

Structure and Function of Toll Receptors and Their Ligands

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Key Words

leucine-rich repeat, pathway cross talk, signaling mechanism, Toll-IL-1 receptor identity region

Abstract

The Toll family of class I transmembrane receptors recognizes and responds to diverse structures associated with pathogenic microorganisms. These receptors mediate initial responses in innate immunity and are required for the development of the adaptive immune response. Toll receptor signaling pathways are also implicated in serious autoimmune diseases such as endotoxic shock and thus are important therapeutic targets. In this review we discuss how microbial structures as different as nucleic acids and lipoproteins can be recognized by the extracellular domains of Toll receptors. We review recent evidence that the mechanism of signal transduction is complex and involves sequential changes in the conformation of the receptor induced by binding of the ligand. Finally, we assess the emerging area of cross talk in the Toll pathways. Recent work suggests that signaling through TLR4 in response to endotoxin is modified by inputs from at least two other pathways acting through $\beta 2$ integrins and protein kinase C ϵ .

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INTRODUCTION

The German word *Toll* is difficult to render precisely in English but approximates to fantastic, mad, or amazing. In a scientific context, Nüsslein-Volhard and Anderson first used the word Toll to name a gene that they discovered in a genetic screen of *Drosophila*, the phenotype of which they thought to be *Toll* (1, 2). In

fact, this pioneering work in the early 1980s identified a group of 10 different genes, all of which produced qualitatively similar maternal effect phenotypes, now known as the dorsal group. Null mutations in any of these genes result in the failure to differentiate pattern elements on the dorsoventral axis and lead to early embryonic lethality. During the following 10 years, all the dorsal group genes were cloned, and a compelling picture emerged of how dorsoventral patterning occurred in the *Drosophila* embryo. Shortly after the fertilization of the embryo, a ventrally restricted signal associated with an extracellular membrane structure activates a protease cascade, and the terminal member Easter then processes an inactive precursor of Spätzle, an endogenous protein ligand of Toll, a class I transmembrane receptor. By this mechanism Toll is activated at ventral positions in the embryos, and an intracellular signaling pathway then causes the relocalization of a transcription factor, dorsal, from the cytoplasm to nuclei located at ventral positions in the embryo. This results in the formation of a ventral-dorsal gradient of the transcription factor, and the information contained in this morphogenetic gradient is then used to direct the subsequent differentiation of the dorsoventral body axis (3).

The sequence of Toll determined in 1988 revealed a tripartite structure with an N-terminal region containing tandem arrays of a short leucine-rich motif, the leucine-rich repeat (LRR), a sequence likely to form a single transmembrane helix and a C-terminal domain of unknown structure and function (4). An important development in the early 1990s was the recognition that this C-terminal domain was significantly related to that of the vertebrate interleukin-1 receptor (IL-1R) (5, 6). IL-1R is activated by a cytokine formerly known as endogenous pyrogen that is now named IL-1. IL-1 is part of the acute phase response to infection characterized by fever and the secretion of defense proteins into the circulation by the liver. This discovery was important as it suggested that this domain, now known as the Toll-interleukin receptor (TIR)

domain, was involved in signaling processes not only in the restricted context of insect development but also in the generation of initial responses to infection by human immune-system cells. This conclusion received significant support in 1995 when Hoffmann's group (7) in Strasbourg discovered that Toll and some other members of the dorsal group functioned in innate immune responses to pathogenic fungi and bacteria, as well as in embryonic patterning in the fly. Shortly after this important discovery, the progress of genome projects led to the identification of approximately 10 receptors in vertebrates that were direct homologs of Toll with related extracellular domains as well as cytoplasmic TIRs, and these became known as the Toll-like receptors (TLRs) (8).

Until this time vertebrate immunologists tended to focus on the adaptive response and the generation of diversity in antibody repertoires [see Di Noia & Neuberger (8a) in this volume]. By contrast immunologists considered innate responses to be a less important part of the immune response and studied them little, except perhaps in the context of complement. It took both the identification of the TLRs and the prescience of Janeway to change this picture. Janeway (9) formulated the problem as the "immunologist's dirty little secret," that is, the well-known fact that an adaptive response is only produced if an adjuvant containing a microbial extract is presented to the immune system together with the specific antigen. This observation implied that antibody responses are tightly coupled to a second largely unknown process of activation mediated by the microbial extracts in the adjuvant. Two key results published in 1998 demonstrated the important roles played by TLRs in the recognition of microbial products and the coupling of innate to adaptive immunity. First, activation of the TLR4 pathway induced the expression of costimulatory molecules required for T cell activation in response to antigen (10). Second, cell signaling induced by a powerful immunostimulatory component of gram-negative bacteria,

lipopolysaccharide (LPS), or endotoxin required TLR4 (11). These findings led to a rapid expansion of research into innate immunity, and the TLRs are now established as bona fide pattern-recognition receptors that respond to specific molecules derived from bacteria and viruses (12) (see **Table 1**).

The aim of this review is not to provide a comprehensive overview of TLR research, but rather to focus on three specific but inter-related problems of particular interest to biochemists. First, how are microbial ligands recognized by the TLRs, and what is the mechanism of signal transduction? Second, what are the molecular events that regulate downstream signaling? Third, what are the nature and significance of cross talk between the TLRs and other signaling pathways in cells of the immune system?

OVERVIEW OF TOLL SIGNALING PATHWAYS: EVOLUTIONARY CONSERVATION AND DIVERGENCE

Although elements of the Toll pathways from insects and vertebrates have been conserved in evolution, it is also clear that there are substantial differences in the way that the ligand is detected and in the regulatory networks that control the signaling process.

The Extracellular Pathways in Insects and Mammals

Insects use specific recognition proteins to detect peptidoglycan derived from the cell wall of gram-positive bacteria (**Figure 1**). A glucanase, gram-negative binding protein 1 (GNBP1), is thought to fragment the peptidoglycan and then present it to a peptidoglycan recognition protein (PGRP-SA) with which it forms a complex. Recent work suggests that conformational changes in the PGRP/GNBP complex caused by the breakdown and transfer of peptidoglycan lead to a self-limiting activation of a serine protease cascade (13). In

Class I

transmembrane receptor: a family of signaling molecules that consists of an extracellular domain (ectodomain), a single-membrane-spanning α -helix, and a cytoplasmic domain

LRR: leucine-rich repeat

Toll-interleukin receptor (TIR)

domain: an α/β -structure found in Toll receptors and adaptors, involved in postreceptor signal transduction

TLR: Toll-like receptor

Cross talk:

modification of a signaling response by inputs from distinct signaling pathways

Table 1 Ligands for the Toll-like receptors (TLRs)

TLRs	Ligands	Origin of ligands	Reference(s)
TLR1/2	Triacyl lipopeptides (Pam ₃ CSK ₄)	Bacteria, mycobacteria	68
	Soluble factors	<i>Neisseria meningitidis</i>	125
	OspA	<i>Borrelia burgdorferi</i>	70
	Porin PorB	<i>Neisseria meningitidis</i>	126
TLR2	Lipoprotein/lipopeptides	A variety of pathogens	120, 121
	Diacyl lipopeptides (Pam ₂ CSK ₄ and MALP2SK ₄)	Synthetic ligands	127
	Peptidoglycan	Gram-positive bacteria (not accessible in gram negative)	128
	Lipoteichoic acid	Gram-positive bacteria	128
	Lipoarabinomannan	Mycobacteria	129
	A phenol-soluble modulin	<i>Staphylococcus epidermidis</i>	130
	Glycoinositolphospholipids	<i>Trypanosoma cruzi</i>	131
	Glycolipids	<i>Treponema maltophilum</i>	132
	Porins	<i>Neisseria meningitidis</i>	126
	Zymosan	Fungi	129
	Atypical LPS	<i>Leptospira interrogans</i>	67
	Atypical LPS	<i>Porphyromonas gingivalis</i>	133
	Hsp70	Host	134
	Hyaluronan	Host	135
	Hemagglutinin	Measles virus	136
TLR3	Poly (I-C) dsRNA	Virus	76
TLR4	LPS	Gram-negative bacteria	11
	Flavolipin	<i>Flavobacterium meningosepticum</i>	137
	ER-112022, E5564, E5531	Synthetic compounds	66
	Taxol	Plant	138
	Fusion protein	Respiratory syncytial virus	139
	Envelope proteins	Mouse mammary tumor virus	140
	Hsp60	<i>Chlamydia pneumoniae</i>	141
	Hsp60	Host	142
	Hsp70	Host	134
	Type III repeat extra domain A of fibronectin	Host	143
	Oligosaccharides of hyaluronic acid	Host	144
	Polysaccharide fragments of heparan sulfate	Host	145
	Fibrinogen	Host	146
	αA crystallin and HSPB8	Host (recombinant <i>E. coli</i> -produced proteins)	147
TLR5	Flagellin	Bacteria	58, 148
TLR6/2	Diacyl lipopeptides	Mycoplasma	69
TLR7	Imidazoquinolines (imiquimod, R-848)	Synthetic compounds	79
	Bropirimine	Synthetic compounds	149
	Guanosine analogs	Synthetic compounds	75
TLR8	R-848	Synthetic compounds	75, 150
TLR9	Unmethylated CpG DNA	Bacteria, virus, yeast, insects	149
	Chromatin-IgG complexes	Host	151

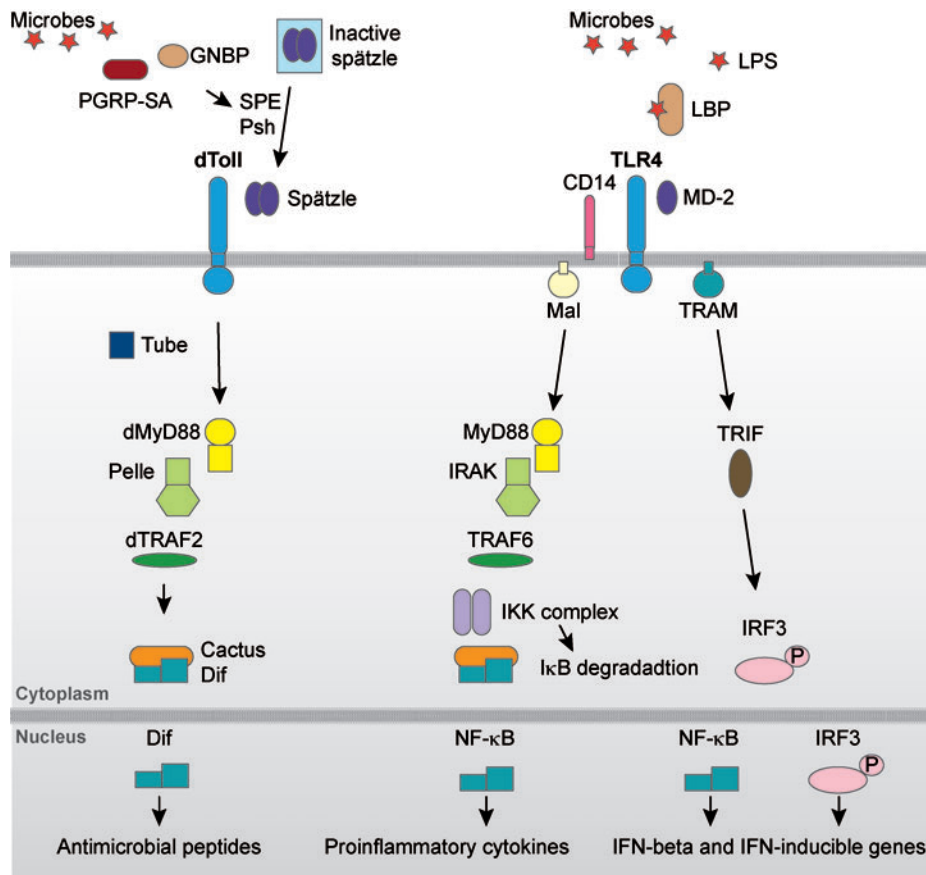


Figure 1

Conservation and divergence in the Toll signaling pathways. Components of the *Drosophila* Toll (left) and the human Toll-like receptor (right) pathways are illustrated schematically. Evolutionarily or functionally conserved elements in the two pathways are illustrated in the same color. Abbreviations: CD14, an extrinsic, PI-glycan modified membrane protein; Dif, dorsal-related immunity factor; dMyD88, *Drosophila* homolog of MyD88; dTRAF2, *Drosophila* homolog of TNF receptor-associated factor 2; GGBP1, gram-negative binding protein 1; IFN, interferon; IKK, IκB kinase; LBP, LPS-binding protein; IRAK, interleukin-1 receptor-associated kinase; IRF3, interferon response factor 3; LPS, lipopolysaccharide; MD-2, co-receptor of TLR4; Mal, MyD88 adaptor-like; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor κ B; Pelle, product of the *Drosophila pelle* gene a protein kinase and homolog of IRAK; PGRP-SA, a peptidoglycan recognition protein; SPE, Spätzle processing enzyme; P, phosphorylation of IRF3; Psh, Persephone, a *Drosophila* serine protease; TLR4, Toll-like receptor 4; TRAF6, TNF receptor-associated factor 6; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing interferon-β.

the immune response, the number and identity of these proteases have not been established, but recent work points to a clip domain serine protease named Spätzle processing enzyme (SPE) as the terminal member (14). SPE acts on the precursor form of Spätzle to generate an activating ligand for the Toll receptor.

The *Drosophila* Toll pathway also responds to the presence of pathogenic fungi, and in this case a different and unidentified recognition protein is required for the response. Antifungal responses also require SPE and a second serine protease, Persephone, that is not necessary for antibacterial responses (15). Thus

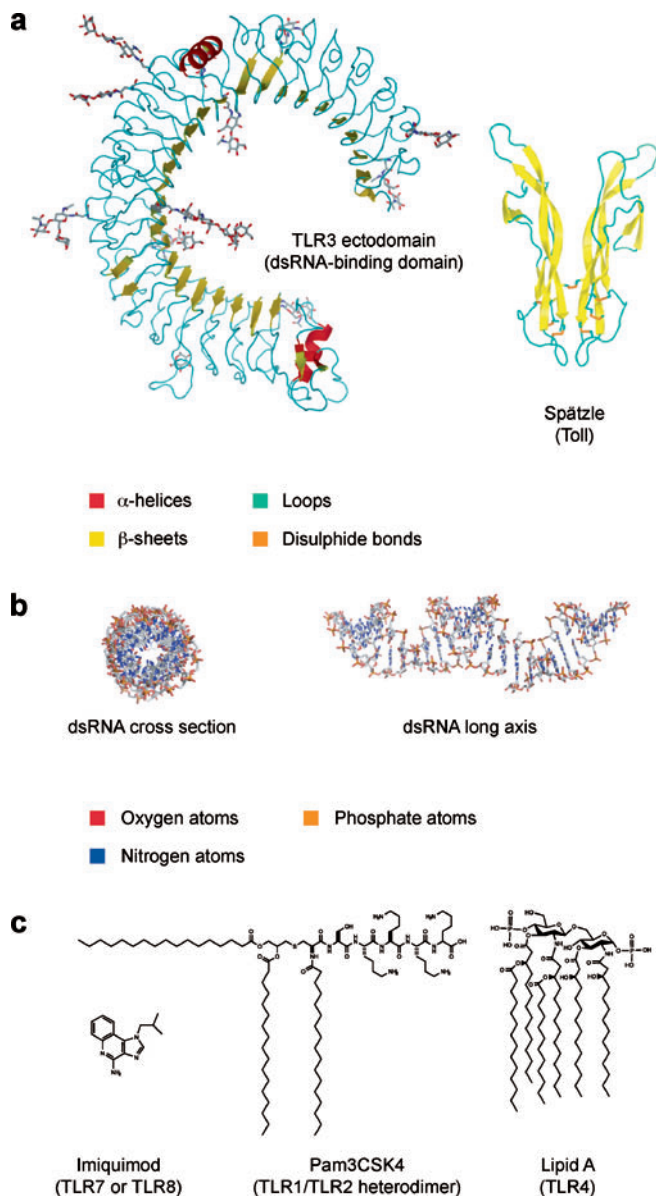


Figure 2

Activating ligands of the Toll and Toll-like receptor family. (a) Human TLR3 ectodomain and *Drosophila* Toll ligand, Spätzle C-106 are shown in protein structures, with β-sheets, α-helices, and loops in ribbon representation. Disulphide bonds are also shown. (b) TLR3 ligand, double-stranded RNA (dsRNA) (152) is displayed as a cross section and along its long axis in stick representation. In nucleic acid and the glycosylations attached to TLR3, oxygen atoms, nitrogen, and phosphate are shown. (c) Imiquimod, Pam3CSK4, and lipid A, the active part of LPS, are displayed in Chemdraw representations. The structures of diverse ligands for Toll receptors are illustrated to scale. Abbreviations: LPS, lipopolysaccharide; Pam3CSK4, a tri-palmitoylated lipopeptide.

microbial signals derived from gram-positive bacteria and fungi converge on a protease that then generates an active protein ligand for the Toll receptor Spätzle.

The Spätzle precursor is synthesized and secreted as a dimer held together by a single intermolecular disulphide bond (**Figure 2**). SPE, or in dorsoventral patterning Easter, protease cleaves the precursor at an arginine residue 106 amino acids from the C terminus to generate a prodomain of approximately 200 amino acids and an active dimeric ligand, C-106. C-106 but not the proprotein binds two molecules of the Toll receptor with high affinity and stimulates downstream signal transduction (16). Spätzle C-106 is related structurally to a family of vertebrate growth factors, the neurotrophins, such as nerve growth factor (NGF) (17). These proteins have a cystine knot fold that has a long antiparallel β-sheet held together by three intersecting intramolecular disulphide bonds (**Figure 2**). Recent work has shown that after cleavage the prodomain of Spätzle remains stably associated with C-106 but is displaced by binding to the Toll ectodomain. This suggests that prior to proteolysis, the prodomain sequesters residues in C-106 that confer specific binding to Toll. After processing, a conformational change occurs that exposes these determinants, an activation mechanism analogous to that of protease zymogens (153).

In view of the evolutionary relatedness of insect Toll and the human TLRs, there was initially an expectation that the mechanisms used for sensing microbial products and for the generation of ligands would be conserved (18). Protease cascades are particularly suitable for this type of process as they can amplify initially small stimuli and can also be regulated by negative feedback loops, causing the response to be spatially and temporally restricted. Thus it was thought that the human genome would encode functional homologs of Spätzle as corresponding endogenous protein ligands for the TLRs, but on completion of the genome-sequence project, none

could be found. In fact activation of TLRs is completely different and involves the sensing of stimuli either directly by the receptors themselves or by receptor/accessory protein complexes (see below and **Table 1**).

The Postreceptor Signaling Pathway

In contrast to extracellular recognition, postreceptor signal transduction by Toll and TLRs requires the function of several conserved proteins (**Figure 1**). Unlike other class I receptors, such as the tyrosine kinases, the cytoplasmic domains of Toll and TLRs do not have an enzymatic activity. Instead stimulus-

induced dimerization or oligomerization is thought to rearrange the TIR domains such that they act as a scaffold for the recruitment of downstream adaptor proteins (see below). In *Drosophila*, the immediate postreceptor events involve the proteins dMyD88, tube, and pelle. dMyD88 is a modular adaptor protein with a TIR domain and a C-terminal death domain (19). Before signal initiation, dMyD88 is present in the cell as a heterodimer with a second adaptor protein tube, and this complex is localized by an unknown mechanism to the plasma membrane. Tube has a death domain (**Figure 3**) but no TIR, and it associates specifically

Adaptor:

cytoplasmic protein that links different elements in a signaling pathway, e.g., an activated receptor and protein kinase

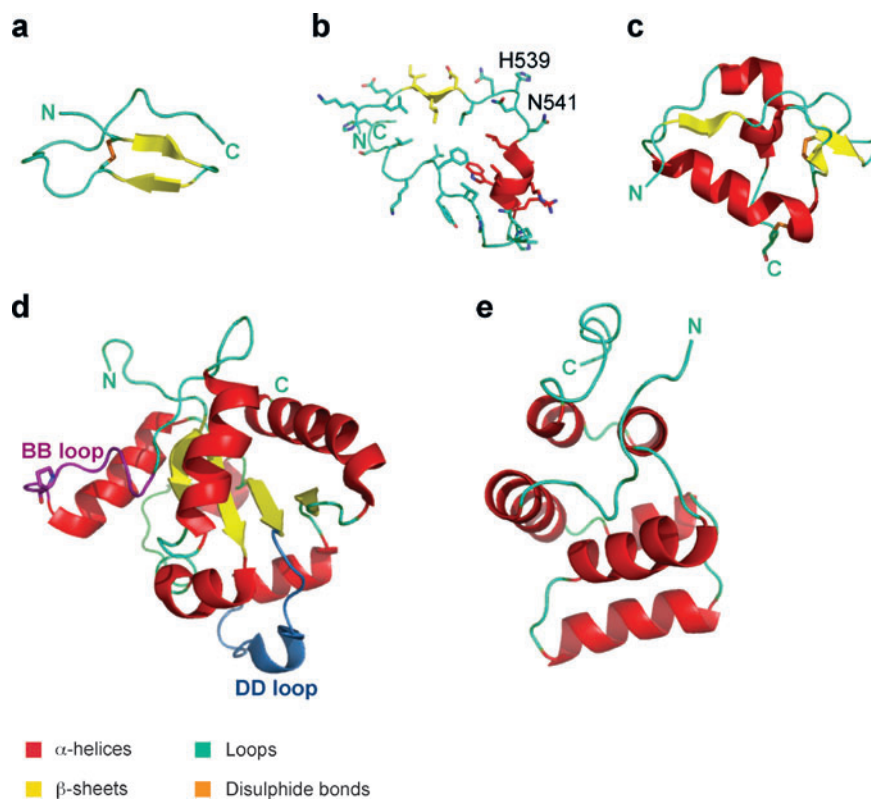


Figure 3

Structures and motifs involved in Toll signaling. (a) N-terminal capping structure taken from the Toll-like receptor 3 (TLR3) ectodomain structure (2A0Z). (b) A single leucine-rich repeat (LRR) unit taken from the TLR3, LRR20 (see **Figure 2**). The short β-sheet (yellow) forms the inside surface of the solenoid. The conserved leucine residues point into the structure to form a hydrophobic core. (c) C-terminal capping structure (taken from TLR3) (see **Figure 2**). (d) The Toll-interleukin receptor (TIR) domain from TLR2. The positions of the BB and DD loops are indicated. (e) The death domain of the pelle protein kinase showing the arrangement of the six α-helical segments.

Protein kinase:

enzyme that transfers phosphate groups from ATP to serine, threonine, and tyrosine residues in other proteins

MyD88: myeloid differentiation primary response protein 88

TNF: tumor necrosis factor

TRAF: TNF receptor-associated factor

Mal/TIRAP: MyD88 adaptor-like/TIR domain-containing adaptor molecule

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adaptor protein inducing interferon- β

with dMyD88 by death domain–death domain interactions. Once the receptor is activated, the dMyD88/Tube dimer is recruited into a ternary complex with the Toll/Spätzle heterotrimer and this in turn recruits pelle (20). Pelle is a serine/threonine protein kinase and also has an N-terminal death domain. The current evidence suggests that the tube death domain is bivalent and has binding sites for both dMyD88 and pelle death domains. Presumably the pelle binding site of tube only becomes available when the tube/MyD88 (myeloid differentiation primary response protein 88) dimer is associated with activated receptor. The signaling complex that contains pelle, tube, dMyD88, and Toll is short lived, and recruitment may activate the pelle protein kinase, causing autophosphorylation and as a consequence the dissociation of pelle from the receptor complex (21).

The events that lie downstream of the pelle protein kinase activity are less well understood in *Drosophila*. However, there is evidence that activated pelle can bind and phosphorylate *Drosophila* tumor necrosis factor (TNF) receptor-associated factor 2 (dTRAF2) and that dTRAF2 is required for the activation of the downstream pathway, ultimately causing the relocalization of dorsal to the nucleus (22). dTRAF2 has both zinc finger and RING finger domains. The latter usually function as an E3 ubiquitin ligase, and TRAF2 appears to be autoubiquitinated. It is not known whether this modification leads to proteasome-mediated degradation of TRAF2 or whether, like its mammalian counterpart TRAF6 (see below), this modification is required for signal transmission. In mammals, signaling by activated TRAF6 involves the kinase TGF β -activated kinase (TAK1) and the adaptors TAB1 and TAB2. Homologs of these proteins are encoded by *Drosophila* and are known to function in an insect pathway similar to that of vertebrate TNF (23) and in responses to gram-negative bacteria mediated by the immune deficiency (IMD) pathway (24). However, they are not essential for Toll

signaling, which suggests either that there is redundancy in the pathway or that unidentified components link dTRAF2 to activation of the dorsal/cactus complex. The final step in Toll signaling involves the regulated nuclear relocalization of the dorsal and dorsal-related immunity factor (DIF) transcriptional regulators to the nucleus. Dorsal and DIF are related to mammalian NF- κ B and are held in the cytoplasm as a complex with cactus, equivalent to I κ B in mammals. Signal-induced phosphorylation likely tags cactus for ubiquitination and degradation by the proteasome pathway, although at present a cactus kinase has not been characterized. However, there are homologs of the mammalian IKKs β , γ , and ϵ encoded by *Drosophila*, but neither β nor γ is required for Toll signaling (25).

Postreceptor signal transduction by the mammalian TLRs uses several proteins that are functional homologs of those described above for the insect pathway (**Figure 1**). However, during evolution there clearly have been duplication, diversification, and a substantial increase in complexity in these pathways. For example, researchers have identified approximately 15 different negative regulators in the TLR pathway (26), and there is growing evidence for cross talk between the TLRs and other pathways (see below). As in *Drosophila*, the first step in TLR signaling is stimulus-induced dimerization or oligomerization. Similarly, the next step is the recruitment of adaptors to the TIR domains of the dimerized receptor, but in contrast to *Drosophila*, TLRs use a total of five adaptor proteins—MyD88, Mal/TIRAP (MyD88 adaptor-like/TIR domain-containing adaptor molecule), TRAM (TRIF-related adaptor molecule), TRIF (TIR domain-containing adaptor protein inducing interferon- β), and SARM (27). MyD88 is a direct homolog of dMyD88, but there is no equivalent of tube in mammals. MyD88 is used exclusively by four of the TLRs (5, 7, 8, and 9), whereas TLR2 requires both Mal and MyD88 (28). TLR4 can use either Mal/TIRAP and MyD88 or TRAM and TRIF to signal to NF- κ B or

antiviral responses mediated through interferon response factor 3 (IRF3), respectively (**Figure 1**) (29). TLR3 uses TRIF exclusively for IRF3-directed signals (30).

Next in the signaling hierarchy are the interleukin-1 receptor-associated kinases (IRAKs). These are homologs of pelle kinase, and there are four of them in mammals. Two, IRAK2 and IRAKM, are not active phosphotransferases (31) and appear to function as negative regulators. IRAK4, conversely, fulfils a role similar to that of pelle, and humans lacking functional IRAK4 are profoundly sensitive to bacterial infections (32–34). Similar to pelle, both IRAK4 and IRAK1 autophosphorylate, and the evidence suggests that they are recruited by association with MyD88 into the postreceptor signaling complex (35; N.J. Gay, unpublished results). The precise events that lead to downstream signaling are not understood, but only kinase-inactive forms of IRAK4 can form a stable complex with MyD88 (36). This suggests a mechanism similar to that for pelle for the binding, activation, autophosphorylation, and dissociation from the postreceptor complex. In the related IL-1 pathway, there is evidence that the initial activation of IRAK4 causes the cross-phosphorylation and activation of IRAK1. IRAK1 then becomes autophosphorylated at multiple sites, dissociates from the postreceptor complex, and binds with TRAF6. However, other studies show that the kinase activity of IRAK4 is not necessary for IL-1 signaling, but the lack of both IRAK1 and -4 abolishes IL-1 signaling (37), suggesting the functional redundancy of the two kinases.

Signaling downstream of the postreceptor complex is critically dependent on TRAF6. As with dTRAF2, TRAF6 has a RING finger domain that functions as an E3 ubiquitin ligase and that can interact with the ubiquitin conjugating complex (E2) Ubc13/Uev1, resulting in the synthesis of polyubiquitin linked through lysine 63 (38, 39). The activation of TRAF6 also causes it to oligomerize through the C-terminal TRAF domain, and this in

turn results in the transfer of the lysine 63 polyubiquitin chains to the TRAF6 oligomer. The polyubiquitin chains do not appear to target TRAF6 for degradation by the proteasome. The next step downstream involves a heterotrimer of the protein kinase TAK1 and the adaptor molecules TAB1 and TAB2 (sometimes called TRIKA2) (40). TAK1 is a member of the MAP kinase kinase kinase family and is required for activation of NF- κ B by TNF, TGF β , as well as by TLRs (41). The roles played by TAB1, TAB2, and a third family member, TAB3, remain unclear as cells from knockout mice respond normally to LPS stimulation. Again, this may indicate redundancy in the pathway. The TAK/TAB trimer does not itself become stably ubiquitinated but may be activated by noncovalent association with the ubiquitin conjugated TRAF6 complexes (42). Thus in this case a stable protein modification different from phosphorylation is essential for signal transduction. The final step in this arm of the TLR pathway is the activation of the I κ B kinase (IKK) by TAK1. The I κ B kinase consists of the two subunits IKK β and the regulatory IKK γ or NEMO (40). Phosphorylation by IKK $\beta\gamma$ targets I κ B for degradation by the proteasome, and this releases NF- κ B, which then relocates to the nucleus. TAK1 is also able to activate a second branch of the TLR pathway mediated through Jun N-terminal kinase (JNK) to the transcription factor AP-1.

In contrast to insects, mammalian cells are able to activate a second pathway in response to some TLR stimuli that leads to antiviral responses such as the production of interferons, chemokines, and anti-inflammatory cytokines. In the case of TLR4, these signals require the adaptor molecules TRAM and TRIF; for TLR3, they require TRIF alone (29, 30, 43). Engagement of these adaptors results in the activation of a complex containing two related protein kinases, TANK binding kinase 1 (TBK1) and IKK ϵ , and the adaptor TANK (TNF pathway with ankyrin repeats) (44, 45). Recent results also suggest that the TRIF adaptor is

IRAK: interleukin-1 receptor-associated kinase

linked to the TBK/IKK ϵ /TANK complex by TRAF3, a homolog of TRAF6 (46). The active TBK/IKK ϵ kinases then phosphorylate the interferon-responsive factors IRF3 and -7, which causes them to dimerize and enter the nucleus (47). IRF3 is ubiquitously expressed and induces the production of β -type interferons. By contrast, IRF7 acts on interferon- α promoters and has a more restricted expression pattern. It is strongly expressed in plasmacytoid dendritic cells (DCs) and is induced in other cell types by interferon- β (48). Activation of IRF7 is mediated by a subset of the TLRs (7, 8, and 9) that responds to nonself-nucleic acids. This subset also induces NF- κ B and requires only the MyD88 adaptor. IRF7 binds to the death domain of MyD88, which results in the activation of the interferon- α promoters. IRF7 also binds to TRAF6, and the ubiquitin ligase activity of TRAF6 is required for the activation process (49). Conversely, a more recent paper suggests that TRAF3 is in fact responsible for the activation of IRF7, perhaps by coordinating the assembly of TBK1 into activated receptor complex (50). In this view TRAF6 would primarily mediate signals to NF- κ B and the production of proinflammatory cytokines.

STRUCTURAL BASIS OF SIGNALING SPECIFICITY IN THE TOLL AND TOLL-LIKE RECEPTOR PATHWAYS

Although only limited progress has been made toward understanding the structural basis of Toll signaling, it is nevertheless possible to derive some general principles from the fragmentary information available. In the past few years a much clearer picture has emerged about the nature of endogenous protein ligands and microbial products that stimulate TLR signaling (see **Table 1**). A certain amount of confusion had been engendered by the use of impure preparations of bacterial products such as peptidoglycan, but most of these problems have now been resolved by the use of chemically synthesized materials.

In view of the clear evolutionary conservation of the Toll and TLR pathways and elements in the signaling cascade, it is perhaps surprising that the activating ligands should be so diverse. **Figure 2** presents the structures of some of these molecules to scale and the extracellular domain of TLR3 for comparative purposes. How then can large protein ligands such as Spätzle and small-molecule stimuli such as imidazoquinolines induce a functionally equivalent dimerization of Toll and TLR ectodomains?

Protein Ligands

Spätzle, the cytokine ligand of the *Drosophila* pathway, is well characterized at the biochemical level, although no structure of the ligand/receptor complex has been solved (16, 51). However, the structure of NGF bound to the p75 and Trk neurotrophin receptors provides a useful analogy (52, 53). In both cases, NGF makes multiple hydrophobic, hydrogen-bonded, and electrostatic contacts with the receptor chains, burying a large surface area of more than 2000 Å². This is reflected by the high affinities of these interactions, between approximately 0.1 and 1 nM (54). Spätzle binds Toll with a similar affinity (0.4 nM), and the alignment of the Spätzle sequence with that of NGF shows that many of the amino acids involved in binding are conserved or are charge reversals (153). Thus the interaction of Spätzle with the Toll ectodomain likely involves an extensive binding area that buries a large hydrophobic surface, stabilized by charge complementarity provided by salt bridges and hydrogen bonds. The two symmetrically related binding sites in Spätzle allow the formation of a high-affinity cross-linked complex at the membrane surface consisting of two receptor chains and one Spätzle dimer, and this initiates signal transduction (see Molecular Mechanism of Signal Transduction, below).

It does not appear that endogenous cytokines equivalent to Spätzle function in vertebrate TLR signaling, but two microbially

derived proteins, flagellin and respiratory syncytial virus (RSV) fusion protein, can activate TLR5 and TLR4, respectively (55, 56). Flagellin is a protein that assembles into the protofilaments that constitute the flagellum, structures that confer on the bacterium motility. Flagellae are also virulence factors required for tissue invasion. TLR5 is activated by a highly conserved sequence in flagellin that is essential for function and is sequestered in the protofilament in assembled flagellae. Thus TLR5 is activated only by monomeric flagellin and responds at low concentrations (less than 100 fM), suggesting a high binding affinity (57). The interaction between flagellin and TLR5 appears to be direct, and the binding site has been mapped to a single LRR in the receptor ectodomain (58). A second microbial protein that may be a direct ligand for TLR4 is the fusion protein of the RSV, a significant pathogen in infants. However, there is no evidence for direct binding, and the MD-2 coreceptor protein is also required for the response (59) (see below).

Lipids and Lipopeptides

The second major class of ligands for the TLRs comprises bacterial lipids and lipopeptides. LPSs from the outer membrane of gram-negative bacteria are the principal agonists for TLR4. LPS derived from *Escherichia coli* consists of six acyl chains of variable length linked together by a phosphorylated diglucosamine head group (lipid A), and this is linked to a polysaccharide chain of variable length and composition (**Figure 2**). The lipid A moiety of LPS is required to activate the TLR4 signaling pathway in conjunction with the coreceptor protein MD-2. MD-2 is a secreted glycoprotein of 160 amino acids and is required for LPS signaling via TLR4 (60). MD-2 interacts with the ectodomain of TLR4 in a constitutive manner (61), and the TLR4/MD-2 complexes form in the endoplasmic reticulum and at the cell surface. In contrast to TLR4, MD-2 binds LPS directly (62, 63). MD-2 belongs to a novel family of

proteins, the MD-2-related lipid-recognition family (64). All members of this family that have been characterized biochemically bind to lipids. They are characterized by a single-domain architecture, belonging to the all-beta class and displaying an immunoglobulin-like β -sandwich fold. It is thought that acyl chains of lipid A intercalate into the β -sandwich structure and that this promotes cross-linking of two TLR4 receptor chains either directly or by inducing a conformational change that allows the association to occur (65). Thus in contrast to *Drosophila* Toll, which binds an endogenous protein ligand, in TLR4 the ectodomain has evolved to form a complex with a specific coreceptor protein (66).

The agonists that signal through TLR2 have obvious similarities to LPS (**Figure 2**). In fact, as well as detecting a variety of lipids, lipoproteins, and lipopeptides derived principally from gram-positive bacteria and parasites, TLR2 can respond to atypical LPS from certain gram-negative bacteria, for example, *Leptospira interrogans* (67). In general TLR2 agonists tend to be less complex than LPS. Lipoteichoic acid (LTA) and macrophage activating lipopeptide 2 (MALP2) have diacyl chains with a glucosamine diphosphate and peptide head groups, respectively. By contrast PAM₃CSK4 has three acyl chains and is connected to a tetra-lysine peptide chain by a cysteine residue (**Figure 2**). Responses to these molecules are mediated by heterodimeric combinations of TLR2 with TLR1 and -6. In general, diacylated molecules require the TLR2/6 combination, whereas the triacylated lipopeptides signal through TLR2/1, although in some in vitro assays TLR2 alone can signal in response to MALP2 and LTA (68–70). The chirality of these molecules also seems important for specificity; for example, the R but not S diastereoisomer of MALP2 is active. Domain-exchange experiments have shown that the ability of the closely related TLR1 and TLR6 to discriminate different agonists resides in LRRs 9–12 in the middle of the ectodomain (71). There are also several distinct coreceptor proteins that are able to

enhance the responsiveness of TLR1/2/6 to stimuli. CD14, an extrinsic membrane protein that also functions in the TLR4 pathway, is thought to bind lipopeptides at the cell surface and deliver them to TLR2 (72). Similarly, the lectin dectin 1 increases the responsiveness of TLR2 to fungal cell-wall components, especially β -glucans (73). Finally, the scavenger receptor CD36 is required for mouse macrophages to respond to low concentrations of MALP2, LTA, PAM₃CSK4, and zymosan (a fungal extract) (74).

At present, it is unclear whether TLR1, -2, and -6 bind directly to these diverse ligands, although the domain-swap experiments outlined above point to this conclusion for the lipopeptides (71). There is also some uncertainty whether peptidoglycan is a bona fide agonist for TLR2 (**Table 1**), owing to possible contamination of the preparations. Clearly the structural information of TLR1/2/6 ectodomains in complex with defined ligands would be useful for understanding the signaling mechanism at a molecular level.

Nucleic Acids

The third major class of molecules that act as specific agonists of TLR pathways comprises nucleic acids and small-molecule drugs that have some structural resemblance to nucleic-acid building blocks, for example, immunostimulatory guanosine analogs and imidazoquinoline compounds (**Figure 2**) (75). The nucleic-acid-responsive subgroup of the TLRs comprises TLR3, -7, -8, and -9, and these receptors are more closely related to each other than to the other Tolls. TLR3 responds to double-stranded (ds)RNA molecules produced during viral infection and also synthetic copolymers such as polyIC (inosine/cytosine) (76). The natural ligand for both TLR7 and TLR8 is U-rich single-stranded RNA exposed to the receptor as a result of viral infection (77, 78). TLR7 and -8 also respond to the imidazoquinolines, small-molecule drugs used to treat genital warts

caused by infection with human papilloma virus and basal-cell carcinomas (79). A number of imidazoquinoline derivatives have been characterized, and some of these display specificity for either the TLR7 or TLR8 pathways in humans (80). Finally, DNA with unmethylated CpG dinucleotides is the agonist for TLR9 (81). In mammals, these sequences are usually methylated at the seven position of cytosine, whereas viral and bacterial DNA do not on the whole have this modification.

An important property of this TLR subgroup is that it functions in acidified intracellular compartments, late endosomes, and lysosomes and that reduced pH is required for signaling function (82). This has led to the suggestion that the protonation of ionizable groups, either histidine residues of the receptor ectodomain or the ligands themselves, is necessary for signaling (83).

Leucine-Rich Repeats and the Structure of the Receptor Ectodomains

It is clear from the diverse nature of the ligands that TLR ectodomains must have features that allow highly variable modes of molecular recognition to evolve from a common structural framework. The key to this problem is the nature of LRR structures. LRRs are units of approximately 24 amino acids length that are characterized by a conserved pattern of hydrophobic residues (**Figure 3**). LRRs are found in a large and diverse group of proteins (approximately 254 in the human genome) with functions ranging from RNA processing (snRNP U2A') and transcriptional regulation (CIITA, activator of MHCII expression) to cell adhesion (chaptin, which is required for eye development in the fly), bacterial pathogenesis (Yop M virulence factor from *Yersinia pestis*), and signal transduction (TLRs and many other type I and 7TM receptors) (84).

Each LRR folds into a secondary structure consisting of a short parallel β -sheet, a turn, and a more variable region. The conserved

hydrophobic residues form the core of this secondary structure (**Figure 3**). The blocks of repeats form a curved, solenoidal structure with the short parallel β -sheets forming the inner (convex) surface of the structure (**Figure 2**). Specific molecular recognition is often achieved by interactions mediated through the side chains of variable residues protruding from the short parallel β -strands, contributed by each LRR, that form the inner (concave) surface of the solenoid. These side chains point out of the structure and can be viewed as a combinatorial code that has evolved to bind specific ligands. Interestingly, chordates, an early divergence of the vertebrate lineage, have evolved an adaptive immune response that uses LRRs instead of the immunoglobulin fold (85). This finding emphasizes the ability of LRR-containing proteins to evolve highly diverse binding modes for proteins and other biological molecules. LRR blocks are often flanked by cysteine-rich domains (86, 87). These domains are called LRRNT and LRRCT when located at the amino- and carboxy-terminal ends of LRR regions, respectively (**Figure 3**). They are crucial capping structures, burying the otherwise exposed hydrophobic residues at the ends of LRR superhelices. N-capping sequences are likely to form a β -hairpin, whereas C-terminal caps adopt a α - β structure. In both cases the fold is stabilized by the formation of disulphide bonds involving pairs of conserved cysteine residues.

The Transmembrane and Toll-Interleukin Receptor Domains

As with other type I receptors, the ectodomains of the TLRs are connected to the cytoplasmic TIRs by a single transmembrane α -helix. Although there is no striking pattern of sequence conservation in these segments (other than hydrophobicity), the transmembrane and juxtamembrane sequence are likely to play critical roles in receptor activation.

Our current understanding of the TIR domain fold is informed by the crystal structures of TLR1 and TLR2 proteins (**Figure 3**) (88). The core sequence adopts an overall β/α -fold comprising a central five-stranded parallel β -sheet surrounded by five α -helices. As with the LRRs, sequence conservation seems to reflect the structural requirements of the fold. Conversely, the surface properties of different receptor and adaptor TIRs are highly diverse, and this diversity may underlie the functional specificity of different modules in the signaling process (89).

A similar fold is seen in CheY, a bacterial chemotaxis protein (8, 90). CheY proteins consist of a single regulatory domain activated by conformational change induced by a phospho-transfer reaction from a histidine kinase to a conserved aspartyl residue. Structural superposition of CheY, TLR1, and TLR2 shows remarkable topological and tertiary structure conservation, especially over the central β -sheet region. Investigators have identified many similar signal response regulators with a β/α -fold in different species, suggesting this could be an evolutionarily conserved signal regulatory conformation.

Death Domains

The multitypic postreceptor complexes formed by the TIRs from receptors and adaptors are coupled to downstream signaling by a second protein-protein interaction motif, the death domain, so named because it is also found in signaling pathways leading to apoptosis (91). The adaptors MyD88 and tube have death domains, as do the IRAK and pelle kinases (33). The tube and pelle death domains associate together to form stable heterodimers, and this association is required for signaling (92, 93). Death domains consist of approximately 120 amino acids and are characterized by a conserved six α -helical bundle fold (**Figure 3**) (91, 94). A crystal structure of the tube/pelle death domain heterodimer (95) and more recently structures of the isolated pelle and IRAK4 death

domains have been solved (96, 97). Together these studies reveal that the binding interface between tube and pelle death domains is extensive, involving at least 27 of the residues in the fold. Helix four of pelle interacts with a groove formed by helices one, two, and six of tube. Additionally, the C-terminal tail of tube interacts with a groove on pelle created by helices two through five. Tube also binds the dMyD88 death domain at a separate site; this interaction is much more stable than that between tube and pelle ($K_d = 150$ nM and 2 μ M, respectively; N.J. Gay & M. Gangloff, unpublished results). To date structural analysis of MyD88/IRAK4 death domain complexes has not been achieved, and the absence of tube in the vertebrate pathways suggests that there are significant differences in the signaling mechanism. It is also unclear how at a structural level autophosphorylation induced during the signaling process can cause the dissociation of death domain–death domain complexes.

MOLECULAR MECHANISM OF SIGNAL TRANSDUCTION

As with other type I transmembrane receptors such as the receptor tyrosine kinases, the basic mechanism for signal transmission by TLRs is ligand-induced dimerization or oligomerization. For example, Spätzle, a symmetrical dimer, cross-links two molecules of *Drosophila* Toll ectodomain into a heterotrimeric complex, and this binding event is necessary and sufficient to induce signal transduction (16). The activation of the vertebrate TLRs likely requires a similar process of ligand-induced dimerization. The evidence for this comes first from domain-swap experiments in which the ectodomain of TLR4 is replaced by that of *Drosophila* Toll. The chimeric receptors are activated by treatment with Spätzle, suggesting that TLR4 will signal if two receptor molecules are arranged in an equivalent symmetrical complex (51). Second, some monoclonal antibodies directed against TLR3 and TLR4 can produce cross-linked complexes

that are unable to signal (98). This suggests that the receptor chains have to be arranged in the correct orientation with respect to each other for signaling to occur.

Recent work also suggests that dimerization promotes conformational changes in the receptor ectodomains and induces stable protein-protein interactions between the receptor chains. In the case of *Drosophila* Toll, the immediate juxtamembrane regions of the ectodomain appear to mediate these receptor-receptor interactions, and ligand binding may be relieving a constitutive autoinhibition that prevents the signaling-competent association of unactivated receptors (51, 99, 100).

The dimerization of receptor ectodomains must also couple to cytoplasmic signaling, enabling the recruitment of cognate adaptors into a postreceptor complex. Thus, receptor dimerization must create a new arrangement of the TIR domains and thereby provide the specificity required for adaptor binding. Two properties of the activated receptor complexes may be relevant to this situation. First, at least in the case of *Drosophila* Toll and probably also TLR4, the receptor complexes are symmetrical. Second, the connections between the transmembrane α -helix, the ectodomain, and TIR are likely rigid, so the ectodomain and TIR cannot rotate independently of each other. As illustrated in **Figure 4** for TLR4, this suggests that symmetry-related surfaces of the receptor TIRs are forced to associate and may undergo a structural reorganization as a consequence. In the case of TLR4, a surface structural feature called the BB loop (see **Figure 3**) that links the second α -helix to the second β -sheet of the fold may be of particular importance for receptor homodimerization. LPS hyporesponsiveness in C3H/HeJ mice is caused by a mutation of a proline residue to histidine in the BB loop of TLR4, and these mutant receptors have dominant negative effects on TLR4 signaling (11). This suggests that they are able to form inactive dimers with wild-type TLR4 that are unable to support downstream signaling. The dimerized receptor TIRs now provide a new

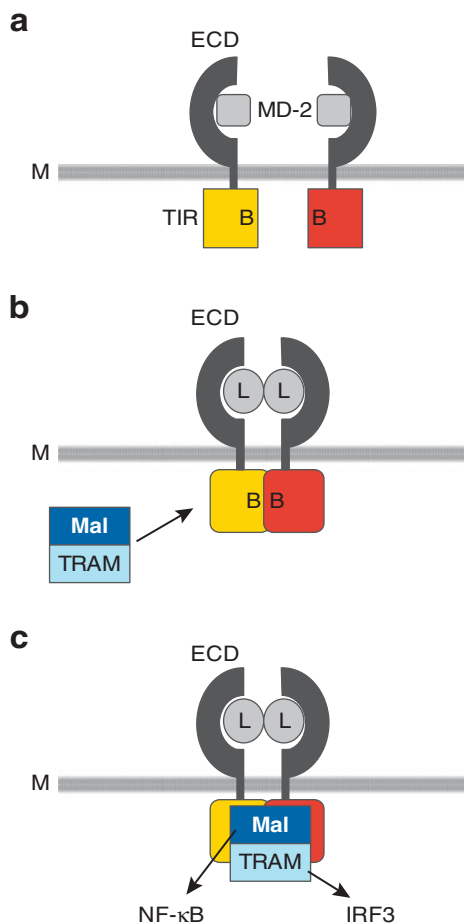
scaffold, which allows the recruitment of the specific adaptor proteins. Although the fold is conserved in both receptor and adaptor TIR domains, the surface electrostatic properties are distinct. TLR2 and TLR4 surfaces are rather basic, whereas the Mal/TIRAP and TRAM adaptors have similar negatively charged surfaces. Thus specificity may be determined by the electrostatic complementarity of the receptor and adaptor TIR domains (89).

The mechanism shown in **Figure 4** can also account for the inhibitory properties of lipid A variants such as the tetra-acylated lipid IVa. Early experiments carried out before the identification of TLR4 as the LPS receptor showed that lipid IVa acted as a competitive inhibitor of LPS signaling in human monocytes. This suggests that it binds to the same site on TLR4/MD-2 as hexa-acylated lipid A

and with comparable affinity (100a). A more recent study demonstrated that hexa-acylated lipid A induces cross-linking of TLR4/MD-2. By contrast, lipid IVa binds to TLR4/MD-2 but does not cause dimerization or oligomerization (100b). Thus, signal transduction by TLR4 depends critically on the ability of lipid A to bind simultaneously to two molecules of MD-2 or alternatively to induce a conformational change in MD-2 that facilitates association of two receptor molecules into a heterotetrameric complex. Synthetic antagonists of TLR4 based on lipid IVa, such as E5531, are effective at blocking responses to endotoxin *in vivo* and are now in Phase III clinical trials for treatment of sepsis (100c, 100d).

Figure 4

Model for signal transduction by Toll-like receptor 4 (TLR4). The curved ectodomains (ECDs) are illustrated with the coreceptor protein MD-2. Two surfaces of the Toll-interleukin receptor (TIR) domains are shown rotated through 180°. The surface containing the BB loop is perpendicular to the plane of the paper. (a) Prior to activation, receptor molecules are able to diffuse in the membrane and may form transient dimers. The ectodomains are rigidly connected to the cytoplasmic TIRs by the transmembrane helix. (b) Activation by lipopolysaccharide (LPS) binding to MD-2 leads to the dimerization of ectodomains, probably stabilized by receptor-receptor interactions in the juxtamembrane region. By analogy with *Drosophila* Toll, which is activated by a dimeric protein ligand, the receptor complexes are likely symmetrical. Thus the TIR domains are constrained to associate by interactions between equivalent surfaces on the TIR to form a symmetrical dimer. The evidence suggests that the surface centered on the flexible BB loop may be the dimerization interface and that association is stabilized by a conformational rearrangement involving this structure. (c) The dimerized TIRs provide a new molecular surface that can bind to adaptor molecules TRAM and/or Mal/TIRAP with high affinity. Abbreviations: B, BB loop; L, LPS; M, membrane.



The structure of the isolated TLR3 ectodomain, solved recently by X-ray crystallography, also provides support for the activation scheme outlined above (101, 102). As expected, the TLR3 LRRs fold into an extended solenoid, and the surface is extensively modified by N-linked glycans (**Figure 2**). The ectodomain behaves as a monomer in solution, but two molecules are arranged as dimers in the crystals used to solve the structure. The dimer interface is on the convex (outer) surface at the C terminus, and this arrangement could represent the conformation adopted during the signaling process. However, as pointed out previously by Kirk & Bazan (103), this interface may be an artifact generated during the crystallization of the protein and therefore may be of uncertain biological significance. Recent data from Segal's lab (104) show that only two residues (H539 and N541) are required for the binding of dsRNA by the TLR3 ectodomain. These are positioned close to the C terminus in LRR20 on a lateral surface that is free of glycan (**Figure 2**). On the basis of these results, the authors propose that binding is mediated by the formation of a hydrogen bond between N541 of TLR3 and the 2' OH group of a ribose in the dsRNA. They further suggest that dsRNA would be able to cross-link two TLR3 ectodomains to form a symmetrical complex, with the first molecule related to the second by a 180° rotation and a short translation. In principal, a single dsRNA molecule could bind together a number of TLR3 ectodomains to produce higher-order oligomers. The binding of dsRNA may also promote protein-protein contacts between two TLR3 ectodomains, although it is not known whether this would induce conformational changes of the type proposed above. Thus, although Spätzle is different chemically from dsRNA, both molecules are able to promote a precise symmetrical arrangement of the receptor ectodomain. Clearly, high-resolution crystal structures of TLR3 in complex with dsRNA and Spätzle bound to Toll are required to address these questions.

In contrast to the situation with Toll and TLR4, recent work suggests that heterodimeric complexes of TLR2 with TLR1 induced by triacyl lipopeptides may have an asymmetrical arrangement of the two receptor chains. These studies identified a cluster of residues in TLR2 located toward the C terminus in the DD loop (connecting the fourth α -helix and β -sheet) and α D region that are required for signaling by PAM₃CSK4, and corresponding residues in TLR1 were located in the BB loop. Docking studies also suggest that the dimerization of the receptor TIRs is mediated by interactions between the BB loop of TLR1 and the DD loop of TLR2 (see **Figure 3**) (105). This implies that the ectodomains also will be arranged in an asymmetric manner by the binding of the triacyl lipopeptide. Another recent study suggests that a different region, the conserved α E helix, is required for homodimerization of TLR2 induced by diacyl lipopeptides (106). This study also revealed a site in the N terminus of the MyD88 TIR that is required for adaptor recruitment and suggests two different modes of engagement between TLR2 and MyD88.

Taken together, these results emphasize the potential of receptor and adaptor TIR domains to mediate a range of signal-induced protein-protein interactions, and this plasticity probably underlies the complexity generated during the TLR signaling process. Our understanding of these mechanisms is currently restrained by the absence of structural studies of receptor and adaptor complexes.

CROSS TALK IN THE TOLL-LIKE RECEPTOR PATHWAYS

An emerging theme in Toll signaling is the regulatory role played by pathway cross talk. Thus, the outcomes of TLR-mediated signals can be modified depending on the cell type and the other stimuli present in the environment.

The Role of Protein Kinase C ϵ and Integrins in TLR4 Signaling

As described above, the activation of TLR4 results in the recruitment of four adaptor proteins (**Figure 5**). Mal/TIRAP is thought to bind directly to the receptor TIRs and acts as a bridging adaptor for recruitment of MyD88. This arm of the pathway mediates rapid activation of NF- κ B responses within a few minutes of stimulation. By contrast, TRAM is

thought to bridge the binding of TRIF to the postreceptor complex, and these adaptors activate the MyD88 independent pathway, leading to the activation of IRF3 and delayed NF- κ B responses (29, 107). At this stage, it is not known if both sets of adaptors can bind simultaneously or whether binding to the dimerized receptor TIRs is mutually exclusive. Both Mal/TIRAP and TRAM are localized to the plasma membrane in unstimulated cells, and

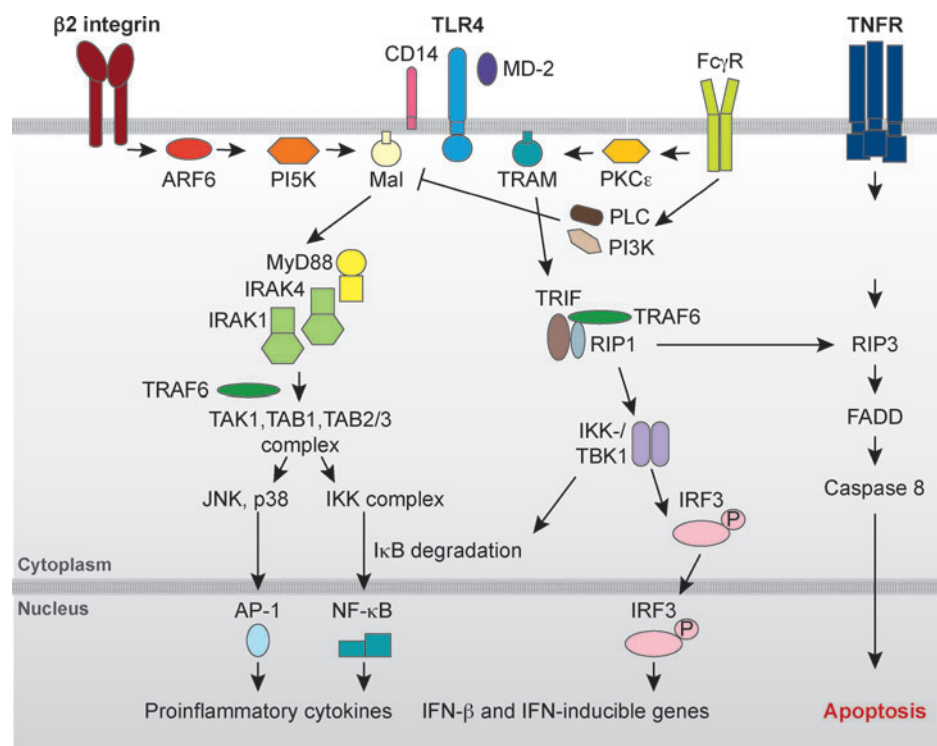


Figure 5

Cross talk in the TLR4 pathway. Model for the modulation of TLR4 signaling by inputs from β 2 integrin and Fc γ receptors. Depending on the inputs received, there will be a bias toward signaling through Mal/MyD88 or TRAM/TRIF, leading to the production of different cytokine profiles. TRIF-directed signaling also favors cross talk to the death pathway through RIP1/RIP3 [adaptors in the tumor necrosis factor (TNF) signaling pathway]. Abbreviations: AP-1, activating protein-1; CD14, an extrinsic membrane protein; FADD, Fas-associated death domain; IFN, interferon; IKK, I κ B kinase; IRAK, interleukin-1 receptor-associated kinase; IRF3, interferon response factor 3; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; MAL, Mal/TIRAP (MyD88 adaptor like), MD-2, a secreted glycoprotein; MyD88, myeloid differentiation primary response protein 88; NF- κ B, Nuclear factor κ B; PKC ϵ , protein kinase C ϵ ; PLC, phospholipase C; PI3K and PI5K, phosphatidylinositol 3 kinase and phosphatidylinositol 5 kinase; TAB1 and TAB2/3, adaptor molecules; TAK1, TGF β -activated kinase; TLR4, Toll-like receptor 4; TNFR, tumor necrosis factor receptor; TRAF6, TNF receptor-associated factor 6; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing interferon- β .

recent studies have shed light on the nature and functional significance of this association.

In the case of TRAM, constitutive binding to the cytoplasmic membrane is caused by the addition of myristate, a 14-carbon fatty-acid chain, to a glycine residue located at the N-terminus of the protein (108). TRAM is therefore one of a group of myristoyl proteins that also includes the src nonreceptor tyrosine kinase, some small G proteins, and a protein kinase C (PKC) substrate protein, MARCKS (109). The anchoring of proteins to the membrane by myristoyl groups is often stabilized by additional electrostatic interactions between basic amino-acid residues close to the site of modification and the head groups of phospholipids in the membrane. Thus, the membrane localization of myristoyl proteins may be reversed by disrupting these stabilizing electrostatic interactions, a so-called myristoyl electrostatic switch (110). In the case of TRAM, a myristoyl electrostatic switch is operated by the PKC isoform PKC ϵ (111).

PKC ϵ phosphorylates a serine residue close to the N terminus of TRAM, which disrupts the electrostatic contacts that stabilize membrane association. The release of TRAM from the membrane is required for function, and this suggests that the activation of this arm of the TLR4 pathway requires a second signal from protein PKC ϵ . Consistent with this idea, mice lacking PKC ϵ are hyporesponsive when stimulated by LPS (112). PKC ϵ activity is regulated by diacyl glycerol produced by rises in intracellular calcium concentration (113), but at this stage it is not known which intrinsic or extrinsic stimuli regulate PKC ϵ in immune-system cells. One possibility, however, is the Fc γ receptor family. These receptors are expressed on many immune-cell types, including B cells, macrophages, and DCs. They signal in response to the Fc region of immunoglobulin G molecules and induce rises in intracellular calcium, leading to the activation of PKC family kinases (114). Ligation of Fc γ R receptors is known to cause a change in the profile of cytokines produced by treat-

ment with LPS (115). Thus the treatment of macrophages with immune complexes causes a polarization of T cell responses away from Th1 toward Th2 (116). A plausible explanation for this effect is that the activity level of PKC ϵ will determine the profile of cytokines produced by TLR4 through the slow NF- κ B and IRF3 responses (TRAM dependent) and the fast NF- κ B activation (Mal/TIRAP dependent). One member of the Fc γ receptor family, Fc γ RIIb, has an inhibitory effect on calcium responses and may exert an antagonistic effect on TRAM-dependent LPS signaling.

The Mal/TIRAP adaptor is also localized to the cytoplasmic plasma membrane but uses a different mechanism to TRAM. Recent work shows that the N terminus of Mal/TIRAP contains a binding site for the inositol phospholipid PIP2 and that Mal/TIRAP binds to the membrane in a PIP2-dependent manner (117). Membrane localization is required for MyD88-dependent signaling through TLR4, and remarkably grafting the Mal/TIRAP PIP2 binding site on to MyD88 confers the ability to signal independently from Mal/TIRAP. This suggests that Mal/TIRAP functions to target MyD88 to the membrane, acting as a sorting adaptor rather than a bridging adaptor as suggested above. The level of PIP2 in the membrane is regulated by β 2 integrin signaling through the small G protein ARF6 and the PI5 kinase. Membrane localization and signaling by Mal/TIRAP but not TRAM are defective in macrophages lacking β 2 integrin. Interestingly, Fc γ receptors activate phospholipase C, which acts on PIP2 to generate IP3 and PI3 kinases that convert PIP2 to PIP3. Thus Fc γ receptors could exert a negative effect on Mal/TIRAP-mediated signaling, as well as a positive effect on TRAM. In this context, it is also interesting to note that PIP3 is responsible for recruiting proteins containing PH domains to the membrane. These include the Bruton tyrosine kinase, which phosphorylates Mal/TIRAP at three sites, and phospho-Mal/TIRAP cannot

form complexes with TLR4 and MyD88 but may be targeted for degradation (118, 118a).

The results described above and summarized in **Figure 5** show that signaling in response to LPS can be modified by at least two other signaling systems in response to both endogenous and exogenous conditions. Similar complexity likely exists in other TLR pathways.

Toll-Like Receptor Signaling and Apoptosis

Under certain conditions and in some cell types, TLR signaling can induce apoptosis; therefore it must have the capacity to cross talk with the death signaling pathways. In particular, there is a clear analogy with signaling through the TNF pathway that can both activate NF- κ B and induce apoptotic cell death. For example, in insects, responses to gram-negative bacteria are sensed by the IMD pathway. This pathway uses homologs of TNF pathway components such as Fas-associated death domain (FADD), DREDD (caspase 8), and relish (NF- κ B), although the IMD pathway itself does not lead to apoptosis (119). Similarly, components of the vertebrate TNF pathway are used promiscuously in TLR signaling (e.g., TRAF6, TAK, and IKK kinases) (41).

The activation of TLR2 with several bacterial lipoproteins can induce apoptosis in both transfected human embryonic kidney (HEK293) cells and a monocyte-derived cell line (MHP1) (120). However, a related study with HEK293 cells did not observe cell death, suggesting that the activation of apoptosis is sensitive to culture conditions and the nature of the treatment (121). It appears that apoptosis induced by TLR2 signaling involves an interaction between the death domains of MyD88 and the TNF-pathway-component FADD, leading in turn to the activation of caspase 8 (122). Thus the TLR2 pathway bifurcates at the level of MyD88 with branches leading to either the activation of NF- κ B or apoptotic cell death. In addition to TLR2,

TLR3 and TLR4 can induce cell death both *in vivo* and in culture cells. For example, LPS can induce both the maturation and apoptosis of DCs. DCs are professional antigen-presenting cells that couple innate and adaptive immunity. Apoptosis may be an important way of limiting infection and downregulating DC-mediated immune responses in sepsis. Recent work has shown that in contrast to TLR2, apoptosis mediated by both TLR3 and TLR4 requires the adaptor TRIF (123, 124). TRIF is a large adaptor protein with a TRAF6 interacting domain and a receptor interacting protein homotypic interaction motif (RHIM), as well as a TIR domain. In DCs, the RHIM domain is required for TLR4-mediated apoptosis, and it interacts with two proteins implicated in TNF signaling, RIP1 and RIP3. As RIP1 is involved in activating NF- κ B, it is unclear how the RIP1/3/TRIF complex promotes apoptosis. One possibility is that RIP1, which has a death domain, can recruit FADD when in complex with RIP3 and TRIF. The involvement of TRIF in LPS-induced cell death is intriguing as it suggests that the activation of PKC ϵ , which should channel LPS signaling through TRAM/TRIF, may also bias cells toward apoptosis.

CONCLUSIONS AND PERSPECTIVE

The discovery of the TLRs 10 years ago led to the development of a large research field and has changed our understanding of the function and importance of innate processes in the human immune response. The initial phase of characterization driven by reverse genetics in the mouse and the use of *in vitro* culture-based signaling systems are close to completion. However, this knowledge provides a basis to address more difficult questions of mechanism at the molecular level and the complexities of regulation and pathway cross talk *in vivo*. In this regard, Toll receptors and pathway components have proved especially challenging as targets for structural studies, and the picture obtained to date

remains partial and fragmentary. However, as with other biochemical systems, the evidence suggests that the signaling process is dynamic and involves concerted and sequential protein conformational changes. Another challenge for the future is to elucidate further the

complexity of pathway cross talk for TLR4 and other TLRs. It is likely that effective intervention in endotoxic shock, for example, requires a more detailed understanding of the other signaling inputs that drive responses to LPS in a pathological direction.

SUMMARY POINTS

1. Toll receptors have evolved to recognize highly diverse ligands derived from pathogenic microorganisms.
2. Insect Toll receptors are activated by an endogenous protein ligand generated indirectly by exposure to microbial stimuli. Vertebrate TLRs probably bind directly to microbial ligands.
3. The signaling mechanism of Toll and TLRs involves the dimerization of two receptor chains induced by ligand.
4. Ligand binding is likely to induce conformational changes that facilitate receptor-receptor interactions.
5. The association of the cytoplasmic TIR domains of the activated receptors produces a scaffold that can bind specifically to postreceptor adaptor proteins.
6. The signaling output of TLR4, the endotoxin receptor, can be modified by cross talk with other pathways.
7. Cross talk may determine the cytokine profile produced by immune system cells and thus the type of adaptive immune response that is generated.

FUTURE ISSUES

1. Structural studies of receptor ectodomains in complex with ligands are required to establish the principles of molecular recognition by TLRs.
2. Membrane biochemistry and structural analysis of activated whole receptor complexes are needed. Structures of isolated ecto- and TIR domains are unlikely to elucidate the conformational changes that provide specificity to the signaling process.
3. Further elucidation of cross talk in the TLR and other pathways is needed, and the significance of cross talk for immune system function in vivo should be explored.
4. Biochemical and structural information should be exploited for the development of therapy in TLR-associated diseases (e.g., endotoxic shock, autoimmunity, viral infection).

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