Review

Dissecting negative regulation of Toll-like receptor signaling

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Toll-like receptors (TLRs) sense invading microbial pathogens and play crucial roles in the activation of innate and adaptive immunity. However, excessive TLR activation can disrupt immune homeostasis, and may be responsible for the development of autoimmune and inflammatory diseases. As such, the molecules and pathways that negatively control TLR signaling have been intensively investigated. Here, we discuss recent insights into the negative regulation of TLR signaling, with focus on three major mechanisms: (i) dissociation of adaptor complexes; (ii) degradation of signal proteins; and (iii) transcriptional regulation. We also highlight how pathogens negatively target TLR signaling as a strategy to evade the host immune response.

TLR signaling

Innate immunity is the first line of host defense mechanisms against pathogens and is essential for efficient activation of adaptive immunity. During the past decade, dramatic progress has been made in our understanding of how host cells recognize invading microorganisms. Recognition is determined by germline-encoded pattern recognition receptors (PRRs), which detect conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs)[1].

TLRs are the most characterized PRRs [2]. TLRs are type I transmembrane proteins and consist of three types of domains: extracellular ectodomains containing leucine-rich repeats, which have avidity for PAMPs; a transmembrane domain; and an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain, which interacts with downstream adapter proteins. Currently, 12 members of the TLR family have been identified in mammals. These members form homo- or heterodimers and recognize various PAMPs (Table 1).

Engagement of TLRs activates multiple signaling cascades leading to the induction of genes involved in innate immune responses. Binding of ligands followed by dimerization of TLRs recruits TIR domain-containing adapter proteins such as myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP), and TRIF-related adaptor molecule (TRAM). Individual TLRs recruit specific combinations of these adapter molecules to elicit specific immune responses tailored to infectious pathogens. MyD88 is recruited to all TLRs except for TLR3 and associates with IL-1R-associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6), resulting in activation of canonical inhibitor of kappa light polypeptide gene enhancer in B-cells, kinases (IKKs) (IKKα and IKKβ) and nuclear factor (NF)-κBs (Figure 1). In contrast, TRIF is recruited to TLR3 and TLR4, leading to activation of NF-κB as well as noncanonical IKKs (TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) and IKKι) and interferon (IFN) regulatory factor (IRF)3 via TRAF proteins (Figure 2). TIRAP functions as a sorting adapter that recruits MyD88 to TLR2 and TLR4, whereas TRAM functions as a bridge adapter between TLR4 and TRIF.

TLR signaling leads to production of proinflammatory cytokines and type I IFNs and these responses are crucial for host defensive responses against pathogens. However, the aberrant activation of TLR signaling may be responsible for the pathogenesis of autoimmune, chronic inflammatory and infectious diseases (Table 1). Furthermore, increasing evidence has indicated that TLRs respond to endogenous molecules, most of which are released from dead cells, and are often referred to as damage-associated molecular patterns (DAMPs) [3], suggesting that TLRs can survey danger signals and are associated with sterile inflammation [4].

To avoid harmful and inappropriate inflammatory responses, TLR signaling is negatively controlled by multiple mechanisms. In this review, we summarize our current understanding of negative regulation of TLR signaling and its association with autoimmune and inflammatory diseases. Negative regulators are often induced by TLR ligands to terminate activation of signaling pathways. Here, we classify regulatory mechanisms into three major categories: (i) dissociation of adaptor complexes; (ii) degradation of signal proteins; and (iii) transcriptional regulation. To date, many negative regulators have been identified and characterized [5], and we focus on the most recent findings. We also summarize the negative regulation of TLR signaling by pathogens, which is an important strategy for escape from host innate immune responses.

Dissociation of adaptor complexes

TLRs and TIR domain-containing adaptor proteins have several variants that act as antagonists to prevent association among intact forms of adaptors, and block downstream signaling pathways. TRAM adaptor with GOLD domain (TAG), identified as a variant of TRAM, competes

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Table 1. TLR ligands and related diseases.

TLR	PAMPs	DAMPs	Disease
TLR1	(w/TLR2) triacyl lipoprotein	n.d.	
TLR2	Lipoproteins (w/TLR1) triacyl lipoprotein (w/TLR6) diacyl lipoprotein, LTA, zymosan	(w/TLR6) HMGB1, HSPs, ECM	Candidiasis
TLR3	dsRNA	mRNA	WNV
TLR4	LPS, viral envelop proteins	HMGB1, HSPs, ECM, Ox-phospholipids, β-defensin 2 (w/TLR6) Amyloid-β, Ox-LDL	Sepsis, EAE, Atherosclerosis, COPD, Asthma
TLR5	Flagellin	n.d.	
TLR6	(w/TLR2) Diacyl lipoprotein, LTA, Zymosan	(w/TLR2) HMGB1, HSPs, ECM	
mTLR7/hTLR8	ssRNA	ssRNA (immune complex)	
TLR9	DNA, hemozoin	DNA (immune complex)	Malaria, SLE
TLR10	Unknown	n.d.	
TLR11	Profilin-like molecule Uropathogenic bacteria	n.d.	

COPD, chronic obstructive pulmonary disease; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; HMGB1, high mobility group box 1; HSPs, heat shock proteins; LTA, lipoteichoic acid; n.d., not determined; Ox-LDL, oxidized low-density lipoprotein; SLE, systemic lupus erythematosus; WNV, West Nile virus.

with TRAM for TRIF binding and inhibits the TRIF-dependent pathway [6]. TAG localizes to the late endosomes and is required for TLR4 degradation after lipopolysaccharide (LPS) treatment, indicating that TAG may mediate destabilization of TLR4 by delivery to lysosomes, as well as inhibiting TRIF binding.

MyD88, TIRAP, TRIF and TRAM are required for activation of TLR signaling [7], whereas another TIR domain-containing protein, sterile alpha- and armadillo-motif-containing protein (SARM), can block TRIF complex formation by directly binding to TRIF after LPS treatment [8]. Although this inhibitory function requires the sterile α-motifs (SAM) domain, the molecular mechanisms of how this domain suppresses the TRIF-dependent pathway remain to be elucidated.

IRF5 directly interacts with MyD88 to induce a set of TLR-inducible genes [9]. IRF4 is induced by TLR activation and competes with IRF5 for binding to MyD88, resulting in shutdown of IRF5-dependent gene induction [10]. IRF4-deficient mice are hypersensitive to DNA-induced shock accompanied by increased cytokine production.

A tumor necrosis factor, alpha-induced protein 8 (TNFAIP8), TNFAIP8-like 2 (TNFAIP8L2; also known as TIPE2) (TNFAIP8L2) binds to caspase 8 and regulates activator protein (AP)-1 and NF-κB activation [11]. Caspase 8 plays essential roles in immune cell activation through various receptors including TLRs, as well as regulating apoptosis [12,13]. TIPE2-deficient cells and mice are hypersensitive to TLR stimulation, which is blocked by a caspase 8 inhibitor. This result suggests that TIPE2 negatively regulates TLR signaling via inhibition of caspase 8.

Certain NOD-like receptors (NLRs) function as intracellular PRRs and are associated with inflammation [14]. Nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family member X1 (NLRX1) was previously identified as an inhibitor of IPS-1, an adaptor for RIG-I-like receptors (RLRs), which can suppress TLR signaling through interactions with TRAF6 and IKK complex [15]. NLRX1 undergoes K63-linked polyubiquitination after LPS treatment and dissociates from TRAF6, resulting in binding to the activated kinase domain of IKKβ via the leucine-rich repeat (LRR) domain. NLRX1-knockdown mice show hyperactivation of NF-κB, elevated production of inflammatory cytokines, and increased susceptibility to LPS-induced septic shock, suggesting that NLRX1 functions as a negative regulator of TLR signaling *in vivo*. However, there is some disagreement concerning *in vivo* functions of NLRX1, at least in RLR signaling [16]. In contrast, another NLR protein, NLRC5, negatively regulates TLR and RLR signaling through IKK inhibition [17]. However, NLRC5-deficient mice develop normal responses against bacterium and virus infection [18]. Further studies are required to evaluate the biological significance of these proteins.

Post-translational modifications such as phosphorylation and ubiquitination also play important roles in signal transduction by regulating interactions among adaptor proteins. TANK was identified as a TRAF-binding protein and shown to activate both NF-κB and IRFs *in vitro*. However, TANK-deficient mice have increased NF-κB activation in response to TLR ligands [19]. Loss of TANK leads to spontaneous development of fatal glomerulonephritis in mice. TRAF6 ubiquitination, which is required for NF-κB activation, is enhanced in TANK-deficient cells, indicating that TANK binds to TRAF6 and inhibits its ubiquitination.

Recently, the orphan nuclear receptor, small heterodimer partner (SHP, also known as NR0B2) has been identified as a negative regulator of TLR signaling by inhibiting TRAF6 ubiquitination [20]. SHP-deficient cells show elevated expression of tumor necrosis factor (TNF), IL-1β and IL-6 following LPS treatment. Furthermore, bone-marrow-derived cells expressing SHP protect SHP-deficient mice from LPS-induced lethal shock. It has also been demonstrated that TLR stimulation induces SHP expression through AMP-activated protein kinase (AMPK) activation-dependent intracellular Ca^{2+} influx mediated by TLRs [20].

Mitogen and stress activated protein kinase (MSK) 1 and 2 activated in the mitogen-activated protein kinase (MAPK) cascade limit the proinflammatory effects of TLR4 signaling [21]. Loss of MSK1 and MSK2 prevents the binding of phosphorylated transcription factors cAMP responsive element binding protein 1 (CREB) and activating transcription

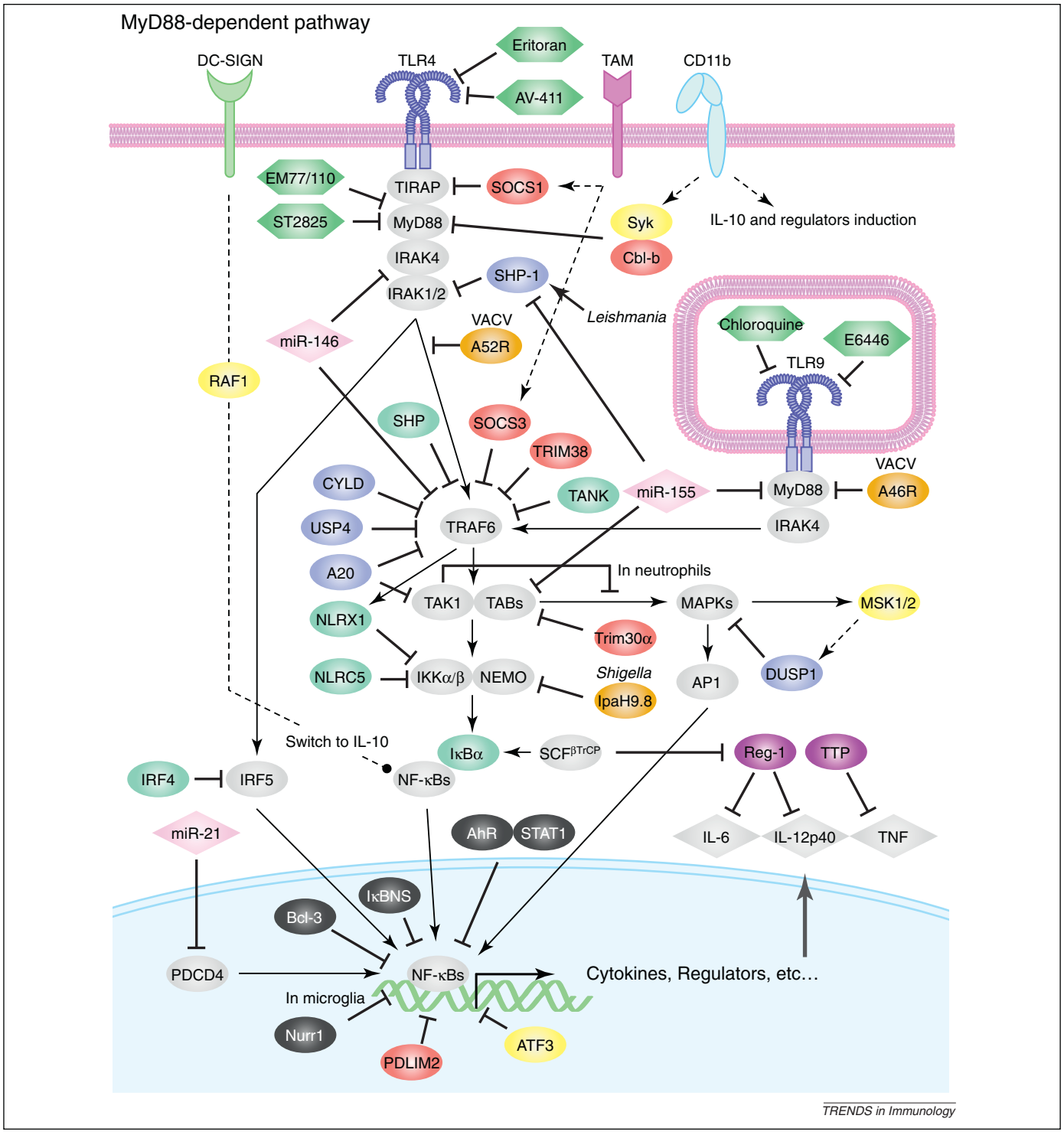

Figure 1. MyD88-dependent TLR pathway and its negative regulators. TLRs recognize their respective ligands at the cell surface or endosomes, leading to recruitment of MyD88. MyD88 interacts with IRAKs and activates TRAF6, resulting in induction of inflammatory responses by activation of NF-κB, MAPK and IRF5. Formation of polyubiquitination on TRAF6 is inhibited by TANK and SHP and once formed the polyubiquitin chain is removed by A20 and CYLD. NLRX1 and NLRC5 attenuate activation of the IKK complex. IRF4 competes with IRF5. SHP-1 mediates activities of IRAKs, which is promoted by *Leishmania* infection. SOCS proteins induced by TAM receptor signaling promote degradation of TIRAP and TRAF6. Integrin signaling induces several regulators and mediates degradation of MyD88 by Syk and Cbl-b combination. In neutrophils, TAK1 blocks TAB-induced activation of p38 MAPK. MSK1 and MSK2 are activated by MAPK and increase MAPK phosphatase DUSP1 expression. PDLIM2 and Trim30a mediate ubiquitination and degradation of p65 and TAK1–TAB complex, respectively. ATF3 promotes histone deacetylation and limits access of transcriptional factors. IκBNS, Bcl-3 and Ah receptor regulate NF-κB activity at promoter regions of target genes. Nurr1 recruits the CoREST complex and removes NF-κB from promoter regions. TTP enhances deadenylation and degradation of TNF-α mRNA. Zc3h12a degrades IL-6 and IL-12p40 mRNA. miR-155 and miR-146 target mRNA of indicated molecules. miR-21 controls cytokine production by regulating PDCD4 expression. IpaH9.8 targets NEMO for degradation. VACV A46R disturbs complex formation consisting of TIR-domain containing adapters. VACV A52R impedes interaction between IRAK2 and TRAF6. DC-SIGN recognizes mycobacterial components and induces IL-10 production by mediating acetylation of p65. Eritoran and AV-411 are TLR4 antagonists. EM77/110 and ST2825 inhibit formation of MyD88–IRAK complex. Chloroquine and E6446 block TLR9 signaling. Gray, signal molecule; lime green, competitor; red, ubiquitin ligase or autophagy protein; blue, DUB or phosphatase; black, transcriptional regulator; purple, regulator of RNA stability; pink, miRNA; brown, pathogen derived; green, artificial antagonist; yellow, other categories.

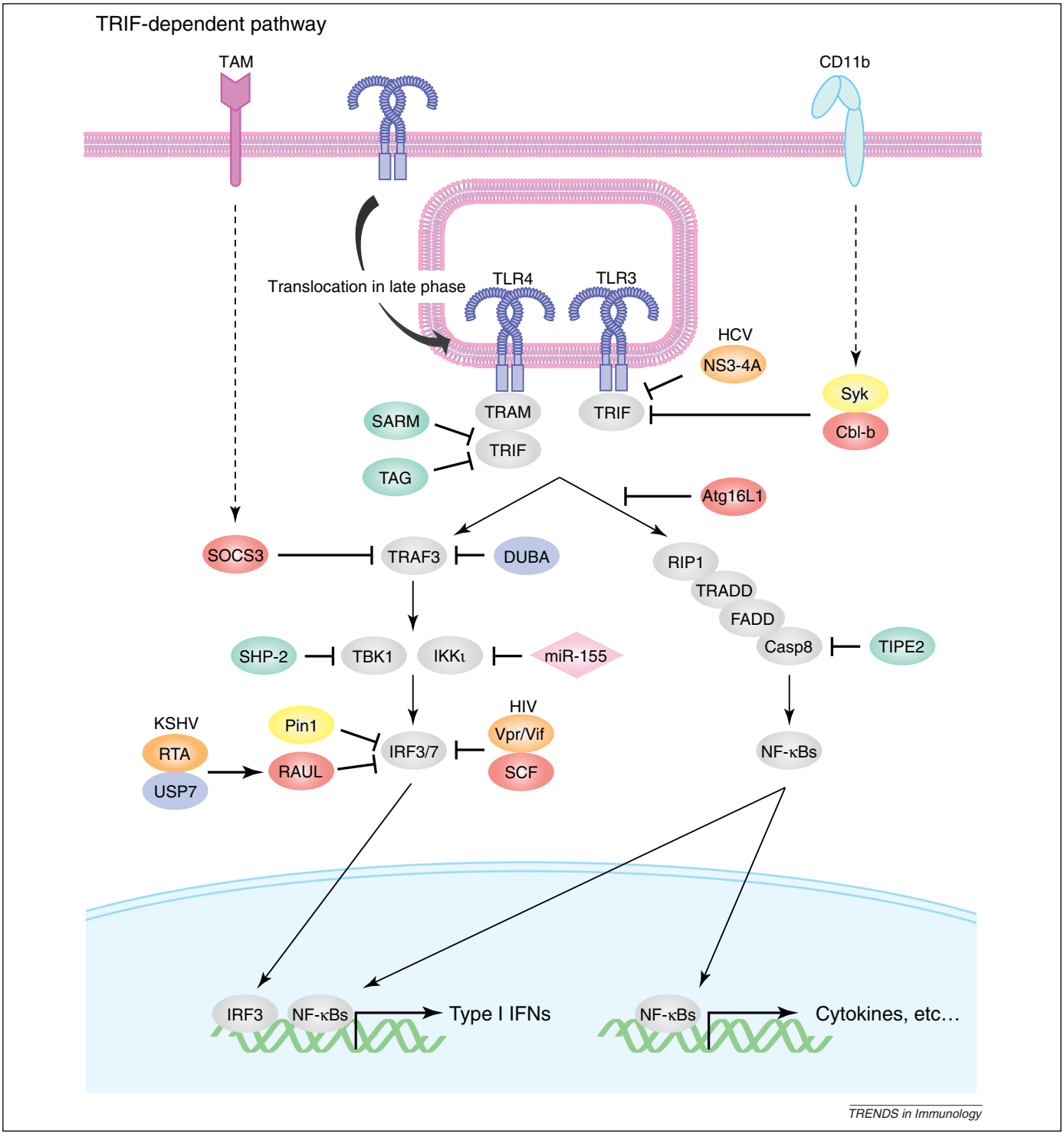

Figure 2. TRIF-dependent TLR pathway and its negative regulators. TLR4, which translocates to the endosome after LPS stimulation, and TLR3, which recognizes dsRNA, activate the TRIF-dependent pathway. Although signal complexes containing receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP1), TNFRSF1A-associated via death domain (TRADD), Fas (TNFRSF6)-associated via death domain (FADD), and caspase 8 activate NF-κB, TRAF3 induces type I IFNs by activating noncanonical IKKs (TBK1 and IKKι) followed by IRF3/7 activation. TAG and SARM negatively regulate this pathway at the TRIF level. TIPE2 prevents caspase 8 from forming a signal complex. SHP-2 binds to TBK1 and blocks downstream signaling. DUBA cleaves the polyubiquitin chain on TRAF3. SOCS3 mediates degradation of TRAF3. IRF3 degradation is regulated by Pin1, RAUL, and SCF complex. Virus-derived proteins such as KSHV RTA or HIV vpr/vif exploit these systems. Inhibitory regulation by Atg16L1 is dependent on the TRIF pathway. miR-155 targets IKKι. HCV NS3-4A cleaves TRIF.

factor 1 (ATF1) to the promoters of the anti-inflammatory cytokine IL-10, and MAPK phosphatase dual specificity phosphatase 1 (DUSP1) that promotes p38 deactivation after LPS stimulation. MSK1- and MSK2-double knockout mice are hypersensitive to LPS-induced endotoxin shock and develop prolonged inflammation in a model of toxic contact eczema induced by phorbol 12-myristate 13-acetate [21].

TGF-β-activated kinase 1 (TAK1) is an essential kinase that activates MAPK and NF-κB in several signaling

pathways. Although the role of TAK1 as a positive regulator for NF-κB and MAPK in lymphocytes and hematopoietic cells has been assessed, the specific functions of TAK1 in myeloid cells have only recently been confirmed [22]. Surprisingly, TAK1 is dispensable for TLR responses in myeloid cells (neutrophils and macrophages). In neutrophils, ablation of TAK1 enhances phosphorylation of p38 and production of cytokines and reactive oxygen species (ROS) induced by LPS. Myeloid lineage-specific TAK1-deficient mice show splenomegaly and lymphadenopathy, and are susceptible to LPS-induced septic shock. These phenotypes are rescued by specific ablation of p38, suggesting that TAK1 negatively controls p38 activation.

Several phosphatases and deubiquitination enzymes (DUBs) participate in negative regulation of TLR signaling. The Src homology 2 domain-containing protein tyrosine phosphatase-1 and -2 (SHP-1, -2) are involved in TLR signaling. SHP-1-deficient mice develop inflammatory lesions in a MyD88-dependent manner [23]. SHP-1 suppresses IRAK1 and IRAK2 activities, resulting in decreased production of proinflammatory cytokines and increased production of type I IFN. This observation is consistent with the fact that the kinase activity of IRAK1 is required for activation of NF-κB and inhibition of type I IFN. *Leishmania* infection promotes binding between SHP-1 and the conserved immunoreceptor tyrosine-based inhibition motif (ITIM), Kinase Tyrosyl-based Inhibitory Motif (KTIM), in the kinase domain of IRAK-1 to suppress innate immune responses [24]. SHP-2 negatively regulates TRIF-dependent type I IFN production [25]. Although SHP-2 interacts with TBK1, its phosphatase activity is dispensable for TBK1-activated gene expression. Thus, SHP-2 acts as an antagonist rather than a phosphatase for TBK1.

A20 is described as a negative regulator in TLR receptor signaling. A20-deficient mice display multiorgan inflammation, which is rescued by co-deficiency with MyD88. The administration of antibiotics suppresses cachexia caused by loss of A20, indicating that A20 might inhibit TLR activation induced by commensal bacteria [26]. A20 functions as a DUB to remove K63-linked polyubiquitin chains from TRAF6 and protect mice from LPS-induced endotoxin shock. Moreover, A20 has been shown to inhibit IKK activation by TAK1 without DUB activity, suggesting that A20 regulates NF-κB activation via multiple mechanisms [27].

A tumor suppressor DUB, cylindromatosis (CYLD), has been shown to inhibit TLR2 signaling [28]. The induction of CYLD by TLR2 activation removes polyubiquitin chains from TRAF6 and TRAF7, both of which are required for NF-κB and MAPK activation by TLR2 ligands.

Recently, USP4 has been identified as a negative regulatory DUB, which binds to TRAF6 and suppresses IL-1β induced NF-κB activation [29]. Ubiquitin specific peptidase 4 (USP4) removes polyubiquitin chains on TRAF6 in a DUB activity-dependent manner. Loss of USP4 enhances cytokine production mediated by LPS and IL-1β.

DUBA (deubiquitinating enzyme A), an ovarian tumor domain containing DUB, has been identified as a negative regulator of type I IFN production [30]. DUBA selectively binds and cleaves K63-linked polyubiquitin chains on TRAF3 to suppress TLR-induced type I IFN production but does not affect NF-κB activation.

Taken together, competitors of adaptors, phosphatases and DUBs disrupt the formation of adaptor complexes. Interestingly, multiple regulators target TRAF6. It is still unknown whether these regulators collaborate with each other.

Degradation of signal proteins

Ubiquitination is a versatile post-translational modification mechanism and regulates various cellular processes, including immune responses [31]. Suppressor of cytokine signaling (SOCS) proteins of the E3 ubiquitin ligase family are well characterized regulators that promote degradation of TIRAP/MyD88-adaptor-like (MAL) or TRAF proteins [32]. Dendritic cells (DCs) deficient in Tyro3, Axl, and Mer (TAM) receptor tyrosine kinases produce elevated levels of inflammatory cytokines when treated with TLR ligands compared with control mice, and stimulation of DCs with TAM receptor ligands suppresses TLR-induced cytokine production. Further analysis has shown that TAM receptor signaling upregulates SOCS1 and SOCS3 expression through signal transducer and activator of transcription (STAT)1, offering an explanation of TAM receptor-mediated inhibition of TLR-induced cytokine production.

Immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors regulate immune responses through crosstalk with other signaling pathways including TLRs [33]. Calcium signaling induced by β2 integrins and Fcγ receptors causes upregulation of IL-10 and signaling molecules involved in the inhibition of innate immune responses, resulting in indirect inhibition of TLR signaling [34]. Additionally, the integrin, CD11b, activates spleen tyrosine kinase (Syk), which phosphorylates MyD88 and TRIF and leads to these proteins binding to the E3 ubiquitin ligase Casitas B-lineage lymphoma (Cbl)-b, which promotes their degradation through the ubiquitin–proteasome system [35]. This study also demonstrated interesting aspects of this regulation, in which TLR-induced activation of PI3K activated outside-in integrin signaling and triggered negative regulation. By contrast, it was shown that CD11b is required for control of TIRAP recruitment to the plasma membrane via phosphatidylinositol 4,5-bisphosphate (PIP2) and activation of cell surface-localizing TLRs [36], suggesting that integrin signaling has both positive and negative roles in TLR signaling.

PDZ and LIM domain protein 2 (PDLIM2), identified as an inhibitor of Janus kinase (JAK)/STAT, also inhibits TLR signaling by degradation of the NF-κB component p65 [37]. PDLIM2 promotes K48-linked polyubiquitination on p65 and sequesters it to nuclear promyelocytic leukemia (PML) bodies enriched for 26S proteasome. Indeed, after TLR ligand treatment, PDLIM2-deficient cells fail to degrade p65, resulting in increased production of proinflammatory cytokines.

Trim30α, a member of the tripartite-motif containing (TRIM) protein superfamily, is induced by TLR ligands to promote degradation of the TAK1 binding protein 2 (TAB2)–TAB3–TAK1 signal complex [38]. Experiments using chemical inhibitors have indicated that degradation

is dependent on lysosomes rather than proteasomes. A decrease in expression of TABs caused reduced NF-κB activation and cytokine production. Consistent with these observations, TRIM30α transgenic mice and *in vivo* knockdown studies have shown that Trim30α protects mice from endotoxin shock induced by LPS. Recently, another TLR-induced TRIM protein, TRIM38, has been shown to act as a negative regulator of TLR signaling [39]. TRIM38 interacts with TRAF6 and promotes formation of K48-linked polyubiquitin chain, resulting in proteasomal degradation of TRAF6.

IRF3 and IRF7 are also regulated by ubiquitination and degradation. The peptidyl-prolyl isomerase Pin1 promotes polyubiquitination and proteasomal degradation of activated IRF3, resulting in suppression of type I IFN and antiviral responses [40]. Pin1 specifically binds to phosphorylated IRF3, consistent with previous characterization studies, suggesting that Pin1 binds phosphorylated serine or threonine followed by proline via WW domain and catalyzes conformational change of the substrate. It is likely that the conformational change facilitates ubiquitination of IRF3, although Pin1 itself does not directly catalyze it as an E3 ubiquitin ligase. By contrast, it has been shown that IRAK1 is activated by Pin1 in plasmacytoid DCs (pDCs) during TLR7 and TLR9 signaling [41]. IRAK1 activation leads to production of type I IFN. Thus, in pDCs, Pin1 functions as a positive regulator for type I IFN production. Recently, homologous to E6-AP carboxyl terminus (HECT) type replication and transcription activator (RTA)-associated ubiquitin ligase (RAUL) has been shown to catalyze directly ubiquitination of IRF3/7 and negatively regulate type I IFN responses [42]. Kaposi's sarcoma-associated herpesvirus (KSHV) RTA promotes deubiquitination of RAUL self-ubiquitination by recruiting USP7 (also known as herpesvirus-associated ubiquitin-specific protease, HAUSP), leading to the stable function of RAUL and effective degradation of IRFs to mute antiviral responses.

In addition to the ubiquitin–proteasome system, autophagy is a major degradation system and plays crucial roles in host defense [43]. Loss of Atg16L1, a Crohn's disease risk allele, leads to generation of high levels of ROS, IL-1β and IL-18 induced by LPS [44]. These responses are dependent on TRIF, suggesting that Atg16L1 negatively regulates the TRIF-dependent pathway leading to caspase 1 activation. It has been shown that ATG16L1-deficient Paneth cells show increased expression of genes involved in intestinal injury responses [45].

As described above, degradation of signal proteins mediated by the ubiquitin–proteasome and autophagy systems plays crucial roles in negative regulation of TLR signaling. Unlike in the case of disruption of adaptor complexes, these degradations are irreversible, suggesting that this mechanism contributes largely to termination of TLR signaling.

Transcriptional regulation

Recent knowledge about epigenetic regulation of gene expression has uncovered control of inflammatory responses by chromatin structures [46]. Cyclic AMP-dependent transcription factor (ATF3) recruits histone deacetylase 1 (HDAC1) to the promoter region of proinflammatory cytokine genes, promoting histone deacetylation and limiting access of transcriptional factors [47]. Macrophages derived from ATF3-deficient mice release large amounts of IL-12p40, IL-6 and TNF-α in response to LPS.

A TLR-inducible IκB protein, inhibitor of kappa light polypeptide gene enhancer in B cells, delta (IκB delta; also known as IκBNS), negatively regulates induction of genes such as IL-6 and IL-12p40, but not TNF-α [48]. IκBNS is selectively recruited to the IL-6 promoter but not the TNF-α promoter, and regulates NF-κB activation. IκBNS-deficient mice are highly susceptible to LPS-induced endotoxin shock and intestinal inflammation, indicating that IκBNS controls inflammatory responses by mediating induction of a subset of NF-κB target genes.

B-cell CLL/lymphoma 3 (Bcl-3), a member of the IκB family, is essential for TLR tolerance [49]. Repeated or prolonged stimulation through TLRs can cause insensitivity or hyporesponsiveness, but Bcl-3-deficient macrophages and DCs pretreated with LPS produce large amounts of cytokines compared to wild-type cells. In Bcl-3-deficient cells, NF-κB subunit p50 ubiquitination and degradation after stimulation with LPS are markedly increased, suggesting that Bcl-3 limits duration of TLR responses by stabilizing p50, which occupies an NF-κB DNA binding site.

Nuclear receptors such as the glucocorticoid receptor regulate diverse aspects of the immune system by controlling gene expression. An orphan receptor, nuclear receptor related 1 protein (Nurr1), is induced by LPS and recruited to p65, followed by recruitment of the CoREST complex and transcriptional repression [50]. Microglia cells are myeloid lineage cells in the central nervous system and respond to infection and tissue injury. Nurr1-knockdown in microglia cells results in a marked increase of cytokine production, indicating that Nurr1 protects neurons by preventing exaggerated inflammatory responses in microglia.

The transcription factor aryl hydrocarbon (Ah) receptor is a cytosolic sensor that recognizes chemical compounds and plays a role in immune responses [51]. In macrophages, LPS stimulation induces Ah receptor, which interacts with STAT1 and NF-κB in the IL-6 promoter to suppress IL-6 production [52]. Ah receptor-deficient mice are highly sensitive to LPS-induced lethal shock compared to wild-type mice, indicating that Ah receptor is an inducible negative regulator that acts to avoid hyperimmune responses triggered by TLR activation.

Several RNA-binding proteins containing CCCH-type zinc finger motif are reported to control gene expression negatively by promoting the mRNA decay of TLR target genes. Tristetraprolin (TTP) binds to AU-rich elements in the 3′ untranslated region (UTR) of TNF-α mRNA and promotes deadenylation by deadenylase recruitment, leading to degradation of mRNA in the exosomes [53]. A TLR-inducible RNase, Zc3h12a (also known as Regnase-1 or MCPIP1) interacts with IL-6 and IL-12p40 mRNA and degrades them with its RNase activity [54]. Zc3h12a-deficient macrophages produce higher levels of IL-6 and IL-12p40 than wild-type cells after treatment with various TLR ligands. Additionally, Zc3h12a-deficient mice show elevated autoantibody production. Thus, Zc3h12a regulates

TLR-induced immune responses and prevents autoimmunity. Another study using independently developed Zc3h12a-deficient mice also has demonstrated negative regulation of responses by Zc3h12a [55]. In that study, Zc3h12a exhibited DUB activity to remove polyubiquitin chains from TRAF proteins. Thus, Zc3h12a may control immune responses via multiple mechanisms. Recently, Zc3h12a has been shown to be regulated by IKKs through phosphorylation and proteasomal degradation [56]. This observation may explain a regulatory mechanism for rapid cytokine production during infection.

Non-coding RNAs, including miRNAs, that are induced by TLR signaling have been identified [57]. The miRNAs have emerged as fine tuners for TLR signaling by targeting mRNA encoding TLRs, intracellular signaling proteins and cytokines. These miRNAs often have several targets and a bidirectional function, similar to miR155. TLR-inducible and negative functional miRNAs are described in Figures 1 and 2.

miR-155 is a TLR-inducible miRNA [58] and has both positive and negative aspects in immune responses [59–66]. To suppress TLR signaling, miR-155 targets MyD88, TAB2 and IKKι [59–63]. By contrast, miR-155 targets SHP-1 [66] and enhances signaling, suggesting that miR-155 fine tunes TLR signaling.

miR-146a was identified as an LPS-induced miRNA with the potential to target the 3′ UTR of TRAF6 and IRAK1 mRNA [67]. miR-146a-deficient mice exhibited myeloid sarcomas and chronic myeloproliferation caused by dysregulation of NF-κB [68]. Another study showed that miR-146a was upregulated in intestinal epithelial cells (IECs) soon after birth, which repressed IRAK1 expression levels and protected the gut mucosa from bacterium-induced damage [69].

Programmed cell death protein (PDCD)4, an apoptotic stimulus-induced tumor suppressor, has proinflammatory functions by regulating NF-κB activity and IL-10 production. It has been demonstrated that PDCD4 is crucial for regulation of responses to LPS [70]. miR-21 is induced by LPS stimulation and negatively regulates inflammatory responses by decreasing PDCD4 expression levels.

Unlike other mechanisms for TLR inhibition, transcriptional regulation often enables control of a particular subset of TLR target genes without termination of TLR signaling. This property possibly contributes to regulate the balance of immunity beyond suppression of TLR signaling.

Evasion by pathogens

Pathogens have developed strategies to evade TLR signaling [71]. Hepatitis C virus (HCV) protein NS3-4A has serine protease activity that cleaves TRIF [72]. Vaccinia virus (VACV) protein A46R contains a TIR domain and inhibits the formation of complexes consisting of endogenous TIR-domain containing adaptors [73]. Another VACV protein A52R negatively regulates TLR signaling through interaction with TRAF6 and IRAK2 [74].

Pathogens use the host ubiquitin system for evasion [75]. IpaH9.8 protein from *Shigella flexneri* acts as an E3 ligase that targets NFκB essential modulator (NEMO) for degradation, and inhibition of NF-κB activation [76]. Pathogens also exploit Skp1–Cul1–F-box (SCF)-type ubiquitin ligase complexes involved in various cellular processes through multiple adaptors capturing target proteins [77]. For example, Vpr and Vif proteins encoded by HIV, target IRF3 for degradation presumably by the SCF complex [78].

Pathogens possess not only PAMPs but also virulence factors to subvert host immune systems. These factors manipulate host receptor crosstalk but sometimes aggressively utilize TLR signaling to inhibit immune cell maturation [79]. The *Mycobacterium tuberculosis* wall component, mannose-capped lipoarabinomannan (ManLAM), induces IL-10 production and impairs DC maturation by carbohydrate-specific signaling through the C-type lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) [80]. Mannose-containing ligands such as ManLAM trigger DC-SIGN to activate v-raf-leukemia viral oncogene 1 (Raf1), leading to acetylation of p65, which prolongs p65 DNA binding and enhances IL-10 transcription [81]. Interestingly, in contrast to mannose, fucose-containing ligands such as Lewis X, from *Helicobacter pylori*, dissociate Raf1-containing signal complexes and enhance IL-10 expression but downregulate IL-12 expression (in the case of mannose containing-ligands, IL-12 is also upregulated). Thus, DC-SIGN ligands have various affects on the modulation of TLR-induced cytokine responses [82].

Concluding remarks

The importance of TLR signaling for both immune homeostasis and for defense mechanisms against pathogens has emerged in the past decade. As such, it is clear that TLR function must be tightly regulated and many negative regulators of TLR signaling have been identified. These findings raise the question: why are there so many negative regulators to control TLR signaling? Loss of individual negative regulators of TLRs leads to hyperactivation of TLR signaling, indicating that individual negative regulators play a nonredundant role. Although overactivation of TLR signaling due to a loss of one negative regulator possibly increases expression of other negative regulators, these fail to suppress overactivation. This implies that each regulator is necessary but not sufficient to terminate TLR signaling, and that combinational or synergistic effects among negative regulators are required for full suppression. In addition, in many cases, multiple negative regulators target the same positive regulator, such as TRAF6, suggesting co-operation, which may explain the inability of different negative regulators to compensate for each other.

Although several negative regulators that target TIR-domain containing adaptors specifically control TLR signaling, other regulators such as A20 regulate TNF receptor signaling in addition to TLR signaling. Thus, a comprehensive analysis of the signaling crosstalk between TLRs and other receptors will be required to understand the complex regulation of TLR signaling by negative regulators. Furthermore, pathogens adopt deliberate strategies of subversion by suppressing TLR signaling.

Given their importance for immune homeostasis, it can be expected that polymorphisms of negative regulators are associated with autoimmune and inflammatory diseases.

Box 1. Therapeutic potential of TLR inhibitors

As mentioned previously in this review, negative regulation by specific regulators is crucial for immune homeostasis and its collapse often causes various diseases. As the importance of inhibitory TLR regulation is unveiled, our interest has focused on therapeutic manipulation of TLR signaling for the treatment of diseases that are derived from overactivation of innate immunity [83].

TLR antagonists, structural analogs of TLR ligands that interact with receptors but fail to initiate signal transductions, are being developed to treat excessive or chronic inflammation and autoimmunity [84]. One evident target example is sepsis characterized by whole-body inflammation caused by microbial infection. The most promising TLR4 antagonist, eritoran can limit excessive inflammatory responses induced by LPS and improve survival in mouse septic models [85]. Structural analysis suggests that antagonism by eritoran results from hydrophilic interactions between TLR4 and MD-2 [86], a TLR4-binding protein on the cell surface and crucial for eliciting TLR4 signaling [87]. Another example of a TLR4 antagonist, ibudilast (AV-411) has potential for treatment of neuropathic pain [88]. Ibudilast, which had been characterized and used as an anti-inflammatory drug based on phosphodiesterase inhibition, can also suppress glial cell activation by induction of IL-10.

TLRs that recognize nucleic acids (TLRs 3, 7, 8 and 9) have been implicated in the development of autoimmune disease by the aberrant recognition of self-derived nucleic acids [89]. The antimalarial drug chloroquine (a quinoline derivative) is a TLR9 antagonist and is currently used for treatment of systemic lupus erythematosus and rheumatoid arthritis. The affinity between TLR9 and CpG-DNA is affected by pH, suggesting that chloroquine blocks TLR9 signaling by perturbation of endosomal pH [90]. Other derivatives of quinoline and quinazoline can also inhibit TLRs [91,92] and autoimmune disease progression in animal models. Recently, a benzoxazole derivative, E6446, specifically inhibited TLR9 signaling and inhibited cytokine production [93]. E6446 also prevents severe experimental cerebral malaria induced by *Plasmodium berghei* ANKA and prolongs mouse survival. In addition, short DNA sequences and DNA-based compounds are also under development [94].

Adaptor proteins involved in TLR signaling are also targets for clinical treatment. Chemical compound screening has identified molecules that function as MyD88 inhibitors by mimicking the BB-loop structure in the TIR domain of MyD88. Although EM77 and EM110 presumably interrupt association between IL-1R and MyD88 [95], ST2825 inhibits MyD88 homodimerization [96]. These compounds may provide a new strategy for suppression of TLR signaling in addition to direct antagonism of TLRs.

Among these regulators, genetic variants around *TNFAIP3* (encoding A20) gene are well studied in relation to human diseases such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, diabetes, atherosclerosis and B-cell lymphomas [97]. It will be interesting to determine further how A20 and other negative regulators intersect with human disease.

Taken together, understanding negative regulation of TLR signaling may be helpful to develop methods of artificial manipulation of TLR signaling to restore inflammatory diseases, overcome uncontrolled inflammation, and make countermeasures against infection. Chemical inhibitors that suppress essential components for TLR signal activation have been developed (Box 1), however, to date, there has been little progress in therapeutic applications that target negative regulators of TLRs.

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