

## Toll-like receptor function and signaling

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Mammals sense pathogen invasion through pattern-recognition receptors. A group of transmembrane proteins, Toll-like receptors (TLRs), play critical roles as pattern-recognition receptors. They are mainly expressed on antigen-presenting cells, such as macrophages or dendritic cells, and their signaling activates antigen-presenting cells to provoke innate immunity and to establish adaptive immunity. Each TLR has common effects, such as inflammatory cytokine induction or upregulation of costimulatory molecule expression, but also has its specific function, exemplified by type I IFN-inducing ability. These immunoadjuvant effects are not only critical in antimicrobial immunity but are also involved in manifestations of autoimmunity. Furthermore, some TLR agonists are now promising therapeutic tools for various immune disorders, including allergy. Therefore understanding molecular mechanisms on TLRs should be quite useful in the development of therapeutic maneuvers against allergy and autoimmune diseases. (*J Allergy Clin Immunol* 2006;117:979-87.)

**Key words:** Pattern-recognition receptor, Toll-like receptor, dendritic cell, type I IFN, MyD88, allergy, adjuvant, plasmacytoid dendritic cell, CpG DNA

Host defense in mammals copes with pathogens through 2 types of immunity: innate and adaptive immunity. Innate immunity functions as a pathogen sensor and contributes to the eradication of pathogens and the

### Abbreviations used

|        |                                                             |
|--------|-------------------------------------------------------------|
| APC:   | Antigen-presenting cell                                     |
| CARD:  | Caspase activation and recruitment domain                   |
| DC:    | Dendritic cell                                              |
| ds:    | Double stranded                                             |
| IKK:   | I $\kappa$ B kinase                                         |
| IPS-1: | IFN- $\beta$ stimulator 1                                   |
| IRAK:  | IL-1 receptor-associated kinase                             |
| IRF:   | IFN regulatory factor                                       |
| MAMP:  | Microorganism-associated molecular pattern                  |
| MAPK:  | Mitogen-activated protein kinase                            |
| NF:    | Nuclear factor                                              |
| NOD:   | Nucleotide-binding oligomerization domain                   |
| PDC:   | Plasmacytoid dendritic cell                                 |
| PRR:   | Pattern-recognition receptor                                |
| RF:    | Rheumatoid factor                                           |
| RIG-I: | Retinoic acid-inducible gene I                              |
| RIP:   | Receptor interacting protein                                |
| SARM:  | Sterile $\alpha$ and armadillo motifs                       |
| SLE:   | Systemic lupus erythematosus                                |
| TAK:   | TGF- $\beta$ -activated kinase                              |
| TBK1:  | TANK-binding kinase 1                                       |
| TIR:   | Toll/IL-1 receptor homologous                               |
| TIRAP: | TIR domain-containing adapter protein                       |
| TLR:   | Toll-like receptor                                          |
| TRIF:  | TIR domain-containing adapter protein inducing IFN- $\beta$ |

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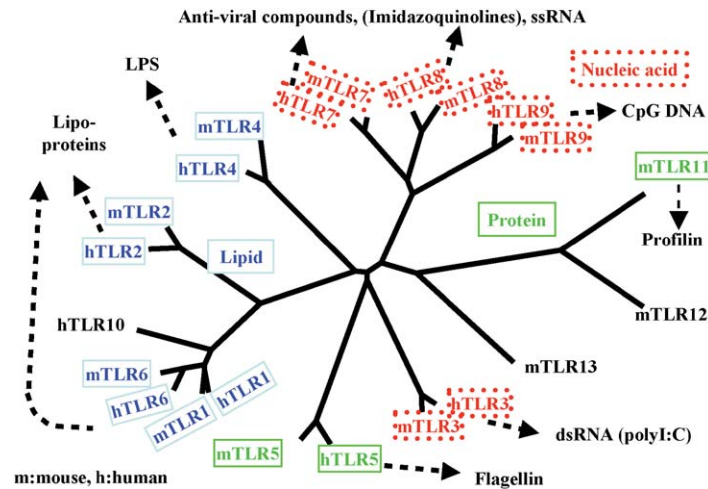
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establishment of adaptive immunity. These functions heavily depend on pattern-recognition receptors (PRRs).<sup>1</sup> Among PRRs, a group of transmembrane proteins, Toll-like receptors (TLRs), are featured by their potent immunoadjuvant ability to activate antigen-presenting cells (APCs).<sup>2,3</sup> TLRs include 10 (TLRs 1-10) and 12 (TLRs 1-9 and 11-13) family members in human subjects and mice, respectively (Fig 1). Most TLR ligands have been identified as a variety of molecular components derived from microorganisms. They can be categorized into lipid, protein, and nucleic acids, according to their ingredients. Importantly, all of these TLR ligands function as immune adjuvants. Principally, TLR signaling activates APCs to support T<sub>H</sub>1 cell differentiation. Blocking or augmenting TLR function can modify T<sub>H</sub>1/T<sub>H</sub>2 balance and



**FIG 1.** Phylogenetic tree of human and murine TLRs. Human and murine TLRs are connected with *solid lines* on the basis of the phylogenetic analysis of their amino acid structures. Branch length is proportional to evolutionary distances. *Dotted arrows* indicate representative ligands. Human TLR8 can function as a sensor, whereas there are no reports on the function of murine TLR8. *h*, Human; *m*, murine.

manipulate a variety of immune disorders, such as cancer, allergy, and autoimmunity. In this review we will summarize current knowledge of TLR function and signaling by relating them to some immune disorders.

## TLRs AS TRANSMEMBRANE SIGNALING PRRs

PRRs can be functionally classified into 2 classes: non-signaling and signaling PRRs. Nonsignaling PRRs include soluble factors or transmembrane proteins. Acute-phase proteins, such as C-reactive protein or lectins, are important soluble molecules that can bind to the invading microorganisms. Those microorganisms bound by these molecules are vulnerable to phagocytosis or recognized by the complement system followed by activation of protease cascades. Transmembrane proteins, such as scavenger receptors, also bind to the microorganisms. Then the organisms are internalized and transported to lysosomal compartments. These recognition systems do not principally activate the signaling cascades in innate immune cells.

Innate immune cells carry the molecules that can not only recognize the microorganisms but can also trigger the signaling pathways, leading to production of inflammatory cytokines or type I IFNs. These signaling PRRs include transmembrane and cytosolic proteins. TLRs are representative as transmembrane-signaling PRRs. Their extracellular domain includes a repetitive structure rich in leucine residues, called leucine-rich repeat, that is involved in the ligand recognition. The intracellular region contains a common structure in TLR and IL-1 receptor family members, called Toll/IL-1 receptor homologous (TIR) domain, which is essential for signal transduction.

The nucleotide-binding oligomerization domain (NOD) molecules Nod1 and Nod2 are cytosolic signaling PRRs.<sup>4</sup> Both Nod1 and Nod2 have leucine-rich repeat regions at

their carboxy terminus, thereby recognizing bacterial peptidoglycans. They also carry a Nod domain and a caspase activation and recruitment domain (CARD), which can stimulate signaling pathways that lead to the activation of nuclear factor (NF)  $\kappa$ B or mitogen-activated protein kinases (MAPKs). Retinoic acid-inducible gene I (RIG-I) and Mda-5 are also cytosolic signaling PRRs that can recognize double-stranded RNA (dsRNA; see below).<sup>5</sup>

## TLR LIGANDS

TLRs can recognize common molecular structures detected in certain groups of microorganisms (Fig 1). These were originally called pathogen-associated molecular patterns, but here we call them microorganism-associated molecular patterns (MAMPs) because they are found not only in pathogenic but also in nonpathogenic commensal microorganisms. Principally, toxins, which are involved in pathogenicity, are not recognized by TLRs. Furthermore, MAMPs are not expressed in the host and behave as nonself, although nucleic acids can act as nonself to some extent (see below).

MAMPs are generated through metabolic pathways specific for a group of microorganisms. MAMPs are required for the survival of microorganisms and therefore are highly conserved over the course of evolution. They are not as heterogeneous as viral proteins, the structure of which can be rapidly changed to escape immune surveillance. Therefore the structural stability of MAMPs enables germline-encoded PRRs to recognize MAMPs with a limited repertoire.

Representative MAMPs are bacterial cell-wall components. The cell walls of gram-negative bacteria contain LPS, an immune adjuvant identified first as a TLR ligand.<sup>6</sup> LPS is recognized by TLR4 and a TLR4-associated soluble factor, MD-2, derived from the host.<sup>7</sup> Gram-positive

bacteria do not carry LPS, but their cell walls contain a thick layer of peptidoglycans and a number of lipoproteins or lipopeptides. Mycoplasma does not possess cell walls, but its plasma membrane also contains several lipopeptides. These components also function as adjuvants and are recognized by TLR2 and its relatives, such as TLR1 or TLR6.

TLRs can also recognize proteins. TLR5 recognizes a protein, flagellin, which is a component of the bacterial flagella. Murine TLR11 recognizes a protozoan-derived profilin-like protein. These proteins are not as heterogeneous among the microorganisms.

Nucleic acids are also important MAMPs recognized by TLRs.<sup>8,9</sup> Bacterial and viral DNA contains a high frequency of unmethylated CpG motif, and this sequence confers immune adjuvant activity through TLR9. TLR7 and TLR8 are closely related to TLR9 and are involved in recognizing virus-derived single-stranded RNAs or synthetic antiviral compounds, such as imidazoquinoline derivatives.<sup>10</sup> Furthermore, dsRNA, which is generated in virally infected cells, is recognized by TLR3.

## ADJUVANT EFFECTS OF TLRs

The main functions of TLRs are induction of inflammation and establishment of adaptive immunity. TLR signaling can induce robust production of inflammatory cytokines, including IL-6 and TNF- $\alpha$ . These cytokines then activate surrounding cells to produce chemokines or adhesion molecules, thereby recruiting various inflammatory cells into the infection sites. Recruited macrophages or neutrophils are activated and ingest invading pathogens through internalizing PRRs. Subsequently, those cells kill them by producing nitric oxide, reactive oxygen species, or defensins. Thus inflammation is a critical local response to resolve infection. However, inflammation is a double-edged sword because excessive amounts of cytokines can be lethal for the host, as is the case in endotoxin shock.

TLR-activated APCs enhance expression of surface molecules and activate T cells, together with antigen presentation. CD4<sup>+</sup> T cells differentiate into T<sub>H</sub>1 or T<sub>H</sub>2 cells.<sup>11</sup> T<sub>H</sub>1 cells produce IFN- $\gamma$  and mediate antiviral or antibacterial immunity, whereas T<sub>H</sub>2 cells secrete IL-4, IL-13, or both and are involved in allergic reactions or immunity against helminths. TLR2 and TLR5 agonists can provoke T<sub>H</sub>2 immune responses in certain conditions. Furthermore, LPS can induce T<sub>H</sub>2 responses at low doses or when inhaled.<sup>12</sup> Most TLR ligands, however, stimulate APCs to produce T<sub>H</sub>1-inducing cytokines, such as IL-12 and IL-18, and to support T<sub>H</sub>1-skewed immune responses. Mutant mice lacking a TLR adapter, MyD88 (see below), fail to generate T<sub>H</sub>1 responses and rather mount T<sub>H</sub>2 responses when challenged with LPS.<sup>13</sup>

## MANIPULATION OF ALLERGIC DISEASES

Allergic diseases, such as asthma, are polygenic diseases, and their cause depends on both genetic and

environmental factors. In particular, increase of incidence for the last several decades should be caused mainly by environmental factors because it is hard to reconcile that genetic factors changed so rapidly. The hygiene hypothesis has proposed that a T<sub>H</sub>1 deviation on early exposure to bacterial or viral infection protects against allergic diseases by reducing the expression of T<sub>H</sub>2 cytokines and that increase of allergy incidence results from a decrease of infectious diseases in the early life of individuals because of improvement in lifestyle or sanitary conditions.<sup>14</sup> This hypothesis is, although controversial, supported by various epidemiologic or immunologic studies. On the basis of the hygiene hypothesis, microbial infection can shift the immune status to T<sub>H</sub>1 conditions and can reduce allergic T<sub>H</sub>2 reactions. In this context activation of TLR signaling instead of microbial infection is important as a therapeutic maneuver for allergy. In particular, TLR9 signaling activated by CpG DNA has the strongest activity to induce T<sub>H</sub>1 cell differentiation.<sup>15</sup>

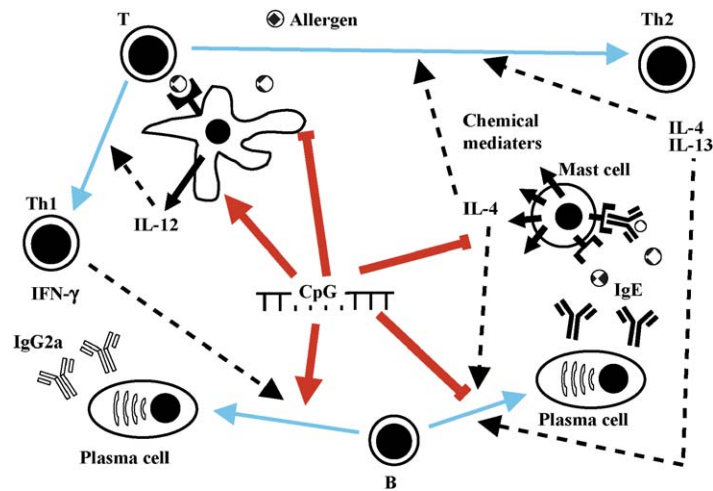
A number of reports support successful effects of CpG DNA in murine and primate models for allergen-induced airway hyperresponsiveness.<sup>16-19</sup> CpG DNA inhibited airway eosinophilia and airway hypersensitivity and deviated cytokine balances to T<sub>H</sub>1 status. This activity lasted 6 weeks in ragweed-induced asthma.<sup>18</sup> When conjugated with antigen, CpG DNA showed more potent and longer effects.<sup>20,21</sup> Conjugation also led to a diminished ability of antigen to react with IgE. Histamine release induced by antigen-CpG DNA conjugates was much lower than that induced by antigen alone. Thus conjugation is beneficial also in reducing the allergenicity.

It still remains unknown how CpG DNA improves manifestations (Fig 2). CpG DNA can activate APCs to promote T<sub>H</sub>1 cell support and inhibit T<sub>H</sub>2 cell activation by preventing APCs from presenting allergens to T<sub>H</sub>2 cells. CpG DNA can also inhibit IgE-dependent release of T<sub>H</sub>2 cytokines, especially IL-4, from mast cells.<sup>22</sup> It is also possible that CpG DNA can directly act on B cells to inhibit IgE production.<sup>23</sup>

Contrary to the hygiene hypothesis, certain infections can promote allergy status. For example, influenza virus infection enhanced allergen-specific asthma, possibly through T<sub>H</sub>1 responses during the acute viral infection.<sup>24</sup> Furthermore, it is important to note that T<sub>H</sub>2 responses themselves do not mean vulnerability or susceptibility to allergy. Helminth infection can manifest a protective effect against allergic responses.<sup>25-27</sup> Regulatory T-cell generation, rather than T<sub>H</sub>2-to-T<sub>H</sub>1 shift, is possibly involved in alleviating allergic inflammation.<sup>28</sup>

## NUCLEIC ACID TLR LIGANDS AND AUTOIMMUNITY

Compared with lipid or protein TLR ligands, nucleic acid TLR ligands, including CpG DNA, have distinct features in several aspects. Notably, nucleic acid TLR ligands show ambiguous distinction between the host and microorganisms. For example, LPS and flagellin are



**FIG 2.** Allergy pathogenesis and effects of CpG DNAs. Allergen induces  $T_H2$  cell differentiation.  $T_H2$  cytokines facilitate further  $T_H2$  cell differentiation and activate B cells to produce IgE. Allergen-IgE complexes bind to mast cells through the high-affinity Fc $\epsilon$ R and induce production of chemical mediators or IL-4.  $T_H2$  cell- or mast cell-derived IL-4 accelerates the vicious cycle to generate disease status. CpG DNAs act on several points in a positive or negative manner and dampen the manifestations.

apparently exogenous and are not found at all in the host. However, unmethylated CpG motifs can be found in mammals, although the frequency is much lower than in bacteria or viruses. Furthermore, host-derived single-stranded RNAs and dsRNAs can also function as TLR7 and TLR3 stimulators, respectively. Thus the structural and functional differences between pathogen and host nucleic acids are not very prominent. Host-derived nucleic acids are quite unstable but can be accessible to recognition by TLRs. For example, host cells are incorporated by certain APCs after they die, and nucleic acids from ingested cells are then released into endosomes or lysosomes. This can cause potential danger for autoimmunity. Such danger, however, is evaded through several mechanisms.

First, nucleic acid TLR ligands are recognized in different cellular compartments from lipid or protein TLR ligands. Lipid or protein TLR ligands are recognized on the plasma membrane, whereas nucleic acids are recognized by TLRs in the endosome. If manipulated to express on the plasma membrane, TLR9 can be stimulated by self DNA.<sup>29</sup> Thus endosomal expression of TLR7/9 can prevent self DNA from being recognized, although it is beneficial for recognizing viral DNA. Second, TLR7 expression is very low in cells with high phagocytic activity.<sup>30</sup> Third, the host possesses certain DNA sequences that can inhibit TLR9 signaling, for example in the telomere.<sup>31</sup> Fourth, compared with bacterial RNA, host-derived RNA carries abundantly modified nucleosides. These modified RNAs are much less potent in dendritic cell (DC) activation than unmodified RNA.<sup>32</sup> Furthermore, TLR7/9 expression and released nucleic acids can be localized in different cell compartments, although there is no formal proof of this. Thus several failsafe mechanisms prevent an autoimmune reaction.

Nucleic acid TLR ligands are also peculiar among TLR agonists in their ability to induce type I IFNs. Type I IFNs

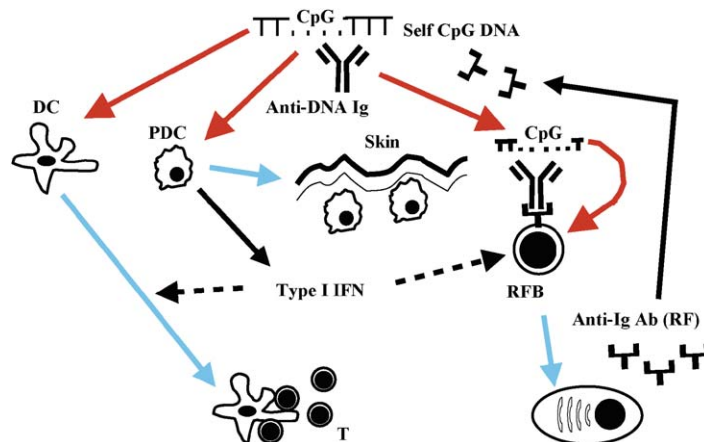
consist of more than 10 IFN- $\alpha$ s and a single IFN- $\beta$  and can induce expression of several antiviral proteins and MHC and induce DC maturation, thereby exerting a critical function in antiviral immunity. Nucleic acid TLR ligands can induce both IFN- $\alpha$  and IFN- $\beta$ . Lipid or protein TLR ligands fail to induce type I IFNs, although LPS is exceptional in that it can induce only IFN- $\beta$  and not IFN- $\alpha$ . This type I IFN-inducing ability greatly contributes to antiviral function in nucleic acid-recognizing TLRs.

Expression of nucleic acid-recognizing TLRs is confined to certain types of cells. Plasmacytoid DCs (PDCs) are a discrete DC subset from conventional DCs and are also known as type I IFN-producing cells.<sup>33</sup> PDCs express TLR7 and TLR9 exclusively among TLRs and secrete vigorous amounts of type I IFN, especially IFN- $\alpha$ , in a TLR7/9-dependent manner. PDCs play critical roles in antiviral immune responses. However, PDCs do not express TLR3 and fail to respond to dsRNA. TLR3-induced type I IFN production depends on other types of cells.

Several studies indicate that TLR activation leading to type I IFNs contributes to the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE).<sup>33</sup> In SLE antinuclear antibodies are produced and nuclear proteins are released from damaged or inflamed tissues. These proteins stabilize nucleic acids to act as efficient TLR agonists (Fig 3). Protein-nucleic acid complexes can activate PDCs to produce IFN- $\alpha$  by coengaging TLR and FcR. Furthermore, the number of activated PDCs is increased in skin lesions in patients with SLE. As a result, serum IFN- $\alpha$  levels are increased in patients with SLE and correlate with the severity of the disease. This hypothesis can also explain deterioration of SLE manifestations after viral infection, which can trigger PDC activation.

Autoreactive B cells can also be activated by protein-nucleic acid complexes (Fig 3).<sup>34</sup> Rheumatoid factor (RF)





**FIG 3.** Autoimmune induction by self nucleic acids. Self DNA containing a CpG motif activates DCs and PDCs. PDCs produce type I IFN and infiltrate in the skin. Type I IFN induces activation of DCs and polyclonal B cells, including autoreactive RF-positive B cells. RF B cells are also stimulated by coengaging anti-Ig B-cell receptor (*RF BCR*) and TLR9. Self RNA also forms a complex with ribonuclear proteins and activates the similar system through TLR7.

is an Ig that can bind to certain types of IgG and that is often found in the sera of patients with autoimmune disorders. DNA–anti-DNA complexes are recognized by RF on the B-cell surface. RF BCR (B-cell receptor) mediates internalization and transport of these complexes into the endosome, where TLR9 is expressed. Then CpG DNA in the complex stimulates TLR9 and, in turn, activates B cells to produce RF. Dual engagement of both BCR and TLR9 is mandatory for this type of autoreactive B-cell activation. Thus TLR signaling is critically involved in autoimmune pathogenesis.

### EFFECTS OF VARIOUS TYPES OF CpG DNA

The biologic effects of TLR9 signaling heavily depend on the nature of the ligands. At least 3 distinct classes of oligonucleotides containing CpG motif have been reported.<sup>35</sup> Conventional CpG DNA, also called K-type CpG DNA or CpG-B, has the potent ability to activate B cells and induce cytokine production from macrophages. K/B-type CpG DNA has multiple CpG motifs, and the activity is stabilized by phosphorothioate backbone. D-type CpG DNA, also called CpG-A, shows a weaker ability to activate B cells or macrophages but a stronger ability to induce type I IFN production from PDCs. D/A-type CpG DNA contains a single CpG motif, and the activity requires a mixed backbone of phosphodiester-phosphorothioate. Especially the region around a CpG motif should be palindromic and phosphodiester linked. In addition, poly-G tail should be included at the 3' end. C-type CpG DNA exhibit type I IFN-inducing, as well as B cell–activating, functions. It is comprised of phosphorothioate backbone with multiple CpG motif and TCG dimer at the 5' end.

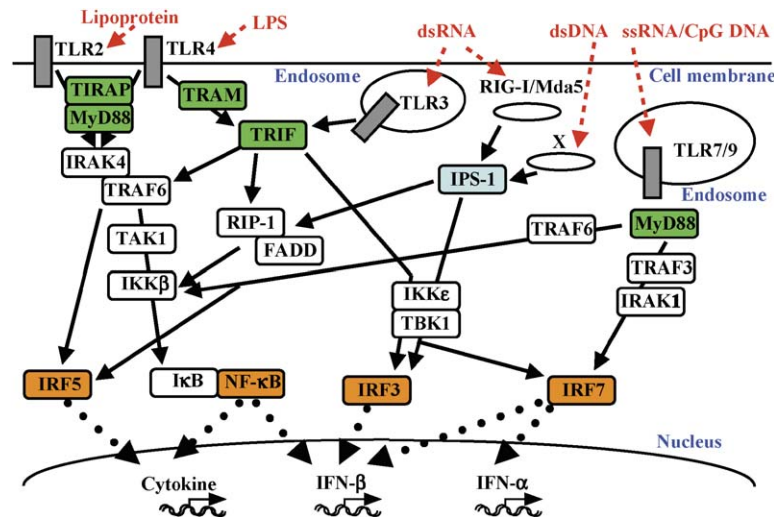
It is notable that the TLR9 system is different between human subjects and mice. First, in human subjects TLR9 expression is restricted to PDCs and B cells, whereas in

mice TLR9 expression is observed also in conventional DCs. Furthermore, responsiveness of PDCs to TLR9 agonists is also different. Human PDCs can secrete IFN- $\alpha$  but do not produce IL-12 in response not only to various TLR9 agonists but also to any other stimuli. Murine PDCs, however, can produce both IFN- $\alpha$  and IL-12 through TLR9 signaling. Interestingly, murine PDCs dominantly produce IFN- $\alpha$  and IL-12 in response to D/A- and K/B-type CpG DNAs, respectively. Despite these differences, the PDC system in human subjects and mice is common in terms of selective expression of TLR7/9 and the unique ability to produce IFN- $\alpha$  in response to TLR7/9 agonists.

It remains unknown how different TLR9 agonists lead to such distinct outcomes. The importance of their intracellular localization has been suggested.<sup>36</sup> K/B-type CpG DNA is rapidly transferred and degraded in the lysosome, whereas D/A-type CpG DNA is retained in the endosome of PDCs, together with signal-transducing molecules, such as MyD88 and IFN regulatory factor (IRF)-7, for long periods. If K/B-type CpG DNA is modified by cationic lipids to be retained in the endosome, it can induce type I IFN production, even from conventional DCs. Through G-tetrad formation of poly-G tail, D/A-type CpG DNA tends to self-assemble to a nanoparticle, which resembles the viral structure.<sup>37</sup> This particular structure contributes to intracellular localization of D/A-type CpG DNA.

### TLR SIGNALING PATHWAYS

TLR signaling can lead to activation of several transcription factors, including NF- $\kappa$ B and IRFs (Fig 4). Subsequently, expression of a variety of immune response genes is induced. Each TLR activates similar signaling pathways, but some TLRs trigger their specific pathways. This differential induction pattern heavily depends on cytoplasmic adapter molecules that can associate with the intracytoplasmic region of TLRs.<sup>38</sup>



**FIG 4.** TLR and RIG-I signaling pathways. TLRs activate the signaling pathways through TIR-domain containing adapters (green). TLR2 and TLR4 trigger the MyD88-dependent pathway, which is dependent also on TIRAP. The TLR9 pathway is MyD88 dependent. TLR3 and TLR4 can stimulate the MyD88-independent, TRIF-dependent pathway. RIG-I/Mda5 activates the IPS-1-dependent (light blue) cytosolic pathway. This pathway is also triggered by another cytosolic PRR (X) that probably recognizes dsDNA. These pathways lead to translocation of various transcription factors (orange) and activation of target genes.

### MyD88-dependent pathway in TLR2/4 signaling

There are 5 intracytoplasmic adapter molecules carrying the TIR domain. MyD88 was first found to be critical for TLR signaling.<sup>39</sup> MyD88 can associate with all TLRs except TLR3 and is required for induction of inflammatory cytokines, including IL-6 and IL-12p40. MyD88 has a death and TIR domain at its N- and C-terminal portion, respectively. A TIR domain is involved in interaction with TLRs, and a death domain recruits IL-1 receptor-associated kinase (IRAK) family members.<sup>40</sup> There are 4 IRAKs: IRAK-1, IRAK-2, IRAK-4, and IRAK-M. Among these, IRAK-4 is most critically involved in the MyD88-dependent pathway. TRAF6 is also critical for this pathway by activating TGF- $\beta$ -activated kinase (TAK) 1 through ubiquitination and the canonical I $\kappa$ B kinases (IKK) IKK $\alpha$  and IKK $\beta$ . The IKKs then phosphorylate I $\kappa$ B and render it degraded, thereby leading to the nuclear translocation and activation of NF- $\kappa$ B. The MyD88-dependent pathway can also lead to activation of MAPKs, such as p38 and JNK (not shown). These chain reactions finally result in gene expression of inflammatory cytokine genes.

TIR domain-containing adapter protein (TIRAP), also known as MyD88 adaptor-like, was subsequently identified.<sup>41,42</sup> TIRAP-deficient mice showed similar impairment to MyD88-deficient mice in terms of TLR2- and TLR4-induced effects. MyD88/TIRAP double-deficient mice did not show further defects compared with single-deficient mice, indicating that TIRAP functions in the MyD88-dependent pathway together with MyD88 downstream of TLR2/4 signaling. TIRAP is not required for TLR7/9 signaling (see below).

### MyD88-independent pathway in TLR3/4 signaling

The diversity of TLR signaling pathway was first revealed by the retained response of MyD88-deficient mice to LPS. TLR4 signaling can activate NF- $\kappa$ B and MAPKs in MyD88-deficient cells, albeit with delayed kinetics.<sup>39</sup> These delayed responses fail to induce gene expression of inflammatory cytokines but are sufficient for DC maturation or expression of IFN- $\beta$  and IFN-inducible genes. IFN- $\beta$  gene expression requires IRF-3 activation, which is detected in LPS-activated MyD88-deficient macrophages.

The function of TIR domain-containing adapter protein inducing IFN- $\beta$  (TRIF), also called TICAM-1, was revealed by means of analysis on TRIF-deficient mice.<sup>43</sup> This analysis first clarified the MyD88-independent pathway in TLR signaling (Fig 2). Through TRIF, TLR3 and TLR4 can activate this pathway, leading to IRF-3 or NF- $\kappa$ B activation. TLR3 can directly associate with TRIF, whereas TLR4 requires another adapter, TRIF-related adapter molecule,<sup>44</sup> also known as TICAM-2, for the association with TRIF. TRIF can interact with the non-canonical I $\kappa$ B kinases IKK $\epsilon$  and TANK-binding kinase 1 (TBK1). IRF-3 is phosphorylated by these kinases, translocates to the nucleus, and induces several target genes, including the IFN- $\beta$  gene.

TRIF can also lead to NF- $\kappa$ B activation through TRAF6. Furthermore, TRIF can also interact with receptor-interacting protein (RIP) 1 through an RIP homotypic interaction motif. This interaction is also critical for NF- $\kappa$ B activation.<sup>45</sup> NF- $\kappa$ B activation leads to gene expression of IFN- $\beta$  and inflammatory cytokines.

Sterile  $\alpha$  and armadillo motifs (SARM) is the fifth adapter containing the TIR domain. A nematode ortholog

of SARM, TIR-1, was found to be critical for TLR-independent innate immunity.<sup>46</sup> However, the function of mammalian SARM is not clear at present.

Both MyD88 and TRIF deficiency lead to defective NF- $\kappa$ B activation in response to TLR4 signaling. However, the underlying mechanism for the defect is different.<sup>47</sup> MyD88 deficiency lacks earlier activation, whereas TRIF deficiency shows impairment in late activation. LPS can induce rapid production of TNF through TRIF-dependent IRF-3 activation. TNF then activates NF- $\kappa$ B in an autocrine manner, which is responsible for delayed activation. This mechanism can account for impairment in late NF- $\kappa$ B in the absence of TRIF, but it is hard to reconcile retained activation of late NF- $\kappa$ B without MyD88 because TNF is not produced from LPS-stimulated MyD88-deficient cells. It can be assumed that trace amounts of TNF in the absence of MyD88 can contribute to late NF- $\kappa$ B activation on the basis of their finding that anti-TNF can decrease nuclear NF- $\kappa$ B activity.<sup>47</sup>

### MyD88-dependent pathway in TLR9 signaling

TLR7 and TLR9 signaling can induce not only inflammatory cytokines but also type I IFNs, including IFN- $\alpha$  and IFN- $\beta$ . All of these effects are dependent on MyD88<sup>48</sup> and do not require any other MyD88-related adapters. TRIF is dispensable for this type I IFN gene induction, although it is essential for TLR4-induced IFN- $\beta$  expression. Furthermore, IKK $\epsilon$  and TBK1 are also dispensable for TLR7/9-mediated induction of type I IFN genes.

Downstream of MyD88, signaling pathways are bifurcated into NF- $\kappa$ B- and IRF-7-activating pathways (Fig 4). The latter pathway is evident in PDCs. NF- $\kappa$ B is required for inflammatory gene expression, whereas IRF-7 is essential for type I IFN gene induction. Both transcription factors reside in the cytoplasm as inactive forms and are translocated into the nucleus on stimulation.

IRF-7 colocalizes with MyD88 in endosomal vesicles, whereas IRF-3 does not.<sup>49,50</sup> IRF-7 can directly associate with MyD88 or TRAF6, forming a large molecular complex that leads to type I IFN production. IRF-7 is critically and selectively involved in this pathway because IRF-7 deficiency severely impaired the ability of TLR7/9-stimulated PDCs to produce type I IFNs but not other cytokines.<sup>51</sup> IRAK-1 also associates with IRF-7 and is critical for IRF-7 activation and induction of type I IFN genes. Similar to IRF-7 deficiency, IRAK-1 deficiency does not lead to defective production of inflammatory cytokines.<sup>52</sup>

It is still unclear how the molecular complex including MyD88, IRAK-1, and IRF-7 leads to NF- $\kappa$ B or IRF-7 activation. Interestingly, TRAF-3 deficiency also led to similar defective phenotype in TLR7/9 signaling to that of IRF-7- or IRAK-1-deficient mice in terms of selective impairment in type I IFN induction.<sup>53,54</sup> TRAF3 seems to function as a molecular rudder to steer into type I IFN induction. TRAF3 deficiency also leads to defective production of IL-10, which in turn causes overproduction of inflammatory cytokines. TRAF3 seems to play broader critical roles not only in TLR7/9 signaling but also in

TLR3/4 and TLR-independent cytosolic pathways (see below) through the interaction with TRIF or IKK $\epsilon$ /TBK1 (not depicted in Fig 4).<sup>53,54</sup>

### IRFs in TLR signaling

In addition to IRF-3 and IRF-7, critical roles of other IRFs have been also revealed. IRF-5 also colocalizes and associates with MyD88 and TRAF6.<sup>55</sup> In TLR7/9 signaling IRF-5 was required for induction of inflammatory cytokines but not for type I IFN induction. NF- $\kappa$ B activation is intact, at least when analyzed with a gel mobility shift assay, in IRF-5-deficient cells, indicating that IRF-5 is dispensable for NF- $\kappa$ B activation and that both IRF-5 and NF- $\kappa$ B are required for production of inflammatory cytokines. Furthermore, IRF-5 is essential for other TLR-induced cytokine production as well.<sup>55</sup>

MyD88 also interacts with another IRF, IRF-4. IRF-4 negatively regulates the signaling pathway.<sup>56</sup> IRF-4 deficiency does not affect the ability of TLR7/9-stimulated PDCs to secrete IFN- $\alpha$  but caused overproduction of inflammatory cytokines. This was accompanied by enhanced activation of NF- $\kappa$ B and MAPKs. This hyperreactivity is observed not only in TLR7/9 but also in TLR2/4 signaling. IRF-4, but not IRF-7, can compete with IRF-5 for association with MyD88, which can account for the phenotype of IRF-4-deficient mice. Thus IRF family members are critically involved in TLR signaling.

### CYTOSOLIC PRRs FOR NUCLEIC ACIDS

The TLR-independent cytosolic PRR system for nucleic acids has been clarified recently<sup>5</sup> and was first described for the dsRNA recognition system.<sup>57</sup> dsRNA is not only recognized by TLR3 but also by the cytosolic molecules RIG-I and Mda5. RIG-I and Mda5 are RNA helicases and carry 2 CARD-like domains and 1 helicase domain. The helicase domain is involved in recognizing dsRNA, and CARD-like domains are critical for activating downstream signaling pathways. The pathways lead to activation of NF- $\kappa$ B and IRF-3, thereby inducing inflammatory cytokines and type I IFNs. IFN- $\beta$  stimulator 1 (IPS-1), also called mitochondrial antiviral signaling protein, virus-induced signaling adaptor, or CARD adaptor inducing IFN- $\beta$ , is a critical adapter molecule that can interact with RIG-I/Mda5 through the homophilic interaction of their CARD-like domains.<sup>5</sup> IPS-1 activates NF- $\kappa$ B through Fas-associated death domain protein and RIP-1. IPS-1-induced IRF3 activation requires IKK $\epsilon$ /TBK-1. Thus IKK $\epsilon$  and TBK-1 are converging points between TLR3/4 and the RIG-I/Mda5 pathway.

Not only dsRNA but also dsDNA can also activate the IPS-1-mediated pathway, although recognition molecules for dsDNA have not been identified.<sup>58</sup> Notably, several pieces of evidence suggest that plasmid DNA vaccines can exhibit the adjuvant activity through a TLR9-independent, as well as a TLR9-dependent, pathway.<sup>59,60</sup> This cytosolic PRR system should be involved in the TLR9-independent pathway.

## CONCLUSION

The TLR system has been rapidly and extensively clarified for the last decades. TLRs are critically involved not only in antimicrobial immunity but also in certain immune disorders, such as autoimmunity. Some TLR ligands, especially nucleic acids, can be synthesized *in vitro* and promisingly applied for antiallergic vaccines. Furthermore, the non-TLR system for nucleic acid recognition has also been gradually revealed. Clarification of these mechanisms should contribute to further development and improvement of therapeutic strategies against various types of immune diseases.

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