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Review

Host Innate Immune Receptors and Beyond: Making Sense of Microbial Infections

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The complexity of the immune system mirrors its manifold mechanisms of host-microbe interactions. A relatively simplified view was posited after the identification of host innate immune receptors that their distinct mechanisms of sensing "microbial signatures" create unique molecular switches to trigger the immune system. Recently, more sophisticated and cooperative strategies for these receptors have been revealed during receptor-ligand interactions, trafficking, and intra- and intercellular signaling, in order to deal with a diverse range of microbes. Continued mapping of the complex networks of host-microbe interactions may improve our understanding of self/non-self discrimination in immunity and its intervention.

Host Innate Immune Receptors

Host-microbe interactions are essential for many aspects of the normal physiologies of both types of organisms, ranging from metabolic activities to immune homeostasis (Dethlefsen et al., 2007). Despite this mutual relationship after long-term coevolution, infectious diseases and their secondary effects have always been one of the biggest threats to humans and are still the second major cause of death (Fauci, 2006). Moreover, economic and environmental changes in human lifestyles have significantly influenced host-microbe interactions in many ways, driving us to face more than 30 emerging pathogens during the past 30 years. Therefore, we urgently need to devise novel strategies for intervening in such pathogen emergence through better understanding of the manifold mechanisms of host immune responses to, and manipulation by, microbial infections.

Over the past decade, a large number of studies in immunology and microbiology have revealed pivotal roles of the host innate immune system in sensing microbial infections via specific innate immune receptors, which are germline encoded and consist of a "recognition" domain and a protein-protein-interacting region for downstream signaling. These receptors act as a molecular switch to trigger innate immune activation and tightly regulate the subsequent adaptive immune responses to microbial infections (Medzhitov, 2007). In mammals, Toll-like receptors (TLRs) are the best characterized examples (Takeda et al., 2003; Beutler, 2004). In addition, nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (Inohara et al., 2005; Fritz et al., 2006; Martinon and Tschopp, 2007), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Yoneyama et al., 2007), and some C-type lectin receptors (CLRs) (Geijtenbeek et al., 2004; Robinson et al., 2006; Willment and Brown, 2008) recognize specific components of microbes and are therefore included among the innate immune receptors (Figure 1).

The innate immune receptors are widely distributed in or on a variety of cell types and can detect a wide range of ligands derived from either pathogenic or nonpathogenic microbial infections. In particular, TLRs are localized at the plasma membrane and can sense microbial products such as bacterial cell wall components or viral proteins at the level of the cell surface or microbial nucleic acids exposed within "vesicular" compartments such as endosomes and/or phagosomes. On the other hand, RLRs and NLRs reside in the cytoplasm, probably in the cytosol, and serve as sensors for intracellular microbial invasion. Interestingly, ligands for RLRs and NLRs are often shared with TLRs (Table 1), although the molecular bases of their receptor-ligand interactions and outcomes are quite different. Although CLRs comprise a large family of proteins containing one or more C-type lectin domains with quite diverse functions, some of them are directly involved in innate immune recognition of microbial products for the innate immune system (reviewed in Geijtenbeek et al., 2004; Robinson et al., 2006; Willment and Brown, 2008). As more evidence accumulates, we continue to learn that receptor-mediated recognition of microbes can cooperate at the level of ligand recognition and the intracellular and intercellular signaling pathways (Table 1). Here, we review recent progress in research on host-microbe interactions through the relatively simplified, yet manifold, recognition machinery of host innate immune receptors.

Molecular Basis of TLR-Ligand Interactions

Toll in *Drosophila melanogaster* was initially discovered as an essential receptor for embryonic patterning and was subsequently identified as a critical component of host defense against fungal and Gram-positive bacterial infections (reviewed in Leulier and Lemaitre, 2008). Since then, its mammalian homologs, designated TLRs, have been identified and functionally characterized as critical components of the innate immune system (Takeda et al., 2003; Beutler, 2004). TLRs are type I transmembrane proteins characterized by an ectodomain that contains varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor

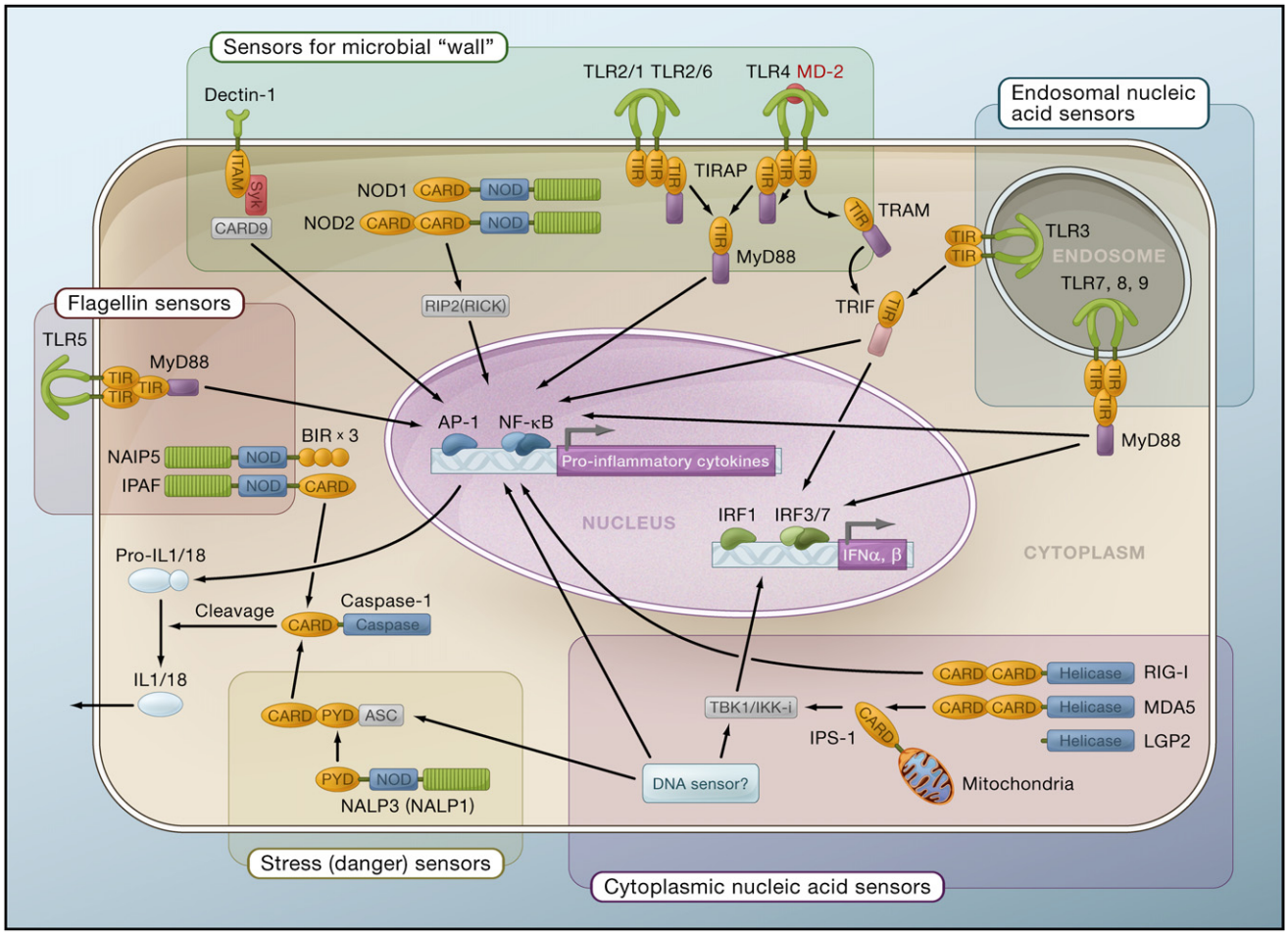

Figure 1. Sensing of Microbial Signatures by Host Innate Immune Receptors

Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs), and C-type lectin receptors (CLRs) are expressed on and in a variety of cells in host tissues that recognize a diverse range of microbial signatures. They cooperate to recognize a variety of microbial infection by sensing microbial cell walls, bacterial motor flagellin, microbial (and modified host) nucleic acids, or stress (danger)-induced molecules on the cell surface, in the endosomes, or in the cytosol. Bacterial or fungal cell wall, for example, can be recognized on the cell surface by TLR2 dimerized with either TLR1 or TLR6 through the TIR-containing adaptor MyD88 and TIRAP, and the CLR dectin-1 through its adaptor CARD9, while their intracellular recognition occurs via NOD1 and NOD2 in a RIP2-dependent signaling pathway, all of which can cooperate to activate cells through activator protein-1 (AP-1), NF-κB, and MAPK activation. TLR4 homodimers with an LPS-binding accessory molecule (MD-2) recognize LPS and viral proteins and activate cells through MyD88 and TIRAP via the cell surface or TRAM and TRIF via the endosomal pathway. Bacterial flagellin is recognized by TLR5 and two NLRs (NAIP5 and IPAF), among which TLR5 is on the cell surface and activates NF-κB pathways through MyD88, whereas NAIP5 and IPAF sense flagellin in the cytosol, resulting in caspase-1 activation and the secretion of IL-1β, IL-18, and possibly IL-33. Other NLRs, such as NALP3, can recognize a variety of microbial signatures or even host molecules derived from damaged or dying cells, including bacterial toxins, nucleic acids, and uric acids. This activation leads to caspase-1 activation along with ASC, inducing processing of pro-IL-1β and pro-IL-18 and resulting in the release of IL-1β and IL-18, respectively. The other innate immune receptors are nucleic acid sensors, among which TLR3, TLR7, TLR8, and TLR9 recognize microbial and/or modified host nucleic acids in the endosome, and RLRs such as RIG-I, MDA5, and LGP2 as well as uncharacterized DNA sensor(s) detect nucleic acids in the cytoplasm, most probably in the cytosol. TLR3- or TLR7/9-mediated signaling pathways are through TRIF and MyD88, respectively, resulting in the induction of proinflammatory cytokines and type I IFNs. RLR-signaling occurs through IPS-1 in the mitochondria to activate NF-κB and IRF3/7 to produce type I IFNs and chemokines. Green-colored horseshoe-like and rectangular shapes indicate receptor dimerization and recognition domain composed of leucine-rich-repeat motifs (LRR). Yellow oval circles (TIR, CARD, PYD, BIRx3, and ITAM) indicate protein-protein-interacting regions for downstream signaling. Pink rounded rectangular shapes indicate adaptor molecules (MyD88, TRIF, TIRAP, TRAM) in the downstream signaling.

(IL-1R), termed the Toll/IL-1R homology (TIR) domain (Figure 2A). Mammalian TLRs consist of at least 11 members that recognize not only microbial-specific elements, including proteins, lipids, and nucleic acids derived from viruses, bacteria, parasites, and fungi, but also damaged host cell components, such as nucleic acids (Table 1).

The issues of why and how TLRs recognize such a diverse range of microbial ligands, which are structurally and biochemically distinct, have been enigmatic. Recently, crystal structural analyses of the ectodomains of some TLRs have revealed their shape and how they bind to their cognate ligands. TLR3 (Choe et al., 2005; Bell et al., 2006), TLR4, and a heterodimer of TLR2 and TLR1 (Kim et al., 2007; Ohto et al., 2007; Jin et al., 2007) were confirmed to have horseshoe-like solenoid shapes consisting of 18 to 25 tandem copies of LRR motifs with 20 to 30 characteristically spaced hydrophobic amino acid residues (Figure 2A). These LRR motifs are responsible for both receptor dimerization and ligand recognition. In fact, direct binding of

Table 1. Host Innate Immune Receptors of Microbial Signatures

	Microbial Signature	TLRs (Transmembrane)	RLRs (Cytoplasm)	NLRs (Cytoplasm)	CLRs (Membrane-Bound)
Viruses	structural proteins (i.e., capsid, envelope proteins)	TLR2, TLR4	N.D.	N.D.	N.D.
	DNA	TLR9	DAI?	NALP3? (through ASC)	FcγR
	RNA	TLR3, TLR7, TLR8	RIG-I, MDA5, LGP2	NALP3	FcγR
Bacteria	cell wall components (LPS, peptidoglycan, lipoteichoic acid, lipoproteins)	TLR2/1, TLR2/6, TLR4	N.D.	NOD1, NOD2, NALP1, NALP3	collectins (MBL)
	flagellin	TLR5	N.D.	IPAF, NAIP5	N.D.
	perotoxins	N.D.	N.D.	NALP3	N.D.
	DNA	TLR9	N.D.	ASC	N.D.
	RNA	N.D.	N.D.	NALP3	N.D.
Protozoan parasites	GPIs	TLR2, TLR4	N.D.	N.D.	N.D.
	malarial hemozoin	TLR9	N.D.	N.D.	N.D.
	proteins (*T. cruzi* Tc52, profilin)	TLR2, TLR11	N.D.	N.D.	N.D.
	DNA	TLR9	N.D.	N.D.	N.D.
Helminths	lipids	TLR2	N.D.	N.D.	N.D.
	RNA	TLR3	N.D.	N.D.	N.D.
Fungi	Cell wall components (i.e., GlcNAc, mannan, β-glucan)	TLR2, TLR4, TLR6	N.D.	N.D.	mannose receptor, DC-SIGN, Dectin 1 and 2, CARD9
	DNA	TLR9	N.D.	N.D.	N.D.

There are specific and shared microbial signatures, such as bacterial cell wall components and nucleic acids, respectively, most (if not all) of which are recognized by host innate immune receptors categorized into TLRs, NLRs, RLRs, and CLRs. N.D., not determined. FcγR, Fc receptors for IgG. GlcNAc, N-acetylglucosamine.

TLR2-TLR1 complexes, TLR3, TLR5, and TLR9 to their cognate ligands has been demonstrated, whereas TLR4 binds lipopolysaccharide (LPS) through an associated molecule (MD-2) (Jin et al., 2007; Leonard et al., 2008; Andersen-Nissen et al., 2007; Haas et al., 2008; Ohto et al., 2007).

Direct binding between TLRs and their cognate ligands is essential, but not sufficient for sequential activation. Although TLRs form homodimers or heterodimers before ligand stimulation (Triantafilou et al., 2006), further structural modifications of these TLR dimers seem to be necessary for their activation of downstream signaling via the TIR domains (Gay et al., 2006) (Figure 2B). Direct binding of triacylated lipopeptides to the C termini close to the ectodomain of the TLR2-TLR1 heterodimer appears to induce rearrangement of the preformed and weakly

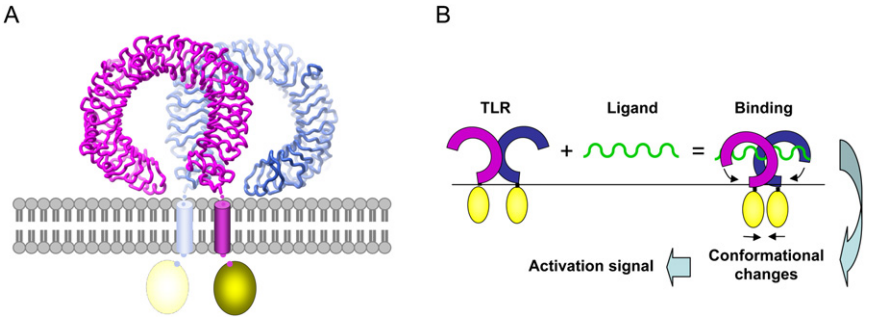

Figure 2. Molecular Basis of TLR-Ligand Interaction

(A) A schematic model of TLR dimer. TLRs are type I transmembrane proteins characterized by an ectodomain that contains horseshoe-like solenoid shapes consisting of 18 to 25 tandem copies of LRR motifs with 20 to 30 characteristically spaced hydrophobic amino acid residues, a transmembrane region, and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain.

(B) TLRs form a dimer through the C-terminal region of the extracellular domain. LRR motifs are responsible for TLR binding of the ligand, but only the agonist, not antagonist, induces conformational changes of the extracellular domain, allowing the subsequent activation through intracellular TIR domain.

bound dimer to a much more rigid and stabilized structure (Jin et al., 2007), which seems to be critical for optimal interactions between the cytoplasmic TIR domains and adaptor proteins, such as myeloid differentiation primary response protein 88 (MyD88). In the case of TLR9, which can bind to single-stranded (ss) DNA containing either agonistic CpG motifs or antagonistic G-rich "inhibitory" sequences (forming a G-tetrad structure), only the agonistic CpG sequence confers conformational changes in the TLR9 ectodomain that brings the two cytoplasmic TIR domains together in the dimer (Latz et al., 2007) (Figure 2B). In this regard, a recent report demonstrated that the sugar-backbone 2′-deoxyribose of DNA binds to TLR9 and is sufficient as a ligand to act as a "mild agonist," and that this agonistic activity is further enhanced by the presence of a CpG motif (Haas et al., 2008). Notably, a phosphorothioate-modified DNA backbone (resistant to deoxyribonuclease [DNase]), on which most studies of TLR9 have been based, actually acts as an antagonist unless the base contains CpG motifs (Haas et al., 2008). As more pieces of evidence continue to accumulate, more questions are being raised, such as why and how agonists, but not antagonists, induce conformational changes of TLRs and how the LRR motifs of each TLR confer ligand-specificity and/or flexibility. Clarification of these issues will be of considerable interest, since the LRR motifs can interact with a variety of ligands such as proteins, lipids, carbohydrates, and nucleic acids.

Factors that Influence the "Microbial Signatures" of the TLR Ligands

Direct recognition of the ligands by TLRs, as described above, were demonstrated; however, there seem to be many other steps in which the host innate immune system distinguishes microbes as nonself and host cells as safe self. In fact, microbial infections change the microenvironments of infected sites in the body, including tissue damage. In such cases, recognition of ligands by the LRR motifs of TLRs can be influenced by many cofactors, which play important roles in regulating TLR-mediated innate immune responses to microbes, or in some cases, to host self-components. In particular, TLR7/8 and TLR9 recognize not only microbial nucleic acids but also host RNA and DNA, and additional factors can skew TLR recognition of microbial versus host nucleic acids (Marshak-Rothstein and Rifkin, 2007). For example, host DNA can form a complex with anti-DNA or anti-nuclear (chromatin) antibodies in the case of systemic autoimmune diseases such as systemic lupus erythematosus, with HMGB1, a nuclear DNA-binding protein released from necrotic cells during tissue damage (Tian et al., 2007), or with the antimicrobial peptide cathelicidin, also known as LL37 (Lande et al., 2007). All of these complex formations can stabilize DNA to protect it against degradation and facilitate its uptake by immune cells, thereby turning host self-DNA, which is generally inert, into a potent TLR9 agonist (Figure 3). As a result, host DNA can trigger, or at least modify, autoimmune responses through increased type I interferon (IFN) production by plasmacytoid dendritic cells (pDCs) and/or polyclonal activation of autoreactive B cells. Recently, cathepsins, which are lysosomal cysteine proteases, were reported to be other factors required for TLR9 responses (Asagiri et al., 2008; Matsumoto et al., 2008). Although the precise mechanisms remain to be fully clarified, the data obtained suggest that the proteolytic activities of these enzymes play roles

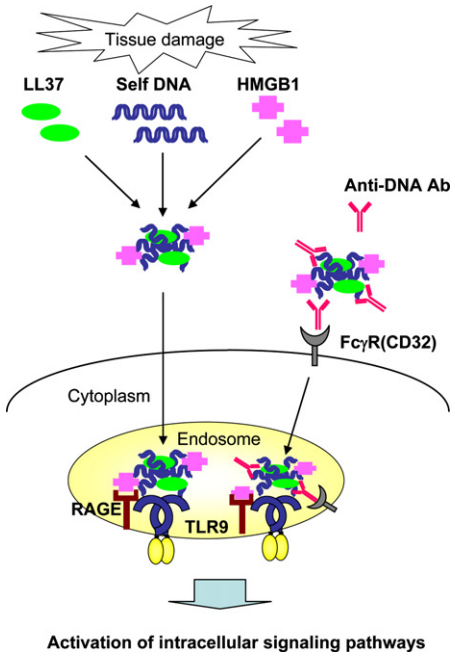

Figure 3. Factors that Influence Microbial Signatures of TLR Ligands
Microbial infections change the microenvironments of infected sites in the body, including tissue damage. Such events can modify the characteristic of TLR ligands. For example, TLR9 activation by host-derived DNA can be enhanced by the presence of cofactors such as HMGB1, a nuclear DNA-binding protein released from necrotic cells during tissue damage (Tian et al., 2007), or the antimicrobial peptide cathelicidin, also known as LL37 (Lande et al., 2007), or in the presence of anti-DNA or anti-nuclear (chromatin) antibodies (Ab). HMGB1's receptor RAGE and the FcγR receptor (CD32) for IgG are involved in this process.

in TLR9-DNA recognition. Thus, the agonistic, inert, or antagonistic activities of TLR-ligand interactions do not seem to be determined by the origins of the ligands, which are rather controlled by the immunological milieu of the tissue microenvironment and may instead depend on a variety of factors including the type, magnitude, and duration of the infection as well as damage to the host tissues and cells.

Spatial Regulation of TLRs: Adaptor Multitasking

As described above, the cytoplasmic TIR domains of TLRs represent a platform for downstream signaling (Figure 1), which involves recruitment and interactions with TIR domain-containing adapters, including MyD88, TIR domain-containing adaptor (TIRAP, also known as MAL), TIR domain-containing adaptor inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2), and sterile alpha and HEAT-Armadillo motifs (SARM) (O'Neill and Bowie, 2007). While all TLRs activate nuclear factor-kappaB (NF-κB) through MyD88- or TRIF-dependent pathways (TLR3) or both pathways (TLR4) for the production of proinflammatory cytokines, type I IFN (IFN-α, β) inductions are differentially regulated, since TLR4 and TLR3 activate interferon regulatory factor 3 (IRF3) through TRIF, while TLR7 and TLR9 activate IRF7 through MyD88 (Figure 4).

Recent studies have revealed new roles for TIRAP and TRAM, in which they function as sorting adaptors for the recruitment of

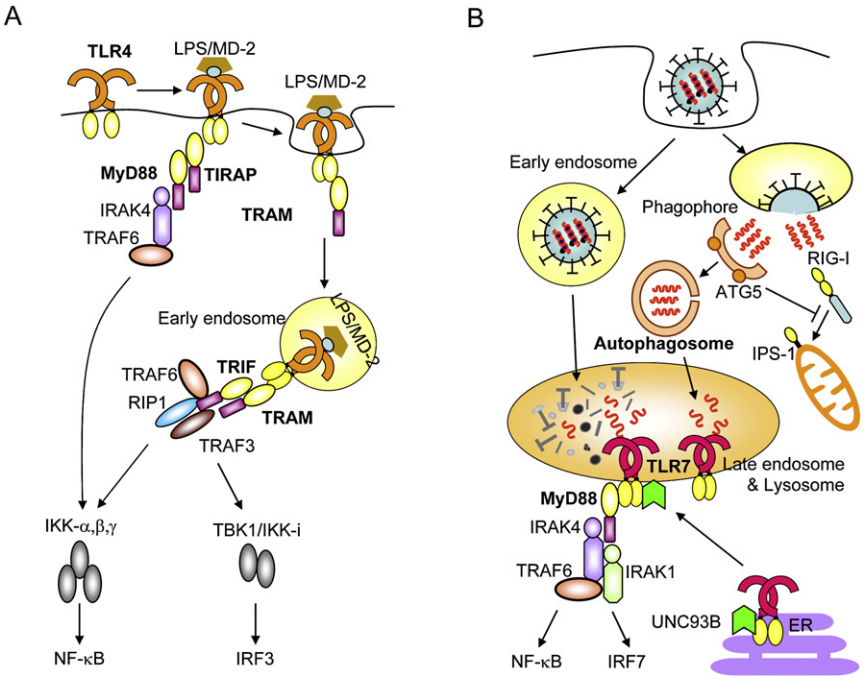

Figure 4. Intracellular Trafficking of TLRs and Their Ligands

(A) Controlled by cell surface sorting adaptor TIRAP, TLR4 can be engaged with LPS-MD-2 complex on the cell surface, then signal through the MyD88 complex with the kinase IRAK4 and signal transducer TRAF6 to activate NF-κB early through IKK-α,β,γ kinase. TRAM, another adaptor protein, acts as sorting adaptor for TLR4 into the early endosome and subsequent recruitment of TRIF and the activation of "late" NF-κB via TRAF6 and RIP1, and that of IRF3 via the signal transducer TRAF3.

(B) In the case of viral infection (e.g., VSV), viral ssRNA is recognized by TLR7 from plasmacytoid DCs (pDCs) or by RIG-I from most other cell types. Viral RNA was delivered to a TLR7-expressing vesicle via endosomal uptake, but pDCs have constitutive autophagy induction that can capture viral RNA replicated in the cytosol via an ATG5-dependent manner and deliver the RNA into a TLR7-expressing late endolysosome. ATG5 also acts as a negative regulator of RIG-I-mediated viral RNA recognition, and type I IFN is subsequently produced through IPS-1. UNC93B is a necessary molecule for recruiting TLR7 (and TLR3 and TLR9) from the endoplasmic reticulum (ER) to the endosome. Once ssRNA is recognized by TLR7, NF-κB and IRF7 are activated via a MyD88 complex with IRAK4, IRAK1, and TRAF6.

MyD88 to TLR4 on the cell surface and TRIF to endosomal TLR4, respectively, via unique mechanisms, and these roles can explain their distinct signaling cascades in spatial and temporal manners (Kagan and Medzhitov, 2006; Kagan et al., 2008) (Figure 4A). Moreover, TIRAP was shown to possess another function, involving its cleavage by caspase-1. Specifically, caspase-1 is required for the ability of TIRAP to activate NF-κB, whereas TIRAP is not required for caspase-1 activation, suggesting intracellular crosstalk between TLR signaling and caspase-1, while its physiological role remains to be confirmed in vivo (Miggin et al., 2007). SARM inhibited the TRIF-dependent pathway in human cell lines (Carty et al., 2006), and its physiological relevance also remains to be determined. All of these newly revealed functions of TLR adaptor molecules not only suggest the importance of the location in which the initial signal occurs, but also imply their potential interactions with other innate immune signaling and/or membrane sorting machineries.

TLR Trafficking from the Endoplasmic Reticulum to Endosomes and Autophagosomes

Distinct from TLRs on the cell surface such as TLR1, TLR2, TLR4, TLR5, TLR6, and probably TLR10 and TLR11, TLRs that recognize nucleic acids such as TLR3, TLR7, TLR8, and TLR9 are not on the cell surface, but rather found in the endoplasmic reticulum (ER). These nucleic acid-sensing TLRs are thus delivered to subcellular location (Figures 3 and 4B). In this regard, the recently identified ER protein UNC93B1 found by a forward genetic approach (Tabeta et al., 2004) turns out to be associated with TLR3, TLR7, TLR9, and TLR13 (Brinkmann et al., 2007) and essential for trafficking of TLR7 and TLR9 from the ER to the endosome, whereby their ligands are delivered from the outside of cells (Kim et al., 2008b). UNC93B1 was also found to be physiologically relevant to herpes encephalitis, a rare complication of infection with herpes simplex virus (HSV), in which TLR3 plays preventive roles (Casrouge et al., 2006; Zhang et al., 2007). It is therefore conceivable that TLR-mediated recognition of microbial nucleic acid ligands through endosomal pathways is a "safety mechanism" by which ER-retained TLRs must be delivered to endosomes to meet their ligands, although the trigger for this TLR trafficking from the ER to endosomes before TLR-mediated recognition of and activation by nucleic acids remains to be elucidated.

While TLR trafficking from the ER or cell surface via endosomes to the lysosome may be constitutive in cells under steady-state conditions, recent evidence has emerged from another biological process for membrane trafficking known as autophagy. Autophagy is a cellular process for recycling cytoplasmic constituents and is often linked to cell death, but can be used for detecting and/or clearing invading microbes in the cytosol, referred to as xenophagy (Levine, 2005). While many biological stressors, including TLR ligands, can induce autophagy directly and indirectly (Xu et al., 2007; Delgado et al., 2008), it is unclear whether autophagy itself plays a role in recruiting TLR-membrane trafficking, and its physiological relevance to host defense is therefore being actively investigated. A recent report suggested that pDCs may utilize this pathway of TLR sorting of their ligands. Recognition of cytoplasmic RNA by TLR7 during viral replication requires autophagy-related proteins, such as ATG5 and ATG7 (Lee et al., 2007), suggesting that viral RNAs in the cytosol are trapped by autophagosomes and carried to the lysosome where TLR7 is activated (Figure 4B). The noncanonical roles of ATG5 and ATG7 in antiviral responses, however, are opposite in the case of viral infection of nonimmune cells. ATG5 and ATG7 are in fact negative regulators of type I IFN responses through direct interactions with the caspase recruitment domains (CARDs) presented by RIG-I and IFN-β promoter stimulator 1 (IPS-1) (Jounai et al., 2007) (Figure 4B). The physiological relevance of these autophagy-related proteins is of considerable

interest, not only to TLR-membrane trafficking, but also to immunological outcomes in vivo. Proteins of interest include the other ATG protein family members and LC3, a frequently used specific marker for autophagosomes. However, a more careful interpretation seems to be required for discriminating the effect of autophagy (or autophagosome formation) from conventional endophagocytosis and phagocytosis. In fact, a recent report suggested that LPS-conjugated particles internalized by macrophages trigger rapid recruitment of the autophagosome marker LC3 to phagosomes in a manner that is dependent on the autophagy pathway proteins ATG5 and ATG7 (Sanjuan et al., 2007).

Integrated Roles of TLR and NLR Recognition in Bacterial Infection

Numerous studies have demonstrated that homologous and heterologous cooperation among TLRs is important for detecting and triggering innate immune responses to microbial infections (Trinchieri and Sher, 2007). On the other hand, another set of evidence suggests that innate immune recognition of microbial, especially bacterial, products involves not only TLRs but also the other innate immune receptors known as NLRs (reviewed in Inohara et al., 2005; Fritz et al., 2006; Martinon and Tschopp, 2007).

Briefly, NLR genes consist of three domains with (1) pyrin N-terminal homology domain (PYD), CARD, or Baculoviral IAP repeat (BIR) domains at the N terminus; (2) NOD, NACHT, or dinucleotide (NAD) domains at the intermediary region; and (3) a C-terminal LRR domain (Figure 1). NLR genes were recently subclassified into the following five groups according to their N-terminal domains: NLRA (also known as CIITA), NLRB (NAIP), NLRC3–5 (and NOD1- and NOD2-containing CARD), NLRP1-14 (NALP), and NLRX (Ting et al., 2008). NLR-mediated recognition of ligands occurs in the cytosol, in which NOD1 and NOD2 activation lead to NF-κB activation, whereas the other NLRs form a large intracellular complex, referred to as the "inflammasome" (Martinon and Tschopp, 2007), which leads to the activation of caspase-1 (also known as IL-1-converting enzyme [ICE]) to cleave pro-IL-1β, thereby releasing active IL-1β outside of cells. The contribution of this inflammasome in bacterial infections has been demonstrated using mice lacking caspase-1 and/or ASC (an adaptor for NALP1 and NALP3), which are unable to mount active IL-1β responses and are highly susceptible to intracellular bacteria such as *Shigella flexneri* and *Francisella tularensis* (Sansonetti et al., 2000; Mariathasan et al., 2005). Although TLRs and NLRs have distinct intracellular localizations and independent signaling pathways, they nevertheless share their ligands, such as bacterial cell wall components and flagellin, in the case of bacterial infections, and further interact at the level of their intracellular signaling cascades, thereby enabling synergetic or concurrent innate immune responses.

Recognition of Bacterial Cell Wall Components by TLRs and NLRs

Bacterial cell walls consist of many immunostimulatory elements. LPS is recognized by MD-2-TLR4 complexes, while the diversity of LPS structures and differential recognition of these structures by TLR4 are associated with the outcomes of several bacterial diseases (Miller et al., 2005). Peptidoglycan (PGN) is recognized by TLR2, although the major agonistic activity of TLR2 is attributed to other cell wall components, possibly lipopeptides or lipoteichoic acids (Travassos et al., 2004). Specific elements of PGN are instead sensed by the NLR system, since dipeptide g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) is sensed by NOD1, while muramyl dipeptide (MDP) is recognized by NOD2. It is of note that such activity of PGN is easily exploited by bacteria; for example, *Listeria monocytogenes* encodes a PGN-deacetylase gene, *pgdA*, which confers virulence by masking NOD1 sensing of PGN (Boneca et al., 2007).

Stimulation of NOD1 and NOD2 induces the recruitment of RIP2 (receptor-interacting protein 2, also known as RICK) and CARD9, which promote NF-κB-dependent gene activation and mitogen-activated protein kinase (MAPK)-signaling pathways, respectively (Park et al., 2007; Hsu et al., 2007). In fact, the critical roles of NOD1/2 recognition and signaling in protective immune responses to *L. monocytogenes* have been demonstrated in vivo, especially in the gut (Kobayashi et al., 2005; Park et al., 2007; Hsu et al., 2007).

Interactions between TLR- and NOD-mediated innate immune recognition and signaling have been described. Recently, CARD9 was shown to participate not only in NOD1/2-mediated MAPK activation, but also in cell type-specific TLR-signaling pathways. Dendritic cells (DCs), but not macrophages or B cells from Card9$^{-/-}$ mice, exhibit impaired TLR signaling (Hara et al., 2007), whereas RICK is not involved in TLR-mediated signaling in macrophages (Park et al., 2007). While synergy or crosstolerance between TLRs and NODs may occur depending on the dose of the stimulatory ligand (Borm et al., 2008), Kim et al. clearly demonstrated that NOD1 and NOD2 ligands and TLR ligands can induce self-tolerance, but not crosstolerance, to each other in vivo (Kim et al., 2008a). Under a TLR-tolerant status, NOD1 and NOD2 are critical for host defense against intraperitoneal infection with *L. monocytogenes* (Kim et al., 2008a). These data suggest that TLRs and NLRs cooperate to recognize and respond to bacterial infections by sharing their ligand but maintaining concurrent signaling pathways.

Recognition of Bacterial Flagellin by TLRs and NLRs

Flagellin, a protein component of bacterial flagella, is recognized by both TLR5 and NLRs (NAIP5 [NLRB] and IPAF [NLRC4]). TLR5-mediated recognition of flagellin occurs on the cell surface membrane and plays important roles in the protective immune responses in Legionnaire's diseases and typhoid fever in humans (Miao et al., 2007). On the other hand, two NLRs come into play for flagellin detection in the cytosol. NAIP5 (also known as BIRC1) is involved in detection of flagellin in the cytoplasm and in caspase-1-dependent control of *Legionella pneumophila* infection (Ren et al., 2006; Zamboni et al., 2006). IPAF, another CARD-containing NOD-LRR protein, recognizes *L. pneumophila* and *Salmonella typhimurium* and also results in caspase-1 activation (Miao et al., 2006; Franchi et al., 2006).

Although the precise mechanisms underlying this flagellin access and recognition by cell surface TLR5 and cytosolic NAIP5 as well as IPAF have not been fully clarified, current evidence suggests that they divide this job in an efficient manner. For example, TLR5 recognition of flagellin may be restricted to bacteria-invading tissues, rather than those among the normal flora, by the polarized expression of TLR5 in the basal side of epithelial cells and its cell type-specific expression in lamina propria CD11c+ DCs of the gut (Uematsu et al., 2006) and possibly in the lungs. Another example is that cytosolic access to flagellin

may be mediated by specific virulence factor transport systems, known as the SPI1 type III secretion system (T3SS) and Dot/Icm type IV secretion system (T4SS) (Miao et al., 2007). Thus, it is reasonable that NAIP5 and IPAF may detect flagellin in the cytosol and cooperate for the recognition of this bacterial component with TLR5 in a synergistic or concurrent manner.

Innate Immune Recognition of Viral Components

Viral infections require host cells for virus survival and multiplication and therefore have many strategies for interacting with host machineries. In turn, the host immune system has evolved to detect and interfere with such viral infections at the levels of cell entry, replication, packaging, and exit. Host innate immune receptors are engaged in the detection of viruses or viral intermediate products to trigger the infected cells as well as other immune cells to produce antiviral effector molecules, including proinflammatory cytokines and type I IFNs. While viral proteins are recognized by TLRs, such as TLR2 and TLR4, that trigger proinflammatory responses, their contributions to either protective or pathological immune responses largely depend on the type of virus, route of infection, and other host factors (Finberg et al., 2007). However, protective responses against viral infections largely rely on the induction of a set of antiviral proteins, including type I IFNs, by infected cells and/or neighboring or recruited immune cells such as DCs (Garcia-Sastre and Biron, 2006).

Such responses are mediated by a variety of innate immune receptors, including TLR3, TLR7, TLR8, TLR9, RIG-I, melanoma-differentiation-associated gene 5 (MDA5), and LGP2, which can directly recognize and mediate the immunostimulatory properties of viral nucleic acids derived from viral genomes or generated during their replication (Kawai and Akira, 2006). Such recognition triggers NF-κB and IRF activation via distinct signaling pathways, leading to the production of antiviral proteins in a cell type-specific manner. While these events are thought to play important roles in both innate and adaptive immune responses to viral infections, viruses often have strategies for exploiting these processes through virulence factors (Pichlmair and Reis e Sousa, 2007). Generally, viral nucleic acids recognized by these innate immune receptors can be divided into the following four groups: ssRNA, double-stranded (ds) RNA, ssDNA, and dsDNA (Ishii and Akira, 2005). The sequence specificities of these ligands are described in detail elsewhere, and this section will focus on natural nucleic acid recognition during viral infection.

Intracellular Recognition of Viral RNA by RLRs

Viral RNA can be sensed by TLR3, TLR7 or TLR8, in which TLR3 recognizes dsRNA generated during viral replication, and TLR7 and TLR8 recognize genomic ssRNA. These TLRs are in the endosome of specialized immune cells such as DCs and are thought to detect viral RNA delivered from outside of cells. On the other hand, three homologous DExD/H box RNA helicases designated RLRs were identified as cytoplasmic sensors for viral RNA within both immune and nonimmune cells. Two family members, RIG-I (also called DDX58) and MDA5 (also called Helicard), share two N-terminal CARDs followed by an RNA helicase domain (Yoneyama et al., 2007). RIG-I binds and mediates innate immune activation through the 5′-triphosphate of ssRNAs (Pichlmair et al., 2006; Hornung et al., 2006) and short dsRNAs (Takahasi et al., 2008). In contrast, MDA5 preferentially recognizes longer dsRNAs, including synthetic poly-IC (polyinosinic and polycytidylic acid) (Takeuchi and Akira, 2008). It is of note that another well-known antiviral protein, RNase L, can cleave and turn single-stranded portions of not only viral RNAs but also self-RNAs into preferentially dsRNA ligands for RIG-I and MDA5 (Malathi et al., 2007).

Crystal and NMR structural studies of RIG-I have demonstrated that its C-terminal domain containing a zinc-binding domain conserved in MDA5 and LGP2 is essential for binding to ligands as well as the subsequent signaling, while the helicase domain is critical for ATPase activity (Cui et al., 2008; Takahasi et al., 2008; Gee et al., 2008). A third RIG-like receptor protein, LGP2, shares homology with RIG-I in the helicase domain but lacks a CARD. It was proposed and demonstrated that LGP2 acts as a negative regulator of RIG-I and MDA5 signaling in vitro. However, a recent report using LGP-deficient mice has suggested that LGP2 predominantly represses the RIG-I pathway rather than the MDA5 pathway but plays a positive regulatory role in the recognition of encephalomyocarditis virus (EMCV), whose replicative intermediate activates MDA5 (Venkataraman et al., 2007). Taken together, RLRs may play important roles in detecting cytoplasmic RNA, depending on the microenvironment and whether the origin of the RNA is from microbes or host cells, provoking innate immune responses if RNA has double-stranded structure or 5′-tryphosphate end.

Intracellular Recognition of Viral DNA

While TLR9 used to be the only known innate immune receptor that recognized DNA, viral DNA was recently shown to be recognized by TLR9-independent, but uncharacterized recognition machineries in the cytoplasm (Wagner and Bauer, 2006; Ishii and Akira, 2006). When DNA enters the cytoplasm, it triggers robust activation of most cell types through TBK1 (TANK-binding kinase 1) and IRF3, in contrast to the TLR9-mediated recognition of DNA via the endosomal pathway in specialized immune cells, such as DCs and B cells (Ishii et al., 2006; Stetson and Medzhitov, 2006). The DNA sources are not restricted to viruses, but rather comprise a broad range from viruses and bacteria to damaged host cells, with the greatest activation by the most common double-stranded right-hand B-form DNA, lower activation by the unusual left-handed Z-form DNA, and no activation for ssDNA (Ishii et al., 2006).

Although the physiological significance may vary, cytoplasmic DNA recognition seems to be involved in many viral and bacterial DNA-mediated innate immune responses, including those against adenoviruses (Nociari et al., 2007; Zhu et al., 2007), vaccinia virus (Waibler et al., 2007; Ishii et al., 2006), cytomegalovirus (Delale et al., 2005), HSV (Hochrein et al., 2004), *Listeria* sp. (Stockinger et al., 2004; Stetson and Medzhitov, 2006), and *Legionella* sp. (Opitz et al., 2006). Recently, DAI (also known as ZBP1 or DLM1), which contains two Z-DNA binding domains, was shown to be a potential cytoplasmic DNA sensor (Takaoka et al., 2007). Nevertheless, DAI knockout mice respond to B-DNA in vitro and in vivo and mount normal type I IFNs and DNA-vaccine-induced adaptive immune responses, suggesting its redundant role (Ishii et al., 2008). Although further studies are necessary, potential cytoplasmic DNA sensors may be connected to not only host defenses against infections, but also to pathogenesis of autoimmune diseases, or even to DNA damage-induced biological responses, which may be referred to as "genome defense."

NLRs Mediate Innate Immune Activation by Intracellular Nucleic Acids

NLRs also come into play in nucleic acid-mediated innate immune activations. NALP3, an NLR sensing a variety of ligands, mediates the caspase-1 activation triggered by both ssRNAs and dsRNAs derived from viral and bacterial origins and their synthetic versions (Kanneganti et al., 2006a, 2006b). In addition, these RNA stimulations lead to the activation of caspase-3, thereby linking their physiological relevance to the induction of apoptosis of virus-infected cells (Rintahaka et al., 2008). Moreover, some NLRs participate in RNA- and DNA-mediated innate immune activation through caspase-1 activation (Kanneganti et al., 2006b; Muruve et al., 2008) and NF-κB activation toward IFN-β production via synergistic pathways activated by NOD2 (Leber et al., 2008). Thus, these results suggest that not only type I IFNs, but also IL-1-β produced via inflammasome activation, participate in nucleic acid-induced innate immune activation, although the physiological roles of nucleic acid detection via inflammasome in either protective or pathological immunity awaits further investigations. It will also be of interest to study intra- or intercellular crosstalk between RLRs (including unidentified DNA sensor[s]) and NLRs, and their manipulation by microbial infection.

Differential Roles of TLRs and RLRs in Antiviral Responses

dsRNA, a well-known viral signature, is generated within cells during viral replication but does not normally exist in host cells. While dsRNA activates macrophages and DCs via TLR3 to secrete proinflammatory cytokines, especially IL-12, most virus-infected cells, such as fibroblasts, produce type I IFNs in a TLR3-independent manner. Instead, MDA5, but not RIG-I, recognizes synthetic dsRNA, poly-IC, and the positive-strand ssRNA picornavirus encephalomyocarditis virus, which generates dsRNA during replication, resulting in type I IFN-mediated antiviral responses (Takeuchi and Akira, 2008). Deletion of both pathways in mice deficient for both TLR3 and MDA5, or TRIF and IPS-1, leads to a failure to elicit any innate or adaptive (adjuvant) responses to poly-IC (Kato et al., 2006; Kumar et al., 2008). Regarding other synthetic dsRNAs, poly-AU and certain siRNAs were found to stimulate pDCs to produce type I IFNs in a TLR7-dependent manner (Hornung et al., 2005; Sugiyama et al., 2008). Of interest, cell type-specific RNA recognition was observed for IL-12 production, similar to the observation that poly-AU (polyadenylic and polyuridylic acid) stimulates $CD11b^{high}$ conventional (myeloid) DCs (cDCs) in a TLR7-dependent manner, whereas $CD24^{high}$ cDCs are stimulated by TLR3 at the same time (Sugiyama et al., 2008).

ssRNA is recognized by TLR7 (or TLR8 in humans) and RIG-I in a cell type-specific manner. TLR7 and TLR8 recognize GU or AU-rich sequences of the ssRNAs of many RNA viruses, such as influenza virus and HIV (human immunodeficiency virus), through TLR7-expressing cells such as pDCs or TLR8-expressing cells such as cDCs or monocytes, although it remains unclear whether their sequence specificities are receptor specific or cell type specific (Diebold, 2008; Forsbach et al., 2008). In contrast, RIG-I is expressed in most cell types and recognizes the 5′-triphosphate of ssRNAs from many positive-strand ssRNA viruses such as Japanese encephalitis virus and negative-strand ssRNA viruses such as Newcastle disease virus, vesicular stomatitis virus (VSV), Sendai virus, and influenza virus.

In the case of influenza A virus, its negative-sense ssRNA genome is recognized by TLR7 expressed in pDCs through MyD88 (Diebold, 2008). However, it is also recognized by RIG-I ubiquitously expressed in most cell types, such as fibroblasts or cDCs, in vitro and probably alveolar macrophages in vivo, similar to other RNA viruses (Kumagai et al., 2007), through an adaptor protein, IPS-1. Both recognition pathways trigger robust type I IFN productions in vitro, but the situation is different when mice are infected with influenza A virus in their lungs. In this case, cell type-specific innate immune recognitions by TLR7 and RIG-I through distinct signaling pathways are concurrently required, especially for the initial type I IFN production in the lungs (Koyama et al., 2007). Surprisingly, the adaptive immune responses to airway influenza A virus infection are strictly governed by the TLR7-MyD88 pathway, but not the RIG-I-IPS-1 pathway (Koyama et al., 2007), suggesting differential roles of TLR7 and RIG-I in innate and adaptive immune responses to airway infection by influenza A virus.

It is noteworthy that an interplay between these innate immune receptors is not necessarily beneficial to the host. For example, TLR3, which is known to be a receptor for dsRNA, plays a pathological role in influenza A virus infection in vivo (Le Goffic et al., 2006). In addition, TLR desensitization is observed after lung infection with influenza A or the respiratory syncytial virus, which results in impaired immune responses to subsequent bacterial infections (Didierlaurent et al., 2008). Moreover, viral factors often manipulate host innate immune receptor-mediated signaling. NS1 protein can inhibit the functions of IPS1 and RIG-I (Mibayashi et al., 2007), while V protein of paramyxoviruses can bind and inhibit the activation of MDA5 (Andrejeva et al., 2004). Taken together, TLR- and RLR-mediated recognition of viral infections via the detection of viral nucleic acids play critical, but often differential, roles in protective antiviral responses, and some of these are involved in the pathological outcomes and can be manipulated by viral virulence factors.

Innate Immune Recognition of Eukaryotic Microbes

Symbiotic relationships between hosts and eukaryotic microbes (e.g., parasites) are often silent and chronic, in part by their sophisticated machineries that can manipulate the host immune system. Accumulating evidence suggests that the host innate immune system nevertheless detects some of the specific components derived from these microbes. In particular, immunomodulatory components unique to parasites or fungi are often found to be ligands for innate immune receptors such as TLRs and CLRs, which cooperate to induce phagocytosis of these relatively larger microbes and activate innate immune signaling pathways.

Recognition of Fungal Pathogens

Among the TLRs, TLR2, TLR4, and TLR9 are involved in sensing many fungal components, such as zymosan, phospholipomannan, mannan, and fungal DNA, respectively (Netea et al., 2008). Although studies have shown that adaptor molecule MyD88-deficient mice are highly susceptible in vivo to infections with various fungi (i.e., *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Pneumocystis carinii*), the physiological roles of the individual TLRs in fungal infection are still controversial.

On the other hand, CLRs have been shown to be involved in the recognition and subsequent immune responses to fungal infections. For example, dectin-1 is the major receptor for soluble and particulate β-1,3- and/or β-1,6-linked glucans, which are carbohydrate polymers mainly found in the cell walls of fungi. Dectin-1 also recognizes zymosan, a stimulatory cell-wall extract of *Saccharomyces cerevisiae* composed mainly of β-1,3-glucan (but also mannans, chitin, protein, and lipids). The binding of zymosan to dectin-1 induces phagocytosis, production of reactive oxygen species in macrophages, and cooperation with TLR2, resulting in optimal immune activations such as the production of proinflammatory cytokines (Gantner et al., 2003). Dectin-1 possesses an immunoreceptor tyrosine-based activation-like (ITAM-like) motif (Underhill and Goodridge, 2007) that engages spleen tyrosine kinase (Syk) and signals through CARD9. This distinct innate immune activation mediated by dectin-1 plays important roles in the host defense against *P. carinii*, but not against *C. albicans* (Saijo et al., 2007), and in the development of T helper 17 (Th17) responses to fungal infections (Gross et al., 2006; LeibundGut-Landmann et al., 2007). The other CLRs, including mannose receptor (CD206), DC-SIGN (SIGN-R1 for mouse), DEC-205 (CD205), and BDCA-2, are expressed on DCs that play roles not only in their intertissue trafficking, but also in endocytic antigen (ligand) uptake, both of which enable DCs to act as professional antigen-presenting cells (Willment and Brown, 2008).

TLR-Mediated Recognition of Parasite Components

Glycosylphosphatidylinositols (GPIs) are found in all eukaryotes and are composed of highly conserved glycolipid molecules that anchor certain cell surface proteins to plasma membranes. However, some parasites such as *Trypanosoma*, *Leishmania*, *Toxoplasma*, and *Plasmodium* species express 10 to 100 times more GPIs that are structurally distinct from those of the host cells covering the surface of the parasites (Gowda, 2007). The parasite GPIs with distinctive free lipids or those linked to proteins activate immune responses via TLR2 and/or TLR4 (Debierre-Grockiego et al., 2007; Gowda, 2007).

Recently, a 17.5 kD profilin-like protein derived from *Toxoplasma gondii*, now designated TgPRF, was identified as a potent agonist for TLR11 in mice and is responsible for potent IL-12 production within the parasite-soluble fraction (known as STAg) by mouse splenic DCs (Yarovinsky et al., 2005). Critical roles for TgPRF in both host immune responses and parasite survival have been revealed. For the host, TgPRF is an immunodominant antigen in the $CD4^+$ T cell responses to the parasite (Yarovinsky et al., 2006). For the parasite, TgPRF, previously known to function as an actin-binding protein and well conserved among eukaryotes, is also required for gliding, invasion, and egress, all of which contribute to parasite virulence (Plattner et al., 2008). *T. gondii*, *Plasmodium falciparum*, and *Cryptosporidium parvum* have all been shown to activate the innate immune system via TLR11 (Yarovinsky et al., 2005). Although the human TLR11 gene has a stop-codon within an open reading frame and thus appears to be nonfunctional, the above findings provide many insights into host-microbe interactions through innate immune receptors.

TLR9 has been suggested to recognize not only parasite DNA, but also the heat-labile fraction and hemozoin derived from *P. falciparum* (Pichyangkul et al., 2004; Coban et al., 2005; Parroche et al., 2007). Hemozoin, a polymerized form of heme, is an abundant but unique product of blood-feeding parasites and is generated after parasite digestion of hemoglobin as a byproduct of the heme detoxification system in malaria infection and captured by the host reticuloendothelial system. Despite the controversy surrounding the issue of whether hemozoin is pro- or anti-inflammatory, hemozoin including its synthetic version is immunologically active, acting as TLR9 agonist and a potent adjuvant for many vaccine applications (C.C., unpublished data), which does not necessarily exclude the possibility of other TLR9 ligands such as *Plasmodium* DNA and an unknown heat-labile TLR9 ligand in *P. falciparum* (reviewed in Coban et al., 2007). Moreover, a recent report indicated the importance of TLR9, since infection of mice with the rodent malaria parasite *Plasmodium yoelii* activates regulatory T cells (Tregs), leading to enhancement of their suppressive function (Hisaeda et al., 2008). TLR9-deficient mice are partially resistant to lethal infection, suggesting that malaria parasites may require TLR9 to activate Tregs for immune escape. Continuous mapping of the molecular and cellular mechanisms that determine or influence the outcome of these host-microbe interactions is necessary, although such mechanisms seem more complex, especially in the case of eukaryotic, multicellular parasites, than those of prokaryotic bacteria or viruses.

Concluding Remarks

Compared to the situation 10 years ago, it is now impossible to cover all the new findings that demonstrate novel mechanisms or the critical importance of innate immune receptors for host defense against microbial infections in one review article. Studies are shifting from those analyzing functions of single receptor interaction with a pure, single ligand derived from the microbe, toward those analyzing more complex host-microbe interactions between multireceptors of the host immune system and whole microorganisms. Continuing to improve our knowledge of host-microbe interactions and gain insights into their mechanisms, however, may enable us to develop new therapeutic approaches for many infectious diseases and other immunological disorders, or to improve current technologies in order to develop vaccines and their adjuvants with greater specificity and safety.

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