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Recognition of Lipopolysaccharides by TLR4 and its Accessory Proteins

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Lipopolysaccharide (LPS) is a bacterial glycolipid that is the major component of the outer membrane of gram-negative bacteria. It serves as an early warning signal of infection by initiating a potent immune response. Lipid A, the lipid part of the LPS, is responsible for the majority of the immunological activity of LPS and binds to the cell surface receptor, TLR4-MD-2 heterodimer. LPS binds to the hydrophobic pocket in MD-2 and induces the dimerization of TLR4. Efficient activation the TLR4 signal *in vivo* requires accessory proteins, LBP and CD14. LBP is a serum glycoprotein that can extract LPS from bacterial membranes or vesicles released from it. CD14 accepts a monomeric form of LPS from LBP and delivers it to the TLR4-MD-2 complex. The structures of these LPS recognition proteins in a complex with LPS and related molecules provide us insight into how our immune system recognizes bacterial infections and initiates efficient defense mechanisms. In this review, we will summarize recent structural studies of these LPS receptors and accessory proteins.

INTRODUCTION

Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria (Raetz, 1990; Raetz and Whitfield, 2002). It is a potent activator of the human innate immune response by activating toll-like receptor 4 pathways. Minute amount of LPS released to the blood stream serves as an early signal of infection and alarms the immune system to prepare against further infection. LPS is recognized by a cascade of four proteins, LBP, CD14, MD-2 and TLR4 (Figure 1). LBP extracts LPS from bacterial membranes and transfers it to CD14. LPS is monomerized by CD14 and delivered to the TLR4-MD-2 complex. LPS binding induces the dimerization of the TLR4 intracellular domain and initiates intracellular signaling (Hailman et al., 1994; Schumann et al., 1990; Tsukamoto et al., 2010).

TLR4 and associated accessory proteins are essential for LPS recognition. The C3H/HeJ strain of mice, which tolerates lethal doses of LPS and has altered inflammatory responses, was found to have a missense mutation in the conserved region of TLR4 (Poltorak et al., 1998; Qureshi et al., 1999). CD14-deficient mice are highly resistant to septic shock initiated by an injection of either LPS or live bacteria (Haziot et al., 1996). LBP-deficient mice were found to display reduced responsiveness to small amounts of LPS and increased susceptibility to peritoneal infection by viable *Salmonella typhimurium* (Fierer et al., 2002).

The LPS recognition system should be tightly controlled because over-activation can lead to a fatal septic syndrome. Small-molecule or protein therapeutics against TLR4 signaling system show beneficial effects against severe sepsis and are being developed as anti-sepsis drugs. The immunological activity of LPS has been studied for more than a century (Galanos et al., 1985). However, high-resolution structures of TLR4 and its accessory proteins have been available only for the last ten years. These structural studies have revealed how these pattern-

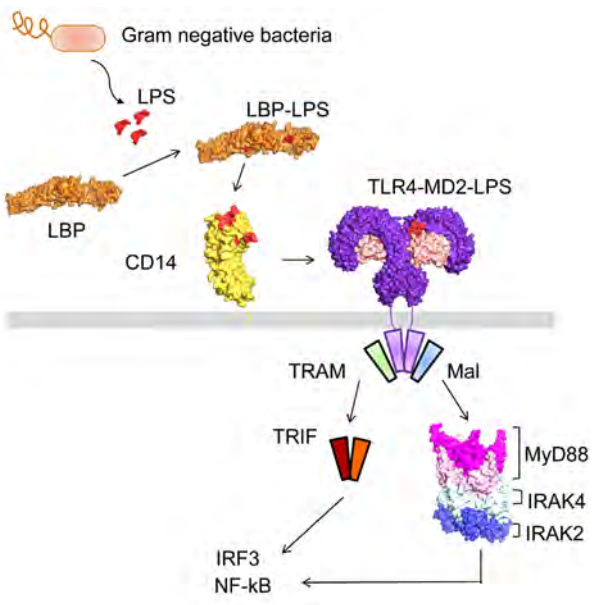

FIGURE 1 | LPS signaling pathway. LPS is recognized by a cascade of proteins, LBP, CD14 and TLR4-MD-2.

recognition receptors bind to LPS and initiate a proper immune response against bacterial infection.

CHEMICAL STRUCTURE OF LIPOPOLYSACCHARIDE

LPS is a phosphorylated bacterial glycolipid with a long and branched carbohydrate chain attached to five to seven lipid chains (Figure 2) (Raetz, 1990) (Raetz and Whitfield, 2002). The lipid part of the LPS is referred to as lipid A. The backbone of the lipid A is composed of two glucosamine residues connected by the β1-6 linkage. Typically, five to seven lipid chains are linked to the glucosamine backbone. *E.coli* LPS normally contains six lipid chains. Four of them are directly connected to the glucosamine backbone by ester or amide bonds and the remaining two are indirectly linked to the glucosamine backbone by ester bonds to the hydroxyl group of the main lipid chains. Lipid A is negatively charged because two phosphate groups are bonded to the glucosamine backbone at the 1 and 4' carbon positions, respectively (Erridge et al., 2002; Rietschel et al., 1994). The carbohydrate chain of LPS is attached to the lipid A by an acid labile bond and can be released from lipid A by a mild acid treatment. This carbohydrate region can be divided into two sub-regions. The core region contains unusual carbohydrate residues such as Hep and Kdo that are not produced by host cells. The core region is also negatively charged due to the frequent phosphorylation of the carbohydrate residues. The O-antigen region is composed of many copies of repeating units containing a variety of sugar residues, including galactoses, glucoses, and rhamnoses. The chemical structure and copy number of the repeating units can vary in different bacterial cells.

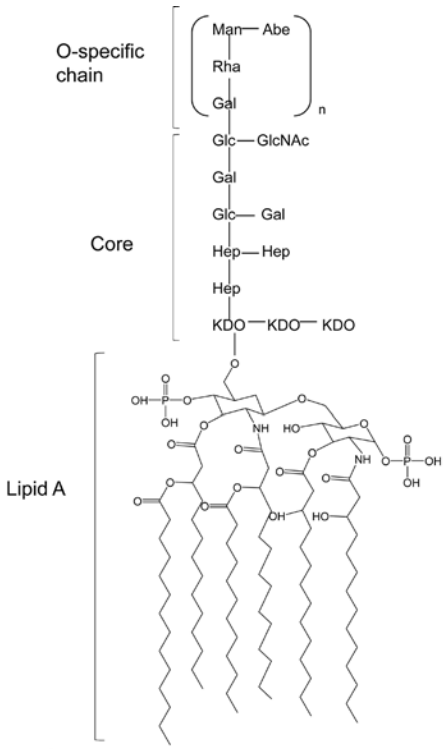

FIGURE 2 | Chemical structure of a typical *E.coli* LPS. LPS is composed of lipid A, core oligosaccharide, and O-specific chain. Abe: abequose, Gal:galactose, GluNHAc: N-acetylgalatosamine, Glu: glucose, Hep: L-glycero-D-mannoheptose, Kdo: 3-deoxy-D-manno-oct-2-ulosonic acid, Man: mannose, Rha: rhamnose

LPS is not a single type of molecule but a collection of molecules with a common structural pattern. Different bacterial species produce LPS with different structures. Sometimes even the same bacteria under a different growth condition can produce different LPS molecules. Although lipid A is a relatively well conserved part of LPS, bacteria produce a large variety of lipid A. Both the number and structure of the lipid chains can be varied. The phosphate groups are frequently modified by an additional phosphate, phosphoethanolamine, or 4-amino-4-deoxy-L-arabinose groups. Although most of the lipid chains in lipid A are linear and fully saturated, lipid chains from some bacteria can have double bonds or carbons branched out. The carbohydrate part of LPS also shows high structural diversity. The number of Kdo or Hep residues can change, and phosphate and other groups can become attached at several places in the core region. The O-specific chain is the most variable part of LPS. Many of these structural differences in the lipid have a modulatory effect on the inflammatory activity of LPS (Erridge et al., 2002).

A mild acid treatment releases lipid A from a water-soluble carbohydrate chain. Early immunological research showed that this lipid A region is responsible for the majority of the inflammatory activity of LPS. The structure and activity relationship of lipid A has been studied extensively by chemical modification, and several factors governing inflammatory activity have been identified. These studies show that lipid A with six lipid chains and two phosphate groups appears to be optimal for immunological activity (Rietschel et al., 1994; Rietschel et al., 1993). The deletion or addition of lipid chains or phosphate groups in the lipid A region can reduce the activity by hundreds of times. Monophosphoryl lipid A (MPLA) is produced by a mild acid treatment of *Salmonella* LPS (Qureshi et al., 1982). MPLA is currently approved for use as a vaccine adjuvant, as it has only mild toxicity while maintaining an intact stimulatory effect for an adaptive immune response (Mata-Haro et al., 2007).

STRUCTURE OF THE LIPOPOLYSACCHARIDE BINDING PROTEIN

LPS is an amphipathic molecule and exists as aggregated micelles or vesicles when released from a bacterial membrane. Multimeric clusters of LPS are extracted from bacterial membranes or from the vesicles released from them by a serum glycoprotein known as the lipopolysaccharide binding protein (LBP) (Tobias et al., 1986). LBP is an acute response protein whose expression is transiently increased by hundreds of times

shortly after infection. LBP is a boomerang-shaped extended protein that is composed of three domains, the N-terminal, central, and C-terminal domains (Figure 3). Both the N and C-domains share a similar fold with a barrel-shaped β sheet supported by a long α helical spine. Mutagenesis experiments suggest that the primary LPS binding site of LBP is located near the N-terminal tip of the boomerang-like structure (Han et al., 1994; Lamping et al., 1996). The tip is composed of two loops connecting antiparallel β hairpins. Their sequences contain multiple lysines and arginines that can interact with negatively charged phosphate groups in the LPS aggregates. In LBP, both the N- and C-terminal barrels have phospholipid-binding pockets. The N-terminal pocket contains a well-fixed phospholipid molecule. The long hydrophobic channel in the pocket surrounds the lipid chain and a number of positively charged amino acid residues near the opening of the pocket interact with the negatively charged phosphate group in the bound phospholipid. The C-terminal pocket is more open and loosely bound to a lipid molecule. The biological role of the phospholipid pockets remains unclear, although it has been proposed that they may interact with the lipid chains of the LPS.

LBP belongs to a small family of lipid-binding proteins, including CETP, PLTP, BPI and several uncharacterized proteins. Among these, BPI is the protein most closely related to LBP. BPI and LBP share ~50% sequence similarity, and the sequences of the two proteins can be aligned with only two single amino acid gaps (Beamer et al., 1998). Both BPI and LBP can bind to LPS, although only LBP can transfer bound LPS to CD14 (Ooi et al., 1987). As expected from this high sequence homology, individual N- and C-terminal domains show a clear structural homology with that of BPI (Ekert, 2013). However, the relative orientation of the two domains shows unexpectedly large deviation (Figure 3C). When the N-terminal domain of LBP is superimposed on that of BPI, the C-terminal domain of LBP is bent by ~20 degrees and rotated by ~30 degrees. This large rearrangement of the C-terminal domain appears to be caused by relatively small changes in the sequences connecting the N- and C-terminal domains. Therefore, the A' helix connecting the N-terminal domain and the C-terminal domain becomes a loop known as the A' loop, and the B' helix that connects the C-terminal domain to the central domain is reoriented by ~10 degrees in LBP. A single nucleotide polymorphism (SNP) is found in a hydrophobic core near the B' helix (Ekert, 2013). LBP with this SNP leads to an altered structure and defects in the LPS response. Populations with this SNP show increased mortality rates during sepsis and pneumonia. This finding demonstrates that the structural integrity of the C-terminal domain and the relative orientation of the N- and C-terminal domains are critical for normal LBP functioning.

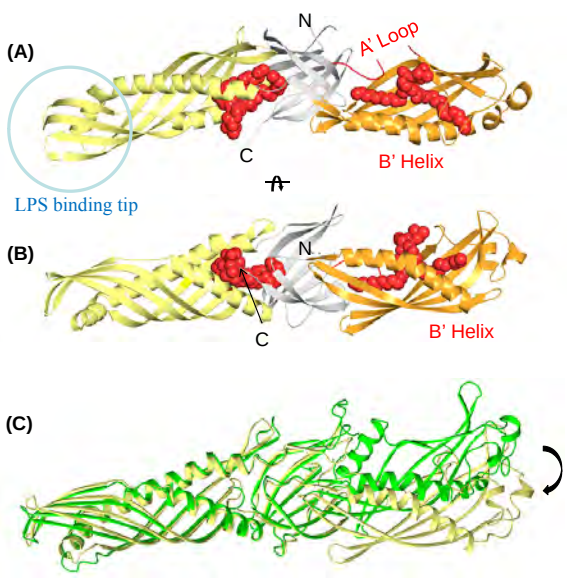

FIGURE 3 | Structure of LBP. (A) Schematic diagram of the N-terminal (yellow), central (grey) and C-terminal (orange) domains of LBP. Bound phospholipid molecules are shown by red space-filling models. (B) The view is rotated by 270°. (C) Structural comparison of LBP and BPI. Ribbon diagrams of LBP and BPI are colored in yellow and green, respectively. The N- and central domains of the two proteins are superimposed. The A' loop and B' helix that connect the N- and C-terminal ends, respectively, of the C-terminal domain to the central domain are labeled.

STRUCTURE OF CD14

CD14 is a glycoprotein that can be attached to the cell membrane using a GPI link (Ulevitch and Tobias, 1995). LPS extracted by LBP is transferred to an N-terminal hydrophobic pocket in CD14 (Kim et al., 2005). CD14 belongs to leucine-rich repeat (LRR) family (Figure 4). LRR-family proteins consist of many copies of LRR modules. An individual LRR module is 20~30 amino acids long and contains a highly conserved LxxLxLxxN motif (Kajava, 1998; Kobe and Kajava, 2001). This conserved part of the module forms a β strand. The β strands from the neighboring LRR modules stack in parallel, forming a large β sheet, and the overall structures of LRR-family proteins share a characteristic horseshoe shape (Gay and Gangloff, 2007). The leucines in the conserved motif point inside, forming a hydrophobic core. The variable X residues in the motif are exposed to the solvent area. Some of these variable residues play a functional role by interacting with ligands. The LRR modules are covered by two specialized modules, LRRNT and LRRCT, in the N- and C-terminal ends, respectively. The LRRNT and LRRCT modules do not have LRR motifs in their sequences. Instead, they often have multiple disulphide bridges that stabilize the structures of these special modules (Kajava, 1998; Kobe and Kajava, 2001; Matsushima et al., 2007). LRR-family proteins play diverse physiological roles in the cell by interacting mostly with protein ligands. The most frequent site for protein interaction is the concave side of the protein. CD14 is an exception and its ligand-binding site is located in the convex part of the structure.

CD14 has a large hydrophobic pocket between the LRRNT and the first LRR module (Figure 4A) (Kim et al., 2005). The convex surface between these two modules is split open, and

some of the internal hydrophobic residues are exposed to the solvent area. Previous mutagenesis and other biochemical experiments strongly suggest that this pocket is responsible for LPS binding (Cunningham et al., 2000; Dziarski et al., 2000; Juan et al., 1995a; Shapiro et al., 1997; Stelter et al., 1997; Stelter et al., 1998; Viriyakosol and Kirkland, 1995). The size of the pocket is large enough to bind at least part of the lipid chains of LPS. Interestingly, the opening area of the pocket does not have positively charged residues, suggesting that the CD14 pocket is not fully optimized for the binding of negatively charged LPS. Instead, it appears that CD14 is a fairly nonspecific lipid binder. This structural observation is consistent with biochemical data showing that CD14 is involved in recognizing not only LPS but also many lipid-containing molecules, such as lipoproteins and a variety of bacterial glycolipids. Some of them do not have negatively charged chemical groups. A mutagenesis study also identified three regions that are important for an LPS transfer to TLR4-MD2 (Juan et al., 1995b; Muroi et al., 2002; Stelter et al., 1999). These sites are clustered in a small area near the LPS binding pocket (Figure 4B). This surface may be responsible for transient interaction with the TLR4-MD-2 complex for efficient LPS transfer.

STRUCTURE OF THE TLR4-MD-2 COMPLEX

LPS monomerized by CD14 is transferred to TLR4 for signal initiation. TLR4 is tightly associated with MD-2, forming a stable heterodimeric complex (Figure 5A) (Kim et al., 2007b; Nagai et al., 2002; Park et al., 2009; Schromm et al., 2001; Shimazu et al., 1999; Viriyakosol et al., 2001). LPS binding is mediated mostly by the MD-2 part of the receptor complex. The lipids of LPS undergo hydrophobic interaction with a large internal pocket in MD-2 and the phosphate groups of lipid A undergo multiple charge interactions with lysines or arginines in MD-2 and TLR4. TLR4 is the signaling unit of the receptor complex because it has an intracellular signaling domain. LPS binding induces the dimerization of the TLR4-MD-2 heterodimer. The overall shape of the receptor heterotetramer resembles the letter “m,” where the N-termini of the two TLR4s stretch outward and the two C-termini juxtapose in the middle. The MD-2-LPS complex, having two TLR4 binding sites, plays a bridging role. The primary binding interface does not require LPS; however, the dimerization interface involves LPS and mediates the LPS-dependent dimerization of TLR4.

TLR is a type-1 transmembrane protein containing an extracellular ligand-binding domain, a single transmembrane domain and an intracellular signaling domain (Gay and Gangloff, 2007; Medzhitov et al., 1997). The extracellular domain of TLR4 responsible for LPS binding belongs to the LRR family, like CD14. The TLR4 LRR domain belongs to the “typical” subfamily. LRR proteins in the typical subfamily contain 24 amino acids in individual LRR modules with conserved LxxLxLxxN motives (Kajava, 1998; Kobe and Kajava, 2001). TLR4 has 22 LRR modules protected by one LRRNT module in the N-terminal

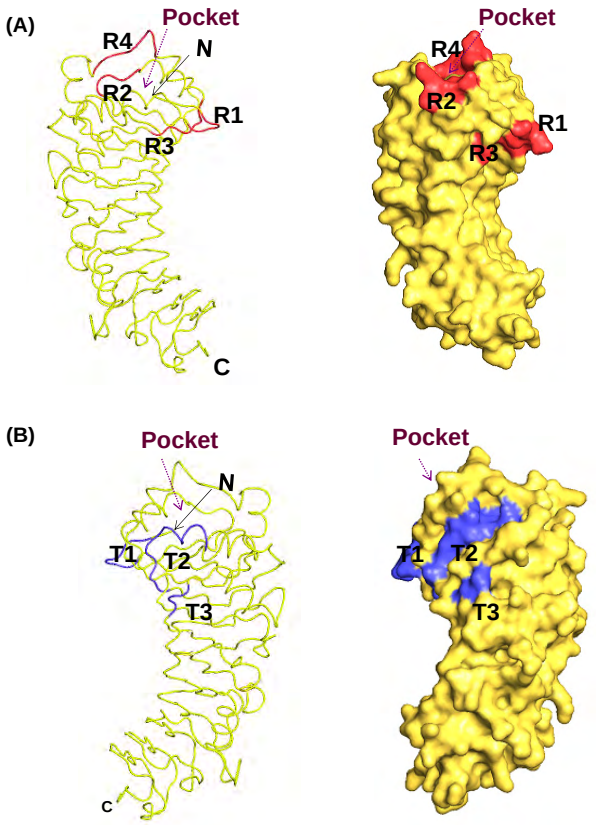

FIGURE 4 I Ribbon and surface representation of CD14. (A) Region critical for LPS binding are colored in red and labeled R1-R4. The position of NH_2-terminal pocket is marked by N. (B) The view is rotated by 180°. The regions critical in LPS signaling are colored in blue and labeled T1-T3.

end and one LRRCT module in the C-terminal end (Kim et al., 2007b). The central β sheet of the typical subfamily has uniform twist and tilt angles. Interestingly, the central β sheet of TLR4 has two sharp structural transitions and the LRR domain can be divided into three subdomains, the N-terminal, the central and the C-terminal subdomains. The domain boundary between the N-terminal and the central domains plays an important role in MD-2 binding.

MD-2 is a ~14k Da serum glycoprotein and the LPS binding unit of the TLR4-MD-2 complex. MD-2 belongs to a small family of lipid-binding proteins known as the β-cup family (Derewenda et al., 2002; Friedland et al., 2003; Kim et al., 2007b; Ohto et al., 2007; Wright et al., 2003). Proteins in this family are involved in either the transport or storage of lipid molecules using a large hydrophobic pocket. The β-cup fold is composed of two antiparallel β sheets, similar to the immunoglobulin fold. In the immunoglobulin fold, the two β sheets are held by a conserved disulfide bridge. In contrast, the β-cup fold proteins do not have this structurally important disulfide bridge, and two β sheets

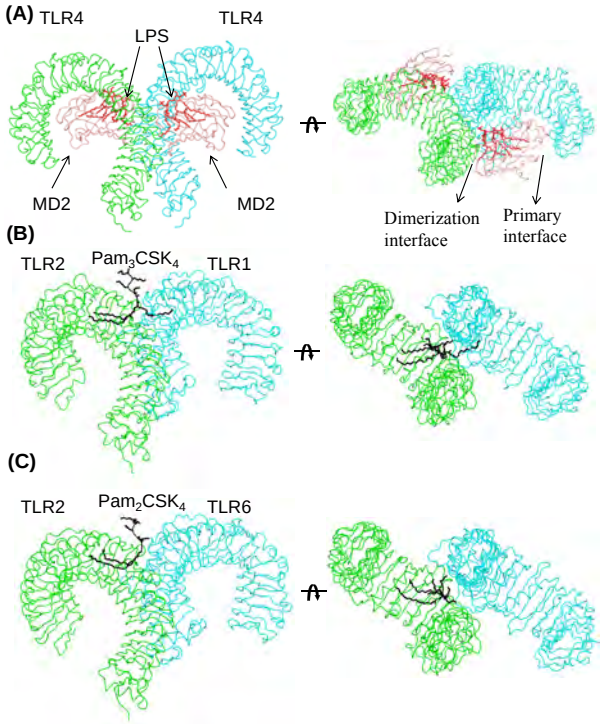

FIGURE 5 | Structures of pocket binding TLRs. Structures of the TLR4-MD-2-LPS (PDB entry 3FX1) (A), TLR1-TLR2-triacyl lipopeptide (PDB entry 2Z7X) (B), TLR2-TLR6-diacyl lipopeptide (PDB entry 3A79) (C)

can be separated. Hence, the hydrophobic core of the protein is exposed to the solvent area and is used for the binding of the lipid chains of the ligands. Because a large portion of the protein internal core is disturbed for ligand binding, the β-cup fold proteins contain multiple disulfide bridges that reinforce the stability of the protein. Among the β-cup proteins, MD-2 has a particularly large pocket that is suitable for the binding of large and flat hydrophobic ligands such as LPS.

The primary MD-2 binding site of TLR4 is located on the concave surface of the horseshoe-like structure and can be divided into two chemically distinctive areas termed the A and B patches (Kim et al., 2007b). The A patch is composed of predominantly negatively charged amino acids provided by the N-terminal domain of TLR4. It interacts with the positively charged surface of MD-2. The B patch is predominantly positively charged and composed of residues from the N- and central domains of the TLR4 extracellular domain. The majority of the TLR4 and MD-2 interaction is charge and hydrogen bond interaction caused by hydrophilic residues. Only a few hydrophobic residues are found on the TLR4-MD-2 interaction surface, and they do not seem to contribute significantly to the protein interaction.

RECOGNITION OF LPS BY THE TLR4-MD-2 COMPLEX

Lipid A of LPS binds to the hydrophobic pocket of MD-2. The LPS used in the crystallographic study was isolated from *E.coli* and contains six lipid chains and two phosphate groups (Park et al., 2009). All of the lipid chains of lipid A are inserted into the hydrophobic pocket of MD-2. The volume of the MD-2 pocket is large enough to contain all the lipid chains of LPS. The two phosphate groups of LPS undergo several charge and hydrogen bonding interactions with MD-2 and TLR4. Carbohydrate groups in the core part of the LPS have only limited interactions with the protein and do not make a significant contribution to the TLR4 binding.

MD-2 with bound LPS has two TLR4 binding interfaces, the primary and the dimerization interfaces (Figure 5A) (Park et al., 2009). Because the MD-2-LPS complex has these two interfaces, it can bridge two TLR4 molecules, creating a symmetric TLR4-MD-2 heterotetramer. The primary interface of the TLR4-MD-2 heterodimer does not require bound LPS. In contrast, the dimerization interface is generated only after LPS binding because a lipid chain of LPS directly participates in the dimerization interface. When bound to the MD-2 pocket, five of the six lipid chains of the *E.coli* LPS are fully submerged inside the hydrophobic pocket. However, the remaining sixth lipid chain is partially exposed to the MD-2 surface and interacts with a small hydrophobic surface composed of the phenylalanines and leucines of the TLR4 molecule. Hydrophilic amino acid residues of MD-2 at the opening of the LPS pocket support the central hydrophobic part of the dimerization interface and contribute to the TLR4 dimerization.

Decades of extensive research have been performed to determine structure-activity relationships associated with LPS (Rietschel et al., 1994; Rietschel et al., 1993; Teghanemt et al., 2005). These studies demonstrated that the optimal inflammatory activity requires six lipid chains and two phosphate groups attached to the glucosamine backbone of lipid A. The core and O-specific chain of LPS has minimal impact on the immunological activity of LPS (Erridge et al., 2002; Galanos et al., 1985; Rietschel et al., 1994). The structure of TLR4-MD-2-LPS complex explains these biochemical and immunological data. The volume of the MD-2 pocket appears to be optimized for six lipid chains. LPS with four lipid chains has minimal inflammatory activity, although binding to the TLR4-MD-2 complex remains possible. In fact, some of them are strong antagonists to TLR4-MD-2. This structure suggest that LPS with less than six lipid chains has reduced activity because all of the lipid chains are submerged inside the pocket and cannot provide an adequate hydrophobic interaction surface necessary for the stable binding of TLR4. LPS with more than six lipid chains has additional lipid chains that may disturb the optimal interaction distance between MD-2 and TLR4. The two phosphate groups attached to the glucosamine backbone also play an indispensable role in the LPS activity. Deletion of any of these phosphates can reduce the inflammatory activity of LPS by more than one hundred fold.

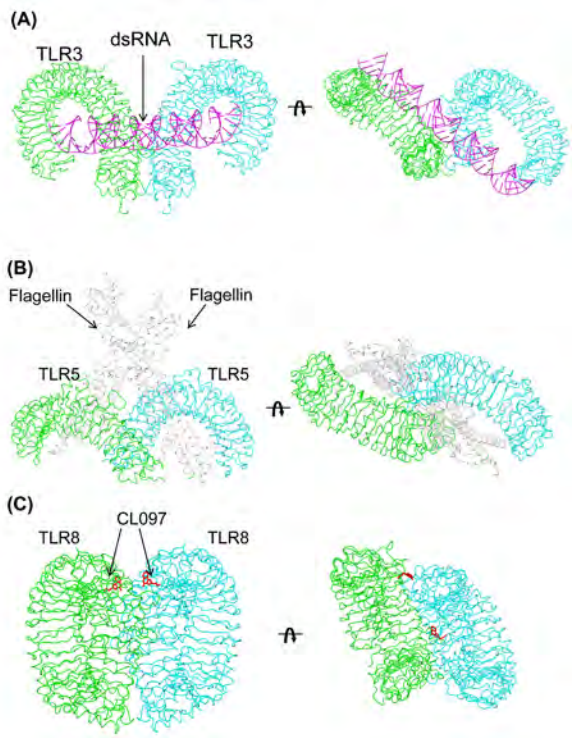

FIGURE 6 | Structures of surface binding TLRs. TLR3-dsRNA (PDB entry EC1Y) (A), TLR5-flagellin (PDB entry 3V47) (B), and TLR8-CL097 (PDB entry 3W3J) (C)

TLR4-MD-2 AND ANTAGONIST INTERACTION

The structures of two antagonists of TLR4, Eritoran and lipid IVa, have been determined in a complex with TLR4-MD-2 and with MD-2 alone, respectively(Kim et al., 2007b; Ohto et al., 2007). Eritoran is an experimental drug being developed as an anti-sepsis drug and is a powerful antagonist of TLR4 (Hennessy et al., 2010; Mullarkey et al., 2003; Rossignol and Lynn, 2005; Savov et al., 2005). Lipid IVa is an intermediate of LPS biosynthesis (Galanos et al., 1984). It functions as an antagonist to human TLR4 (Akashi et al., 2001; Lien et al., 2000). Both Eritoran and lipid IVa have four lipid chains and two phosphate groups attached to a di-glucosamine backbone. Regardless of the substantial structural discrepancies in their lipid chains, the positions of the phosphate groups and the glucosamines are practically conserved in both structures. Compared to hydrogen bonds and other hydrophilic interactions, the hydrophobic interaction between the lipid chains and the MD-2 pocket is not very sensitive to the distance and orientation of the interacting groups. Therefore, it appears that the total interaction surface area, not the actual chemical structures of the lipid chains, is important for MD-2 binding. This observation is consistent with experimental data showing that variation of the chemical structure of the lipid chains can be tolerated, although the total number of lipid chains or the chain length can have a serious effect on the inflammatory activity of LPS.

When compared to the structure of LPS bound to MD-2, the positions and orientation of antagonists bound to MD-2 show interesting differences. In the antagonist-bound structure, the glucosamine backbone positions move down inside the pocket by ~ 5 angstroms and the 1 and 4' phosphate groups swap their positions. The down shift of the glucosamine backbone position is understandable because the antagonists have fewer lipid chains. Because the shape of the MD-2 pocket is not changed by bound ligands, the glucosamine positions need to move down to occupy the pocket space with fewer lipid chains. It is not clear why the phosphate groups swap positions. Careful calculations of the charge interaction network between the phosphate groups and protein amino acids are required to explain the phosphate positions.

STRUCTURES OF OTHER TLR-FAMILY PROTEINS

Other TLR-family proteins are involved in the recognition of a variety of microbial products and contribute to the innate immune defense system against infection. Humans have ten TLR members (Akira and Hemmi, 2003; Gay and Gangloff, 2007; West et al., 2006). TLR2 forms a heterodimeric complex with either TLR1 or TLR6 to bind to lipopeptides or lipoproteins released from the bacterial membrane (Jin et al., 2007; Kang et al., 2009; Takeuchi et al., 2001; Takeuchi et al., 2002). TLR3, 7, 8, 9 are localized in the intracellular organelles and recognize various forms of microbial nucleic acids (Tanji et al., 2013; Witte et al., 2008). TLR5 is responsible for the recognition of the bacterial protein flagellin (Yoon et al., 2012). It contains 20~26 LRR, one LRRNT and one LRRCT modules. Over the last several years, the structures of most of these TLR proteins in complexes with their corresponding ligands have been determined (Figures 5 and 6). TLR-family proteins show surprisingly diverse ligand-binding mechanisms. TLR2-TLR1 and TLR2-TLR6 heterodimers use an internal pocket for the binding of lipoproteins. TLR3, TLR5 and TLR8 use a solvent-exposed surface for ligand binding. However, their ligand-binding sites are located in completely different parts of proteins. Regardless of this large variation of the ligand recognition mechanism, the overall shapes of the TLR-ligand complexes show a striking resemblance. In all reported TLR structures, ligand binding induces the homo- or hetero-dimerization of TLRs with an overall shape that resembles the letter "m." In this m-shaped complex, the two N-termini stretch outward and the two C-termini are close in the middle of the complex. This structural observation leads to a hypothesis about TLR activation: ligand binding induces the dimerization of extracellular domains, thus bringing the intracellular TIR domains into close proximity. The forced dimerization of the intracellular domains increases the binding affinity of intracellular signaling adaptors, MyD88 and TRIF, to the receptor and initiates signaling pathways (Jin et al., 2007; Kang and Lee, 2011).

STRUCTURE OF THE INTRACELLULAR SIGNALING DOMAIN OF TLR4

The intracellular domain of TLR4 is ~180 amino acids long and is termed the TIR (Toll/IL-1R homology) domain (Gay and Keith, 1991; Schneider et al., 1991). Its sequence and structure are conserved not only among the TLR family but also among several inflammatory interleukin receptors, including IL1 and IL17 receptors. Four adaptor proteins are recruited as TLR receptor proteins after activation by the binding of the corresponding ligands in their extracellular domains (Figure 1) (Kenny and O'Neill, 2008; O'Neill and Bowie, 2007; Watters et al., 2007). These adaptor proteins, MyD88, MAL, TRIF and TRAM, also contain TIR domains in their sequences. The TIR domains in the receptors and adaptors are responsible for adaptor aggregation at the receptor TIR domain via the creation of homo- and heterotypic TIR complexes. Different TLR families have unique requirements of these TIR-containing adaptor proteins. MyD88 is activated by every TLR except TLR3. TLR4 signaling requires MAL, TRIF and TRAM in addition to MyD88. TLR3 recruits TRIF for signaling.

The structure of the TIR domain of TLR4 remains unknown. However, the structures of three TLR TIR domains have been reported, and these structures can be used for the homology modeling of TLR4 TIR domains (Figure 7) (Chan et al., 2009; Nyman et al., 2008; Xu et al., 2000). All TIR domain structures share a similar arrangement of five parallel β strands surrounded by 4~6 α helices. Several areas in these structures critical for TLR signaling have been identified by site-directed mutagenesis experiments. The BB loop connecting the second β strand and the second α helix and the DD loop connecting the fourth strand and the fourth helix protrude from the protein core, likely playing a central role in TIR-TIR interactions.

MyD88 plays a central role in the TLR signaling pathway because it is required for all TLRs except TLR3. The amino acid sequence of MyD88 contains a death domain in addition to the TIR domain. The death domain of MyD88 creates complexes with death domains of downstream kinases, IRAK2, IRAK1 and IRAK4. The structure of this death domain complex has been determined (Lin et al., 2010). The structure shows that the death domain complex is composed of a large left-handed helical arrangement of six MyD88 death domains, four IRAK4 death domains, and four IRAK2 death domains. Based on this structure, it has been proposed that the juxtaposition of IRAK kinases by the formation of the death domain complex initiates intracellular signaling by the cross-phosphorylation of the IRAKs and the recruiting downstream signaling molecules. The structures of TIR-TIR complexes have not been reported; therefore, how this death domain aggregation process is controlled by ligand-induced receptor multimerization is still unknown.

HYBRID LRR TECHNIQUE

Crystallographic study of some TLR-family proteins has been difficult because the production of functionally and structurally intact proteins in quantities large enough for this type of study is difficult. This is a general problem related to modern crystallography, as protein production is often the most difficult step in protein crystallization. Furthermore, the crystallization of some TLRs, including TLR4, is not possible even after a sufficient amount of protein becomes available. These problems could be solved by a novel technique known as the "Hybrid LRR Technique" (Kim et al., 2007b). In this technique, the nonfunctional part of the target LRR protein is replaced by LRR modules from other LRR-family proteins. While combining the two proteins, the sequence conservation pattern of the two LRR modules should be maintained because the spacings between the leucines are critical for the proper positioning of the hydrophobic core. This technique can only be applied to LRR-family proteins, as they are composed of multiple LRR modules and because the assembly of the modules is enabled during the evolution of this protein family. Combining two non-LRR family

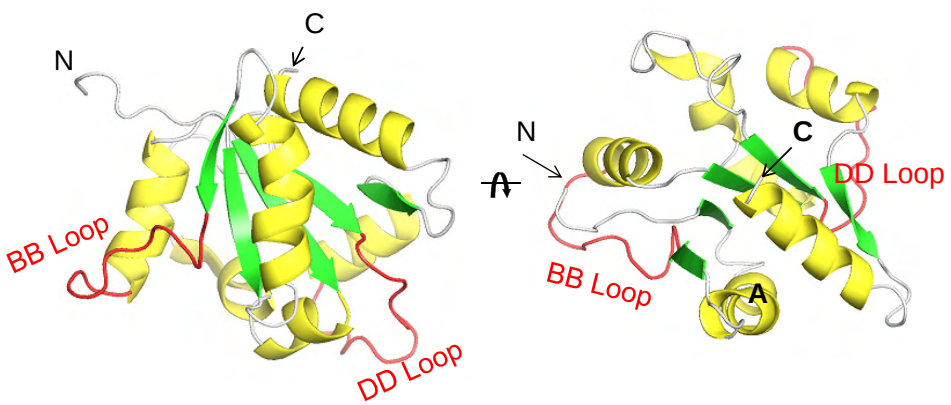

FIGURE 7 | Structure of TLR2 TIR domain. Structure of TLR2 TIR domain (PDB entry 1FYW) is schematically drawn. The α helices and β sheets are colored in yellow and green, respectively. The BB and DD loops critical in signaling are colored in red.

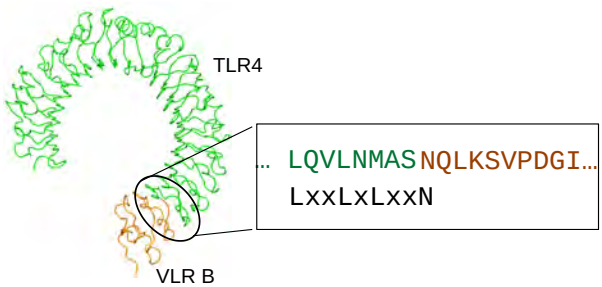

FIGURE 8 | Hybrid LLR Technique. Human TLR4 and VLR of hagfish is fused while maintaining conservation pattern of the LRR modules. TLR4 and VLR sequences at the fusion sites are written in green and dark brown, respectively.

proteins is not possible because the structural compatibility of the two proteins cannot be predicted in other families of proteins. For the LRR family, the structure of the conserved LxxLxLxxN part of the module can be predicted with high confidence, and this structural information can be used for protein fusion.

In principle, any LRR-family protein can be used as fusion partner in a hybrid LRR as long as it can easily be produced and crystallized. For TLR research, the variable lymphocyte receptor (VLR) from hagfish was chosen as the fusion partner (Figure 8) (Kim et al., 2007a; Pancer and Cooper, 2006). VLRs are adaptive immune receptors of jawless fish such as lamprey and hagfish. VLRs belong to the LRR family, and they show the nearly ideal sequence conservation patterns typical of the LRR family. VLR diversity is generated by the shuffling of several hundreds of LRR modules. A single hagfish can generate millions of diverse VLR genes by somatic recombination. Therefore, in principle, VLR proteins suitable for fusion with TLR can be isolated from this diverse VLR gene pool. The success rate of VLR-TLR fusion was surprisingly high, and ~50% of the fusion proteins designed could be produced in a functionally and structurally intact form. Some of them could be crystallized even though the original TLR protein was impossible to crystallize.

This technique has been successfully used for crystallization of TLR4-MD-2-Eritoran complex and TLR5-flagellin complex (Kim et al., 2007b; Yoon et al., 2012). The first crystallographic study RP105 also benefited from this technique (Yoon et al., 2011). RP105 is a LRR family protein with substantial structural and sequential homology with TLR4.

CONCLUSION

Crystallographic study of TLR4 and associated accessory proteins provide clues how diverse LPS molecules are recognized by our immune system. Stringency of ligand specificity gradually increases while it is being transferred from LBP to the TLR4-MD-2 complex. LBP does not have clear ligand binding pocket and charge complementarity appears to have major role in ligand binding. CD14 has more stringent binding specificity because it has a hydrophobic binding pocket and only monomeric form of LPS can bind to this pocket through mostly nonspecific hydrophobic interaction. MD-2 has highest stringency in ligand selection. Charge and hydrogen bonding near the MD-2 pocket allow only LPS-like molecules to bind and induce TLR dimerization. To gain further insight in LPS recognition, we need to determine structure of LBP-LPS and CD14-LPS complexes. We also need to know how LPS is transferred from LBP to CD14 and from CD14 to TLR4-MD-2 in the future. This structural study will have important role in future development of anti-inflammatory agents.

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