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Review

Structures of the Toll-like Receptor Family and Its Ligand Complexes

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Toll-like receptors (TLRs) play central roles in the innate immune response by recognizing conserved structural patterns in diverse microbial molecules. Here, we discuss ligand binding and activation mechanisms of the TLR family. Hydrophobic ligands of TLR1, TLR2, and TLR4 interact with internal protein pockets. In contrast, dsRNA, a hydrophilic ligand, interacts with the solvent-exposed surface of TLR3. Binding of agonistic ligands, lipopeptides or dsRNA, induces dimerization of the ectodomains of the various TLRs, forming dimers that are strikingly similar in shape. In these "m"-shaped complexes, the C termini of the extracellular domains of the TLRs converge in the middle. This observation suggests the hypothesis that dimerization of the extracellular domains forces the intracellular TIR domains to dimerize, and this initiates signaling by recruiting intracellular adaptor proteins.

Introduction

Ten human Toll-like receptors (TLRs) have been discovered since TLR4 was first identified as the ortholog of *Drosophila* Toll (Gay and Keith, 1991; Medzhitov et al., 1997). Toll and the TLRs are critical for the innate immune response in *Drosophila* and mammals, respectively. TLRs are type I transmembrane glycoproteins composed of extracellular, transmembrane and intracellular signaling domains (Gay and Gangloff, 2007). The extracellular domains have repeated leucine-rich repeat (LRR) modules and are responsible for binding so-called "pathogen-associated molecular patterns" (PAMPs) (Janeway, 1989; Medzhitov, 2001). For example, lipoproteins or lipopeptides are recognized by TLR2 in complex with TLR1 or TLR6, viral double-stranded RNA are recognized by TLR3, lipopolysaccharide are recognized by TLR4, flagellin are recognized by TLR5, single-stranded RNA are recognized by TLR7 or TLR8, and microbial DNAs are recognized by TLR9 (Uematsu and Akira, 2008). Ligand-induced dimerization of TLRs is believed to trigger recruitment of adaptor proteins to the intracellular TIR (Toll/interleukin-1 receptor) domains to initiate signaling (O'Neill and Bowie, 2007). Intracellular TIR domains are composed of ~150 amino acid residues. The signaling cascades via the TIR domains are mediated by specific adaptor molecules including MyD88, MAL (also known as TIRAP), TRIF, and TRAM (O'Neill and Bowie, 2007). These adaptor proteins also contain TIR domains and TIR-TIR interactions between receptor-receptor, receptor-adaptor, and adaptor-adaptor are critical for activating signaling (Pålsson-McDermott and O'Neill, 2007).

Structural studies of TLR-ligand complexes have been an attractive area of research given that structural information is critical for understanding innate immunity as well as designing novel drugs. In 2005, Choe et al. (2005), and later Bell et al. (2005), first reported crystal structures of human TLR3 in atomic detail. These structures did not contain bound ligands. Subsequently, three structures of TLR-ligand complexes, TLR1-TLR2-lipopeptide, TLR4-MD-2-Eritoran, and TLR3-double-stranded RNA (dsRNA), have been determined by others and by us, and they provide experimental insights into how these receptors recognize a remarkably large variety of ligands, and how the agonists induce dimerization of the receptors (Jin et al., 2007; Kim et al., 2007b; Liu et al., 2008). In this review, we summarize the reported structures of the TLR complexes and discuss their implications for our understanding of TLR activation.

Structure of the Extracellular Domains of the TLR Family

The extracellular domains of TLR family proteins contain 16–28 LRRs (Matsushima et al., 2007). The LRR family comprises approximately 6000 proteins in the Pfam database (http://pfam.sanger.ac.uk). They are involved in a wide variety of physiological functions including immune responses, signal transduction, cell-cycle regulation, enzyme regulation, etc. (Dolan et al., 2007; Matsushima et al., 2005). All known LRR family proteins contain multiple LRR modules. The shorter LRR proteins have two to three LRR modules but the longer ones can have more than 40. The individual LRR module is 20–30 amino acids long and is composed of a conserved "LxxLxLxxN" motif and a variable part (Kajava, 1998; Kobe and Kajava, 2001). Nearly 50 crystal structures of the LRR family have been reported in the Protein Data Bank database. All these structures have a common horseshoe-like shape (Gay and Gangloff, 2007). The hydrophobic core, formed by the conserved leucines and hydrophobic residues in the variable regions, extends throughout the entire protein. The LRRNT and LRRCT modules in the N and C termini do not have LRR motives but frequently contain clustered cysteines forming disulfide bridges (Kajava, 1998; Kobe and Kajava, 2001; Matsushima et al., 2007). These modules stabilize the protein by protecting its hydrophobic core from being exposed to solvent. The unique horseshoe-like shape is due to conserved sequence patterns in the LRR modules. The "LxxLxLxxN" motives are located in the inner concave surfaces of the horseshoe-like structure formed from parallel β strands. The variable parts of the modules form the convex surface generated by helices, β turns, and/or loops.

The production and crystallization of some LRR proteins including TLRs have proven to be extremely difficult. To overcome these problems, we developed a method termed the "hybrid LRR technique" (Jin et al., 2007; Kim et al., 2007b). This technique was applied to the TLR family and enabled us to determine the crystal structures of three TLR proteins, TLR1, TLR2, and TLR4, and their complexes with ligands. Hagfish variable lymphocyte receptors (VLRs) were chosen as the fusion partners, and the TLR and the VLR fragments were fused at their conserved "LxxLxLxxN" motifs so that local structural incompatibility could be minimized (Kim et al., 2007a). Analysis of seven TLR-VLR hybrids demonstrated that the fusion strategy did not disturb the structure or function of any of the proteins (Jin et al., 2007; Kim et al., 2007b).

On the basis of their sequences and structural patterns, LRR family proteins can be classified into seven subfamilies, "typical," "RI-like (ribonuclease inhibitor-like)," "CC (cysteine containing)," "PS (plant specific)," "SDS22-like (SDS22$^+$ protein-like)," "bacterial," and "TpLRR (*Treponema pallidum* LRR)" (Kajava, 1998; Kobe and Kajava, 2001; Matsushima et al., 2007). TLRs are "typical" subfamily proteins. These have LRR modules of 24 amino acids with the conserved motif "xLxxLxxLxLxxNxLxxLPxxxFx." In addition to the β sheet in the concave region common to all LRR family, the typical subfamily member contains parallel 3_{10} helices (a form of secondary structure) in the convex region of the horseshoe-like structure. The LRR modules of TLR1, TLR2, and TLR4 deviate substantially in conformation from those of other "typical" subfamily proteins (Jin et al., 2007; Kim et al., 2007b). The three TLRs have two sharp structural transitions in the β sheet, and therefore their LRR domains can be divided into three subdomains, N terminal, central, and C terminal. The central domains have radii, twist and tilt angles that deviate markedly from the standard values of the "typical" subfamily member. These atypical structures seem to be caused by unusual LRR modules concentrated in their central domains. First, the central domains of TLR1, TLR2, and TLR4 lack asparagine ladders that stabilize the overall horseshoe-like structure by forming a continuous hydrogen-bond network with backbone oxygens of the neighboring β strands. The broken asparagine ladders in the central domains may allow the unusual structural distortions found in TLR1s, TLR2s, and TLR4s. Second, the LRR modules of the central domains vary considerably in numbers of residues, which range from 20 to 33 residues. The LRR modules in the majority of the LRR proteins are of uniform length (Kajava, 1998; Kobe and Kajava, 2001; Matsushima et al., 2007). LRR subfamilies with shorter LRR modules have loops in the convex area, and those containing longer LRR modules have bulkier α helices. Helices require more space than loops and therefore subfamilies with α helices have smaller radii compared to those with loops that generate enough space in the convex region. Therefore, the large variation in the length of the central domain should contribute to the structural anomalies found in the TLRs. Third, the central domains of TLR1, TLR2, and TLR4 have one or more α helices inserted into the convex area. The insertion of the bulkier α helices in some of these LRR modules should increase the curvature of the domains and contribute to the unusually small and nonuniform radii of the central domains. The structural alterations in TLR1, TLR2, and TLR4 are associated with their biological functions because they play essential roles in binding ligands or coreceptors (see Structure of TLRs and Their Ligand Complexes). The N- and C-terminal domains agree well with consensus structure of the typical subfamily: The length of LRR modules varies little around a value of 24 amino acid residues, and the structurally important asparagine ladder and phenylalanine spine are conserved.

In contrast to TLR1, TLR2, and TLR4, TLR3 has a flat horseshoe-like shape with uniform conformational angles comparable to those of other LRR proteins of the "typical" subfamily (Bell et al., 2005; Choe et al., 2005; Liu et al., 2008). The asparagine ladders are intact throughout the TLR3 molecule, and the lengths of the LRR modules vary considerably less than those of TLR1, TLR2, or TLR4. Although the structures of TLR5, TLR7, TLR8, and TLR9 have not yet been reported, their LRR modules appear to have continuous asparagines ladders and relatively uniform module lengths, suggesting that they belong to the single domain subfamily. In contrast, TLR6 and TLR10 have LRR modules with broken asparagine ladders and greater variation in module length in the central part of the protein. They probably belong to the three-domain architecture found in TLR1, TLR2, and TLR4.

Structures of the Intracellular TIR Domains of TLRs and Signaling Adapters

The structures of the TIR domains of TLR1, TLR2, and TLR10 have been studied by X-ray crystallography (Nyman et al., 2008; Xu et al., 2000). These domains have a common fold containing a five-stranded β sheet surrounded by five α helices. Mutational and modeling studies indicate that the BB loop connecting the second β sheet and the second α-helix plays an important role in TIR dimerization and/or adaptor recruitment. Mutation Pro681His in the TLR2 BB loop abolished signal transduction in response to stimulation by yeast and Gram-positive bacteria (Underhill et al., 1999). The Pro681His mutation did not cause noticeable structural changes but disrupted the physical interaction between the TIR domains of TLR2 and MyD88 (Xu et al., 2000). Modeling and docking analyses predict that electrostatic complementarity plays the main role in the interaction between TIR domains (Dunne et al., 2003; Gautam et al., 2006). Interestingly, in a recent crystal structure, the BB loop of the TIR domain of TLR10 was shown to be involved in the homodimeric interaction with a neighboring TIR domain in the crystal (Nyman et al., 2008). However, it is not certain whether the homodimeric structure seen in the crystal corresponds to a physiologically relevant dimer of the TLR10 TIR domains because the TIR domain of TLR10 exists as a monomer in solution.

Experimental structure determination of the TIR multimer is severely hampered by the low affinity between isolated TIR domains in solution (Xu et al., 2000). Therefore, several modeling studies have been performed to predict the structures of TIR multimers. Gautam et al. proposed that the DD loop of TLR2 connecting the fourth β sheet and the fourth α-helix is positioned in close contact with the BB loop of TLR1 (Gautam et al., 2006). In support of the modeling result, a Gly676Leu substitution in the BB loop of the TLR1 TIR domain was shown to interfere with signaling. Other groups have proposed models for the TIR domain of TLR4 with the structure of the TIR domains of TLR2 as template. For example, Dunne et al. suggests in their model that MAL and MyD88 bind to different regions in TLR4, thereby forming a heterotetrameric receptor-adaptor complex (Dunne et al.,

2003). In a more recent report, the authors proposed another model using the crystal structure of the TLR10 TIR domain as template (Núñez Miguel et al., 2007). They hypothesized that the TIR dimers found in crystals of the TLR10 TIR domain mimic physiological dimers and built a model of dimeric TLR4 TIR on the basis of this idea. In their model, the BB loops of the two TLR4 TIRs interact in a symmetric fashion. They also proposed that MAL and TRAM TIRs interact at the dimeric interface between the two TLR4 TIR domains.

Structure of TLRs and Their Ligand Complexes: TLR1-TLR2-Ligand Interaction

Recently, three crystallographic structures of the extracellular domains of TLRs and their ligand complexes have been reported (Jin et al., 2007; Kim et al., 2007b; Liu et al., 2008). Two of them were complexed with agonistic ligands and the remaining one was complexed with a coreceptor and an antagonistic ligand. They provide the first clues as to how these pattern-recognition receptors recognize ''patterns'' in the ligands. They also suggest an activation mechanism that may be common to all TLR family receptors.

TLR2 in association with TLR1 or TLR6 is essential for recognizing bacterial lipoproteins and lipopeptides. Lipoproteins are found in more than 400 proteins from all kinds of bacteria (Babu et al., 2006). They are anchored to the cellular membrane via lipid chains attached to conserved N termini and induce strong proinflammatory responses from macrophages (Chambaud et al., 1999). Most bacteria except mycoplasmas produce lipoproteins with three lipid chains (Hantke and Braun, 1973; Muhlradt et al., 1997; Shibata et al., 2000). Their invariant N-terminal cysteines are covalently attached to a diacylglyceryl chain via a thioether bond, and they are further modified by the third acyl chain's being connected by an amide linkage to the N-terminal cysteine (Figure 1A). The lipopeptides produced by mycoplasmas do not have the amide-linked acyl chain and therefore contain only two acyl chains. Palmitoyl groups are by far the most common lipids in the lipoproteins, and the composition of the lipid chains closely resembles that of membrane phospholipids (Belisle et al., 1994; Braun, 1975; Mizuno, 1979; Zlotnick et al., 1988). Other than the lipid modifications and the shared sequence patterns found in the N termini, bacterial lipoproteins do not have any similarities of sequence or function. The acylglyceryl cysteine appears to be the structure recognized by TLRs because synthetic lipopeptides that have most of their protein residues except the N-terminal cysteine removed still retain full immune stimulatory activity (Berg et al., 1994; Bessler et al., 1985; Seifert et al., 1990; Wiesmuller et al., 1992). Previous studies have shown that TLR2 is the main receptor recognizing lipoproteins and lipopeptides. Triacylated lipoproteins are recognized by the TLR1-TLR2 complex (Shimizu et al., 2007; Takeuchi et al., 2002), but diacylated lipopeptides, lacking the amide-bound lipid chain, can activate both of the TLR1-TLR2 and TLR2-TLR6 complexes (Buwitt-Beckmann et al., 2005; Takeuchi et al., 2001).

We recently determined the crystal structure of the extracellular domain of TLR2 in association with TLR1 and a synthetic triacylated lipopeptide, Pam_3CSK_4 (Jin et al., 2007). Pam_3CSK_4 is a synthetic derivative of triacylated lipoproteins that retains most of immune stimulatory activity of full-length lipoproteins. Without

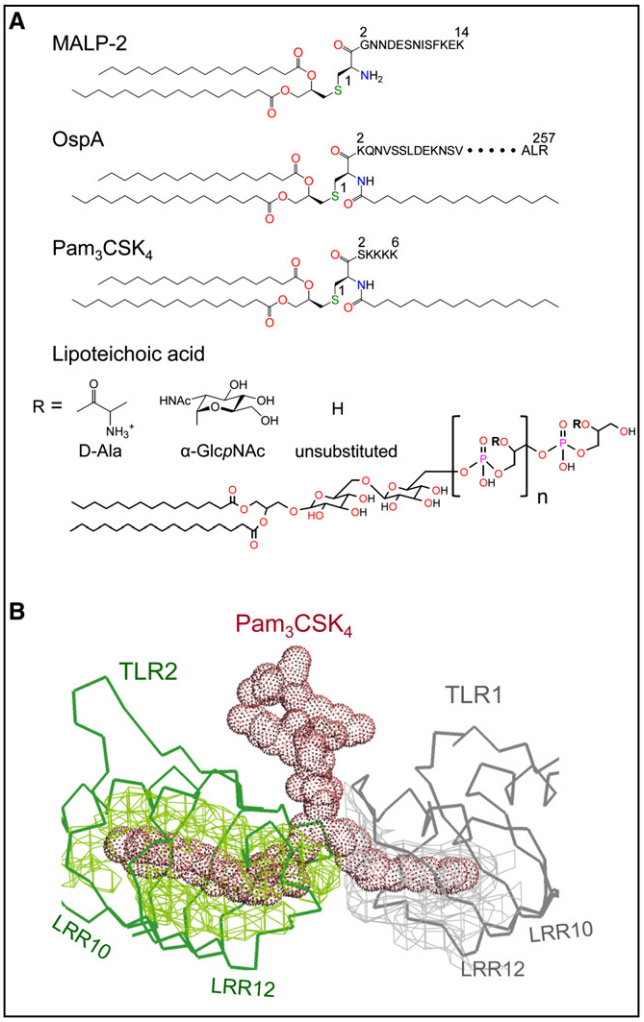

Figure 1. TLR1, TLR2, and Ligand Interaction

(A) Chemical structures of a diacylated lipopeptide, MALP-2 from *M. fermentans*, and triacylated lipoproteins-natural Outer surface protein A (OspA) from *B. burgdorferi*, and synthetic Pam_3CSK_4, and lipoteichoic acid (LTA) from *S. aureus*, are shown. Residue numbers are written above the sequence. The bold Rs in LTA represent substituting groups.

(B) The ligand-binding sites of the TLR1-TLR2 complex are shown in gray and green, respectively. The molecular surface of the residues directly involved in pocket formation is represented in mesh. Pam_3CSK_4 is drawn in red as a space-filling model.

bound ligand, both the TLR1 and TLR2 extracellular domains eluted as monomers in gel-filtration chromatography. However, after addition of the agonistic lipopeptide, the TLRs formed a stable heterodimer in solution as well as in the crystals. In the crystal structure, the lipid chains of the ligand bridge the TLRs; two of the three lipid chains are inserted into an internal pocket in TLR2, and the remaining amide-bound lipid chain is inserted into the narrower channel in TLR1 (Figure 1B). The ligand-binding pockets of TLR1 and TLR2 are located at the boundary of the central and C-terminal domain in the convex region. The flexible loops at the domain boundaries are separated, forming crevices that are connected to large internal pockets. The pockets of TLR1 and TLR2 are bridged by the bound ligand and therefore form a long continuous hydrophobic pocket. Structural flexibility

at the domain boundary ought to be crucial for ligand recognition because LRRs are very rigid structural frameworks, and structural transitions of this magnitude have not been reported. The heterodimeric complex of TLR1 and TLR2 is further stabilized by extensive protein-protein interactions near the ligand-binding pocket. Interestingly, the Pro315Leu substitution in a polymorphic variant of TLR1 is located in the protein-protein interaction interface (Omueti et al., 2007). This change blocks TLR1 signaling in response to pathogenic lipoproteins, confirming that the heterodimeric interaction shown in the crystal is essential for lipoprotein signaling.

The crystal structure of the TLR1-TLR2-lipopeptide complex provides a reasonable template for structure prediction of the TLR1-TLR2-lipoteichoic acid (LTA) complex. LTAs are potent inducers of innate immune responses via the TLR2-dependent pathway (Deininger et al., 2003; Han et al., 2003; Schroder et al., 2003). The LTAs of *S. aureus* are composed of two lipid chains attached to glycerophosphate units repeated typically from 4 to 25 times (Figure 1A and Morath et al., 2001). In solution, LTA can induce the formation of a stable heterodimeric complex between the purified extracellular domains of TLR1 and TLR2 (unpublished data). Because the two thioester-linked lipid chains of the synthetic lipopeptides, Pam_2CSK_4 and Pam_3CSK_4, are inserted into the TLR2 pocket in practically the same conformation, it is highly probable that the two lipid chains of LTAs are inserted into the binding pocket in TLR2 in a similar fashion. Because LTA has only two lipid chains, the hydrophilic sugars and repeating units must also interact with TLR1 in order to stabilize the TLR1-TLR2 complex in the absence of stabilization of the TLR dimer by a third lipid chain.

TLR3-Ligand Interaction

TLR3 has been shown to recognize dsRNA produced during viral replication (Alexopoulou et al., 2001). The crystal structure of TLR3 bound to a dsRNA ligand has been reported (Figure 2 and Liu et al., 2008). The dsRNA interacts with both N-terminal and C-terminal sites on the lateral side of the convex surface of TLR3. The N-terminal interaction site is composed of LRRNT and LRR1-3 modules, and the C-terminal site is composed of LRR19-21 modules. The positively charged residues of the termini of TLR3 make the major contributions to the interaction with the sugar-phosphate backbones of the dsRNA ligand. Only a minor TLR3-TLR3 interaction is located near the LRRCT, demonstrating that the ligand-protein interaction is the main driving force for TLR3 dimerization. The ligand interaction sites of the TLR3 homodimer are separated by ~120 Å, which accounts for why a minimum of 40–50 base pairs is required for stable binding of dsRNA to TLR3 (Leonard et al., 2008). However, there have been reports that dsRNA of substantially less than 40 bp can initiate TLR3 signaling (Kariko et al., 2004; Kleinman et al., 2008). These reports raise the possibility that the N-terminal interaction site may not be essential for efficient TLR3 signal initiation in some experimental conditions. The interactions between TLR3 and dsRNA are very different from those between TLR1-TLR2 and lipopeptides. Hydrophobic interactions make the main contribution to ligand binding by the TLR1-TLR2 complex (Jin et al., 2007). In contrast, the dsRNA interaction sites in TLR3 are located on the surface of the protein, and ionic and hydrogen bonds with the sugar-phosphate backbones of the ligand play the major role (Figure 2).

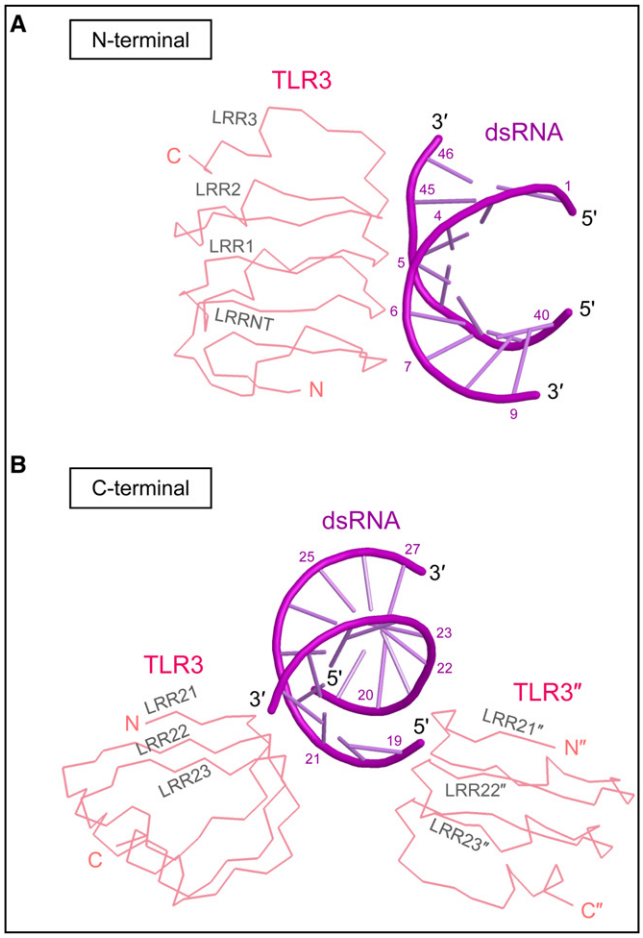

Figure 2. TLR3-Ligand Interaction
Structures of the N-terminal (A) and C-terminal binding sites (B) of TLR3 are shown. Cα traces of the LRR modules directly interacting with RNA are drawn in pink. Residues of the RNA are numbered. The second TLR3 molecule in the TLR3 homodimer is marked with a double apostrophe.

TLR4-Ligand Interaction

TLR4 in company with its coreceptor MD-2 is specific for LPS recognition (Shimazu et al., 1999; Viriyakosol et al., 2001). Lipopolysaccharides (LPSs) are outer-membrane glycolipids of Gram-negative bacteria and are well-known inducers of the innate immune response (Erridge et al., 2002). They are composed of a hydrophobic lipid A component and a hydrophilic polysaccharide component. The lipid A portion is composed of phosphorylated diglucosamine and four to seven acyl chains (Figure 3A). Chemically synthesized lipid A has the same biological activity as full-sized LPS, demonstrating that it possesses the same configuration (pattern) as LPS and is the main inducer of biological responses to LPS (Tanamoto et al., 1984). Both the 1 and 4′ positions of the glucosamine backbone of lipid A are frequently phosphorylated. Phosphorylation is important for the biological activity of LPS because diphosphorylated lipid A is more than 1000-fold more active than monophosphorylated lipid A (MPL) (Rietschel et al., 1987). The phosphate group can

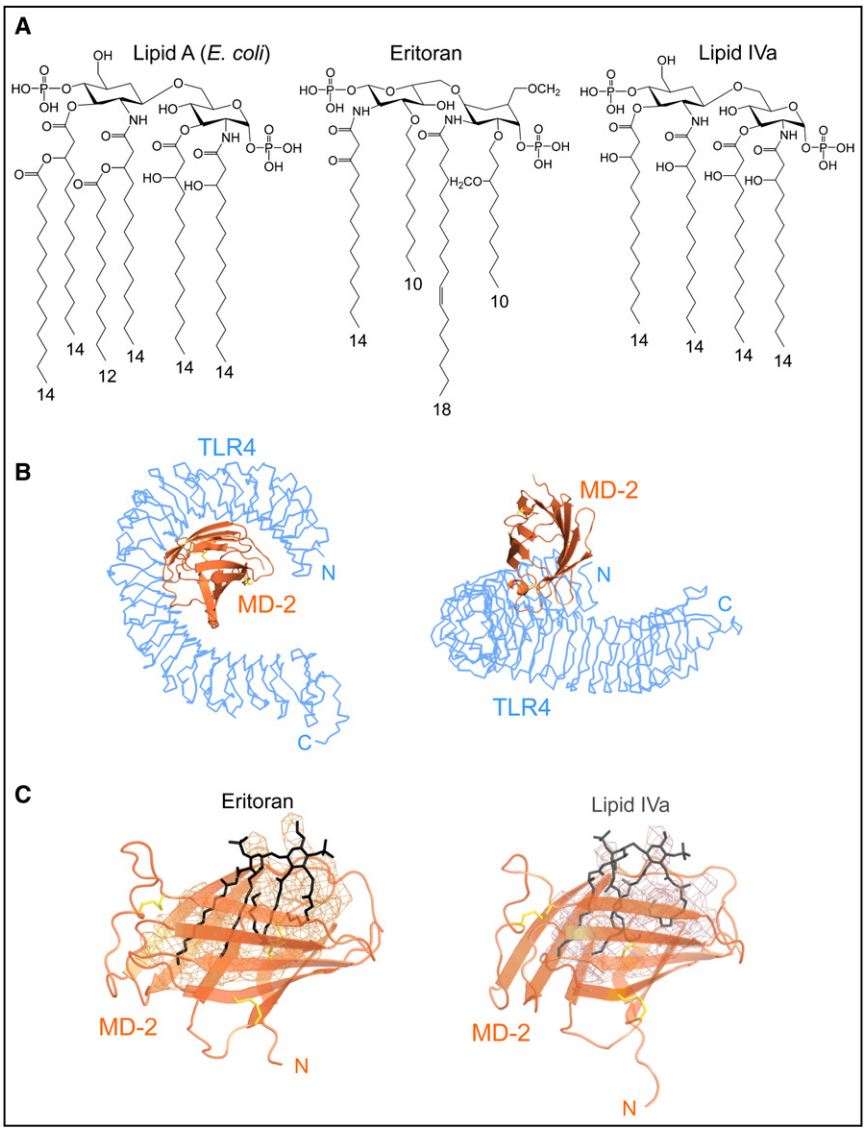

Figure 3. TLR4-MD-2-Ligand Interaction
(A) Chemical structures of lipid A of *E. coli*, the synthetic antagonist, Eritoran, and lipid IVa. Numbers of carbons in the lipid chains are written below.
(B) Overall structure of the TLR4-MD-2 complex. The left shows the top view, and the right shows the side view.
(C) Structures of MD-2 complexed with the antagonistic ligands, Eritoran (left) and lipid IVa (right). MD-2, Eritoran, and lipid IVa are colored orange, black, and gray, respectively. Disulfide bridges are represented as yellow lines. The molecular surfaces of residues involved in formation of the hydrophobic pocket of MD-2 are drawn in mesh.

be further modified by other chemical groups. Four to seven mostly saturated lipid chains are linked to the sugar backbone of lipid A through either ester or amide linkages. The lipid A moiety is connected to the core oligosaccharide part of LPS. The core sugar chain contains unusual Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) and heptose saccharides not normally found in humans and is connected to the highly variable O-specific chain composed of repeating oligosaccharide units (Erridge et al., 2002). The O-specific sugar chains are highly variable in structure compared to the core sugar. The number of repeating units can be anywhere from 1–50 in the same bacterium. The sugar composition, sequence, chemical linkage, substitution, and ring form of the repeating units can vary dramatically, thereby making LPS of almost unlimited diversity. Experiments using synthetic variants of lipid A demonstrate that *E.coli* lipid A containing six lipid chains and two phosphate groups has a nearly optimal structure for endotoxicity (Erridge et al., 2002; Rietschel et al., 1991). Lipid A with five or seven acyl chains is 100-fold less active, and structural changes in the sugar or lipid chains invariably lead to substantially reduced activity.

Several synthetic derivatives of lipid A have been developed as candidate drugs against sepsis and septic shock syndrome. Eritoran (or E5564) is a synthetic molecule derived from the lipid A component of the nonpathogenic LPS of *Rhodobacter sphaeroides* (Figure 3A and Mullarkey et al., 2003). It is a strong antagonist of TLR4-MD-2 and is currently in a phase III clinical trial against severe sepsis. The diglucosamine backbone and the phosphate positions of LPS are conserved in Eritoran. However, the number and chemical structure of the lipid chains have noticeable differences that make it an antagonist. For example, Eritoran has only four lipid chains and one of them contains a double bond in *cis* configuration. Lipid IVa, also called compound 406, is an intermediate in LPS biosynthesis (Figure 3A and Kusumoto et al., 2003). Interestingly, it is an antagonist of human TLR4-MD-2 but a mild agonist of the mouse form (Means et al., 2000; Tanamoto and Azumi, 2000). Both Eritoran and lipid IVa bound to MD-2 have been crystallized, providing the first glimpse of the structural flexibility of the ligands recognizing the TLR4-MD-2 complex (Kim et al., 2007b; Ohto et al., 2007).

MD-2 adopts a β cup fold with two antiparallel β sheets that are separated on one side, and with internal hydrophobic residues exposed for ligand binding (Kim et al., 2007b). The internal pocket of MD-2 is completely lined with hydrophobic residues, but the open region has positively charged residues. Therefore, the overall shape and electrostatic characteristics of MD-2 seem to be suitable for binding negatively charged amphipathic ligands such as LPS. Interaction between TLR4 and MD-2 is mediated mainly by ionic and hydrogen bonds in two oppositely charged patches. The negatively charged A patch of TLR4 interacts with basic residues in MD-2. On the other hand, the positively charged B patch that is located in a less conserved area of TLR4 interacts with negatively charged residues in MD-2 (Figure 3B).

A hybrid of TLR4-VLR complexed with Eritoran has been crystallized (Kim et al., 2007b). As with TLR1 and TLR2, the "hybrid LRR technique" made a crucial contribution to crystallization of the TLR4 complexes (discussed above). In the crystal structure, Eritoran binds to the hydrophobic pocket in MD-2, with its four acyl chains occupying almost all the available space in the pocket. There is no direct interaction between Eritoran and TLR4. Because Eritoran mimics the structure of LPS and the two compete for MD-2, these data suggest that MD-2 is the only LPS-binding component of the TLR4-MD-2 complex (Visintin et al., 2005). Another complex of human MD-2, this time with bound lipid IVa, has been recently reported by Ohto et al. (2007). Their structure contains no TLR4 subunit, and comparison of their MD-2 structure with our TLR4-bound MD-2 structure indicates that TLR4 binding causes only minor changes in the loops of MD-2. The comparison also reveals that the mode of binding of lipid IVa is remarkably similar to that of Eritoran despite the structural differences between their lipid chains (Figure 3C). The four lipid chains and phosphate groups occupy identical sites in MD-2 and the glucosamine backbone is only shifted by two angstroms.

As noted, both Eritoran and lipid IVa have tetra-acylated lipid chains, whereas agonistic LPS has more or longer lipid chains (Figure 3A). Because the lipid chains of Eritoran and lipid IVa occupy almost all the available space in the MD-2 pocket, LPS must bind to MD-2 in a different fashion from Eritoran and lipid IVa. Several structural adjustments of MD-2 can be envisaged. In the first model, LPS may be able to enlarge the MD-2 pocket. Because one edge of the β sandwich of MD-2 is pulled away, and no intersheet disulfide bridge is present, it appears to be possible to enlarge the pocket without excessive structural changes. In the second model, structural changes of MD-2 are minimized, whereas four of the chains of LPS are inserted into the MD-2 pocket with the remaining two chains protruding from the pocket and able to interact with a second TLR4 molecule to promote dimerization. Both of these modes of binding LPS should provoke some structural changes in MD-2, and these may play an important role in dimerization of the TLR4-MD-2 complex. Because the phosphate groups of lipid A are known to be crucial for the high potency of *E.coli* LPS, they may also have a direct role in dimer formation.

Several lines of evidence support the hypothesis that ligand binding induces aggregation of the TLR4-MD-2 complex and that this initiates signaling. Miyake and colleagues discovered that residues Phe126 and Gly129 of MD-2 are crucial for aggregation of the TLR4-MD-2 complex (Kobayashi et al., 2006). In addition, we have conducted mutation and deletion experiments to help build a reliable model of TLR4 dimerization and activation (Kim et al., 2007b). We showed that binding of agonistic LPS to purified TLR4-MD-2 complexes induces dimerization of the complexes. Truncation of the C terminus of TLR4 prevented dimerization. We also demonstrated using purified proteins that Phe126 and His155, located on the same side of MD-2, are essential for dimerization. These results can be explained by a model in which LPS induces dimerization of TLR4-MD-2 by altering the conformation of the Phe126 surface of MD-2 and exposing otherwise hidden interaction sites for binding to the C-terminal domain of the second TLR4 molecule. Interestingly, in this model, the two TLR4 molecules form an "m"-shaped complex similar to that of the TLR1-TLR2 heterodimer and TLR3 homodimer (Kim et al., 2007b). Recent biochemical data with horse TLR4 and MD-2 supports our model (Walsh et al., 2008).

Many previous reports demonstrate that, in addition to MD-2, TLR4 needs two accessory proteins, LBP and CD14, to recognize LPS at physiological concentrations (Fujihara et al., 2003; Miyake, 2003; Schumann et al., 1990). LBP is required for extracting LPS from the outer membrane of Gram-negative bacteria or vesicles liberated by them, and for transferring it to CD14 in a monomeric form. Because CD14 has no intracellular signaling domain, LPS must ultimately be delivered to the TLR4-MD-2 complex to initiate immune responses. LBP belongs to the lipid transfer or lipopolysaccharide binding protein (LT-LBP) family (Mulero et al., 2002). Other current members of the family are bactericidal and permeability-increasing protein (BPI), cholesterol ester transfer protein (CETP), phospholipid transfer protein (PLTP), and a few poorly characterized proteins. LBP and BPI are especially similar in sequence (45% sequence identity) and function. They both bind to LPS and control its biological effects. BPI is a plasma protein that neutralizes LPS. It is a boomerang-shaped molecule composed of a central β sheet and barrel-shaped domains at its termini (Beamer et al., 1997; Beamer et al., 1999). Each barrel contains a long α helix surrounded by a five-stranded antiparallel β sheet and a short α helix. It also contains a hydrophobic pocket that binds a phospholipid molecule, mainly by interacting with its acyl chains. The head group of the phospholipid lies at the entrance of the pocket and is exposed to solvent. Although the structure of LBP bound to LPS has not been reported, the structure of BPI allowed homology modeling of LBP (Beamer et al., 1998); the two proteins are highly homologous and can be aligned with only two single residue gaps. Therefore, LBP is predicted to have the same boomerang-like two-barrel structure with two hydrophobic phospholipid pockets. Mutational and domain-swapping experiments have suggested that the N-terminal domain of LBP is responsible for interaction with LPS and the C-terminal domain mediates the transfer of LPS to CD14 (Abrahamson et al., 1997; Iovine et al., 2002; Theofan et al., 1994). Although the two barrels have an almost identical structural architecture, their electrostatic characteristics appear to differ markedly. Whereas the surface of the N-terminal barrel of LBP is strongly positively charged, the C-terminal barrel does not seem to have any particular overall charge. The expected charge distribution may play a role in the transfer of LPS to CD14 because BPI with its uniformly positive charge can bind LPS but cannot transfer it to CD14.

CD14, which is found in soluble form or as a GPI-anchored protein on the cell membrane, is another essential accessory protein for LPS recognition (Ulevitch and Tobias, 1995). Our crystallographic analysis shows that it is an LRR family protein with 11 LRR modules (Kim et al., 2005). Unlike other LRR family members, CD14 does not contain an LRRCT module protecting its hydrophobic core. Instead, the C terminus of the LRR modules of CD14 interacts with the C terminus of another CD14 forming a dimer. Therefore, their hydrophobic cores are covered by the opposing monomers in the CD14 dimer. Interestingly, because dimeric CD14 contains 22 LRR modules in total, its overall shape and size appear to be comparable to those of TLRs. The LPS interaction site of CD14 is located at the boundary of the

LRRNT and the first LRR module, providing additional support for the hypothesis that the domain boundaries are crucial for pocket formation in LRR family proteins. As in the TLR1-TLR2 complex, the LPS-binding pocket of CD14 is located in the convex region formed by opening a crevice composed of flexible loops and helices. The pocket is covered with purely hydrophobic residues except for the rim of the pocket, which contains hydrophilic and flexible residues. Several laboratories have reported interesting mutational analyses showing that a distinct region near the LPS-binding pocket of CD14 is crucial for LPS signaling but not for LPS binding (Juan et al., 1995; Kim et al., 2005; Muroi et al., 2002; Stelter et al., 1999). The data suggest that this LPS signaling area may make direct and transient contact with TLR4-MD-2 to achieve efficient LPS transfer. Additional structural and biochemical studies are required to identify the CD14 contact area in TLR4-MD-2 and ultimately to build a reliable model of the mechanism of LPS transfer between CD14 and the TLR4-MD-2 complex.

Ligand-Induced Activation of TLR Family Proteins

TLR family receptors interact with an unusually diverse variety of ligands, ranging from hydrophilic nucleic acids to hydrophobic LPS or lipoproteins (Akira and Hemmi, 2003; Gay and Gangloff, 2007). They also vary greatly in size from small synthetic molecules to macromolecules. Regardless of these differences, the structures of the TLR1-TLR2-lipopeptide, TLR3-dsRNA complexes and the model of the TLR4-MD-2-LPS complex all have an "m"-shaped dimeric architecture, suggesting that all the other TLRs undergo similar dimerization upon binding agonists (Figure 4 and Jin et al., 2007; Kim et al., 2007b; Liu et al., 2008). This observation immediately suggests the hypothesis that dimerization of the extracellular domains brings about dimerization of the intracellular TIR domains and so initiates signaling. It is known that, in a membrane environment, full-length TLRs exist as homomultimers or heteromultimers even without their ligands (Akira and Takeda, 2004; Latz et al., 2007; Ozinsky et al., 2000; Triantafilou et al., 2006). However, these preligand complexes cannot induce intracellular signaling, probably because their TIR domains have an inappropriate orientation or distance for signaling. Dimerization of the extracellular domains may lead to proper orientation of the TIRs, recruitment of adaptor proteins, and initiation of intracellular signaling. Sequence alignment shows that only a few residues are able to act as flexible linkers between the extracellular and transmembrane domains and between the transmembrane region and the intracellular domain (Bell et al., 2003). Therefore, close apposition of the C termini of the ectodomains is very likely to encourage juxtaposition of the intracellular TIR domains, as shown in the model.

Conclusions

TLR family receptors have a common structural architecture. The extracellular domains of TLRs belong to the well-known LRR family with multiple LRR modules. Sequence and structure analyses demonstrate that TLR1, TLR2, TLR4, TLR6, and TLR10 belong to the three-domain subfamily; they bind to and are activated by hydrophobic ligands such as lipoproteins, LTA, LPS, etc. Conversely, TLR3, TLR5, TLR7, TLR8, and TLR9 belong to the single-domain subfamily. These TLRs interact with hydrophilic proteins or nucleic acids. The structures of the TLR1-TLR2 heterodimer and TLR3 homodimer induced by binding of agonistic ligands have revealed a common "m"-shaped architecture. In these dimeric arrangements, the C termini of the extracellular domains of TLRs converge in the center, and such a convergence should bring the two intracellular TIR domains close together and so promote their dimerization.

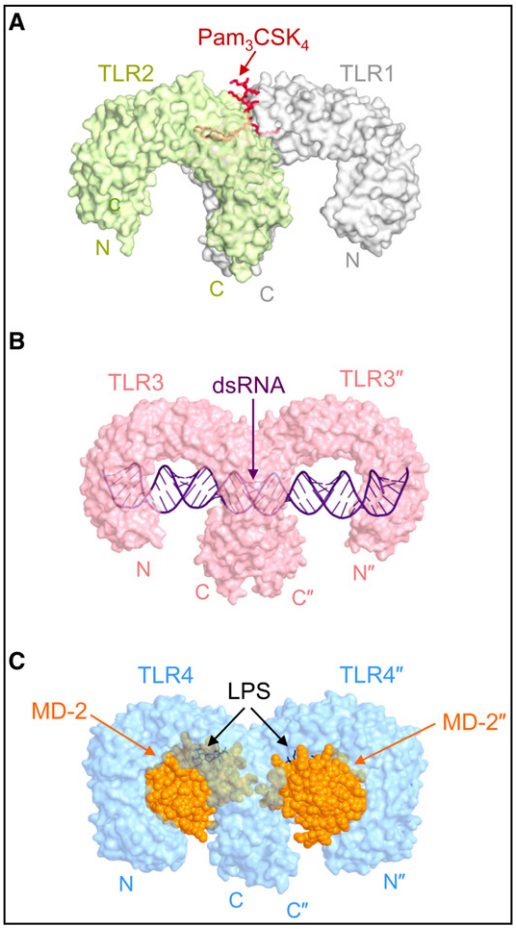

Figure 4. Ligand-Induced TLR Dimers
The "m"-shaped TLR dimers induced by binding of agonistic ligands. The crystal structures of (A) TLR1-TLR2-Pam_3CSK_4 and (B) TLR3- dsRNA are colored gray, green, red, pink, and purple, respectively. (C) shows a model of the TLR4-MD-2-Eritoran complex. TLR4, MD-2, and Eritoran are colored light blue, orange, and black, respectively. Double apostrophes are used to mark the second TLR4 or MD-2 in the receptor complex.

Additional structural studies of the TLR system are needed to address several key issues in innate immune research: (1) TLRs recognize "pattern" in structurally diverse ligands. Previous studies have shown that even minor modifications to ligands may lead to unpredictable changes in the immune responses that they evoke. We need to define precisely the meaning of "pattern" in TLR ligands, not only to better understand the innate immune system but also to be able to design improved antagonists and agonists for clinical use. For this purpose, structural studies of TLRs complexed with diverse ligands are essential. (2) Common structural principles of TLR dimerization and activation appear to emerge from structural studies of the TLR2 and TLR3 complexes. It will be important to see whether other

TLRs complexed with different ligands dimerize in a similar fashion. (3) There are several models of TIR multimers. Therefore, experimental structure analysis of multimers composed of the TIR domains of TLRs and adaptor proteins is crucial for improving our understanding of the TLR signal pathway.

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