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*J Immunol* 2004; 173:7401-7405; doi: 10.4049/jimmunol.173.12.7401

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TLR2 Recognizes a Bacterial Lipopeptide through Direct Binding

Thierry Vasselon,²* Patricia A. Detmers, † Dominique Charron,* and Alain Haziot²*

The TLRs play an important role in the initiation of cellular innate immune responses to a wide range of bacterial products, including LPS and lipoproteins. Although rapid progress has been made on signaling functions of activated TLRs, the molecular mechanisms that lead to TLR activation are still poorly understood. We report in this study that the extracellular domain of TLR2 interacts directly with synthetic bacterial lipopeptide (sBLP), a potent analog of bacterial lipoproteins. Using fluorescently labeled sBLP complexed to soluble recombinant CD14 (rsCD14), we observed specific binding of sBLP to the surface of cells expressing TLR2 transgenes and to a recombinant soluble form of the TLR2 ectodomain. TLR2-mediated binding of sBLP at the cell surface did not require prior induction of intracellular signals. In addition, using a chimeric TLR2/TLR4 construct, we showed that the leucine-rich region of TLR2 carries the specificity for binding of the agonist and for initiating signaling. Specific binding of fluorescent sBLP to purified sTLR2 required sCD14. However, sCD14 was not part of the complex formed by soluble TLR2 and sBLP. Together, these data provide evidence that TLR2 recognizes sBLP through direct binding. *The Journal of Immunology, 2004, 173: 7401–7405.

The innate immune system detects the presence of pathogens, provides the first line of host defense, and initiates the adaptive immune responses (1). Recent studies have revealed that the TLR family plays an important role in recognition of microorganisms and initiation of innate immune responses (1). TLRs are transmembrane proteins with an ectodomain consisting of leucine-rich repeats (LRRs)³ and one or two cysteine-rich regions (CRR). The intracellular segment contains a Toll/IL-1R homology domain, which is required for signaling. Identification of agonists of TLRs and of the signaling pathways induced by activated TLRs has progressed rapidly. Most TLRs are activated by structures that are found conserved in large groups of microorganisms. For example, TLR4 is activated by LPS of most Gram-negative bacteria (2) and TLR2 by lipoproteins, which are nearly ubiquitous constituents of bacterial cell walls (3–5). Activation of TLRs by microbial products leads to intracellular signals through common as well as TLR-specific pathways.

In contrast, the initial events that lead to TLR activation by their agonists are still poorly understood. Close proximity of LPS with a group of receptors that included TLR4, MD2, CD14, heat shock protein 70 (Hsp70), Hsp90, and CXCR4 has been observed using biophysical methods such as fluorescence resonance energy transfer (6, 7). In addition, the observation that cells transfected with TLR4 from different species reacted to LPS-partial structures with the species specificity of the transgene provided evidence that TLR4 is involved in recognition of the bacterial structure (8, 9). However, it remains unclear whether recognition of bacterial product is mediated through specific binding of these products to the TLR they activate. To address this question, we have used the synthetic bacterial lipopeptide (sBLP) Pam₃CysSerLys₄, which is an agonist for TLR2. sBLP is an analog of triacylated lipoproteins that is exempt from contamination by other bacterial products. TLR2-mediated responses to sBLP and lipoproteins are greatly enhanced both by membrane-bound CD14 and soluble CD14 (sCD14), with which lipoproteins have been shown to form complexes (5, 10–12). Using fluorescent sBLP/ sCD14 complexes, we observed that sBLP specifically bound to the surface of TLR2-transfected cells without the need for prior intracellular signaling. Using purified proteins, we show direct interaction of sBLP with soluble rTLR2 (rsTLR2). Together, our results suggest that TLR2 recognizes sBLP through direct binding at the cell surface.

Materials and Methods

Materials

Human embryonic kidney 293 and 293T cells were obtained from American Type Culture Collection (Manassas, VA). Recombinant human sCD14 and 6×His-tagged sCD14 were purified, as described (12). sBLP, a palmitoylated version of N-acetyl-S-diacylglyceryl-Cys-Ser-Lys₄, was from Boehringer Mannheim (Mannheim, Germany). The fluoroprobe Alexa Fluor 488 was coupled to sBLP using a labeling kit from Molecular Probes (Eugene, OR). Briefly, 1 mg of sBLP was incubated with the reactive dye for 1 h at 19°C. Every 5 min, the mixture was sonicated for 30 s to disrupt sBLP aggregates. Conjugated sBLP was then separated from unconjugated dye by repeated washes in PBS. LPS from Escherichia coli O111:B4 (Sigma-Aldrich, St. Louis, MO) was re-extracted twice with phenol, as described (13). Alexa Fluor 488-labeled LPS (A₄₈₈-LPS) was from Molecular Probes (Eugene, OR).

Complexes between sCD14 and sTLR, Alexa Fluor 488-labeled sBLP (A₄₈₈- sBLP), LPS, or A₄₈₈-LPS were formed by incubating sBLP (8 µg/ml), A₄₈₈-sBLP (8 µg/ml), LPS (4 µg/ml), or A₄₈₈-LPS (4 µg/ml) with sCD14 (100 µg/ml) overnight at 37°C in Dulbecco’s PBS containing 0.05% pyrogen-free human serum albumin (Centeon, Kankakee, IL) or BSA (Sigma-Aldrich). In these conditions, the great majority of LPS is found under...
a soluble form bound to sCD14 (14). In contrast, only 20–25% of $\text{A}_{\text{ss}}$-sBLP is found under a soluble form bound to sCD14 (data not shown). For affinity measurements, $\text{A}_{\text{ss}}$-sBLP/sCD14 complexes were separated from $\text{A}_{\text{ss}}$-sBLP aggregates by centrifugation (12,000 x g, 1 min), and the total amount of $\text{A}_{\text{ss}}$-sBLP fluorescence added to beads determined before use.

TNF-α was from R&D Systems (Minneapolis, MN). The CD14 mAb 60b and the CD11b mAb 44a were purified from ascites (14), and the CD14 mAb MEM-18 was from Caltag Laboratories (Burlingame, CA). The fluorophore Alexa Fluor 594 was coupled to the anti-Flag M2 mAb (Sigma-Aldrich) using a labeling kit from Molecular Probes.

**Native PAGE assays**

To assess $\text{A}_{\text{ss}}$-sBLP binding to purified sCD14, sCD14 (100 μg/ml) was incubated overnight at 37°C with 8 μg/ml $\text{A}_{\text{ss}}$-sBLP in PBS in the presence or absence of 750 ng/ml CD14 mAb 60b or control CD11b mAb 44a and then analyzed by electrophoresis on native 10% polyacrylamide gels. Bands were scanned for $\text{A}_{\text{ss}}$-sBLP fluorescence using a Storm 860 fluorescence imager (Amersham, Saclay, France).

**Plasmid preparation**

TLR2 and TLR2 tagged with an NH$_2$-terminal Flag epitope (Flag-TLR2) were cloned into pcDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA), as described (12). The P681H mutation was generated by PCR using the Flag-TLR2 construct. Human nsTLR2 was obtained by cloning the region coding for AA 1-588 of TLR2 at the BamHI site of pcDNA3.1/myc-His (Invitrogen Life Technologies). Human cDNAs encoding TLR4 and MD-2 were cloned into pcDNA3.1 vectors. Human TLR1 was obtained from Genemanex (Stratagene, La Jolla, CA). At 24 h posttransfection, cells were harvested and redistributed into a 96-well plate. At 48 h posttransfection, cells were incubated with various stimuli for 6 h at 37°C. Supernatants were assayed by ELISA (BD Pharmingen, San Diego, CA).

**Expression and purification of sTLR2**

The 293 transfectants were cultured for 24–48 h before experiments in complete culture medium on glass coverslips precoated with 0.5% gelatin. The cells were washed twice with PBS containing 0.05% BSA (PBS-BSA) and incubated at 37°C in PBS-BSA with or without $\text{A}_{\text{ss}}$-sBLP/sCD14. At the end of incubation, coverslips were washed several times with PBS-BSA and either used for live microscopy or fixed for 30 min in PBS containing 4% paraformaldehyde and mounted. Images were obtained using either a Bio-Rad (Hercules, CA) MRC 1024 confocal microscope or a fluorescence microscope equipped with a CoolSNAP HQ charge-coupled device (CCD) camera (Koppe Scientific, Trenton, NJ).

**IL-8 ELISA**

Semiconfluent 293 cells were transfected with various constructs using Genejumper (Stratagene, La Jolla, CA). At 24 h posttransfection, cells were harvested and redistributed into a 96-well plate. At 48 h posttransfection, cells were incubated with various stimuli for 6 h at 37°C. Supernatants were assayed by ELISA (BD Pharmingen, San Diego, CA).

**Expression and purification of sTLR2**

The 293T cells were transfected with pcDNA3.1/TLR2/myc-his or control pcDNA3.1/myc-his using calcium phosphate (Invitrogen Life Technologies) and cultured in DMEM containing 10% FCS for 24 h. The medium was then replaced with protein-free 239 medium (Invitrogen Life Technologies). At 72 h posttransfection, conditioned medium was harvested. To purify sTLR2, conditioned medium was first subjected to fast protein liquid chromatography (FPLC) on a resource Q column (Amersham). FPLC fractions were used to amplify the vector containing the deleted TLR2 gene. All constructs were verified by sequencing.

**Fluorescence microscopy**

The 293 transfectants were cultured for 24–48 h before experiments in complete culture medium on glass coverslips precoated with 0.5% gelatin. The cells were washed twice with PBS containing 0.05% BSA (PBS-BSA) and incubated at 37°C in PBS-BSA with or without $\text{A}_{\text{ss}}$-sBLP/sCD14. At the end of incubation, coverslips were washed several times with PBS-BSA and either used for live microscopy or fixed for 30 min in PBS containing 4% paraformaldehyde and mounted. Images were obtained using either a Bio-Rad (Hercules, CA) MRC 1024 confocal microscope or a fluorescence microscope equipped with a CoolSNAP HQ charge-coupled device (CCD) camera (Koppe Scientific, Trenton, NJ).

**Results**

Expression of TLR2 in 293 cells confers ability to bind a lipopeptide

Human epithelial kidney 293 cells become responsive to purified bacterial lipoproteins and the triacylated synthetic bacterial lipopeptide analog Pam$_3$CysSerLys$_3$ (sBLP) upon transfection with TLR2 (3, 5). TLR2-mediated responses to lipoproteins are greatly increased when cells express a membrane-bound CD14 that is co-transfected with TLR2 (5, 11) or when they are incubated in the presence of sCD14 (4, 12). In particular, we have previously shown that sBLP activity on 293-TLR2 cells is enhanced >100-fold upon preincubation with sCD14 (12). To visualize binding of sBLP at the surface of responsive cells, we generated a fluorescent derivative of the lipopeptide sBLP by labeling it with the fluorophor Alexa 488. The resulting molecule termed A488-sBLP was preincubated with sCD14, and, as shown in Fig. 1A and similarly to other microbial lipoproteins (11), A488-sBLP and sCD14 formed complexes detected as a broad band in native PAGE (lane 2), A488-labeled bacterial agonists presented under various forms. After washing, A488-sBLP and A488-LPS fluorescence at the surface of beads was determined using a fluorescence microscope equipped with a CCD camera. Fluorescence (arbitrary units) of beads was calculated from images using the Fluorchem 8800 software. Amounts of $\text{A}_{\text{ss}}$-sBLP bound to TLR2 were calculated from fluorescence values of known amounts of $\text{A}_{\text{ss}}$-sBLP bound to beads via His-tagged sCD14.

**FIGURE 1.** A Fluorescent $\text{A}_{\text{ss}}$-sBLP forms stable complexes with sCD14. $\text{A}_{\text{ss}}$-sBLP (8 μg/ml) was incubated with 100 μg/ml rsCD14 in the absence (lane 2) or presence of CD14 mAb 60b (lane 3) or control mAb 44a (lane 4), as described in Materials and Methods. Lane 1. Contains $\text{A}_{\text{ss}}$-sBLP in the absence of additional protein. Mixtures were run on native polyacrylamide gels, and $\text{A}_{\text{ss}}$-sBLP fluorescence detected by scanning gels with a fluorescence imager. $\text{A}_{\text{ss}}$-sBLP aggregates alone did not enter the gel (lane 1). Upon preincubation with sCD14, a band corresponding to $\text{A}_{\text{ss}}$-sBLP/sCD14 complexes was observed (lane 2). Mobility of $\text{A}_{\text{ss}}$-sBLP in $\text{A}_{\text{ss}}$-sBLP/sCD14 complexes was shifted upon incubation with anti-CD14 mAb (lane 3). Positions of $\text{A}_{\text{ss}}$-sBLP aggregates, $\text{A}_{\text{ss}}$-sBLP/sCD14 complexes, and $\text{A}_{\text{ss}}$-sBLP/sCD14/anti-CD14 mAb are indicated. B. TLR2-dependent binding of $\text{A}_{\text{ss}}$-sBLP presented as $\text{A}_{\text{ss}}$-sBLP/sCD14 complexes at the cell surface of 293 cells. The 293 cells transiently transfected with human TLR2, TLR4 and MD-2, TLR1, or empty pcDNA3 vector were incubated in 100 μl of PBS for 15 min at 37°C with $\text{A}_{\text{ss}}$-sBLP/sCD14 (80 ng/ml $\text{A}_{\text{ss}}$-sBLP) complexes. CCD images of $\text{A}_{\text{ss}}$-sBLP fluorescence in representative cells are shown. Bars, 10 μm.
Moreover, cells transfected transiently expressed various levels of sBLP/sCD14 complexes and free A488-sBLP were used to assess binding to TLR2-transfected 293 cells. No specific binding of sBLP presented alone was detected at concentrations up to 1 µg/ml. Raising sBLP concentrations above that led to high nonspecific binding that precluded analysis (data not shown). As seen in Fig. 1B, 293 cells transiently transfected with Flag-TLR2 strongly bound A488-sBLP presented as sBLP/sCD14 complexes. Binding of A488-sBLP was also observed with 293 transfectants transiently expressing TLR2 without the Flag epitope (data not shown). In contrast, 293 cells transfected with empty vector or expressing either TLR4 and MD2 or TLR1 did not bind A488-sBLP (Fig. 1B). These results indicate that expression of TLR2 is required for the binding of A488-sBLP to 293 cells.

To further document the involvement of TLR2 in the binding of A488-sBLP, the localization of A488-sBLP and Flag-TLR2 at the cell surface was analyzed. There was colocalization of A488-sBLP and Flag-TLR2 at the cell surface (Fig. 2A, TLR2, merge panel). Moreover, cells transfected transiently expressed various levels of Flag-TLR2 at their cell surface. Intensity of A488-sBLP and Flag-TLR2 fluorescence at the cell surface was found to be proportional (Fig. 2B), indicating that the amount of A488-sBLP bound to the surface of cells depends on the amount of TLR2.

To rule out the possibility that A488-sBLP binding was dependent on a receptor whose expression would be induced by cell activation via TLR2, binding of A488-sBLP was analyzed on cells expressing TLR2 with the P681H point mutation. Structural and functional studies have shown that this mutation in the cytoplasmic tail of TLR2 does not disturb the structure of the protein, but abolishes signal transduction (15). As shown in Fig. 2A, binding of A488-sBLP to P681H-TLR2 was strong and similar to wild type. Altogether, these results suggest that sBLP binds directly or indirectly to TLR2 at the cell surface, and that the binding does not require prior induction of intracellular signals.

**The leucine-rich domain of TLR2 carries the specificity for binding and activation**

The ectodomain of TLR2 comprises a leucine-rich domain consisting of LRRs, and a CRR that links to the TM region (1). To determine which regions of TLR2 were involved in recognition of sBLP, we engineered chimeric constructs in which regions of the ectodomain of TLR2 were replaced with their equivalent in TLR4 (Fig. 2C). We observed that expression of a form of TLR2 in which CRR and TM domains were replaced by that of TLR4 (LRR2/CRR4-TM4) allowed 293 cells to bind A488-sBLP (Fig. 2A) and to secrete IL-8 in response to the agonist (Fig. 3). As the CRR and TM domains of TLR2 do not confer the specificity for the binding of sBLP to the cell surface, these results suggest that the LRR domain of TLR2 carries the specificity, allowing both binding of sBLP and signaling.

**TLR2 directly binds sBLP**

To determine whether isolated TLR2 could bind sBLP, we produced a recombinant soluble form of the ectodomain of TLR2 (sTLR2). For this, we expressed in 293T cells a truncated form of TLR2, which consisted of the first 588 residues corresponding to the LRR and CRR region of the protein, and which carried a V5-DHFR epitope. We used a recombinant soluble form of the ectodomain of TLR2 (sTLR2) for this, which consisted of the first 588 residues corresponding to the LRR and CRR region of the protein, and which carried a

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**FIGURE 2.** The 293 cells transiently transfected with human TLR2, TLR2 P681H mutant, or LRR2/CRR4-TM4 chimera were incubated in 100 µl of PBS for 15 min at 37°C with A488-sBLP/sCD14 (80 ng/ml A488-sBLP, 1 µg/ml sCD14) complexes. Cells were then fixed, and the various flag-tagged TLR2 proteins were labeled with Alexa Fluor 594 anti-Flag Ab (A488-anti-Flag). A, sBLP colocalizes with TLR2, TLR2 P681H mutant, and LRR2/CRR4-TM4 chimera at the cell surface. CCD images for A488-sBLP and TLR2, or TLR2 P681H or LRR2/CRR4-TM4 fluorescence are shown for representative cells in the left and center panels, respectively, and the merged images are shown in the right panels. Bars, 10 µm. B, Intensities of A488-sBLP and Flag-TLR2 fluorescence are proportional. Cell surface fluorescence intensities of A488-sBLP and Flag-TLR2 were measured from CCD images using the Scion image software and expressed as arbitrary units. C, Schematic representation of the TLR2/TLR4 chimera. The domain structure of TLR2 and TLR4 is shown. Numbers indicate residues at the boundaries of regions that have been swapped in TLR2/TLR4 chimera.

**FIGURE 3.** IL-8 production induced by sBLP and LPS in 293 cells expressing TLR2, TLR4 and MD2, or a TLR2/TLR4 chimera. The 293 cells transfected with the indicated constructs were stimulated for 6 h with buffer alone ( ), LPS/sCD14 complexes (40 ng/ml LPS, ), or sBLP/sCD14 complexes (80 ng/ml sBLP, ). IL-8 production was measured in supernatants by ELISA. Results are expressed as mean of triplicate wells ± SEM.
COOH-terminal 6×His tag. sTLR2 was purified from culture supernatants and, as seen in Fig. 4, it migrated as an 83-kDa protein. To test its binding capacity, sTLR2 was captured at the surface of agarose beads through its histidine tag, and the beads were incubated with various agonists. As shown in Fig. 4B, sBLP in suspension did not bind to TLR2-coated or control beads. In contrast, sTLR2-coated beads strongly bound A488-sBLP presented as a complex with sCD14 (Fig. 4, B and C). Binding was dose dependent and reached saturation with an apparent dissociation constant (Kd) of 3.5 × 10⁻⁸M for the sBLP/sTLR2 complex (Fig. 4C). This binding was dependent on the presence of TLR2, as no binding could be measured on control beads (Fig. 4B, and data not shown). In addition, binding of A488-sBLP to sTLR2 was inhibited by a 20-fold excess of unlabeled sBLP/sCD14 and was specific for the lipopeptide, as A488-LPS complexed to sCD14 did not bind to sTLR2 (Fig. 4B). These results show that TLR2 interacts with its agonist sBLP in the presence of CD14.

To determine whether sCD14 is part of a complex in association with sTLR2 and sBLP, we probed the beads for the presence of sCD14 by Western blot. Fig. 5A shows the Western blot analysis of material eluted from beads assayed for fluorescence in Fig. 5B. No sCD14 was found associated with TLR2-coated beads that bound sBLP upon incubation with A488-sBLP/sCD14 complexes (Fig. 5A). To confirm that sCD14 would be detectable if it was responsible for binding A488-sBLP as part of a complex with sTLR2, we directly bound increasing amounts of 6×His-tagged sCD14/A488-sBLP complexes to fresh beads, and analyzed by Western blot and fluorescence the material bound on beads. As
shown in Fig. 5B, beads with a wide range of fluorescence intensities were obtained, reflecting the density of 6XHis-sCD14-A488-sBLP on the bead surface. Analysis of beads with fluorescence intensity similar to that of sTLR2/A488-sBLP showed that sCD14 was strongly detected in material eluted from the beads (Fig. 5A). Even when the intensity of 6XHis-sCD14-A488-sBLP beads was equal to only one-third of that of sTLR2/A488-sBLP beads, sCD14 was still easily detectable. Together, these results strongly suggest that sCD14 is not the major sBLP-binding molecule on sTLR2-coated beads, implying that sTLR2 is able to interact directly with its agonist sBLP.

Discussion

The present study provides evidence that TLR2 recognizes triacylated lipopeptides through direct binding. We have demonstrated that the extracellular domain of TLR2 is involved in this interaction, and that the LRRs of TLR2 carry the specificity, allowing both binding of sBLP and signaling. Binding of sBLP to sTLR2 and to membrane-bound TLR2 was observed when sBLP was presented to TLR2 as sBLP/sCD14 complexes. As sCD14 does not form a stable ternary complex with sTLR2 and sBLP, it may act by facilitating the binding of sBLP to sTLR2. In this regard, it is interesting to note that, while it is still not known whether recognition of LPS involves a direct interaction between LPS and TLR4, transfer of LPS from CD14 to soluble rMD2, a protein that binds membrane-bound TLR4, led to cell activation (16). Similarly, CD14 has been shown to mediate transfer of LPS to a cell surface complex that contains both TLR4 and MD2 (17). Although our studies with purified proteins indicated that no other proteins than sCD14 were required to transfer sBLP to TLR2, other proteins may be required in addition to CD14 to mediate efficient transfer of sBLP to TLR2 at the cell surface. Recent studies proposed a model in which LPS dissociates from CD14 to come in close proximity with Hsp70 and Hsp90, and to induce the recruitment of receptors such as TLR4, and CXCR4 to lipid rafts (6). By analogy, a larger molecular complex may be involved at the cell surface for optimal transfer of lipoproteins to TLR2. TLR2 is activated by a variety of bacterial products, including some that depend on CD14 for sensitive responses. Recently, binding of sTLR2 to insoluble peptidoglycan, a chemically ill-defined preparation that induced cell responses at high concentrations, was reported (18). In this study, we used a purified synthetic structural analog of bacterial lipoproteins and showed that it interacted directly with purified rTLR2 and with TLR2 at the surface of cells. This interaction was observed when the agonist was presented at low concentration and under a form that stimulated cell responses very efficiently. Altogether the demonstration of a direct interaction between TLR2 and one agonist of defined structure at low concentrations is a critical advance in understanding the molecular bases of bacterial recognition by the innate immune system. A role for TLR1 in TLR2-mediated responses to triacylated lipoproteins has been reported (19, 20), raising the possibility that TLR2 and TLR1 cooperate for optimal response to these agonists. Our study with rTLR2 indicated that a high affinity interaction between a lipopeptide and TLR2 can be observed in the absence of TLR1. Moreover, binding of sBLP at the surface of 293 cells was found to be directly proportional to the amount of TLR2 expressed at the membrane (Fig. 2B). Because 293 cells expressed no detectable TLR1 protein and no TLR1 transcript (data not shown), these observations suggest that TLR2 can recognize sBLP in the absence of TLR1. We have shown that in contrast to TLR2, overexpressing TLR1 is not sufficient to confer 293 cells the ability to bind sBLP at their surface (Fig. 1B). In addition, when cells were cotransfected with equal amounts of TLR2 and TLR1, binding of sBLP was found to be similar to that of cells transfected with equal amounts of TLR2 and control vector (T. Vasselon and A. Haziot, unpublished observations). TLR2 appears thus as the primary mediator for sBLP binding. Further studies will be necessary to evaluate the contribution of TLR1 to TLR2-dependent recognition of lipopeptides, and to determine the role of TLR1 in following events leading to signaling in responses to triacylated lipopeptides. Although the structural requirements for binding of agonists to TLR2 remain to be elucidated, demonstration of a stable direct interaction between TLR2 and sBLP will help devise strategies for the development of agonists and antagonists to modulate inflammatory and immune responses for therapeutic intervention.

Acknowledgments

We thank Rolf Thieringer for providing rsCD14 and Samuel D. Wright for critical reading of the manuscript.

References