

TOLL-LIKE RECEPTORS AND INNATE IMMUNITY

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Toll-like receptors have a crucial role in the detection of microbial infection in mammals and insects. In mammals, these receptors have evolved to recognize conserved products unique to microbial metabolism. This specificity allows the Toll proteins to detect the presence of infection and to induce activation of inflammatory and antimicrobial innate immune responses. Recognition of microbial products by Toll-like receptors expressed on dendritic cells triggers functional maturation of dendritic cells and leads to initiation of antigen-specific adaptive immune responses.

One of the most fascinating problems in immunology is understanding how the host organism detects the presence of infectious agents and disposes of the invader without destroying self tissues. This problem is not trivial given the enormous molecular diversity of pathogens and their high replication and mutation rates. In response to this challenge, multicellular organisms have evolved several distinct immune-recognition systems. In vertebrate animals, these systems can be broadly categorized as 'innate' and 'adaptive'.

Adaptive immune recognition relies on the generation of a random and highly diverse repertoire of antigen receptors — the T- and B-cell receptors (TCR and BCR) — followed by clonal selection and expansion of receptors with relevant specificities. This mechanism accounts for the generation of immunological memory, which provides a significant adaptive fitness to vertebrate animals. However, the adaptive immune response has two main limitations. First, randomly generated antigen receptors are unable to determine the source and the biological context of the antigen for which they are specific. Second, a clonal distribution of antigen receptors requires that specific clones expand and differentiate into effector cells before they can contribute to host defence. As a result, primary adaptive immune responses are delayed, typically for 4–7 days, which is too much of a delay to combat quickly replicating microbial invaders. However, the adaptive immune system does not function independently. Indeed, almost every aspect of the adaptive immune response is controlled by a combination of

permissive and instructive signals, which are provided by the evolutionarily ancient and more universal innate immune system. As will be discussed, the innate immune system detects the presence and the nature of infection, provides the first line of host defence, and controls the initiation and determination of the effector class of the adaptive immune response.

Although the innate immune system was first described by Elie Metchnikoff over a century ago, progress in its analysis has been largely overshadowed by the fascinating intricacies of adaptive immunity. Nevertheless, the discoveries of antimicrobial peptides, complement and dendritic cells (DCs), as well as studies in plant and invertebrate immunity, have all greatly contributed to our current understanding of the innate immune system. The recent discovery and characterization of the Toll-like receptor (TLR) family have incited new interest in the field of innate immunity. It is already clear that these receptors have a vital role in microbial recognition, induction of antimicrobial genes and the control of adaptive immune responses. Indeed, recent studies have shown that TLRs have a crucial role in the recognition of 'molecular signatures' of microbial infection, in engaging differential signalling pathways, and in controlling DC maturation and differentiation of T helper (T_H) cells.

Innate immune recognition

The strategy of innate immune recognition is based on the detection of constitutive and conserved products of

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Box 1 | **PAMPs and virulence factors**

Pathogen-associated molecular patterns (PAMPs) and virulence factors are not equivalent. PAMPs did not evolve to interact with the host immune system; they evolved to perform essential physiological functions. Pattern-recognition receptors evolved to recognize PAMPs, and therefore to detect the presence of infection. Virulence factors, by contrast, developed as a microbial adaptation to the unique environment within the host. As PAMPs are essential for microbial survival, they are incapable of sustaining mutations. As a result, they are conserved within a class of microbes. Virulence factors are produced by pathogens in order to interact with the host: to invade host cells, to form colonies, to avoid host immune responses, or to adjust to new nutrient sources. Because each group of pathogens has developed a unique strategy for survival within the host, there are multiple virulence factors that can vary between different strains and species of pathogens. Virulence factors are typically encoded by 'pathogenicity islands', which are associated with several features characteristic of mobile DNA and can be acquired by, or deleted from, the microbial genome. Furthermore, unlike PAMPs, which in most cases are expressed constitutively, the genes encoding virulence factors are turned on and off depending on the stage of the infection cycle. The lack of conservation and the inducible expression of virulence factors are two probable reasons why, at least in animals, they were not selected during evolution as targets for innate immune recognition.

It should be noted, however, that in plants, the distinction between PAMPs and virulence factors might not hold. In addition to PAMP recognition, some plant host-defence receptors are thought to interact with virulence factors, in particular with the effectors of the TYPE III SECRETION SYSTEM.

microbial metabolism. Many metabolic pathways and individual gene products are unique to microorganisms and absent from host cells. Some of these pathways are involved in housekeeping functions and their products are conserved among microorganisms of a given class and are essential for their survival. For example, lipopolysaccharide (LPS), lipoproteins, peptidoglycan and lipoteichoic acids (LTAs) are all molecules made by bacteria, but not by eukaryotic cells. Therefore, these products can be viewed as molecular signatures of microbial invaders, and their recognition by the innate immune system can signal the presence of infection^{1,2}. One important aspect of innate recognition is that its targets are not absolutely identical between different species of microbes. However, although there are several strain- and species-specific variations of the fine chemical structure, these are always found in the context of a common molecular pattern, which is highly conserved and invariant among microbes of a given class. For example, the lipid-A portion of LPS represents the invariant pattern found in all Gram-negative bacteria and is responsible for the pro-inflammatory effects of LPS, whereas the O-antigen portion is variable in LPS from different species of bacteria and is not recognized by the innate immune system. Because the targets of innate immune recognition are conserved molecular patterns, they are called pathogen-associated molecular patterns (PAMPs). Accordingly, the receptors of the innate immune system that recognize PAMPs are called pattern-recognition receptors (PRR)¹.

PAMPs have three common features that make them ideal targets for innate immune recognition. First, PAMPs are produced only by microbes, and not by host cells. Therefore, recognition of PAMPs by the innate immune system allows the distinction between 'self' and 'microbial non-self'. Second, PAMPs are invariant

between microorganisms of a given class. This allows a limited number of germ-line-encoded PRRs to detect the presence of any microbial infection. So, recognition of the conserved lipid-A pattern in LPS, for example, allows a single PRR to detect the presence of almost any Gram-negative bacterial infection. Third, PAMPs are essential for microbial survival. Mutations or loss of PAMPs are either lethal for that class of microorganisms, or they greatly reduce their adaptive fitness. Therefore, 'escape mutants' are not generated.

These properties of PAMPs indicate that their recognition must have emerged very early in the evolution of host-defence systems. Indeed, many PAMPs are recognized by the innate immune systems not only of mammals, but also of invertebrates and plants.

It is important to note that PAMPs are actually not unique to pathogens and are produced by both pathogenic and non-pathogenic microorganisms. In fact, none of the gene products that are unique to pathogens — the so-called 'virulence factors' — are known to be recognized by the mammalian innate immune system (BOX 1). This means that PRRs cannot distinguish between pathogenic and commensal microorganisms. This distinction, however, is vitally important. We live in constant contact with commensal microflora, and continuous activation of inflammatory responses by commensals would have potentially lethal consequences for the host. This, however, does not occur under normal physiological conditions. The exact mechanisms that allow the host to 'tolerate' non-pathogenic microorganisms are largely unknown. Presumably, compartmentalization (for example, confinement of microflora to the luminal side of intestinal epithelium), as well as anti-inflammatory cytokines, such as transforming growth factor- β (TGF- β) and interleukin (IL)-10, have an important role in this process.

The innate immune system uses various PRRs that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. The principal functions of PRRs include: opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling pathways and induction of apoptosis^{1,3} (TABLE 1).

Toll-like receptors

The Toll-like receptors are PRRs that have a unique and essential function in animal immunity. TLRs comprise a family of type I transmembrane receptors, which are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain⁴⁻⁶.

LRRs are found in a diverse set of proteins in which they are involved in ligand recognition and signal transduction⁷. The characteristic feature of the LRRs is the consensus sequence motif, L(X₂)LXL(X₂)NXL(X₂)L(X₂)L(X₂), in which X is any amino acid. The LRR region in the TLRs is separated from the transmembrane region by a so-called 'LRR carboxy-terminal domain', which is characterized by the consensus motif CXC(X₂₃)C(X₁₇)C.

TYPE III SECRETION SYSTEM
A specialized multisubunit secretion apparatus found in many Gram-negative bacterial pathogens. It allows the bacteria to secrete various effector proteins directly into the cytosol of the host cells, where they have several functions, such as induction of apoptosis and stimulation of phagocytosis.

The TIR domain of Toll proteins is a conserved protein–protein interaction module, which is also found in a number of transmembrane and cytoplasmic proteins in animals and plants⁸. Interestingly, most of the TIR domain-containing proteins in animals and plants have a role in host defence (FIG. 1).

In transmembrane proteins, the TIR domain is also present in the cytoplasmic portions of members of the IL-1 receptor (IL-1R) family, including IL-1R and IL-18R. Instead of LRR domains, IL-1R and IL-18R have three immunoglobulin domains in their extracellular portions. In mammals, the TIR domain is also present in several cytoplasmic proteins, including two signalling adaptors, MyD88 (REFS 9–12) and TIRAP¹³ (TIR domain-containing adaptor protein), both of which function in TLR signal transduction (FIG. 1; and see below).

TLRs in *Drosophila* immunity

The first identified member of the Toll family, *Drosophila Toll*, was discovered as a maternal-effect gene that functions in a pathway that controls dorsoventral axis formation in fruitfly embryos^{4,14}. Other genes in this pathway encode the Toll ligand Spätzle, the adaptor protein Tube, the protein kinase Pelle, the nuclear factor-κB (NF-κB)-family transcription factor Dorsal, and the Dorsal inhibitor and mammalian inhibitor of κB (IκB) homologue Cactus¹⁵. Spätzle is secreted as a precursor protein that has to be processed by serine proteases before it can activate Toll¹⁵. It should be noted that although genetic studies clearly show that Spätzle functions upstream of Toll, direct binding of Spätzle to Toll has yet to be shown.

The similarity between the *Drosophila* Toll pathway and the mammalian IL-1R pathway indicated that the Toll pathway might function in fruitfly immunity, as well as in developmental patterning. This was shown in Toll mutant *Drosophila*, which rapidly succumb to fungal infection, due to a failure to induce the antifungal peptide Drosomycin¹⁶. Similarly, fruitflies with loss-of-function mutations in *spätzle*, *tube* or *pelle* were also highly susceptible to fungal infection¹⁶. Therefore, the Toll pathway controls not only dorsoventral patterning in embryos, but also the antifungal immune defence in adult fruitflies. One difference between the two pathways is that a different member of the *Drosophila* NF-κB family, Dif (*Drosophila* immunity factor), rather than Dorsal, is involved in the antifungal response in adult fruitflies^{17,18}. Interestingly, *Drosophila* Toll does not function as a PRR, in that it does not seem to recognize pathogens directly. Instead, the processing of Spätzle into a biologically active form is induced on infection and leads, in turn, to the activation of the Toll pathway¹⁹. This is shown both by the requirement for Spätzle for antifungal responses, and by the analysis of mutations in the *necrotic* gene. *necrotic* encodes a serine protease inhibitor of the serpin family. Mutations in this gene result in the spontaneous activation of the Toll pathway and constitutive induction of the *Drosomycin* gene¹⁹. These results indicate that in *Drosophila*, the pattern-recognition event occurs upstream of Toll and triggers a protease cascade, analogous to complement activation by the lectin pathway in mammals. Interestingly, the Toll pathway can also be activated in response to Gram-positive infection, indicating that several pattern-recognition molecules might function upstream

Table 1 | **Pattern-recognition receptors**

PRR	Protein/domain family	Ligands	Function	References
Secreted PRRs				
MBL	C-type lectin	Terminal mannose residues	Activation of the lectin pathway of complement	102
CRP, SAP	Pentraxins	Phosphorylcholine on microbial membranes	Opsonization, activation of classical complement pathway	103,104
LBP	Lipid-transfer protein family	LPS	LPS recognition	41
Cell-surface PRRs				
CD14	Leucine-rich repeats	LPS, peptidoglycan	Co-receptor for TLRs	42
Macrophage mannose receptor	C-type lectin	Terminal mannose residues	Phagocytosis	105
Macrophage scavenger receptor	Scavenger receptor cysteine-rich domain	LPS, dsRNA, oxidized LDL, anionic polymers	Phagocytosis, LPS clearance, and lipid homeostasis	106
MARCO	Scavenger receptor cysteine-rich domain	Bacterial cell walls	Phagocytosis	107
Intracellular PRRs				
PKR	dsRNA-binding domain, protein kinase domain	dsRNA	Activation NF-κB and MAP kinases; inhibition of translation and induction of apoptosis in virally infected and stressed cells	74
NODs	Leucine-rich repeats, Nucleotide-binding domain, CARD domain	Ligands for most NOD proteins are unknown. NOD1 and NOD2 were shown to recognize LPS	Activates NF-κB and MAP kinases; some family members may be involved in the induction of apoptosis. The exact function is unknown.	108,109

CARD, caspase-recruitment domain; CRP, C-reactive protein; LBP, lipopolysaccharide (LPS)-binding protein; LDL, low-density lipoprotein; MAP, mitogen-activated protein; MARCO, macrophage receptor with collagenous structure; MBL, mannan-binding lectin; NF-κB, nuclear factor-κB; PKR, double-stranded RNA (dsRNA)-activated protein kinase; PRR, pattern-recognition receptor; SAP, serum amyloid protein; TLRs, Toll-like receptors.

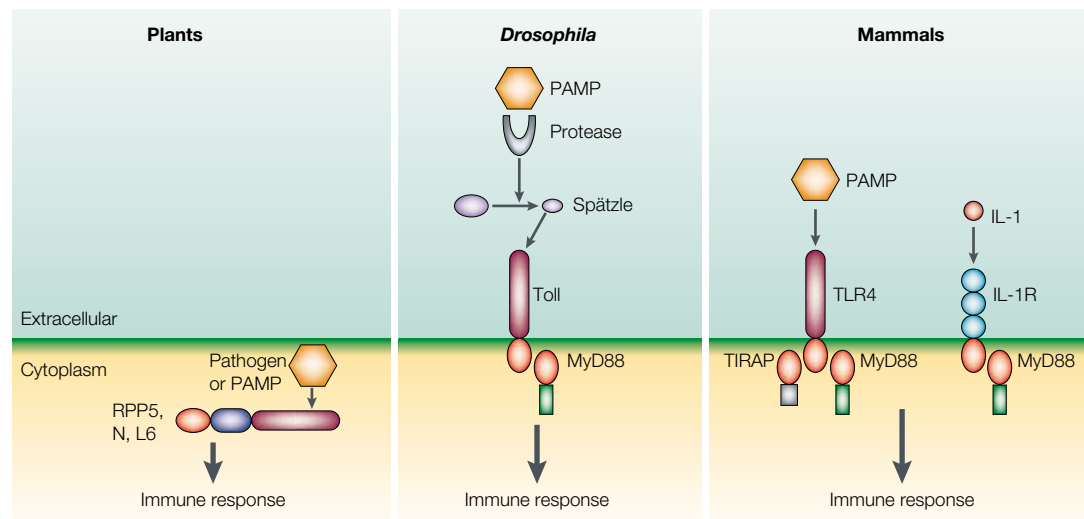


Figure 1 | TIR domain in host-defence pathways. The Toll/interleukin-1 (IL-1) receptor (TIR) domain is a protein-interaction module found in transmembrane and cytoplasmic proteins involved in animal and plant immunity. RPP5, N and L6 are prototypic examples of intracellular plant-disease-resistance proteins that contain an amino-terminal TIR domain as well as a nucleotide (ATP or GTP)-binding domain and leucine-rich repeat (LRR) domains. *Drosophila* has two types of protein with TIR domains: Tolls and MyD88. At least one out of nine Tolls in *Drosophila*, as well as MyD88, are involved in host defence. Toll is activated by a proteolytically processed form of the Spätzle protein. The cleavage of Spätzle is triggered by an unknown pattern-recognition molecule responsive for fungal and Gram-positive bacterial pathogens (see text for details). Mammals have at least four types of proteins with TIR domains: members of the Toll-like receptor (TLR) and IL-1 receptor (IL-1R) families, MyD88 and TIRAP (TIR domain-containing adaptor protein). In TLRs and IL-1Rs, the TIR domain is carboxy-terminal to LRRs and immunoglobulin domains, respectively. Both mammalian and *Drosophila* MyD88 contain carboxy-terminal TIR domains and amino-terminal death domains and function as adaptor proteins. TIRAP is another adaptor protein that does not have a *Drosophila* homologue. TIR has a carboxy-terminal TIR domain, but lacks a death domain. The amino-terminal region of TIRAP does not share similarity with any known protein. PAMP, pathogen-associated molecular pattern.

of the protease cascade that controls cleavage of Spätzle²⁰. The upstream cascade that generates active Spätzle in response to infection has not yet been identified.

Despite their profound defect in antifungal immunity, fruitflies harbouring mutations in *Toll* and the other components of the Toll pathway show normal resistance to infection by Gram-negative bacteria¹⁶. Similar to wild-type fruitflies, they produce antimicrobial peptides specific for Gram-negative bacteria, such as **Diptericin**¹⁶. *Drosophila* therefore discriminates between different classes of pathogens, such that the antifungal peptide Drosomycin is selectively produced on fungal infection, whereas Diptericin is made in response to Gram-negative bacteria²¹. Furthermore, although the Toll pathway regulates antifungal defence, resistance to Gram-negative infection is conferred by a distinct pathway, which was defined by a mutation in the *imd* (immune-deficient) gene²². *imd* mutants fail to induce the antibacterial peptide Diptericin and, therefore, have a profound defect in resistance to Gram-negative bacterial pathogens, although remaining essentially normal with regard to fungal and Gram-positive infection²³.

The *imd* gene has recently been identified and shown to encode an adaptor protein with a DEATH DOMAIN²⁴. So, Imd presumably functions downstream of a putative receptor responsible for sensing Gram-negative bacteria²⁴. Genetic analyses led to the identification of five additional *Drosophila* genes that function in the Imd pathway: *Dredd*^{25,26}, *dIKK-β*^{27,28} (IκB kinase-β),

*dIKK-γ*²⁹, *dTAK1* (a homologue of TGF-β-activated kinase 1)³⁰ and *Relish*³¹. Mutations in any of these genes yield phenotypes very similar to *imd* mutants — susceptibility to Gram-negative bacterial infection due to impaired induction of antibacterial peptides, such as Diptericin²³. *Dredd* is a *Drosophila* caspase that was previously implicated in the control of apoptosis during fruitfly development³². *Drosophila* IKK-γ and IKK-β are homologues of human IKK-γ — also known as NEMO (NF-κB essential modulator) — and IKK-β. In human cells, IKK-β and NEMO are essential regulators of NF-κB activation³³. *Relish* is a *Drosophila* homologue of the mammalian Rel/NF-κB family members, **p100** and **p105** (REF. 34).

Interestingly, the Toll and Imd pathways use different NF-κB transactivators that are activated by distinct mechanisms²⁰. *Dif*, similar to its mammalian homologues **p50** and **p65**, is activated on stimulus-dependent degradation of its inhibitor *Cactus*^{17,18}. *Relish*, in contrast, is homologous to mammalian p105, and is activated by a proteolytic processing event that removes its autoinhibitory ankyrin repeats²⁸. *Dredd* was shown to function downstream of *Drosophila* IKKγ and IKKβ, but its involvement in *Relish* processing has not yet been shown²⁶. Interestingly, the Imd pathway lacks an IκB-like molecule, an obvious target of *Drosophila* IKK-β phosphorylation that would be analogous with the mammalian NF-κB pathway, as *Cactus* seems to function exclusively in the Toll pathway²⁰. Conversely, how

DEATH DOMAIN
A protein–protein interaction domain found in many proteins that are involved in signalling and apoptosis.

Toll activation results in Cactus degradation is not yet clear, as no Cactus kinase has yet been identified. Therefore, although there are similarities in the Toll/NF- κ B pathways in *Drosophila* and mammals, there are also intriguing differences.

One of the main questions in *Drosophila* immunity that remains unresolved is the identities of the pattern-recognition molecules that trigger processing of Spätzle in response to fungal and Gram-positive infection (FIG. 2). Another very important question is the identity of the receptor that controls activation of the Imd pathway in response to Gram-negative bacterial infection. As there are nine TLRs in *Drosophila*³⁵, an attractive possibility is that one of them might be responsible for the activation of the Imd pathway. A mutation in **18 Wheeler**, another Toll family member, was shown to affect expression of several antibacterial peptides³⁶. However, 18 Wheeler does not seem to function in the Imd pathway^{23,35}. Moreover, none of the *Drosophila* Tolls could induce activation of the *Diptericin* promoter in *Drosophila* cell lines, and only Toll and **Toll-5** were able to activate Drosomycin³⁵. Therefore, it is likely that a receptor unrelated to Toll might control the Imd pathway and function as a sensor for Gram-negative PAMPs such as LPS.

TLRs in mammalian immunity

In mammalian species there are at least ten TLRs, and each seems to have a distinct function in innate immune recognition. In the past few years, dozens of TLR ligands have been identified³⁷. Many more ligands are yet to be identified, both for those TLRs that already have assigned ligands and those with no known ligands. TLR ligands are quite diverse in structure and origin. However, several common themes are emerging based on the available information. First, most TLR ligands are conserved microbial products (PAMPs) that signal the presence of infection (FIG. 3). Second, many, and perhaps all, individual TLRs can recognize several, structurally unrelated ligands. Third, some TLRs require accessory proteins to recognize their ligands. Finally, although the actual mechanism of ligand recognition is still not known, available evidence indicates that mammalian TLRs recognize their ligands by direct binding and therefore function as PRRs.

TLR4. Human TLR4 was the first characterized mammalian Toll⁵. It is expressed in a variety of cell types, most predominantly in the cells of the immune system, including macrophages and DCs⁵. TLR4 functions as the signal-transducing receptor for LPS^{38–40}. This discovery was made by positional cloning of the *Lps* gene in the LPS-non-responsive C3H/HeJ mouse strain^{38,39}, and was confirmed in *Tlr4* knockout mice⁴⁰. C3H/HeJ mice are unresponsive to LPS due to a point mutation in the TIR domain of *Tlr4*, which abrogates downstream signalling^{38,39}.

Recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS is first bound to a serum protein, **LBP** (LPS-binding protein), which functions by transferring LPS monomers to **CD14** (REF. 41). CD14 is a high-affinity LPS receptor that can either

be secreted into serum, or expressed as a glycosylphosphatidylinositol (GPI)-linked protein on the surface of macrophages⁴². CD14-deficient mice have a profound defect in responsiveness to LPS, showing the importance of CD14 in LPS recognition⁴³. Another component of the LPS receptor complex is **MD-2** (REF. 44). MD-2 is a small protein that lacks a transmembrane region and is expressed on the cell surface in association with the ectodomain of TLR4 (REF. 44). Although its precise function is not known, MD-2 is required for LPS recognition by TLR4 (REF. 45). The molecular mechanism of TLR-mediated recognition is one of the most challenging issues in Toll biology. Several lines of evidence indicate that TLR4 might, in fact, interact with LPS directly^{46,47}; however, this interaction is clearly aided by CD14 and MD-2 (REF. 48).

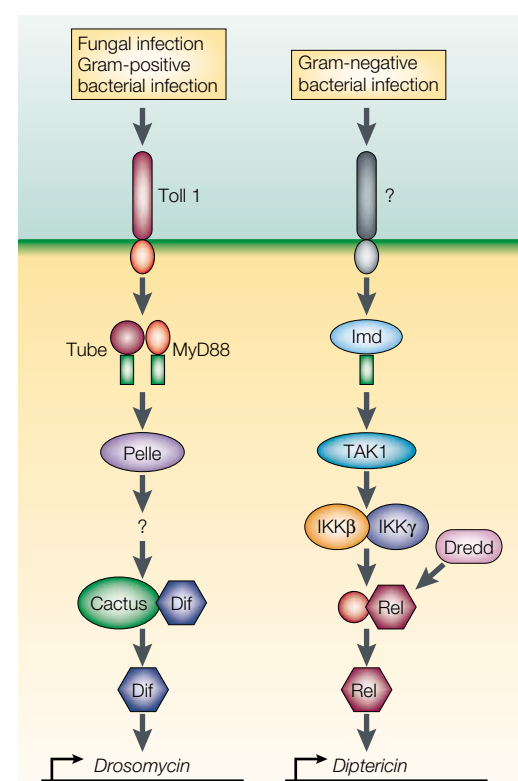


Figure 2 | *Drosophila* Toll and Imd pathways. The *Drosophila* Toll pathway is activated by fungal and Gram-positive bacterial pathogens and induces production of antifungal peptides, such as Drosomycin. Toll signals through two adaptor proteins, Tube and MyD88, which function upstream of the protein kinase Pelle. The signalling components immediately downstream of Pelle are not known. Activation of this pathway leads to degradation of Cactus and release of the nuclear factor- κ B (NF- κ B) family transcription factor Dif (*Drosophila* immunity factor). Dif, in turn, activates the transcription of Drosomycin and other antimicrobial peptides. The Imd pathway is triggered in response to Gram-negative bacterial infection through an unknown receptor. In addition to Imd, this pathway involves the *Drosophila* homologue of the protein kinase TAK1 (TGF- β -activated kinase), the IKK- γ /IKK- β protein kinase complex, the caspase Dredd and the NF- κ B family transcription factor Relish. This pathway is responsible for the induction of antibacterial peptides, such as Diptericin and Drosocin, in response to bacterial infection. Rel, Relish.

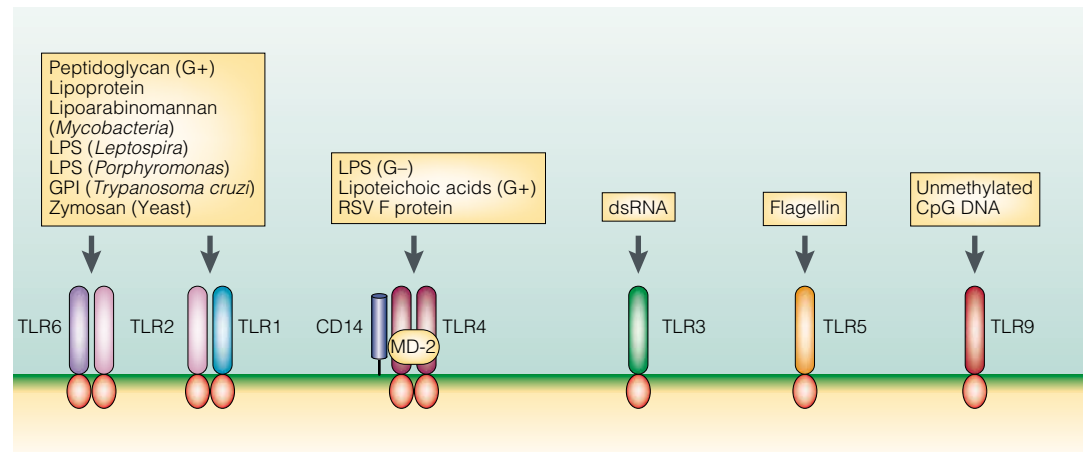


Figure 3 | Ligand specificities of TLRs. Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns (PAMPs). Recognition of lipopolysaccharide (LPS) by TLR4 is aided by two accessory proteins: CD14 and MD-2. TLR2 recognizes a broad range of structurally unrelated ligands and functions in combination with several (but not all) other TLRs, including TLR1 and TLR6. TLR3 is involved in recognition of double-stranded (dsRNA). TLR5 is specific for bacterial flagellin, whereas TLR9 is a receptor for unmethylated CpG motifs, which are abundant in bacterial DNA. G+, Gram-positive; G-, Gram-negative; GPI, glycosphosphoinositol; RSV, respiratory syncytial virus.

Another protein that seems to cooperate with TLR4 in LPS recognition is **RP105**. RP105 is an LRR-containing protein expressed almost exclusively on the surface of B cells⁴⁹. The extracellular region of RP105 is related to the ectodomain of TLR4; however, RP105 lacks the TIR domain and instead has a short cytoplasmic region with a tyrosine-phosphorylation motif⁴⁹. Ligation of RP105 leads to activation of SRC-family tyrosine kinases, including **LYN**⁵⁰. Similar to TLR4, RP105 is associated with an accessory protein, **MD-1**, which is a homologue of MD-2 (REF. 51). Deletion of the *RP105* gene results in reduced responsiveness of B cells to LPS⁵². As TLR4-deficient mouse B cells are completely unresponsive to LPS, RP105 and TLR4 presumably cooperate in LPS recognition and signalling in B cells, although the exact nature of this cooperation remains unknown.

In addition to LPS, TLR4 is involved in the recognition of several other ligands, including LTA⁵³, and a heat-sensitive cell-associated factor derived from *Mycobacterium tuberculosis*⁵⁴. TLR4 is also implicated in the recognition of the heat-shock protein **HSP60** (REF. 55). HSP60 is a molecular chaperone that is conserved from bacteria to mammals. It is normally not available for recognition by cell-surface receptors, but presumably can be released from necrotic cells during tissue injury or lysis of virally infected cells. The physiological significance of HSP60 recognition by a TLR is not yet understood, but the inflammatory response induced by necrotic cells (which might be mediated by HSPs and other ligands released from dying cells) might have a role in tissue remodelling and wound healing⁵⁶.

Interestingly, TLR4 and CD14 were also shown to trigger a response to the fusion (F) protein of respiratory syncytial virus (RSV)⁵⁷. It is not clear yet whether the F protein of RSV represents an example of a viral PAMP, in that some conserved feature of the F protein is shared with fusion proteins of other viruses. An alternative

possibility is that the RSV evolved the ability to stimulate TLR4 for its own benefit. More examples of viral interactions with TLRs are likely to be discovered in the near future. Not surprisingly, some viruses evolved the ability to interfere with TLR function. For example, the vaccinia virus encodes two cytoplasmic proteins that block TLR and IL-1R signal transduction⁵⁸.

TLR2. TLR2 has been shown to be involved in the recognition of a broad range of microbial products, including: peptidoglycan from Gram-positive bacteria^{53,59}, bacterial lipoproteins^{60–62}, mycobacterial cell-wall lipoarabinomannan^{63,64}, glycosylphosphatidylinositol lipid from *Trypanosoma Cruzi*⁶⁵, a phenol-soluble modulin produced by *Staphylococcus epidermidis*⁶⁶, and yeast cell walls⁶⁷ (FIG. 3). In addition, TLR2 functions as a receptor for atypical LPS produced by *Leptospira interrogans*⁶⁸ and *Porphyromonas gingivitis*⁶⁹, both of which are structurally different from Gram-negative LPS. This unusually broad range of ligands recognized by TLR2 is explained, in part, by cooperation between TLR2 and at least two other TLRs: **TLR1** and **TLR6** (REFS 70,71). So, the formation of heterodimers between TLR2 and either TLR1 or TLR6 dictates the specificity of ligand recognition^{70,71}. For example, TLR2 cooperates with TLR6 for the recognition of mycoplasma macrophage-activating lipopeptide 2 kDa (MALP-2)⁷¹. Interestingly, it is TLR6 that discriminates between bacterial lipoproteins, which are triacylated at the amino-terminal cysteine residue, and the diacylated mycoplasma lipoprotein MALP-2. This conclusion is based on the finding that TLR2-deficient macrophages are unresponsive to both bacterial and mycoplasma lipoproteins, whereas TLR6-deficient cells are unresponsive to MALP-2, but respond normally to bacterial lipoproteins⁷¹. Therefore, TLR2 cooperates with TLR6 for recognition of MALP-2, but presumably with another TLR for the recognition of triacylated bacterial

lipoproteins. It is not known yet whether TLR2 heterodimerization is induced by appropriate ligands or occurs prior to ligand interaction.

It is interesting to note that both TLR1 and TLR6 are expressed constitutively on many cell types, whereas expression of TLR2 is regulated and seems to be restricted to antigen-presenting cells and endothelial cells⁷². The combinatorial recognition by TLR2 and regulation of its expression might provide an important mechanism to control cellular responsiveness to microbial products. The full repertoire of possible TLR heterodimers is not yet known, but TLR4 and TLR5, at least, are likely to function as homodimers⁷⁰.

TLR3. TLR3 has two interesting features that distinguish it from other mammalian TLRs. First, cloning of human and mouse *TLR3* immediately showed that, unlike all other TLRs, TLR3 does not contain the conserved proline residue in the position equivalent to proline-712 of mouse TLR4. Substitution of this proline residue for histidine in the *Tr4* gene in the C3H/HeJ mouse strain results in unresponsiveness to LPS. Equivalent substitutions in some other TLRs abolish their signalling activities^{67,70}. Therefore, the fact that TLR3 lacks the conserved proline at this crucial position indicated that the TLR3 signalling mechanism might differ from that of other TLRs. The second interesting feature of TLR3 is that it is expressed predominantly, albeit not exclusively, in dendritic cells⁷².

Recent studies have shown that TLR3 functions as a cell-surface receptor for double-stranded RNA (dsRNA) (FIG. 3)⁷³. dsRNA is a molecular pattern produced by most viruses at some point of their infection cycle. It has long been known to have immunostimulatory activity, partly because of its ability to activate the dsRNA-dependent protein kinase, PKR⁷⁴. However, PKR-deficient cells are still able to respond to both dsRNA and its synthetic analogue, polyinosine-polycytosine (polyIC)⁷⁵, indicating the existence of another receptor for dsRNA. This receptor seems to be TLR3, as cells deficient for TLR3 have a profound defect in their responsiveness to polyIC, as well as to viral dsRNA⁷³.

Although a contribution of TLR3 to antiviral defence remains to be shown, the fact that dsRNA — an important viral PAMP — is recognized by a TLR, significantly broadens the range of pathogens that can be detected by the TLRs.

TLR5. TLR5 is involved in recognition of flagellin — a conserved protein that forms bacterial flagella⁷⁶ (FIG. 3). An unusual aspect of this TLR ligand is that, unlike most other PAMPs, flagellin is a protein, and it does not undergo any posttranslational modification that would distinguish it from host cellular proteins. However, the amino- and carboxy-termini of flagellin are extremely conserved, presumably because they form a hydrophobic core of the flagella and have significant structural constraint on variability⁷⁷. This extreme structural conservation and the vitally important function of flagellin for bacterial mobility explain why it was selected as a target for recognition by Toll.

Interestingly, TLR5 is expressed on the basolateral side of the intestinal epithelium, where it can sense flagellin from pathogenic bacteria, such as *Salmonella*⁷⁸. Polarized expression of TLR5 (and presumably other TLRs) on surface epithelia might provide an important mechanism of discrimination between commensal and pathogenic bacteria, as pathogenic, but not commensal microbes, can cross the epithelial barriers.

TLR9. Perhaps the most enigmatic example of pattern recognition is the recognition of unmethylated CpG motifs in bacterial DNA by TLR9 (REF. 79) (FIG. 3). Unmethylated DNA in a particular sequence context (the so-called 'CpG motif') has long been known for its potent immunostimulatory activity⁸⁰. A single nucleotide substitution or methylation of a cytosine residue within the CpG motif completely abrogates the immunostimulatory property of bacterial DNA⁸⁰. Because bacteria lack cytosine methylation, and most CpG is methylated in the mammalian genome, CpG motifs might signal the presence of microbial infection. The essential role of TLR9 in CpG DNA recognition was shown using *Tr9* knockout mice⁷⁹. Interestingly, signalling by CpG DNA requires its internalization into late endosomal or lysosomal compartments⁸¹. The reason for this is not yet known, and it will be important to determine the subcellular localization of TLR9. It is not yet known whether any other TLR ligands need to be internalized in order to activate TLRs. Notably, TLR2 is expressed on the cell surface and is recruited to phagosomes on interaction with yeast cell walls (zymosan)⁶⁷. Additionally, some available data indicate that signalling by LPS might require its internalization⁸².

Another enigmatic aspect of CpG DNA recognition is that the optimal response of mouse versus human cells requires slightly different sequence motifs flanking CpG dinucleotides⁸³. It has recently been shown that CpG DNA that optimally stimulates mouse cells is also a much stronger activator of transfected mouse TLR9 compared with human TLR9; the opposite is true of CpG DNA that preferentially stimulates human cells⁸⁴. These results indicate that TLR9 itself can distinguish between the two immunostimulatory CpG motifs, and therefore can presumably recognize CpG DNA directly⁸⁴.

TLR signalling pathways

Activation of signal transduction pathways by TLRs leads to the induction of various genes that function in host defence, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules. Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthase and antimicrobial peptides, which can directly destroy microbial pathogens⁸⁵.

Although both TLRs and IL-1Rs rely on TIR domains to activate NF- κ B and MAP (mitogen-activated protein) kinases and can induce some of the same target genes, a growing body of evidence points to several differences in signalling pathways activated

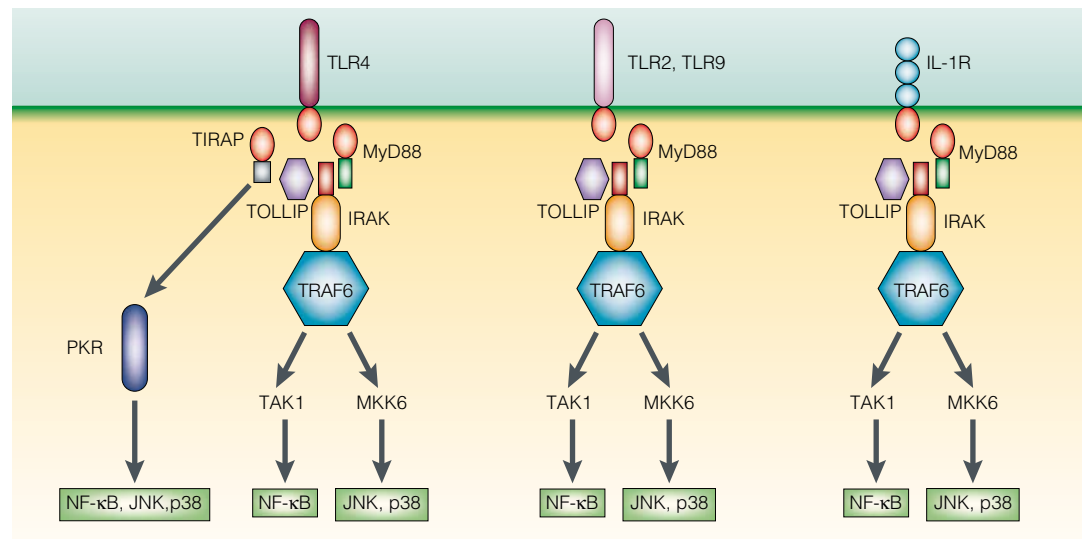


Figure 4 | **Toll signalling pathways.** The Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R)-family members share several signalling components, including the adaptor MyD88, Toll-interacting protein (TOLLIP), the protein kinase IRAK (IL-1R-associated kinase) and TRAF6 (TNF receptor-associated factor 6). TRAF6 can activate nuclear factor- κ B (NF- κ B) through TAK1 (TGF- β -activated kinase), and JNK (c-Jun N-terminal kinase) and p38 MAP kinases through MKK6 (mitogen-activated protein kinase 6). TLR4 signals through another adaptor in addition to MyD88—TIRAP (Toll/interleukin-1 (IL-1) receptor domain-containing adaptor protein), which activates MyD88-independent signalling downstream of TLR4. The protein kinase PKR functions downstream of TIRAP, but its importance in this pathway has not yet been established.

by individual TLRs. Signalling pathways activated by TLRs can be divided into ‘shared’ and ‘specific’. A shared signalling pathway is induced by all TLRs as well as by the IL-1R family. The specific pathways are activated by some, but not other TLRs, and might also account for differences in signalling between TLRs and IL-1Rs.

The signalling pathway that seems to be shared by all members of the Toll and IL-1R families includes four essential components: the adaptor proteins, MyD88 (REFS 9–12) and TOLLIP (Toll-interacting protein)^{86,87}; a protein kinase, IRAK (IL-1R-associated kinase)^{9,10,88}; and another adaptor, TRAF6 (TNF-receptor-associated factor 6)^{9,10,89} (FIG. 4). The essential roles of MyD88 and TRAF6 in TLR and IL-1R signalling have been confirmed by targeted deletion of their genes^{90–92}. MyD88 contains two protein-interaction domains: an amino-terminal death domain and a carboxy-terminal TIR domain. The TIR domain of MyD88 associates with the TIR domain of TLR and the IL-1R, whereas the death domain interacts with the amino-terminal death domain of IRAK and recruits IRAK to the receptor complex^{9–12}. TOLLIP lacks a TIR domain, but contains a C2 domain, which in other proteins is known to interact with membrane lipids⁸⁶. TOLLIP can also associate with IRAK and the TIR domains of the receptors, and recruits IRAK to the receptor complex, although with different kinetics⁸⁶. The functional differences between MyD88 and TOLLIP are not yet understood. On recruitment to the receptor complex, IRAK is autophosphorylated and associates with TRAF6 (REF. 88). TRAF6 induces activation of TAK1 and MKK6 (MAP kinase kinase 6), which, in turn, activate NF- κ B, JNK (c-Jun N-terminal kinase) and p38 MAP kinase, respectively⁹³.

In addition to MyD88-dependent signalling, TLR2 has been shown to engage a signalling pathway that involves protein kinase B (PKB)⁹⁴. The cytoplasmic domain of TLR2 was shown to interact with a RHO family GTPase, RAC1, and phosphatidylinositol 3-kinase (PI3K), which functions upstream of PKB⁹⁴. PI3K and PKB are activated by a wide variety of cell-surface receptors and have several roles in cellular physiology. In the context of TLR2 signalling, PKB was shown to be involved in a pathway that leads to the phosphorylation of NF- κ B, which is required for its transactivation activity⁹⁴. It is not yet known whether this pathway is unique to TLR2, but as NF- κ B phosphorylation is a necessary step in transactivation, it is likely that this pathway might be activated by all TLRs.

Analysis of MyD88-deficient mice showed several unexpected features of the signalling downstream of TLR4 and TLR3. Macrophages and DCs derived from *MyD88* knockout mice do not produce the cytokines IL-1 β , TNF- α , IL-6 and IL-12 when stimulated with LPS, polyIC, MALP-2 or CpG, which signal through TLR4, TLR3, TLR2 and TLR9, respectively^{60,73,92,95}. Consequently, MyD88-deficient mice are completely resistant to ENDOTOXIC SHOCK⁹². However, a detailed analysis of the NF- κ B and MAP kinase signalling pathways has shown that LPS and polyIC, but not CpG or MALP-2, could induce activation of NF- κ B, JNK and p38 in MyD88-deficient cells^{60,73,92,95,96}. Activation of these signalling pathways through TLR4 occurred with delayed kinetics and, importantly, was insufficient for the induction of cytokine gene expression⁹². These unexpected findings indicated that TLR4 and TLR3 use at least two signal-transduction pathways for activation of NF- κ B and MAP kinases. One of the signalling pathways is

ENDOTOXIC SHOCK

A clinical condition induced by hyper-reaction of the innate immune system to bacterial LPS. It is mediated by the inflammatory cytokines IL-1 and TNF- α , which are produced in high amounts due to sustained stimulation of TLR4 by LPS.

COMPLETE FREUND'S ADJUVANT (CFA). A mixture of mycobacterial lysate with mineral oil. When animals are immunized with antigen mixed with CFA, they induce strong immune responses to the antigen.

MyD88-dependent and is used by all TLRs, whereas the other pathway is MyD88-independent and is triggered by TLR4, and possibly by TLR3, but not by TLR2 or TLR9. The IL-1 and IL-18 receptors also fail to induce signalling in the absence of MyD88, indicating that these receptors also lack the MyD88-independent signalling pathway⁹¹.

Another interesting aspect of MyD88-independent signalling is that it can induce DC maturation^{73,96}. When immature bone-marrow-derived DCs (BMDCs) are stimulated with LPS, polyIC or CpG, they produce large amounts of IL-12 and upregulate cell-surface expression of MHC and co-stimulatory molecules. MyD88-deficient BMDCs stimulated with LPS, polyIC or CpG fail to produce IL-12 or IL-6 (REFS 73,95,96). However, they can still be induced to upregulate expression of MHC and co-stimulatory molecules, such as CD80 and CD86, when treated with LPS or polyIC, but not when stimulated with CpG^{73,95,96}. These results show that the MyD88-independent signalling pathway(s) stimulated by TLR4 and TLR3 is sufficient for DC maturation, whereas the MyD88-dependent signalling pathway is required for the induction of IL-6 and IL-12 (REFS 73,96). In addition to the transcriptional events that can be induced through the MyD88-independent pathway, it has been shown that caspase-1 processing of IL-18 into its biologically active form can also be induced by TLR4 independently of MyD88 (REF. 97).

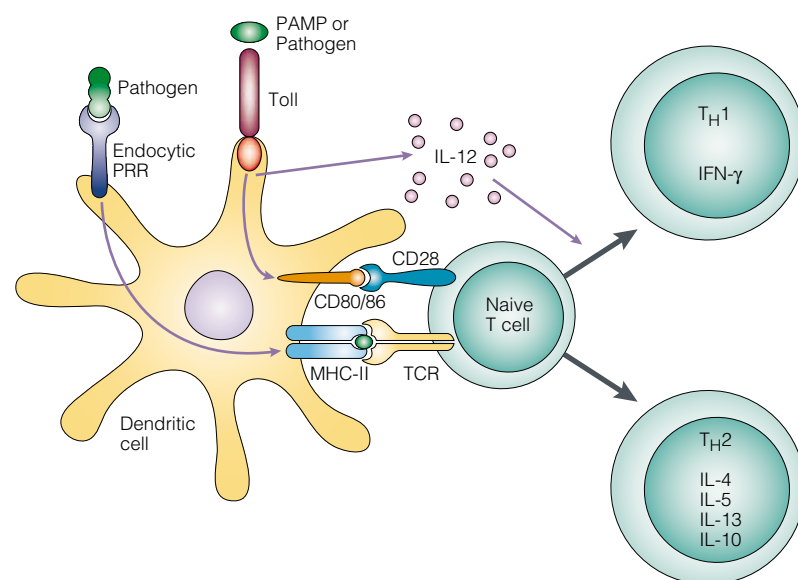


Figure 5 | Role of TLRs in the control of adaptive immunity. TLRs sense the presence of infection through recognition of PAMPs (pathogen-associated molecular patterns). Recognition of PAMPs by Toll-like receptors (TLRs) expressed on antigen-presenting cells (APC), such as dendritic cells, upregulates cell-surface expression of co-stimulatory (CD80 and CD86) molecules and major histocompatibility complex class II (MHC II) molecules. TLRs also induce expression of cytokines, such as interleukin-12 (IL)-12, and chemokines and their receptors, and trigger many other events associated with dendritic cell maturation. Induction of CD80/86 on APCs by TLRs leads to the activation of T cells specific for pathogens that trigger TLR signalling. IL-12 induced by TLRs also contributes to the differentiation of activated T cells into T helper (T_H)1 effector cells. It is not yet known whether TLRs have any role in the induction of T_H 2 responses. IFN- γ ; interferon- γ ; PRR, pattern-recognition receptor.

Recently, a new adaptor protein TIRAP (also called MAL, for MyD88 adaptor-like) was identified and shown to function downstream of TLR4 (REFS 13,98). TIRAP has a carboxy-terminal TIR domain, but unlike MyD88, TIRAP does not have a death domain, and instead has a serine/proline-rich region of unknown function at the amino-terminus. TIRAP associates with the TIR domain of TLR4, and a dominant-negative form of TIRAP inhibits TLR4, but not TLR9 or IL-1R signalling, indicating that TIRAP controls activation of the MyD88-independent pathway¹³. Interestingly, TIRAP also associates with the protein kinase PKR and two PKR-regulatory proteins, PACT (PKR-activating protein) and p58, indicating that PKR functions downstream of TIRAP¹³. Indeed, PKR can be activated by LPS even in the absence of MyD88, indicating its involvement in the MyD88-independent pathway¹³. These results are consistent with a report showing impaired LPS signalling in PKR-deficient cells⁹⁹. Taken together, this indicates that TLR4 uses two adaptors with TIR domains — MyD88 and TIRAP — which control activation of distinct signal-transduction pathways. TLR2 and TLR9, as well as IL-1R, use only MyD88, which accounts for differences in signalling by these receptors and TLR4 (FIG. 4)¹³.

Tolls and control of adaptive immunity

Specificity of the TLRs for products of microbial origin allows them to signal the presence of infection and to direct the adaptive immune responses against antigens of microbial origin. DCs have a key role in coupling innate and adaptive immune-recognition systems. Immature DCs are located in peripheral tissues, including the potential pathogen-entry sites, where they can detect and capture microbial invaders¹⁰⁰. Not surprisingly, immature BMDCs express a full set of TLRs, which, on recognition of their ligands, induce DC maturation. Mature DCs express high levels of MHC and co-stimulatory molecules (CD80 and CD86) and migrate to draining lymph nodes where they present pathogen-derived antigens to naive T cells¹⁰⁰. TLRs also induce expression by DCs of various cytokines, including IL-12, which directs T_H cell differentiation into T_H 1 effector cells (FIG. 5)³⁷.

The role of Toll-mediated recognition in the control of adaptive immune responses was studied using MyD88-deficient mice. When these mice are immunized with ovalbumin mixed with COMPLETE FREUND'S ADJUVANT (CFA), they show a profound block in antigen-specific T-cell proliferation, the production of interferon- γ (IFN- γ) and the generation of ovalbumin-specific IgG2a antibodies¹⁰¹. These results clearly show a crucial requirement for Toll-mediated recognition in the generation of antigen-specific T_H 1 responses. Surprisingly, however, T_H 2 responses in these mice are largely unaffected under the same conditions¹⁰¹. So, B cells in these mice produce the same amounts of antigen-specific IgG1 and IgE as do B cells in wild-type mice, whereas T cells produce even higher amounts of IL-13 on re-stimulation with

antigen¹⁰¹. These results indicate that TLR-mediated recognition is vital for the generation of T_H1, but not T_H2 effector responses¹⁰¹. One possible explanation of these observations is that all of the known TLR ligands are products of either prokaryotic, viral or protozoan metabolism, and T_H1 responses are required for protection against pathogens of these classes. T_H2 responses, by contrast, are protective against multicellular eukaryotic parasites, such as helminths. These pathogens might not produce any ligands for Tolls, and perhaps are recognized by a distinct set of PRRs that could be specific for glycoproteins and glycolipids produced by worms, but not by the host or prokaryotic pathogens. Allergens also lack PAMPs that are recognized by TLRs and might initiate adaptive immune responses by a TLR-independent mechanism. It is also possible that T_H2 responses might be TLR dependent, but MyD88 independent. This is less likely, however, as MyD88 is expressed constitutively in most cell types. Whichever is the case, the complete block of T_H1 responses to antigen administered with CFA clearly shows that adjuvants function by triggering TLRs on DCs and other antigen-presenting cells. Indeed, MyD88-deficient DCs fail to mature and to activate naive T cells when stimulated by mycobacterial lysate, which is the active ingredient of CFA¹⁰¹.

Perspectives

The identification and functional characterization of TLRs in *Drosophila* and mammals have brought our understanding of the innate immune system to a new level. The role of the TLRs in host defence is so fundamental, it is likely that their function affects most aspects of the mammalian immune system. Loss-of-function mutations in TLRs are likely to result in immunodeficiencies, whereas gain-of-function mutations might predispose an individual to inflammatory or autoimmune disorders. The importance of the TLRs in the control of adaptive immune responses also makes them crucial targets for immune intervention.

Although much progress has been made in the characterization of individual TLRs, there are many more fundamental questions to address: what are the full complement of PAMPs and other ligands recognized by TLRs? What are the differences between individual TLRs in the induction of cellular and immune responses? What is the mechanism of ligand recognition by TLRs? Can TLRs detect any features of pathogens that are important for the choice of effector responses? What is the biological significance of differential TLR expression? And why are TLRs not continuously activated by commensal microflora? The answers to these questions will greatly expand our understanding of the complex interactions between pathogens and the host immune response.

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