Leading Edge
Review
Cell

Pathogen Recognition and Innate Immunity

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DOI 10.1016/j.cell.2006.02.015

Microorganisms that invade a vertebrate host are initially recognized by the innate immune system through germline-encoded pattern-recognition receptors (PRRs). Several classes of PRRs, including Toll-like receptors and cytoplasmic receptors, recognize distinct microbial components and directly activate immune cells. Exposure of immune cells to the ligands of these receptors activates intracellular signaling cascades that rapidly induce the expression of a variety of overlapping and unique genes involved in the inflammatory and immune responses. New insights into innate immunity are changing the way we think about pathogenesis and the treatment of infectious diseases, allergy, and autoimmunity.

Introduction

Vertebrates are constantly threatened by the invasion of microorganisms and have evolved systems of immune defense to eliminate infective pathogens in the body. The mammalian immune system is comprised of two branches: innate and acquired immunity. The innate immune system is the first line of host defense against pathogens and is mediated by phagocytes including macrophages and dendritic cells (DCs). Acquired immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory. Acquired immunity is characterized by specificity and develops by clonal selection from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated via a mechanism generally known as gene rearrangement. The innate immune response is not completely nonspecific, as was originally thought, but rather is able to discriminate between self and a variety of pathogens. The innate immune system recognizes microorganisms via a limited number of germline-encoded pattern-recognition receptors (PRRs). This is in contrast to the large repertoire of rearranged receptors utilized by the acquired system.

PRRs possess common characteristics. First, PRRs recognize microbial components, known as pathogen-associated molecular patterns (PAMPs), that are essential for the survival of the microorganism and are therefore difficult for the microorganism to alter. Second, PRRs are expressed constitutively in the host and detect the pathogens regardless of their life-cycle stage. Third, PRRs are germline encoded, nonclonal, expressed on all cells of a given type, and independent of immunologic memory. Different PRRs react with specific PAMPs, show distinct expression patterns, activate specific signaling pathways, and lead to distinct antipathogen responses. The basic machineries underlying innate immune recognition are highly conserved among species, from plants and fruit flies to mammals.

Here we will review the mechanisms of pathogen recognition by the innate immune system, focusing on host PRRs and their signaling pathways.

TLRs as Pattern-Recognition Receptors

TLRs are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals (Akira and Takeda, 2004; Beutler, 2004; Hoffmann, 2003; Janeway and Medzhitov, 2002). Toll, the founding member of the TLR family, was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in *Drosophila*. Later, it was also shown to play a critical role in the antifungal response of flies (Lemaitre et al., 1996). To date, 12 members of the TLR family have been identified in mammals. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neill, 2000). The LRR domains are composed of 19–25 tandem LRR motifs, each of which is 24–29 amino acids in length, containing the motif XLXXLXLXX as well as other conserved amino acid residues (XØXXØXXXXFXXLX; Ø = hydrophobic residue). Each LRR consists of a β strand and an α helix connected by loops. It was imagined that the LRR domain of TLR would form a horseshoe structure with the ligand binding to the concave surface. However, the three-dimensional structure of the human TLR3 LRR motifs has recently been elucidated, and its structure suggests a somewhat different model in which negatively charged dsRNA is more likely to bind the outside convex surface of TLR3 (Choe et al., 2005). How general this mode of

Table 1. TLR Recognition of Microbial Components

Microbial Components	Species	TLR Usage
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

ND = not determined. See text for references.

binding is will be answered with the crystallographic analyses of other TLRs.

Based on their primary sequences, TLRs can be further divided into several subfamilies, each of which recognizes related PAMPs: the subfamily of TLR1, TLR2, and TLR6 recognizes lipids, whereas the highly related TLR7, TLR8, and TLR9 recognize nucleic acids (Table 1). However, the TLRs are unusual in that some can recognize several structurally unrelated ligands. For example, TLR4 recognizes a very divergent collection of ligands such as lipopolysaccharide (LPS), the plant diterpene paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibronectin, and heat-shock proteins, all of which have different structures.

TLRs are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is not static but rather is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses. Furthermore, TLRs may be expressed extra- or intracellularly. While certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as

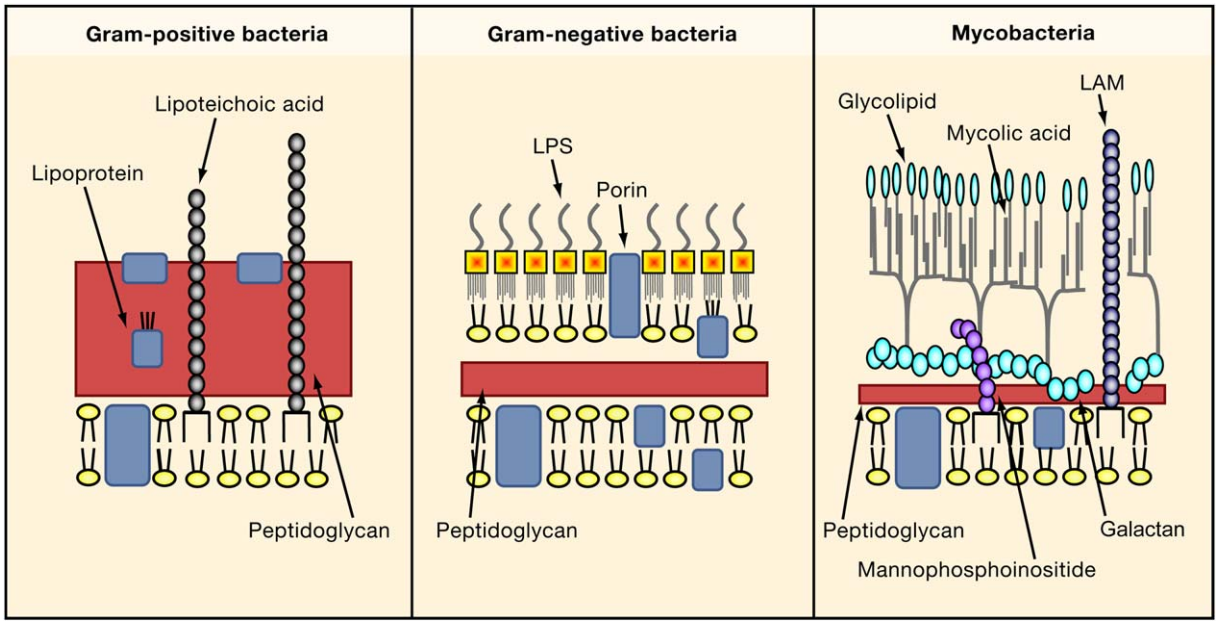

Figure 1. Schematic Representation of Bacterial Cell Walls

Gram-positive bacteria have a thick layer of PG. Lipoteichoic acids and lipoproteins are embedded in this cell wall. The cell wall of Gram-negative bacteria is characterized by the presence of LPS. Mycobacteria have a thick hydrophobic layer containing mycolyl arabinogalactan and dimycolate, in addition to a lipid bilayer and a PG layer. Lipoarabinomannan (LAM) is a major cell-wall-associated glycolipid. Lipoproteins are common structures for various types of bacteria.

endosomes, and their ligands, mainly nucleic acids, require internalization to the endosome before signaling is possible. The transmembrane and membrane-proximal regions are important for the cellular compartmentalization of these receptors.

TLRs activate the same signaling molecules that are used for IL-1R signaling (Akira and Takeda, 2004). Stimulation of cells with a TLR ligand recruits adaptor proteins containing a TIR domain, such as myeloid differentiation factor 88 (MyD88), to the cytoplasmic portion of the TLRs through homophilic interaction of their TIR domains. This results in the triggering of downstream signaling cascades and production of proinflammatory cytokines and chemokines. Cells prominently expressing TLRs include antigen-presenting cells (APCs) such as DCs and macrophages, which ingest and degrade pathogens. The role of TLR signaling in pathogen phagocytosis is controversial. Early papers suggested a role of TLR in phagocyte maturation, whereas a recent paper did not find such a role (Blander and Medzhitov, 2004; Yates and Russell, 2005). APCs also activate the adaptive immune response by migrating from the infection site to the regional lymph node, where they present microbe-derived antigens to naive $CD4^+$ T cells. At the same time, activated DCs express costimulatory molecules essential to T cell activation and can instruct the differentiation of naive $CD4^+$ T cells into T helper 1 (Th1) cells or Th2 cells. Th1 cells produce interferon-γ (IFN-γ) and mediate the elimination of bacterial and viral infection, while Th2 cells, which produce IL-4 and IL-13, are involved in the response against helminth infection. Stimulation of most TLRs leads to Th1 rather than Th2 differentiation. Thus, innate immunity is a key element in the inflammatory response as well as the immune response against pathogens.

Bacterial Recognition by TLRs

Gram-Negative and -Positive Bacterial Cell Wall

Bacteria can be classified into two major groups depending on the different staining characteristics of their cell walls, namely Gram-positive and -negative bacteria (Figure 1). Some of the unique cell-wall components stimulate immune cells and serve as PAMPs, recognized by individual TLRs. LPS, also known to be an endotoxin, is generally the most potent immunostimulant among these cell-wall components. A lipid portion of LPS termed "lipid A" is responsible for most of the pathogenic phenomena associated with Gram-negative bacterial infection such as endotoxin shock. LPS liberated from Gram-negative bacteria associates with LPS binding protein (LBP), an acute-phase protein present in the bloodstream, and then binds to CD14, a glycosylphosphatidylinositol (GPI) linked protein expressed on the cell surface of phagocytes. LPS is then transferred to MD-2, which associates with the extracellular portion of TLR4, followed by oligomerization of TLR4, a key molecule of LPS signaling (Poltorak et al., 1998; Shimazu et al., 1999).

Different bacteria produce structurally different LPS molecules varying in their phosphate patterns, numbers of acyl chains, and fatty-acid composition. These variations are reflected in the varied biological activities of lipid

A, as evidenced by the extremely reduced toxicity of monophosphoryl lipid A. Furthermore, LPS preparations from nonenteric bacteria, such as *Legionella pneumophila* and *Leptospira interrogans*, are reported to act as TLR2, but not TLR4, agonists, although such results should be viewed with caution due to the difficulty of removing impurities from LPS preparations (Werts et al., 2001). In fact, it has been shown that the TLR2-stimulating activity of an LPS preparation from *Porphyromonas gingivalis* was due to the presence of contaminating lipoproteins, not to LPS.

Consistent with their reduced response to Gram-negative bacteria, TLR4-mutated C3H/HeJ mice are highly susceptible to infection by *Salmonella typhimurium* or *Neisseria meningitis*. Interestingly, two cosegregating missense mutations, Asp299Gly and Thr399Ile, have been found in the human *TLR4* gene (Cook et al., 2004). The Asp299Gly polymorphism was found to be associated with impaired LPS signaling, increased susceptibility to Gram-negative bacterial infections, and a lower risk of atherosclerosis.

Components of Gram-positive bacterial cell walls can also stimulate innate immunity. Although these bacteria do not contain LPS, lipoteichoic acid (LTA) seems to function in a similar manner as an immune activator. Lipoproteins and peptidoglycan (PG), which are present in both Gram-positive and Gram-negative bacteria, are also potent immunostimulants. TLR2 plays a major role in detecting Gram-positive bacteria and is involved in the recognition of a variety of microbial components, including LTA, lipoproteins, and PG. TLR2 interacts physically and functionally with TLR1 and TLR6, which appear to be involved in the discrimination of subtle changes in the lipid portion of lipoproteins. LTA, an amphiphilic, negatively charged glycolipid, also contains a diacylated moiety and activates the cells via the TLR2/TLR6 heterodimer (Alexopoulou et al., 2002; Ozinsky et al., 2000; Takeuchi et al., 2001, 2002).

PG is composed of long linear sugar chains of alternating N-acetyl glucosamine (GlcNac) and N-acetyl muramic acid (MurNac) that are interlinked by peptide bridges to form a large macromolecular structure. TLR2 has been reported to recognize PG, but this observation remains controversial (Travassos et al., 2004). Studies using components that have been biochemically purified from bacteria are often inconclusive due to the possibility of contaminations, and chemically synthesized mimics of active components should be used for definitive conclusions. To date, only chemically pure lipopeptides have been unambiguously demonstrated to be TLR2 stimulators.

The importance of TLR2 in the host defense against Gram-positive bacteria has been demonstrated using TLR2-deficient (*TLR2*$^{-/-}$) mice, which were found to be highly susceptible to challenge with *Staphylococcus aureus* or *Streptococcus pneumoniae* (Echchannaoui et al., 2002; Takeuchi et al., 2000). A polymorphism in the human *TLR2* gene (Arg753Gln) has been shown to be associated with a reduced response to different bacterial lipoproteins and septic shock after infection by Gram-positive bacteria, especially staphylococcal septic shock (Cook et al., 2004). This polymorphism was identified in a large group of Caucasian subjects, and 6%–9% of the study population was found to be heterozygous.

Uropathogenic bacteria are recognized by mouse TLR11, although the specific components that are responsible for this activation have not been identified (Zhang et al., 2004). However, human TLR11 is nonfunctional because of the presence of a stop codon in the gene.

Flagellins and TLR5

Flagellin is the major protein constituent of bacteria flagella, the motility apparatus used by many microbial pathogens, and is a potent activator of innate immune responses. A recent analysis of the crystal structure of a *Salmonella* flagellin revealed that the flagellin domains are composed of N- and C-terminal α helix chains (D0), the central α helix chains (D1), and the hypervariable central region with β sheets (D2 and D3) (Yonekura et al., 2003). TLR5 is responsible for the detection of flagellin and specifically recognizes the constant domain D1, which is relatively conserved among different species (Hayashi et al., 2001). TLR5 is expressed by epithelial cells, monocytes, and immature DCs. Since TLR5 is basolaterally expressed on intestinal epithelia, flagellin is recognized by the host only when bacteria have invaded across the epithelia. TLR5 is also highly expressed in the lungs and seems to play an important role in the defense against pathogens of the respiratory tract. Up to 10% of individuals have a point mutation that introduces a stop codon within the ligand binding domain of TLR5 (TLR5^{392STOP}). The TLR5^{392STOP} mutant protein functions as a dominant-negative receptor that severely impairs TLR5-mediated signaling and is associated with susceptibility to pneumonia caused by the flagellated bacterium *L. pneumophila* (Hawn et al., 2003). Some bacteria, such as *Helicobacter pylori* and *Campylobacter jejuni*, produce flagellins that lack proinflammatory properties and therefore escape the flagellin-specific host immune responses (Andersen-Nissen et al., 2005).

Bacterial DNA

Bacterial genomic DNA is also an immunostimulant and is recognized by TLR9 (Hemmi et al., 2000; Krieg, 2002). Its stimulatory effect is due to the presence of unmethylated CpG dinucleotides in a particular base context designated CpG-DNA. Although the CpG motif is abundant in bacterial genomes, its frequency is suppressed and it is highly methylated in mammalian genomes. The methylated CpG motif does not activate mammalian immune cells. CpG-DNA manifests strong immunostimulatory activities, including the induction of inflammatory cytokine production and Th1 immune responses. The particular DNA sequences that provoke an immune response vary between species. Synthetic oligonucleotides containing the CpG motif are equivalent to bacterial DNA in their immunostimulatory activity. Since TLR9 resides in the endosome, bacterial DNA must be delivered to this intracellular compartment, where the acidic and reducing conditions lead to the degradation of double-stranded DNA into multiple

single-stranded CpG-motif-containing regions that subsequently interact directly with TLR9 (Ahmad-Nejad et al., 2002; Latz et al., 2004). Compounds that block endosomal acidification, such as bafilomycin and chloroquine, inhibit CpG-DNA-driven signaling (Hacker et al., 1998). An in vitro experiment also demonstrated that TLR9 interacted with CpG-DNA more strongly at the acidic pH (6.5 or 5.5) condition (Rutz et al., 2004). However, the requirement of acidic pH in CpG recognition has recently been challenged by a study showing that a chimeric TLR9 localized to the cell surface is still capable of responding to CpG-DNA (Barton et al., 2006). The study suggests that the expression of TLR9 in intracellular compartments is important for preventing recognition of self-DNA. Therefore, further studies are required for clarifying the role of endosomal acidification in CpG DNA recognition.

PAMPs in Mycobacteria

Mycobacteria are intracellular bacteria that survive in the host macrophages by a number of elaborate mechanisms. The mycobacterial cell wall is composed of a thick waxy mixture of lipids and polysaccharides and is characterized by a high content of mycolic acid (Figure 1). Purified mycobacterial cell-wall components have been shown to preferentially activate TLR2 and, to a lesser extent, TLR4. Lipomannan (LM) and lipoarabinomannan (LAM) are related powerful immunomodulatory lipoglycans. LM is further arabinosylated to give LAM. The arabinan domain is capped by either mannosyl (ManLAM) or a phosphoinositide residue (PILAM). PILAM is a potent TLR2 stimulator that has been identified in nonpathogenic, fast-growing species such as *Mycobacterium smegmatis* (Gilleron et al., 2003). In contrast, ManLAM is a powerful anti-inflammatory molecule that is found in slow-growing virulent mycobacteria, such as *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, and *Mycobacterium avium*. Since LMs from both pathogenic and nonpathogenic mycobacterial species, independent of their origin, induce inflammatory cytokines in a TLR2-dependent manner, the ultimate response against virulent mycobacteria may be determined by the ManLAM/LM ratio in the cell wall (Quesniaux et al., 2004).

In addition, TLR2 in association with TLR1 can recognize a 19 kDa cell-wall-associated lipoprotein, a secreted antigen of *M. tuberculosis* that is also a potent cytokine inducer of macrophages (Thoma-Uszynski et al., 2001), and TLR9 can be activated by mycobacterial DNA, which may be released during endolysosomal degradation.

The role of individual TLRs in mycobacterial infections has been examined using TLR-deficient mice. In the case of the nonpathogenic species *M. smegmatis*, TLR2 is indispensable for effective clearance of *M. smegmatis* from the pulmonary compartment. In contrast, in the case of infections with virulent mycobacteria, such as *M. avium* and *M. tuberculosis*, mice deficient in TLR2, TLR4, or TLR6 appear to show minor or no defects in the control of infection, although conflicting results have been reported to date.

Mycobacterium leprae is the causative bacterium of leprosy, whose clinical manifestations depend on the host cell-mediated immune response against the pathogen. Tuberculoid leprosy patients manifest a strong Th1 response, resulting in a few localized and often self-healing paucibacillary lesions. In contrast, lepromatous leprosy patients manifest a predominant Th2 response, leading to a disseminated disease involving extended multibacillary lesions of the skin and nerves. The TLR2/TLR1 heterodimers are responsible for the cellular activation mediated by *M. leprae* as well as its triacylated 19 kDa and 33 kDa lipoprotein (Krutzik et al., 2003). TLR2 and TLR1 are strongly expressed on monocytes and DCs in lesions from tuberculoid leprosy patients but not in those from lepromatous leprosy patients. It is reported that a mutation in the intracellular domain of hTLR2 (Arg677Trp) is associated with lepromatous leprosy in a Korean population (Malhotra et al., 2005). These findings demonstrate that TLR2 plays a critical role in the innate immune response to *M. leprae*.

Fungal Recognition by TLRs

The observation that Toll-deficient *Drosophila* are highly susceptible to fungal infection led to the assumption that mammalian TLRs also participate in antifungal immunity. Several fungal PAMPs located in the cell wall or on the cell surface of fungi are recognized by TLR2 or TLR4.

The Th1 response is critical in protection against fungi. Although TLR-mediated signals mostly induce Th1-directed responses, activation of TLR2 is less inflammatory and favors the development of the Th2 response through the induction of IL-10 (Agrawal et al., 2003). Indeed, in vivo infection experiments using mutant mice suggest differential roles of TLR2 and TLR4 in fungal infection. *TLR4*$^{-/-}$ mice showed increased susceptibility to disseminated *Candida* infection, whereas *TLR2*$^{-/-}$ mice showed increased resistance (Netea et al., 2004). Infected *TLR2*$^{-/-}$ mice showed normal production of inflammatory cytokines such as TNF and IL-1 but severe impairment in IL-10 production, indicating that *C. albicans* induces immunosuppression through IL-10. A similar escape mechanism from the host defense is also observed in *A. fumigatus* infection. *A. fumigatus* grows in two forms, conidia and hyphae. TLR2 and TLR4 both recognize conidia, whereas the hyphae are only recognized by TLR2; thus, the phenotypic switch to hyphae from conidia results in the release of IL-10, which impairs the cellular immune response necessary for the *Aspergillus* clearance.

Dectin-1 is a type II transmembrane protein with a C type lectin domain in the extracellular region and an ITAM motif in the intracellular domain. Dectin-1 binds β-glucan and is the primary receptor on macrophages for phagocytosis of various fungi (Brown et al., 2002). It has been demonstrated that dectin-1 can collaborate with TLR2 in response to yeast to elicit a strong inflammatory response via recruitment of the protein tyrosine kinase Syk (Gantner et al., 2003; Rogers et al., 2005; Underhill et al., 2005). However, although β-glucan is presented during *C. albicans* yeast growth, it is not presented

during filamentous growth. As a consequence, dectin-1-mediated antimicrobial defenses are not effective against filaments, which may explain why filaments are more virulent. In addition to dectin-1, other receptors such as the type 3 complement receptor, the mannose receptor, and DC-SIGN are implicated in the recognition and phagocytosis of *Candida* (Takahara et al., 2004).

Protozoan-Parasite Recognition by TLRs

Components of protozoan parasites are also sensed by TLRs. These protozoan parasites include *Trypanosoma cruzi*, *Trypanosoma brucei*, *Toxoplasma gondii*, *Leishmania major*, and *Plasmodium falciparum*. *Trypanosoma*-derived molecules, such glycosylphosphatidylinositol-mucin (tGPI-mucin), glycoinositolphospholipids (GIPLs), and genomic DNA, have been reported to activate TLR2, TLR4, and TLR9, respectively (Gazzinelli et al., 2004). A soluble extract of *T. gondii* tachyzoites (STAg) contains a potent, heat-labile inducer of IL-12. The active component was recently identified as a profilin-like molecule that is recognized by murine TLR11 (Yarovinsky et al., 2005). Profilins are small ubiquitous proteins that were originally described as actin binding proteins. Although their exact cellular functions have not yet been established, their predicted actin binding activities suggest their involvement in parasite motility and/or invasion. However, human TLR11 is nonfunctional due to the presence of a stop codon in the gene.

Blood-stage schizonts or soluble schizont extracts from *Plasmodium* activate plasmacytoid DCs (pDCs) in a TLR9-dependent manner (Pichyangkul et al., 2004). The soluble schizont extracts are heat labile and can be precipitated with ammonium sulfate, unlike DNA. Recently, hemozoin, a heme byproduct, was shown to be a TLR9 stimulant (Coban et al., 2005). However, hemozoin is not heat labile and does not induce type I IFN production by pDCs, in contrast to the heat-labile schizont extracts, implying the existence of an additional TLR9 stimulator, probably a protein in the soluble schizont extracts.

The Th1/Th2 balance is well established to be of critical importance to the fate of parasites. Th1 responses are associated with the elimination of protozoan parasites, whereas Th2 responses are associated with uncontrolled parasite growth. Although mice deficient for individual TLRs do not show impaired responses to live protozoan parasites, mice deficient for MyD88, which causes a more general defect in TLR responses, showed impaired production of proinflammatory cytokines as well as enhanced parasitemia and mortality in response to protozoa infection (Adachi et al., 2001). This indicates that protozoan parasites may be recognized by more than one TLR.

Viral Recognition by TLRs

Viruses contain genetic material composed of either DNA or RNA (but not both) that encodes viral structural components and synthetic and replication enzymes. Various structural components, including viral DNA, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and surface glycoproteins, are recognized as PAMPs by TLRs and other PRRs. Among the TLR family members, TLR3, TLR7, TLR8, and TLR9 are involved in the recognition of viral nucleotides. The recognition of viral components by PRRs commonly induces type I IFN production that can activate target cells in both autocrine and paracrine manners.

Viral DNA Is Recognized by TLR9

DNA viruses, including herpes simplex virus 1 (HSV-1), HSV-2, and murine cytomegalovirus (MCMV), contain genomes that are rich in CpG-DNA motifs, and they activate inflammatory cytokines and type I IFN secretion by stimulation of TLR9 (Hochrein et al., 2004; Krug et al., 2004a, 2004b; Lund et al., 2003; Tabeta et al., 2004). The TLR9-mediated IFN-α response to HSV-1 and HSV-2 is cell-type specific and limited to pDCs, a DC subpopulation characterized by their ability to secrete high levels of IFN in response to viral infection. A group of synthetic oligonucleotides, termed A/D type CpG-DNAs, are also potent type I IFN inducers in pDCs (Krug et al., 2001; Verthelyi et al., 2001). They are characterized by the presence of a phosphorothioate-modified poly G stretch at the 5′ and 3′ ends and a phosphodiester CpG motif in the central portion. In contrast, conventional phosphorothioate-modified CpG-DNAs, termed B/K type CpG-DNAs, do not stimulate pDCs to produce type I IFNs, although they can stimulate cells to produce proinflammatory cytokines. Recognition of HSV-2 by pDCs does not require virus replication. Indeed, both live and UV-inactivated HSV-2 induces identical IFN-α secretion from pDCs. In contrast, in the case of macrophages that produce 5- to 10-fold less IFN-α in response to viral infections, HSV-2-induced expression of TNF and RANTES is mainly dependent on TLR9, whereas type I IFN is induced in a TLR9-independent manner (Hochrein et al., 2004). This is also supported by a study showing that mice lacking TLR9 or MyD88 can still control HSV-1 infection initiated either by footpad or eye inoculation, suggesting that cells other than pDCs may have a TLR9- and MyD88-independent system that exerts effective anti-viral responses(Krug et al., 2004b).

Single-Stranded RNA Is Recognized by TLR7 and TLR8

The *TLR7* and *TLR8* genes show high homology to each other, and are both located on the X chromosome. Mouse TLR7 and human TLR8 recognize synthetic antiviral imidazoquinoline components (R848, Imiquimod, etc.) and some guanine nucleotide analogs (loxoribine etc.) as well as uridine-rich or uridine/guanosine-rich ssRNA of both viral and host origins (Hemmi et al., 2002; Heil et al., 2004; Diebold et al., 2004). Human TLR8 also recognize R848. Although both TLR7 and TLR8 are expressed in mice, mouse TLR8 appears to be nonfunctional. TLR7 and TLR8 are expressed within the endosomal membrane, indicating that the accessibility of ssRNA may be a key factor for cell activation via these receptors. Many enveloped viruses traffic into the cytosol through the endosomal compartment. The phagolysosome is a highly acidified environment containing abundant degradation enzymes

that may damage the viral particles, leading to ssRNA release and recognition by TLR7 or TLR8. Furthermore, when virus-infected apoptotic cells are taken up by phagocytes, viral RNAs may be released from these cells in the phagolysosome. Unlike virus particles whose genomes are sheltered in the capsid, self RNAs are subject to degradation by extracellular RNases when they are released from the cell, and rarely reach the endocytic compartment.

Double-Stranded RNA Is Recognized by TLR3

dsRNA, along with its synthetic analog, polyinosine-deoxycytidylic acid (poly I:C), is a potent inducer of type I IFNs and is recognized by TLR3 (Alexopoulou et al., 2001). dsRNA can be generated during viral infection as a replication intermediate for ssRNA viruses or as a by-product of symmetrical transcription in DNA viruses. TLR3 is specifically expressed in conventional DCs (cDCs) that avidly phagocytose dying cells, but not in pDCs. TLR3 is also expressed in a variety of epithelial cells, including airway, uterine, corneal, vaginal, cervical, biliary, and intestinal epithelial cells, which function as efficient barriers to infection. Expression of TLR3 is rapidly and dramatically upregulated by treatment with poly I:C or IFN-α/β. Interestingly, although uterine epithelial cells or corneal epithelial cells express a wide range of TLRs, only poly I:C stimulates these cells. Unlike DCs, these epithelial cells appear to express TLR3 on their cell surface. Furthermore, TLR3 is strongly expressed in the brain, specifically in astrocytes and glioblastoma cell lines, indicating a specific role in the brain and/or in the response to encephalitogenic viruses. Since dsRNA is a universal viral PAMP, it has been assumed that TLR3 would have a key role in antiviral immunity. However, several lines of evidence have demonstrated that TLR3 is not required for the initial, cell-autonomous recognition of viral infection that induces the first wave of type I IFN production (Lopez et al., 2004). Consistent with this finding, *TLR3*$^{-/-}$ mice fail to show increased susceptibility to many viral infections such as MCMV, VSV, lymphocytic choriomeningitis virus (LCMV), and reovirus (Edelmann et al., 2004). The West Nile virus appears to benefit from its interaction with TLR3, indicating a peripheral inflammatory response via TLR3, which causes a disruption of the blood-brain barrier, and enables virus entry into the brain (Wang et al., 2004). Consistent with this observation, *TLR3*$^{-/-}$ mice are more resistant to lethal West Nile virus infection. Thus, the role of TLR3 in the antiviral response is still largely unclear. TLR3 has been reported to promote crosspresentation of virus-infected cells through engagement of virus-derived RNAs (Schulz et al., 2005). Immunization with virus-infected cells or cells containing synthetic dsRNA leads to a striking increase in CTL crosspriming against cell-associated antigens, which is largely dependent on TLR3 expression by antigen-presenting cells.

Viral Glycoproteins Are Recognized by TLR2 and TLR4

Some viral-envelope proteins can be recognized by TLR4 or TLR2. Detection mostly results in the production of proinflammatory cytokines, but not type I IFNs, implying that the response leads to the inflammation rather than specific antiviral responses. For instance, the fusion (F) protein from RSV has been identified as a viral component that activates TLR4 (Kurt-Jones et al., 2000). The importance of TLR4 activation during RSV infection in vivo was demonstrated with *TLR4*$^{-/-}$ mice, which exhibited lower levels of infiltrating mononuclear cells and reduced production of IL-12 compared to their wild-type counterparts, resulting in a reduced rate of viral clearance. The envelope protein (Env) of mouse mammary tumor virus (MMTV) also activates TLR4. Env directly activates B cells via TLR4 to allow an initial round of infection, and MMTV enhances the expression of its entry receptor, CD71, on DCs to facilitate virus entry (Burzyn et al., 2004). Thus, the MMTV-TLR interaction may favor the virus and represent a strategy to subvert the antiviral response. TLR2 is also activated by viruses or viral components such as measles virus (MV) hemagglutinin protein, human CMV, and HSV-1 (Bieback et al., 2002; Compton et al., 2003). It has been suggested that TLR2-mediated cytokine responses to HSV-1 are responsible for a significant portion of the morbidity and mortality associated with HSV-1 infection (Kurt-Jones et al., 2004).

Thus, various viral components are detected by TLRs, and this leads to the vigorous production of type I IFNs as well as proinflammatory cytokines. Nevertheless, antiviral host defense appears to involve more than just the TLR system. In RNA Helicases and Double-Stranded RNA, we will discuss TLR-independent viral detection mechanisms and their relationships to TLRs.

The TLR Signaling Pathway in General

The engagement of TLRs by microbial components triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense (Figure 2). After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules to the TIR domain of the TLR. There are four adaptor molecules, namely MyD88, TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1 (TICAM1) (Oshiumi et al., 2003; Yamamoto et al., 2002b), and TRIF-related adaptor molecule (TRAM). The differential responses mediated by distinct TLR ligands can be explained in part by the selective usage of these adaptor molecules. MyD88 and TRIF are responsible for the activation of distinct signaling pathways, leading to the production of proinflammatory cytokines and type I IFNs, respectively.

Proinflammatory Cytokine Production via TLRs

MyD88 is critical for the signaling from all TLRs except TLR3. Upon stimulation, MyD88 associates with the cytoplasmic portion of TLRs and then recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 through a homophilic interaction of the death domains (Figure 2). In TLR2 and TLR4 signaling, another adaptor, TIRAP/Mal, is required for recruiting MyD88 to the receptor (Fitzgerald et al.,

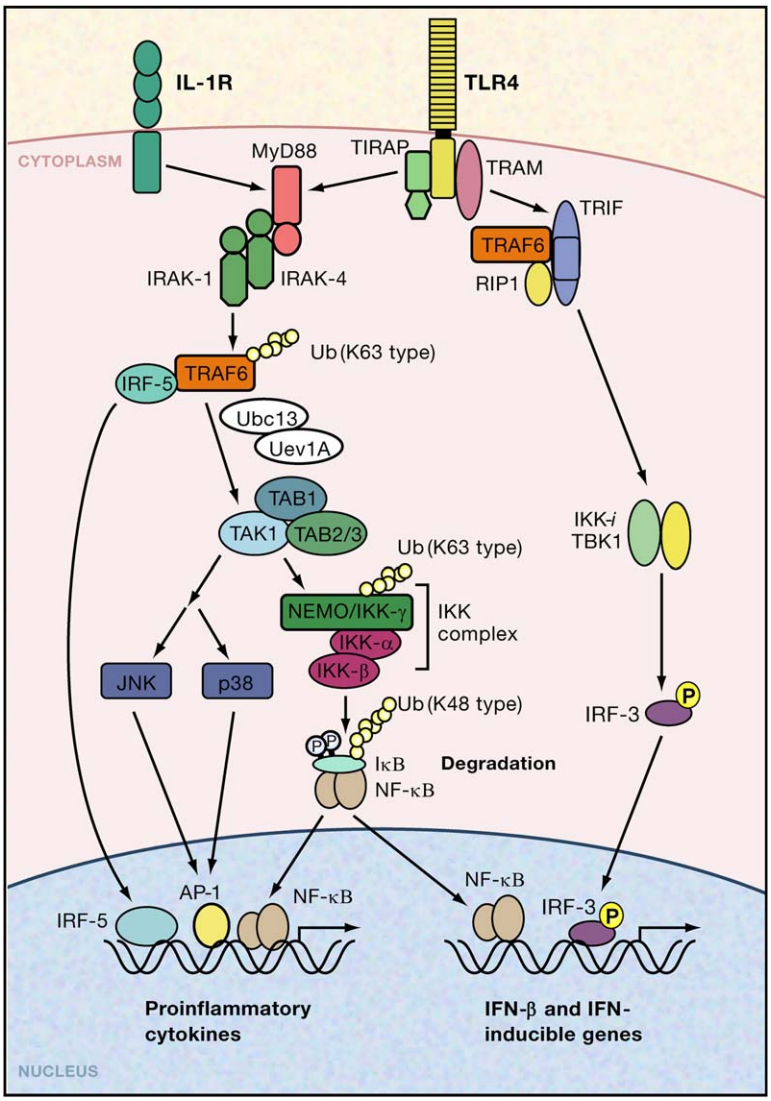

Figure 2. The TLR Signaling Pathway

TLRs and IL-1R share common signaling pathways in general. Stimulation with their ligands recruits TIR-domain-containing adaptors including MyD88 and TIRAP to the receptor, and the subsequent formation of a complex of IRAKs, TRAF6, and IRF-5 is induced. TRAF6 acts as an E3 ubiquitin ligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and UEV1A. This ubiquitination activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IκB undergoes K48-linked ubiquitination and degradation by the proteasome. Freed NF-κB translocates into the nucleus and initiates the expression of proinflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TLR4 triggers the MyD88-independent, TRIF-dependent signaling pathway via TRAM to induce type I IFNs. TRIF activates NF-κB and IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 induce NF-κB activation and TBK1/IKK-*i* phosphorylate IRF-3, which induces the translocation of IRF-3.

2001; Horng et al., 2001, 2002; Yamamoto et al., 2002a). The essential role of IRAK-4 in IL-1R/TLR responses is demonstrated by the defective responses observed in *IRAK-4*$^{-/-}$ mice and by the poor defenses against bacterial infection observed in patients having autosomal recessive amorphic mutations in *IRAK-4* (Picard et al., 2003; Suzuki et al., 2003). After IRAK-1 associates with MyD88, it is phosphorylated by the activated IRAK-4 and subsequently associates with TNFR-associated factor 6 (TRAF6), which acts as an ubiquitin protein ligase (E3) (Li et al., 2002). Subsequently, TRAF6, together with a ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, catalyzes the formation of a K63-linked polyubiquitin chain on TRAF6 itself and on IKK-γ/NF-κB essential modulator (NEMO) (Deng et al., 2000). A complex composed of TGF-β-activated kinase 1 (TAK1) and the TAK1 binding proteins, TAB1, TAB2, and TAB3, is also recruited to TRAF6 (Wang et al., 2001). TAK1 then phosphorylates IKK-β and MAP kinase kinase 6 (MKK6), which modulates the activation of NF-κB and MAP kinases, resulting in induction of genes involved in inflammatory responses.

In addition to NF-κB, the transcription factor IRF-5 regulates the expression of cytokine genes (Takaoka et al., 2005). Upon stimulation with TLR ligands, IRF-5 translocates into the nucleus and binds potential IFN-stimulated response element (ISRE) motifs present in the promoter regions of cytokine genes. IκBζ, an IκB-like molecule, is also indispensable for induction of a subset of genes activated in TLR signaling (Yamamoto et al., 2004). It is rapidly induced by stimulation with TLR ligands, but not TNF, and activates the IL-6, IL-12, and other inflammatory genes by associating with NF-kB p50.

Type I IFN Production via TLRs

TRIF-Dependent Pathway. Stimulation with TLR3, TLR4, TLR7, and TLR9 ligands, but not the TLR2 ligand, induces type I IFN production in addition to proinflammatory signals. TLR3 and TLR4 have the ability to induce IFN-β and IFN-inducible genes in *MyD88*$^{-/-}$ cells. The activity

of these pathways leads to DC maturation, expression of costimulatory molecules, and IFN-α/β secretion (Kaisho et al., 2001). This MyD88-independent pathway is initiated by another TIR-domain-containing adaptor, TRIF (Hoebe et al., 2003; Yamamoto et al., 2003a). TRAM, another TIR-domain-containing adaptor, is specifically involved in TLR4 signaling (Fitzgerald et al., 2003b; Yamamoto et al., 2003b). TRAM associates with TLR4 and TRIF, suggesting that TRAM acts as a bridging adaptor between TLR4 and TRIF.

TRIF interacts with receptor-interacting protein 1 (RIP1), which is responsible for the activation of NF-κB (Meylan et al., 2004). On the other hand, TRIF activates TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1; also known as NAK or T2K) via TRAF3 (Hacker et al., 2006; Oganesyan et al., 2006). TBK1 comprises a family with inducible IκB kinase (IKK-*i*, also known as IKK-ε) and these kinases directly phosphorylate IRF-3 and IRF-7 (Fitzgerald et al., 2003a; Sharma et al., 2003). Analysis of cells lacking TBK1 and IKK-*i* revealed that TBK1 and, to a lesser extent, IKK-*i* are responsible for TRIF-mediated IFN responses (Hemmi et al., 2004; McWhirter et al., 2004; Perry et al., 2004). Phosphorylated IRF-3 and IRF-7 form homodimers, translocate into the nucleus, and bind to the ISREs, resulting in the expression of a set of IFN-inducible genes. Among nine IRF family members, IRF-3 and IRF-7 are essential for the induction of type I IFN production since virus-mediated IFN production is severely impaired in *IRF-7*$^{-/-}$ mice and was abrogated in *IRF-3*$^{-/-}$*IRF-7*$^{-/-}$ cells (Honda et al., 2005b). Interestingly, TLR4-induced type I IFN production is impaired in *IRF-3*$^{-/-}$ mice, suggesting the distinct role of IRF-3 and IRF-7 in TLR- and virus-mediated signaling (Sakaguchi et al., 2003).

TLR7- and TLR9-Mediated Type I IFN Production in pDCs. TLR7 and TLR9 are highly expressed in pDCs, and stimulation of pDCs, but not cDCs, with TLR7 and 9 ligands leads to induction of IFN-α. Intriguingly, TLR9-mediated IFN-α secretion occurs in a MyD88-dependent manner, in contrast to TLR3- or TLR4-mediated IFN responses, which are dependent on TRIF but not on MyD88. In addition, TLR9-mediated IFN production does not depend on TBK1, suggesting that the signaling pathways activated by TLR9 are different from those activated by TRIF.

In pDCs, IRF-7 plays a critical role in the expression of type I IFNs. Upon stimulation, a complex comprised of MyD88, IRAK-4, IRAK-1, TRAF6, and IRF-7 is formed and recruited to the TLR (Figure 3) (Honda et al., 2004; Kawai et al., 2004). pDCs lacking MyD88 or IRAK-4 failed to produce either inflammatory cytokines or IFN-α in response to CpG-DNA stimulation. On the other hand, IRAK-1, which can potentially serve as an IRF-7 kinase, appears to mediate TLR7- and TLR9-induced IFN-α production in pDCs since this response is absent in *IRAK-1*$^{-/-}$ pDCs yet inflammatory cytokines are produced normally (Uematsu et al., 2005). Furthermore, IRF-7 activation by the TLR9 ligand is impaired in *IRAK-1*$^{-/-}$ pDCs in spite of normal NF-κB activation, suggesting that IRAK-1 specifically mediates IFN-α induction downstream of MyD88 and IRAK-4.

A/D type CpG-DNAs are potent inducers of IFN-α in pDCs but not in cDCs, but the molecular mechanism underlying this difference is not understood. One explanation is that pDCs express high amounts of IRF-7, a key transcription factor for IFN-α synthesis, while cDCs express lower levels. A recent paper has proposed an additional explanation: that A/D type CpG-DNAs are retained longer in endosomal vesicles in pDCs but are rapidly transferred to the lysosome in cDCs, thus facilitating encounters between the DNA and TLR9-MyD88-IRF-7 complexes in pDCs (Honda et al., 2005a).

Cytoplasmic Pathogen Recognition System

TLRs recognize pathogens at either the cell surface or lysosome/endosome membranes, suggesting that the TLR system is not used for the detection of pathogens that have invaded the cytosol. These pathogens are detected by various cytoplasmic PRRs, which activate a number of signaling pathways. A large family of cytoplasmic PRRs has been cloned to date. Currently, they are roughly subclassified into the NOD-LRR proteins and the CARD-helicase proteins. These protein families are implicated in the recognition of bacterial and viral components, respectively.

NOD-LRR Proteins and Their Functions

NOD-LRR proteins are implicated in the recognition of bacterial components. Proteins in this family possess LRRs that mediate ligand sensing; a nucleotide binding oligomerization domain (NOD); and a domain for the initiation of signaling, such as CARDs, PYRIN, or baculovirus inhibitor of apoptosis repeat (BIR) domains (Inohara et al., 2005; Martinon and Tschopp, 2005).

Among the large number of NOD-LRR family members, the functions of several proteins have been studied. These proteins include NOD1 and NOD2, which both contain N-terminal CARD domains. NOD1 and NOD2 detect γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), found in bacterial PG, respectively (Figure 4) (Chamaillard et al., 2003; Girardin et al., 2003). Consistently, macrophages lacking either NOD1 or NOD2 fail to produce cytokines in response to the corresponding ligands (Kobayashi et al., 2005). A missense point mutation in the human *NOD2* gene is correlated with susceptibility to Crohn's disease, an inflammatory bowel disease. Ligand binding to NOD1 and NOD2 causes their oligomerization and results in NF-κB activation through the recruitment of RIP2/RICK, a serine/threonine kinase, to the NODs via their respective CARD domains by homophilic interactions.

Infection with bacteria induces activation of caspase-1, which catalyzes the processing of pro-IL-1β to produce the mature cytokines. A complex of proteins responsible for these catalytic processes has been purified and designated the inflammasome (Figure 4) (Martinon et al., 2002). The inflammasome consists of caspase-1; caspase-5; ASC; and members of the NALP family, which are PYRIN-

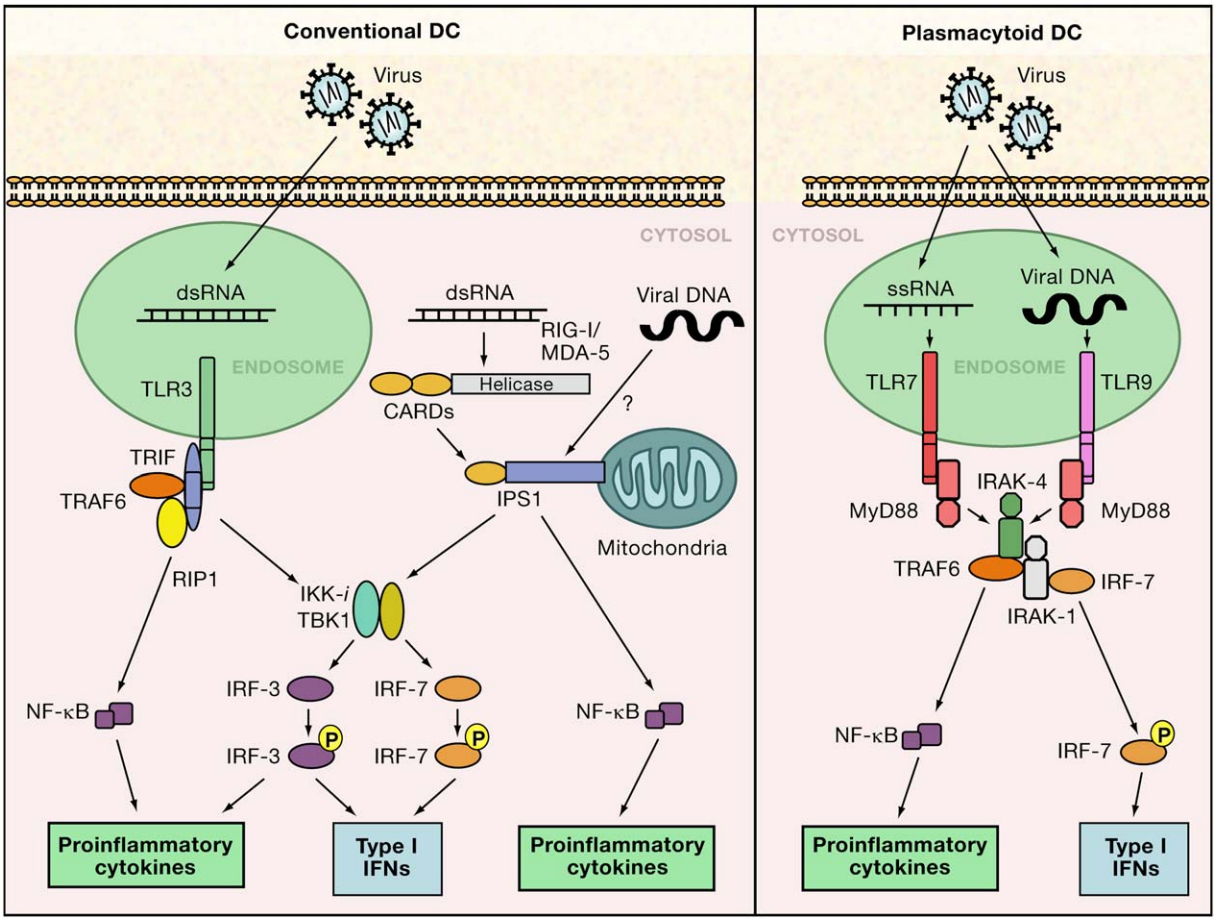

Figure 3. Mechanisms of Viral Detection

In cDCs, TLR3-dependent and RIG-I-dependent pathways operate to detect viral infection. Recognition of dsRNA by TLR3 in the endosomal membrane recruits TRIF to the receptor, which induces proinflammatory cytokines and type I IFNs via the RIP1/TRAF6-NF-κB pathway and the TBK1/IKK-*i*-IRF-3/IRF-7 pathway, respectively. In contrast, detection of dsRNA in the cytoplasm by RIG-I activates TBK1/IKK-*i* through IPS-1, which is localized on the mitochondrial membrane.

In pDCs, TLR7 and TLR9 recognize viral ssRNA and DNA, respectively. Stimulation with TLRs recruits a complex of MyD88, IRAK-4, IRAK-1, TRAF6, and IRF-7. Phosphorylated IRF-7 translocates into the nucleus and upregulates the expression of type I IFN genes.

domain-containing proteins that also contain NOD-LRR. ASC (apoptosis-associated speck-like protein containing a CARD) is an adaptor protein that contains a PYRIN domain and a CARD. NALPs recruit ASC through a homotypic interaction between the PYRIN domains, and ASC in turn recruits caspase-1 via its CARD, leading to the activation of IL-1β and IL-18 processing. $ASC^{-/-}$ macrophages exhibit defective maturation of IL-1β and IL-18 (Mariathasan et al., 2004). The ligands for NALP family members are currently unknown, except for NALP3, which is involved in the recognition of bacterial RNA, ATP, and uric-acid crystals (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006). Ipaf, another CARD-containing NOD-LRR protein, is responsible for *S. typhimurium*-induced, but not TLR-induced, caspase-1 activation (Mariathasan et al., 2004).

NAIP5, a NOD-LRR protein containing BIR domains, is associated with host susceptibility to the intracellular pathogen *L. pneumophila* (Diez et al., 2003). Although NAIP5 is assumed to function as a cytoplasmic sensor of *Legionella*, the specific ligands of NAIP5 and its signaling pathway remain to be identified.

RNA Helicases and Double-Stranded RNA

dsRNA that is synthesized in the cytoplasm of the cell or that is present in viral genomes already released into the cell is not accessible to TLR3, the TLR that recognizes dsRNAs. Indeed, most virus-infected cells produce type I IFNs in a TLR3-independent manner. Moreover, fibroblasts and cDCs lacking MyD88 and TRIF are still capable of inducing type I IFNs after viral infection, indicating that the TLR system is not required for viral detection in at least several cell types (Kato et al., 2005).

Retinoic-acid-inducible protein I (RIG-I) is an IFN-inducible protein containing CARDs and a DExD/H box helicase domain and has been identified as a cytoplasmic dsRNA detector (Figure 3) (Yoneyama et al., 2004).

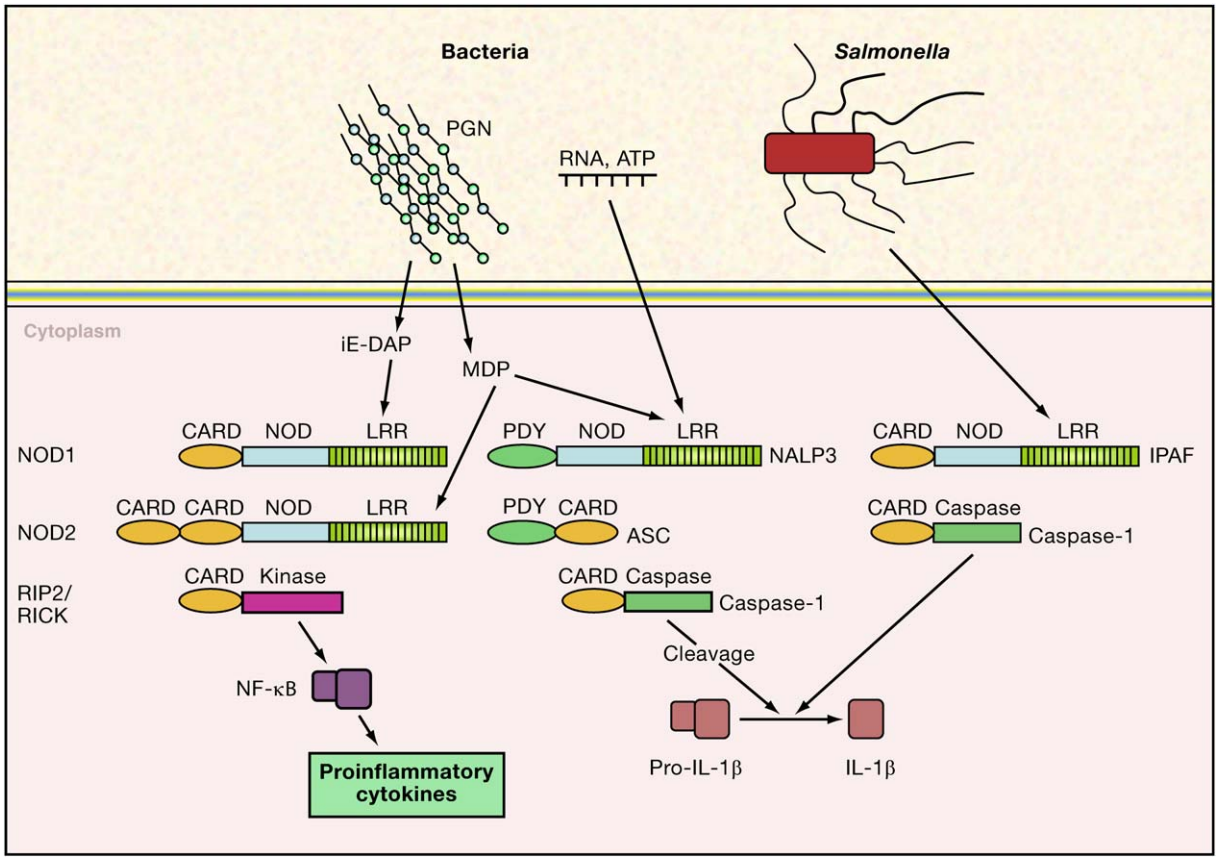

Figure 4. Cytoplasmic Bacterial Detectors and Their Signaling

NOD-LRR proteins recognize bacterial proteins in the cytoplasm and trigger signaling pathways. NOD1 and NOD2 recognize iE-DAP and MDP, respectively, and activate NF-κB via RIP2/RICK. MDP is also recognized by NALP3, which forms an inflammosome comprised of ASC, CARDINAL, and caspase-1. Activated caspase-1 cleaves pro-IL-1β for the maturation of IL-1β. Another NOD-LRR protein, IPAF, is activated by *Salmonella* and induces maturation of IL-1β by associating with caspase-1.

Overexpression of RIG-I has been shown to enhance Newcastle disease virus (NDV)- and dsRNA-mediated IFN responses on the cells. Melanoma differentiation associated gene 5 (MDA5), a molecule showing homology to RIG-I, has also been implicated in the recognition of viral dsRNA (Andrejeva et al., 2004; Kang et al., 2002). In addition, these proteins bind poly I:C. The protein LGP2 also shares homology with RIG-I in the helicase domain, although it lacks a CARD (Rothenfusser et al., 2005; Yoneyama et al., 2005). It has been suggested that LGP2 acts as a negative regulator of RIG-I/MDA-5 signaling. Analyses of *RIG-I*$^{-/-}$ cells revealed that RIG-I is essential for the induction of type I IFN responses after RNA virus infection (Kato et al., 2005).

Recently, expression cloning studies have identified IPS-1, an adaptor protein composed of an N-terminal CARD domain resembling that of MDA-5 or RIG-I (Kawai et al., 2005). This protein was also independently isolated and functionally characterized by several groups and has also been designated, MAVS, VISA, or CARDIF (Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). When expressed in human cells, this protein has the ability to induce the activation of the type I IFN promoter as well as NF-κB. IPS-1 associates with RIG-I or MDA5 via their CARD domains, suggesting that IPS-1 acts as an adaptor for RIG-I and MDA-5. Consistently, RNAi-mediated knockdown of IPS-1 resulted in inhibition of dsRNA- or RNA-virus-induced type I IFN responses. These findings indicate that IPS-1/MAVS/VISA/CARDIF plays an essential role in RIG-I/MDA5 signaling. Interestingly, this protein is present in the outer mitochondrial membrane, suggesting that mitochondria might be important for IFN responses in addition to their roles in metabolism and cell death (Seth et al., 2005). Downstream of RIG-I-IPS-1, TBK1 and IKK-*i* are activated to phosphorylate IRF-3 and IRF-7, indicating that the signaling pathways triggered by TLR stimulation and RIG-I converge at the level of TBK1/IKK-*i*.

FADD and RIP1 have been reported to be required for type I IFN production in response to dsRNA, and *FADD*$^{-/-}$ or *RIP1*$^{-/-}$ MEFs have been shown to be highly susceptible to VSV infection (Balachandran et al., 2004).

IPS-1 interacts with FADD and RIP1 via the non-CARD region to facilitate NF-κB activation. Taken together, this evidence suggests that IPS-1 is an adaptor linking RIG-I and MDA5 to downstream signaling mediators including FADD, RIP1, TBK1 and IKK-*i*. However, virus-induced type I IFN responses are not impaired in *FADD*$^{-/-}$ cells (Yoneyama et al., 2005). Further analyses are therefore required to clarify the roles of FADD and RIP1 in the IFN response to viral infection.

How do RIG-I and TLR3 differ in their recognition of viral components? Given that RIG-I and TLR3 are localized in the cytoplasm and on endosomal membrane, respectively, it is hypothesized that the viral entry route may determine the contribution of each. However, some evidence suggests that the route of infection is not so critical. For example, Sendai virus and VSV infect cells via different routes, namely cell fusion and endosomal entry, respectively, yet both induce the production of type I IFNs in cDCs via RIG-I, but not via TLR3,. These suggest that RNA viruses actively replicating in the cytoplasm are recognized by RIG-I, but not TLR3, irrespective of their route of entry. On the other hand, TLR3 has been suggested to be responsible for the recognition of dsRNA contained in the apoptotic bodies of virus-infected cells taken up by DCs.

Interestingly, pDCs from *RIG-I*$^{-/-}$ and wild-type mice produce a comparable amount of IFN-α in response to NDV stimulation, indicating that pDCs mainly use mechanisms for the induction of IFNs that do not involve RIG-I (Kato et al., 2005). In pDCs, the TLR system appears to play more important role in the induction of type I IFNs. These observations suggest that pDCs have developed specialized mechanisms for the detection of viruses. Given that UV-inactivated virus particles are still capable of inducing type I IFNs via TLRs, it is unlikely that the replication of viruses in pDCs is needed for their recognition (Lund et al., 2003). Moreover, it is well known that pDCs are difficult to infect with viruses. Therefore, viral recognition in pDCs may mainly depend on endocytosis of viral particles rather than direct infection. However, further studies are needed to uncover the mechanisms of the cell-type-specific viral recognition.

As described in Viral DNA Is Recognized by TLR9, infection with DNA viruses such as HSV1 induces type I IFN production in both TLR9-dependent and -independent manners. Similar to dsRNA, viral DNA is also recognized in the cytoplasm independently of the TLRs. Induction of type I IFNs by cytoplasmic DNA is dependent on TBK1/IKK-*i* and IPS-1 and is independent on RIG-I, implying that infected viral DNA is recognized by an unknown PRR that signals via IPS-1 and TBK1/IKK-*i* (Ishii et al., 2005).

Escape of Pathogens from Innate Immune Recognition

Viruses employ multiple strategies to evade the host immune system. A number of viral proteins inhibit host immune responses, prevent viral antigen presentation, and abrogate induction of cell death. Given their roles in detecting viruses, it is not surprising that molecules involved in TLR signaling, particularly TIR-domain-containing adaptors, are the targets of viral immune disturbance. For example, vaccinia virus produces the TIR-domain-containing proteins A46R and A52R, which target host MyD88 and TRIF, and suppresses TLR- or IL-1R-induced NF-κB activation (Bowie et al., 2000; Stack et al., 2005). Vaccinia virus also encodes N1L, a protein that antagonizes TLR signaling at the level of IKKs and TBK1 (DiPerna et al., 2004). Another strategy by which viruses evade antiviral immunity is the processing of host signaling molecules. Hepatitis C virus (HCV) encodes NS3/4A protease, which blocks IRF-3 activation by cleaving TRIF and thereby subverting poly I:C-mediated activation of IFN responses (Li et al., 2005).

Since the RIG-I/MDA-5 system is critical for host defense against RNA viruses, we might expect that its signaling cascades are also targeted by viruses to evade immune responses. In fact, various proteins encoded by RNA viruses have been shown to antagonize the RIG-I/MDA5 pathway to inhibit type I IFN responses. The V proteins of paramyxoviruses associate with MDA-5, but not with RIG-I, and inhibit dsRNA-induced activation of the IFN-β promoter (Andrejeva et al., 2004). HCV NS3/4A protease also targets IPS-1/MAVS/VISA/CARDIF (Meylan et al., 2005). Moreover, various RNA viruses and their proteins suppress IRF activation as well as type I IFN production, although their precise mechanisms have yet to be understood.

DNA viruses have also developed various strategies to inhibit host immune surveillance. First, the genomic DNA of certain adenoviruses contains an unusually low frequency of the immunostimulatory CpG-DNA, thereby avoiding immune surveillance by TLR9. In addition, pathways activating IRFs are suppressed by DNA viruses. The ICP0 and ICP34.5 proteins from HSV1 inhibit IRF-3 activation, and a ubiquitin E3 ligase RTA encoded by the Kaposi's sarcoma-associated herpesvirus (KSHV) promotes IRF-7 ubiquitination and proteasome-mediated degradation (Lin et al., 2004; Yu et al., 2005). These indicate that both RNA and DNA viruses can hamper host type I IFN production and suggest that controlling the IFN response is essential for the survival of a broad range of viruses.

Bacteria and fungi are also able to exploit the TLR system to evade host immune responses. Certain pathogens have modified forms of the normal TLR ligands, such as the LPSs from *H. pylori*, *P. gingivalis*, and *L. pneumophila* and flagellin of *H. pylori* and *C. jejuni* (Andersen-Nissen et al., 2005). Some pathogens modify the TLR signaling pathways for their benefits. *M. tuberculosis* avoids being killed by macrophages by inhibiting IFN-γ-mediated signaling. Prolonged signaling with a 19 kDa lipoprotein from *Mycobacterium*, which stimulates TLR2, inhibits IFN-γ production and major histocompatibility complex (MHC) class II antigen-processing activity (Fortune et al., 2004; Pai et al., 2003). These findings suggest that, at least

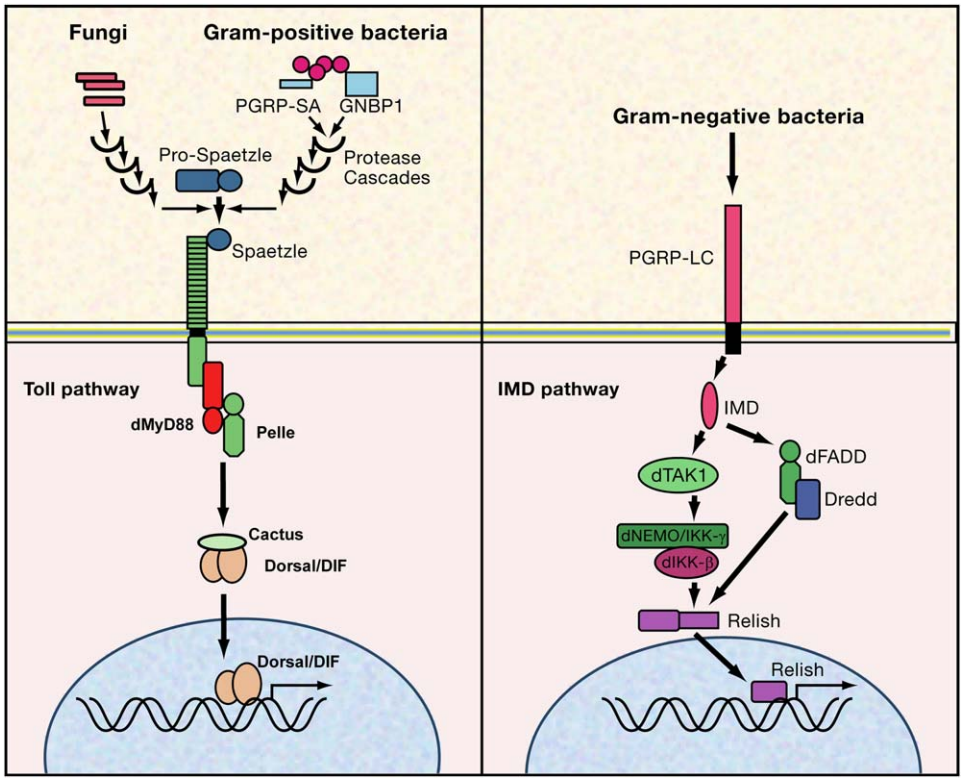

Figure 5. The Toll and Imd Pathways in *Drosophila*

Infection by fungi and Gram-positive bacteria activates the Toll signaling pathway. GNBP1 and PGRP-SA recognize Gram-positive bacteria and trigger activation of protease cascades, leading to the cleavage of Spaetzle. Spaetzle binds Toll, initiates signaling pathways by recruiting dMyD88 and Pelle, and induces nuclear translocation of the Rel-type transcription factors Dorsal and DIF. Gram-negative bacterial infection is sensed by PGRP-LC, and the Imd pathway is activated. Downstream of the *Imd* gene, dTAK1 and dFADD function as a IKK kinase and a Dredd activator, respectively. Relish is phosphorylated by the IKK and cleaved by Dredd, leading to the induction of antimicrobial-peptide genes.

in part, persistent TLR2 signaling enables *Mycobacterium* to evade T cell responses and persist as a long-term infection. Similarly, pathogenic *Yersinia* species release V antigen (LcrV), a virulence factor that stimulates the production of IL-10 via TLR2 and suppresses production of TNF and IFN-γ. As a consequence, *TLR2*$^{-/-}$ mice are actually less susceptible to oral infection with *Yersinia enterocolitica* (Sing et al., 2002).

Evolutionary Aspects of Innate Immunity

Advances in a number of genome projects have revealed that the TLR genes are conserved among many vertebrates, including chicken, *Xenopus*, zebrafish, and the Japanese pufferfish *Takifugu rubripes* (Roach et al., 2005). Mammalian TLRs are subdivided into six families based on their sequence similarities. A bioinformatics analysis revealed that all of these families are conserved among vertebrates from *Takifugu* and *Xenopus* to mammals. Indeed, mammalian TLR ligands, such as dsRNA, CpG-DNA, and flagellin, are immunostimulatory in the fish. This conservation suggests that there is little mutation or variation among the PAMPs recognized by TLRs and that selective pressure to recognize these PAMPs tropic to a broad range of vertebrates maintains the repertoire of TLRs that detect them.

Although it was the initial discovery and characterization of *Drosophila* Toll that led to the identification of vertebrate TLRs, subsequent studies have revealed large differences in the pathogen recognition mechanisms between vertebrates and flies (Hoffmann, 2003). The *Drosophila Toll* pathway is activated in response to infections by fungi and Gram-positive bacteria. It is well known that *Drosophila* Toll activates signaling cascades closely resembling the mammalian TLR signaling pathway. However, unlike the mammalian system, *Drosophila* Toll does not directly recognize fungal and bacterial components but rather interacts with endogenous ligands generated by a protease cascade (Figure 5). Invading Gram-positive bacteria are recognized by PG-recognition proteins (PGRP) followed by the activation of the Toll ligand Spaetzle via the protease cascade (Michel et al., 2001). In *Drosophila*, the Toll family consists of nine members, although other Toll family members may not be involved in antimicrobial immune responses. On the other hand, Gram-negative bacterial infection activates the *Imd* pathway, but not the *Toll* pathway. Gram-negative binding proteins (GNBPs) and

a member of the PGRP family containing a transmembrane domain have been identified as a PRR in the *Imd* pathway (Gottar et al., 2002). The IMD protein contains a death domain that has high homology to that of mammalian RIP (Hoffmann, 2003). *Drosophila* homologs of FADD, TAK1, and IKK-β are also involved in the *Imd* pathway. Activation of the *Drosophila* IKK-β leads to phosphorylation of a NF-κB-like protein, Relish, resulting in its cleavage and subsequent nuclear translocation. In turn, Relish induces the expression of genes encoding antimicrobial peptides against Gram-negative bacteria. *Drosophila* antiviral immune responses are less understood, although it is known that there are JAK-STAT-dependent and -independent pathways modulating transcriptional responses against *Drosophila* C virus infection (Dostert et al., 2005).

TLRs exist in primitive metazoans as well. A single Toll homolog has been identified in *C. elegans* and termed *Tol-1* (Pujol et al., 2001). Tol-1 expression in adults is restricted to the nervous system and is implicated in embryonic development and pathogen recognition. Tol-1 mutants are susceptible to bacterial infection due to impaired pathogen avoidance. These suggest that Tol-1 contributes to the recognition of a specific bacterial component (or components) and elicits in a change in *C. elegans* behavior. Furthermore, it has been shown that a TIR-containing adaptor, TIR-1, is critical for resistance to pathogens via activation of p38 MAP kinase (Couillault et al., 2004). However, the activity of TIR-1 is independent of the single Toll homolog, and the function of its mammalian homolog, SARM1, is not yet understood.

Plants infected with pathogens activate host defense responses, including elimination of infected cells, called the hypersensitive response, and antibiotic production at the site of infection. Although plants do not possess TLRs, a family of multiple NOD-LRR proteins, called resistance (R) proteins, play a critical role in the host defense (Belkhadir et al., 2004). In *Arabidopsis*, effector domains of the R proteins contain TIR, leucine-zipper-like, or coiled-coil domains. *Arabidopsis* has more than 149 genes encoding R proteins in its genome. R protein-mediated signaling induces the hypersensitive response, which is a form of programmed cell death, to prevent the spread of infection. Whereas mammalian NOD-LRR proteins recognize PG components directly, plant R proteins are shown to indirectly detect bacterial proteins (Belkhadir et al., 2004). In addition, the plant genome encodes another type of PRRs that are composed of LRRs, a transmembrane domain, and a serine/threonine kinase domain. The flagellin-sensitive receptor (FLS2) of *Arabidopsis thaliana* is an extensively studied receptor-like kinase (Gomez-Gomez and Boller, 2000). As the name indicates, FLS2 detects flagellin monomers and is functionally equivalent to mammalian TLR5. However, the LRR domain of FLS2 is different in amino acid sequence from that of TLR5 and recognizes distinct regions of flagellin monomers. These results may indicate that the LRR structure has been independently selected in plants and mammals for the detection of flagellin. Downstream of FLS2, a MAP kinase cascade and WRKY transcription factors are activated to induce effector proteins.

Taken together, immune recognition is mediated by PRRs that are composed of a related set of domains throughout different species. However, the details of pathogen recognition and signaling mechanisms differ significantly among species, especially between vertebrate and invertebrate plants or animals. This indicates that ancient innate immunity has branched off into a variety of unique and specialized systems in each species during evolution.

Future Perspectives

There has been considerable recent expansion in our knowledge of host innate immune responses against pathogen infection. These responses involve some very sophisticated mechanisms for detecting various pathogens using a limited number of PRRs and induction of adaptive responses. In particular, dissection of the functions of the various TLRs and their signaling pathways has revealed that mammalian innate immune cells activate distinct signaling pathways depending on the pathogen involved in the infection and mount adaptive cytokine responses for each.

However, there are other systems for recognizing pathogens that do not involve the TLRs: In particular, the detection of pathogens via cytoplasmic PRRs plays a critical role in mounting immune responses. A more complete description of the cytoplasmic PRRs and their functions awaits future study. In addition, the relationships among the TLR system and cytoplasmic PRRs, as well as the details of the mechanisms of TLR-independent activation of adaptive immunity, are quite intriguing issues that remain to be clarified. Accumulating evidence suggests that these different systems function in a cell-type-specific manner. Furthermore, cooperation between immune cells is critical for the rapid elimination of pathogens in vivo. Natural killer (NK) cells are known to detect microbial infection via a non-TLR receptor system, and the interaction between DCs and NK cells is prerequisite for mounting efficient innate immune responses. In summary, the mechanisms of cytoplasmic pathogen recognition and the role of these PRRs in activating immune responses in vivo are important future topics to be investigated.

ACKNOWLEDGMENTS

We thank all of the colleagues in our lab for helpful discussion, M. Lamphier for careful appraisal of the manuscript, and M. Hashimoto for secretarial assistance. This work is supported in part by grants from the Special Coordination Funds of the Japanese Ministry of Education, Culture, Sports, Science and Technology and from the 21st Century Center of Excellence Program of Japan.

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