activation of transcription factors NF-κB, IRF3/7/5, and/or AP-1. These transcription factors collaborate with each other to produce a large number of cytokines, which are barely detectable in resting cells. The multi-transcription factor binding sites in the promoter of a given gene lead to this highly specific activation. The multistage gene regulation by this interaction and the specific transcription factors activated will be discussed below.

NF-κB as double edged sword

The continued research on TLRs has led to the delineation of specificity in the regulation and interaction of transcription factors upon stimulation leading to a highly specific gene expression. NF-κB is the major transcription factor which functions on TLR signaling to control/elicit inflammation. NF-κB was first described as a B cell specific transcription factor that binds the κB site in the Ig κ light chain enhancer (Sen and Baltimore, 1986). Viral promoters contain NF-κB binding sites making it advantageous for its replication. So it is not exaggerating to say that cells which have NF-κB as a sword against the viral infection turn back against to them. NF-κB has often been called a 'central mediator of the immune response'. MAL-MyD88 and TRAM-TRIF pathways stimulate NF-κB activation albeit with different kinetics (Selvarajoo, 2006). NF-κB activity was found to be inducible in all cell types and it is now known that members of the NF-κB/Rel family regulate many genes involved in immune and inflammatory responses (Pahl, 1999; Hayden and Ghosh, 2004). NF-κB can be induced by a variety of stimuli (www.NF-κB.org). For example, recent studies described about the involvement of IkBζ to interact with NF-κB *via* a C terminal ankyrin repeat domain in the nucleus to induce IL-6 (Motoyama *et al.*, 2005; Yamazaki *et al.*, 2005). The differential interaction of transcription factors activated upon TLR stimulation is shown in Figure 4.

Activating protein-1 (AP1)

The JNK and p38 cascades are activated first and foremost in response to inflammatory cytokines, bacterial products, and various stress factors. Activation of TAK1 during TLR signaling results in the activation of MAPKs, including JNK/p38, leading to the activation of AP-1 (Ninomiya-Tsuji *et al.*, 1999; Akira and Takeda, 2004; Sato *et al.*, 2005), which together with NF-κB governs the production of inflammatory cytokines and chemokines (Kawai and Akira, 2006). Activation of these JNK/p38 cascades is associated with selective activation of different AP-1 subunits and transcription factors interacting with AP-1 (Johnson and Lapadat, 2002).

For example, LPS stimulation in precursor B cells and primary dendritic cells leads to JUND and JUNB activation (Krappmann *et al.*, 2004). The authors further show that these complex in turn are required for high-level sustained induction of chemokine receptor 7 (CCR-7) and Ig light chain

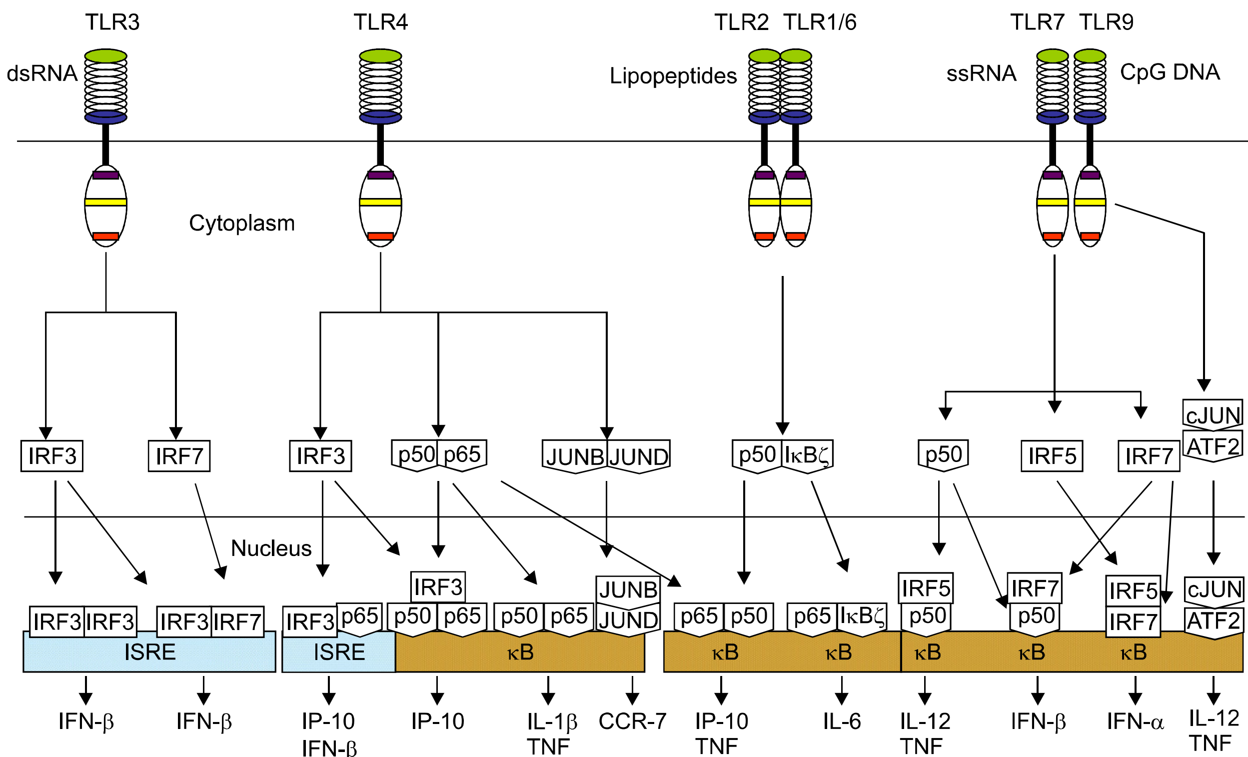

Figure 4. Interaction of transcription factors leading to a highly specific gene expression upon TLR stimulation. ISRE, interferon stimulatory response element; κB, κB site.

expression and production of surface IgM. On the other hand, CpG-DNA induces activation of JNK1/2 and p38 in murine macrophages and dendritic cells leading to the phosphorylation of c-JUN and ATF2 (Hacker *et al.,* 1998). This activation *via* p38 is necessary for the full induction of TNF-α and IL-12 as inhibition of p38 abrogates this biological response. TLR2 stimulation by PAM3CSK4 in DCs leads to the enhanced induction of c-FOS and induces high production of IL-10 but low level of IL-12 (Dillon *et al.,* 2004). All these studies together indicate that it is the differential activation and binding of AP-1 subunits which contribute to the inflammation.

IRFs are the novel regulators of TLR pathway

In recent past, attentions have been diverted to the IRF family of transcription factors which have important roles in the regulation of type I interferon production and a growing list of other genes. Our best understanding of IFN gene regulation comes from studies on TLR3 and TLR4 signaling both known to activate IRF3. The molecular mechanisms responsible for the phosphorylation induced activation of IRF3 have been the subject of intense study. IRF3 and IRF7 have recently been identified as the master regulators of type 1 IFN activation but yet many unanswered questions remain about their biology, structure, function and crosstalk with other important transcription factors. The answers to these questions will undoubtedly have important therapeutic applications for immune responses and modulation.

A major breakthrough recently implicated two IκB kinase-related kinases, IKKε (also known as IKKi) and TBK1 (TANK-binding kinase 1), in the IRF3 pathway (Fitzgerald *et al*., 2003; Sharma *et al*., 2003). TBK1 deficient embryonic fibroblasts are impaired in their ability to activate IRF3 or to induce IFN-β in response to LPS, poly (I-C) or a variety of viruses (Hemmi *et al*., 2004; McWhirter *et al*., 2004; Perry *et al*., 2004). Both IKKε and TBK1 directly phosphorylate IRF3, a property not shared by either IKKα or IKKβ (Sharma *et al*., 2003; McWhirter *et al*., 2004). Recently, phosphatidylinositol 3-kinase has also been implicated in IRF3 activation and seems to be required for full activation of IRF3 following TLR3 signaling (Sarkar *et al*., 2004). However, the IFN regulation seems to differ from cell to cell. Macrophages solely utilize TLR4 for IFN production, but neither TLR3 nor TLR4 is expressed in pDCs (plasmacytoid dendritic cells), therefore, these receptors cannot account for the production of IFN in these cells (Colonna *et al.,* 2004). TLR expression by human and mouse pDCs is restricted to TLR7 and TLR9 making them highly specialized for the detection of viral nucleic acids (Hornung *et al*., 2002). Further experiments in this area would delineate the specificity of IFN production during antiviral signaling.

Stimulation *via* TLR3 leads to the nuclear localization of IRF3 which binds as a dimer to induce IP-10 and IFN-β genes (Fitzgerald *et al*., 2003). IKK activated by TLR4 signaling activates the p65/p50 dimer (Zandi *et al*., 1997). IRF3 activated by TBK1 forms dimer with p65 to induce IP-10 and IFN-β (Wietek *et al*., 2003) and glucocorticoids can also suppress these heterodimers (Ogawa *et al*., 2005). IP-10 and proinflammatory cytokines can be induced by p65/p50 dimers binding to κB site and if IRF3 acts as a co-activator for this complex IP-10 can be induced (Leung *et al*., 2004).

Studies of IFN regulation also suggest that the autocrine/paracrine action of INF-β produced by IRF3 can induce IRF7 *de novo* which activates IFN-α (Au *et al*., 1998; Marie *et al*., 1998). Regulation of IFN production *via* TLRs is entirely distinct in pDCs. pDCs unlike fibroblasts, epithelial and most hematopoietic cells utilize IRF7 (Colonna *et al*., 2004; Diebold *et al*., 2004; Heil *et al*., 2004; Krug *et al*., 2004). Comparison between IRF3$^{-/-}$ pDCs and IRF7$^{-/-}$ pDCs revealed that IFN production by TLR 7, 8 and 9 was normal in IRF3$^{-/-}$ cells, but completely ablated in IRF7$^{-/-}$ cells, indicating that IRF7 is essential for the induction of IFN-α/β genes in pDCs (Honda *et al*., 2005). Moreover, IRF7 was shown to be involved in the induction of CD8^{+} cells responses in pDCs (Honda *et al*., 2005). Hence, IRF7 seems to be a very important and novel player in the development of innate and adaptive immunity in pDCs. Mutation analysis studies showed that IFN-*β* promoter also requires IRF7 for its induction.

IRF5 is shown to be activated only by certain viruses such as Newcastle Disease Virus (NDV), whereas the other virus such as Sendai Virus (SV) or dsRNA [e.g., poly (I-C)] activates IRF3 and IRF7 but not IRF 5 (Barnes *et al*., 2003; Elco *et al.,* 2005). These studies indicate that IRF5 activation is restricted. However, recently Takaoka *et al*. (2005) have found that using hematopoietic cells from mice deficient in IRF5, the induction of inflammatory cytokines such as IL-6, IL-12 and TNF-α in response to the ligands of TLR4, TLR5, TLR7 and TLR9 is severely impaired. The authors suggest that putative IFN-stimulated response elements in the promoters of these inflammatory cytokines bind IRF5. Hence, this new player of TLR signaling awaits further studies to delineate its involvement in controlling inflammation.

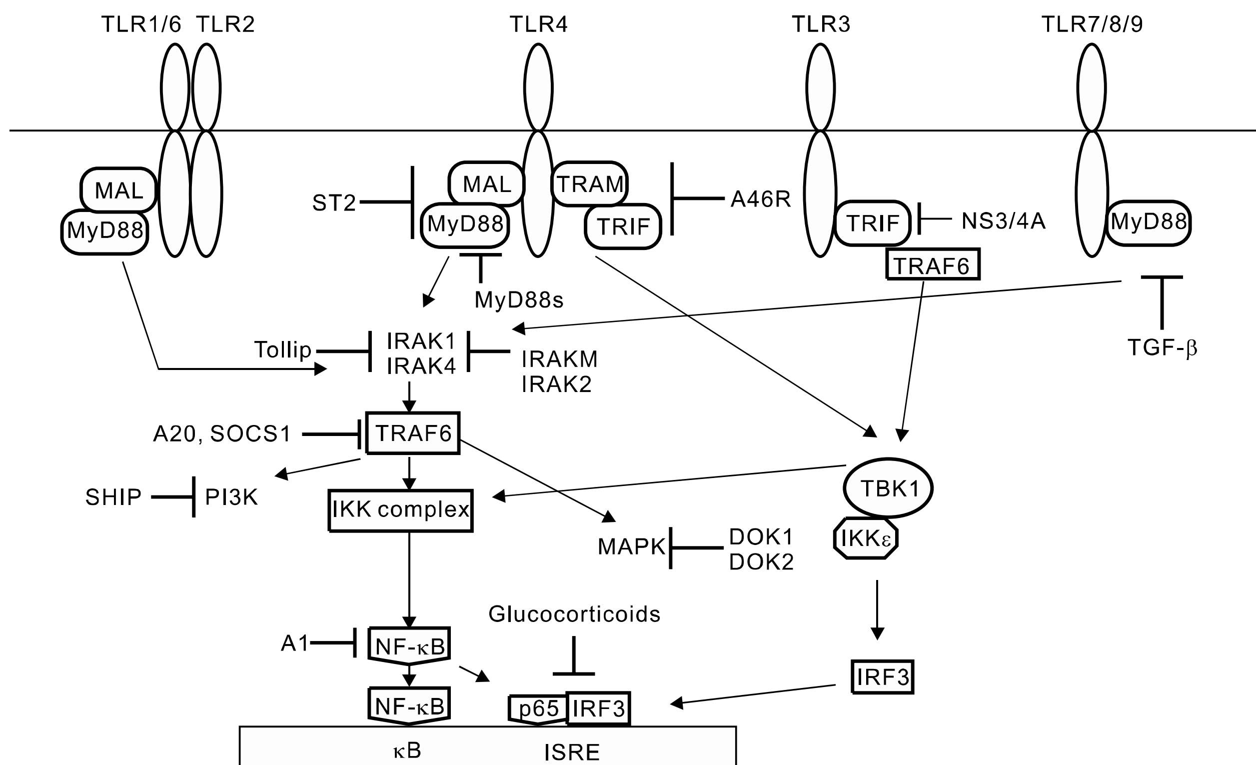

Figure 5. Negative feedback loops have evolved at different steps along the TLR pathway, all serving the same: Keeping the inflammatory responses in check. A1, *BCL-2* homologue; DOK1, downstream of kinase 1; MyD88s, splice variant of MyD88; SHIP, SH2-containing inositol phosphatase; SOCS1, suppressor of cytokine signaling 1; ST2, suppression of tumorigenicity 2.

Negative regulators of TLR signaling

The adapters are the indispensable components of TLR signaling and appear to be Achilles' heels for both exogenous and endogenous inhibition of TLR signaling. The immune system has created checkpoints at various levels of TLR signaling that will limit the amount and/or duration of inflammatory response. Amongst the molecules under checkpoints, the adapters appear to be prone to inhibition. Figure 5 illustrates our current understanding of adaptor inhibition. ST2 (suppression of tumorigenicity 2), also called as interleukin1 receptor-like 1 which is a member of IL-1R subgroup of TIR proteins, sequesters MAL and MyD88, thereby preventing the IL-1R and TLR4 signaling but not TLR3 signaling (Brint *et al*., 2004). A splice variant of MyD88, called MyD88s, acts to displace MyD88 and prevent NF-κB activation (Burns *et al*., 2003). MyD88s does not interact with IRAK4 because it lacks a region that is important for IRAK4 recruitment. So, IRAK4 is not recruited to the IL-1R, thereby preventing the association of IRAK1 and IRAK4 and thus the phosphorylation of IRAK1. As a result, there is no NF-κB activation. TGF-β facilitates ubiquitination and proteasomal degradation of MyD88 and attenuates MyD88-dependent signaling by decreasing cellular levels of MyD88 protein (Naiki *et al*., 2005). This may be a key aspect of the anti-inflammatory effects of TGF-β.

Viruses also target adapters, thus limiting TLR signal transduction to modulate immune responses. Vaccinia virus A46R can block both IL-1 and TLR4 signaling (Bowie *et al.,* 2000). A46R is in fact an inhibitor of multiple TIR adapters by sequestering MyD88, MAL, TRAM and TRIF (Stack *et al*., 2005). Another important observation is that the hepatitis C virus protein NS3/4A causes specific proteolysis of TRIF, an adaptor protein linking TLR3 to kinases responsible for activating transcription factors controlling a multiplicity of antiviral defenses (Li *et al*., 2005). This limits the induction of IFN-β by TLR3 during infection and might contribute to the persistence of the virus. Viruses have therefore evolved mechanisms to target adapters in TLR signaling, pointing to the importance of TLRs in antiviral immunity.

In endothelial cells the zinc finger protein A20 inhibits activation of TRAF6 by interfering with NF-κB signaling (Heyninck and Beyaert, 1999). A1 (*BCL-2* homologue) also inhibits NF-κB activation but this process is a little ambiguous (Karsan *et al.,* 1996). A1 and A20 provide negative feedback

Table 3. Collaboration of TLRs with other receptors.

Interacting receptor	Function of the interacting receptor
Dectins	Aids in phagocytosis of zymosan
Integrins	CR3 binds with LPS
Scavenger receptors	Induced by TLR, phagocytosis of *E. coli*
Fc gamma R	Anti-inflammatory/inhibition of TLR mediated IL-12 production
MD2	Interact with TLR4 for LPS recognition
Card like helicases	Enhanced antiviral responses
NODs	Enhanced antiviral responses
NALPs	Augmented inflammatory responses
GPCRs and TLRs	Increased polymorphonuclear leukocyte (PMN) migration
Adenosine receptors and TLRs	Angiogenesis

signaling for TLR4 as both of them can be up-regulated by LPS induced NF-κB signaling. PI3K plays dual roles after activated from TRAF6 as both positive and negative regulator of signaling. SHIP (SH2-containing inositol phosphatase) can inhibit PI3K signaling (Liu *et al*., 1999). TRAF6 and IRAK association is prevented by IRAKM (Kobayashi *et al.,* 2002). LPS induced ERK activation is prevented by the inhibitory adapter DOK1 (downstream of kinase 1) and DOK2 (Shinohara *et al.,* 2005). SOCS1 (suppressor of cytokine signaling 1) has been reported to be rapidly induced by LPS and negatively regulate LPS signaling, suggesting the suppression of TRAF6 (Kinjyo *et al*., 2002). These studies indicate that it might be possible to design selective inhibitors of adapters that might be used therapeutically.

Collaboration and crosstalk of TLRs with other receptors and pathways

TGF-β signaling modulates LPS-induced NF-κB activation, cytokine release, and suppression of inflammation through MyD88 which is a specific target of TGF-β and reveals novel signaling crosstalk interactions between TGF-β and TLR signaling (Naiki *et al*., 2005). The authors further showed that TGF-β potently inhibited LPS-induced NF-κB activation and TNF-α release from RAW 264.7 cells but had no effect on *IFN-β* promoter activation or RANTES (regulated upon activation, normal T-cell expressed, and secreted) release, indicating that MyD88-dependent pathway is inferred but not the MyD88-independent pathway downstream of TLRs. Ramification of cAMP pathway contributes to the diversity of signaling pathways and PKA phosphorylates p38 with a delayed kinetics forming the crosstalk point between G protein coupled receptors and TLRs (Delghandi *et al*., 2005) (Table 3). p38 which is activated through TLR can also phosphorylate STAT1 forming another crosstalk with IFN signaling pathways (Dalpke *et al*., 2003).

There is a synergistic effect of TLR to induce its neighbors upon appropriate stimulation. For instance, given the effective immune modulation by CpG DNA, regulation of TLR9 expression might play an important role in controlling the overall responses of immune cells to bacteria. When stimulated with TLR4 ligand LPS, macrophages increase TLR9, and thus respond to CpG DNA more effectively (An *et al*., 2002).

Systems biology approach to TLR4 signaling

So far, we have discussed about the main players of the TLR pathways through the sequential signaling process. In reality, however, the signaling behavior is highly complex (Oda and Kitano, 2006) due to the dynamical interaction of the various intracellular molecules and pathways. To approach the non-intuitive character of signaling pathways, we therefore require systematic methodologies that incorporate both biological and theoretical methods to interpret signaling dynamics (Gilchrist *et al.,* 2006; Markevich *et al.,* 2006; Selvarajoo, 2006; Alon, 2007; Basak *et al.,* 2007; Kholodenko, 2007). Recently, there have been such attempts to understand the signaling dynamics of TLRs (Covert *et al.,* 2005; Selvarajoo, 2006). The results of these studies have systematically revealed some important findings regarding the behavior of TLR pathways, not intuitively understood. For example, we demonstrated the delayed activation of NF-κB observed in MyD88 knock-out mice is due to both slower kinetics and missing intermediates acting along the TRIF-dependent pathways (Selvarajoo,

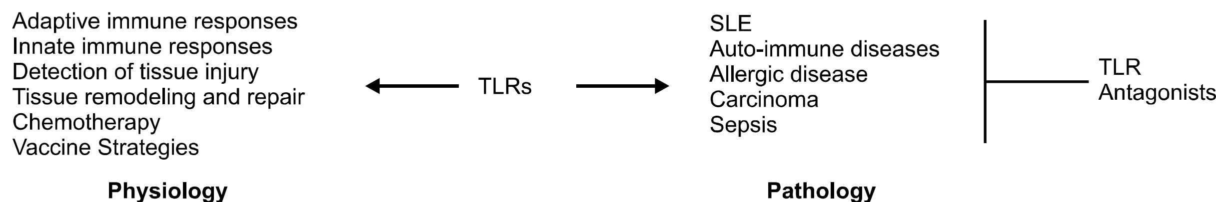

Figure 6. The physiological and pathological effects of TLR stimulation. TLRs have bi-functions and play roles in both physiological responses to pathogen as well as pathology in various diseases.

2006).

On a global front, the analysis of high throughput experimental data has demonstrated more detailed understanding of large scale gene expressions in innate immune system (Ricciardi-Castagnoli and Granucci, 2002; Zhu *et al.,* 2004, 2006; Hirotani *et al.,* 2005; Nilsson *et al.,* 2006). Despite this, we are yet to have a comprehensive view of TLR signaling events due to the lack of understanding in the interplay between the dynamics of gene regulatory networks and signaling pathways. For example, in LPS stimulation the secondary effects such as miRNA regulation (Taganov *et al.,* 2007) or autocrine/paracrine signaling (e.g., TNF-α signaling) after a few of hours of stimulation are not clearly understood. This is perhaps one of the reasons for the failure of the development of drug target for any specific pathways in controlling inflammatory diseases. Therefore, the development, integration and use of systems biology approaches will be necessary for deeper knowledge of TLR signaling pathway.

TLRs in physiology and pathology: TLRs and immune disorders

The role played by TLRs in various physiological and pathological conditions is shown in Figure 6. Impaired vaccine responses and increased morbidity and mortality in aged humans (65 $<$) were associated with 36% decreased surface expression of TLR1 compared with young adults (Duin *et al*., 2007). Certain TLRs expressions are implicated in laryngeal carcinoma (Szczepanski *et al*., 2007). During diabetes in mice, augmented TNF-α production by LPS in macrophages was due to hyperglycemia and increased activation of p38 kinase (Sherry *et al*., 2007). Chemokines and TLRs are important signals in macrophage mediated recognition and rejection of islet xenografts (Chandra *et al*., 2007). In patients with active SLE (systemic lupus erythematosus), the proportion of peripheral blood memory B cells and plasma cells expressing TLR9 is increased. Endogenous nucleic acids released during apoptotic cell death may stimulate B cells *via* TLR9 and contribute to SLE pathogenesis (Papadimitraki *et al*., 2006).

Interestingly a role of TLRs in exercise is also reported. Cell surface expressions of TLRs are reported to be decreased after prolonged exercise contributing post-exercise immunodepression and the reported higher susceptibility to infection in athletes (Gleeson *et al*., 2006). The precise physiological stimulus mediating an exercise-induced decrease in cell-surface TLR expression is not known. However, a number of possible signals have been implicated including anti-inflammatory cytokines, stress hormones and heat shock proteins. TLR ligands are considered as adjuvants for immunotherapy since $CD8^+$ effector cells can constitutively express TLR3 and produce IFN-γ upon poly (I-C) stimulation, which may lead to better responses against tumors or chronic viral infections (Tabiasco *et al*., 2006). TLR4 promotes the trapping of activated $CD8^+$ T cells in the liver (Komatsu *et al*., 2000; John *et al*., 2007) and without TLR4, the liver trapped fewer activated $CD8^+$ T cells, leading to an increase of these cells in the circulation. Hyperthermia (39.5°C) during fever might activate innate immune response by promoting TLR4 expression and signaling but not TLR2 expression (Zhao *et al*., 2007). TLR-mediated induction of type I IFNs and soluble adhesion molecules such as sE-selectin (soluble form of E-selectin) and sICAM (soluble intercellular adhesion molecule) in sera are shown to be operative in retinal vascular endothelial cells (Lee *et al*., 2007). Taken together, all these studies indicate that the old dogma of TLRs would span across the fields of immunity.

Future researches

Though we have come across a lot of information from the remarkable progress which has been made since the discovery of TLRs, yet, many unanswered questions remain. For instance,

whether TLRs can directly recognize their ligands as some studies suggest (Lien *et al.*, 2000; Poltorak *et al.*, 2000; Sato *et al.*, 2003a) or whether an accessory molecule like MD2 or an intermediary similar to *Drosophila* extracellular protein Spatzle performs this function (Akashi *et al.*, 2001; Medzhitov, 2001; Hoffman and Reichhart, 2002). The another intriguing question with TLRs is how the innate immune system manages to recognize synthetic CpG oligonucleotides and bacterial DNA against a possible background of host DNA set generated through trauma, apoptosis or other events that liberate host DNA inside the body. Although a consistent view of the TLR-signaling mechanism is emerging from biochemical and biophysical studies, there is a lack of detailed structural information on the macromolecular complexes involved in the signaling process.

Since the innate immune system is highly complex, systems biological approaches are going to play an ever increasing role to provide understanding of innate immune dynamic regulation. More information of the above mentioned lacunas through continued researches on TLRs will add more understanding in this aspect and help to develop therapeutics in the clearance of pathogen, which is what we can wish for.

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