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Human Toll-Like Receptor 2 Mediates Monocyte Activation by Listeria monocytogenes, But Not by Group B Streptococci or Lipopolysaccharide

Trude H. Flo,* Øyvind Halaas,* Egil Lien,* Liv Ryan,* Giuseppe Teti,‡ Douglas T. Golenbock,‡ Anders Sundan,* and Terje Espevik*‡

Human Toll-like receptor (TLR) 2 has been implicated as a signaling receptor for LPS from Gram-negative bacteria and cell wall components from Gram-positive organisms. In this study, we investigated whether TLR2 can signal cell activation by the heat-killed group B streptococci type III (GBS) and Listeria monocytogenes (HKLM). HKLM, but not GBS, showed a time- and dose-dependent activation of Chinese hamster ovary cells transfected with human TLR2, as measured by translocation of NF-κB and induction of IL-6 production. A mAb recognizing a TLR2-associated epitope (TL2.1) was generated that inhibited IL-6 production from human monocytes by 60%, whereas a CD14 mAb (3C10) reduced the TNF production by 30%. However, coadministering TL2.1 and 3C10 inhibited the TNF response by 80%. In contrast to this, anti-CD14 blocked LPS-induced TNF production from monocytes, whereas anti-TLR2 showed no inhibition. Neither TL2.1 nor 3C10 affected GBS-induced TNF production. These results show that TLR2 can function as a signaling receptor for HKLM, possibly together with CD14, but that TLR2 is unlikely to be involved in cell activation by GBS. Furthermore, although LPS can activate transfected cell lines through TLR2, this receptor does not seem to be the main transducer of LPS activation of human monocytes. Thus, our data demonstrate the ability of TLR2 to distinguish between different pathogens.

B both Gram-positive and Gram-negative bacteria are potent inducers of proinflammatory responses that can eventually cause fatal sepsis syndrome in humans (1, 2). Whereas the membrane glycolipid LPS appears to be the main mediator of immune responses to Gram-negative bacteria, there are several cell surface components mediating cell activation by Gram-positive bacteria. The 53- to 55-kDa cell surface glycoprotein CD14 (3) has been identified as the principle LPS-binding molecule on phagocytic leukocytes, enabling them to be stimulated with picogram amounts of LPS (4). CD14 is linked to the membrane with a glycosylphosphatidylinositol anchor (5), and it has been assumed that CD14 mediates LPS activation by interaction with other signal-transducing molecules (6–8). The recent discovery that human Toll-like receptors (TLRs)3 can signal LPS activation has lent new support to this theory (9–13).

The Toll protein was first described as the mediator of dorsal-ventral patterning in Drosophila embryo, and later a role for Toll and Toll-like proteins in Drosophila innate immunity was found (14–16). By now, six human homologues of the Drosophila Toll have been cloned and designated TLRs 1–6 (17–19). Like Drosophila Toll, the TLRs are type I transmembrane proteins with a leucine-rich repeat (LRR) extracellular domain and a cytoplasmic domain with homology to the mammalian IL-1R (20). Moreover, as for the IL-1R, NF-κB and related transcription factors can be activated by mammalian and Drosophila Toll receptors through similar signaling cascades (20, 21).

Recently, Yang et al. (11) and Kirschning et al. (10) independently described that TLR2 mediates LPS-induced NF-κB activation when overexpressed in human embryonic kidney 293 cells. Although these findings suggested that TLR2 may be the dominant CD14-associated signal transducer in mammalian cells, subsequent reports demonstrated that the LPS hyporesponsiveness of C3H/HeJ and C57BL/10ScCr mice maps to a related gene, Tlr4 (9, 22). A Tlr4 knockout mouse has subsequently been engineered and is similarly hyporesponsive to LPS (13).

Studies of Drosophila mutants suggest that Toll and homologue receptors discriminate between different pathogens (14–16). This prompted us to investigate whether TLR2 can function as a signal transducer for two different heat-killed Gram-positive bacteria, Listeria monocytogenes (HKLM) and type III group B streptococci (GBS). GBS is known primarily as the leading cause of sepsis and meningitis in neonates (23). Although several components isolated from GBS employ CD14-dependent pathways in activating cells, whole heat-killed bacteria preferentially use the β2-integrin CD11b/CD18 (CR3) (M. Cuzzola, et al., manuscript in preparation) (24).

L. monocytogenes is a facultative intracellular bacterium and the causative agent of food-borne listeriosis. Infections with L. monocytogenes are rare, but with high rates of mortality, and the major
risk groups are pregnant women, fetuses, neonates, and immunocompromised individuals (25). Adhesion of *L. monocytogenes* to macrophages results in immediate activation of NF-κB and production of proinflammatory cytokines (26). Both type I scavenger receptor (25) and CD11c/CD18 (CR4) (M. Cuzzola, et al., manuscript in preparation) have been suggested as cell receptors for *L. monocytogenes*, but it is by far still unclear how it signals cell activation.

In this study, we show that TLR2 mediates cell activation induced by HKLM, but not by GBS, suggesting that human TLR2 distinguishes between two Gram-positive pathogens.

Materials and Methods

Reagents

LPS (L-2137) from smooth *Salmonella minnesota* was purchased from Sigma (St. Louis, MO). COH-1, an encapsulated type III GBS strain, was kindly provided by Craig Rubens (University of Washington, Seattle, WA).

*L. monocytogenes* was from a recent clinical isolate. Bacteria were grown to the early stationary phase in Todd-Hewitt broth, killed by heat treatment (60°C for 45 min), washed extensively with distilled water, and lyophilized. Human CD14 mAb 3C10 (IgG2; American Type Culture Collection, Manassas, VA) and the mAb 6H8 (IgG1) that recognizes a widely distributed 180-kDa glycoprotein (T. Espevik and B. Naume, unpublished observation) were purified from supernatants of the respective hybridoma cell lines on Sepharose goat anti-mouse IgG as described by the manufacturer (Zymed, San Francisco, CA). Recombinant human TNF (sp. act. 7.6×10^6 U/mg) was supplied by Genentech (South San Francisco, CA), and recombinant human IL-6 (sp. act. >1×10^6 U/mg) was purchased from Genzyme (Cambridge, MA).

Cell line and culture conditions

The following stable, transfected Chinese hamster ovary (CHO)-K1 fibroblast cell lines were generated or have been described elsewhere: CHO-CD14, CHO-K1 transfected with human CD14 (27); CHO-neo, CHO-K1 transfected with pcDNA3/neo (Invitrogen, San Diego, CA); CHO-TLR2 and CHO-TLR4, TLR2 and TLR4 cDNAs cloned into a pFLAG vector (10), gifts from C. Kirchning and M. Rothe (Tularik, San Francisco, CA), were transfected into CHO-K1 cells along with pcDNA3/neo (28) or pEGFP1/neo (Clontech, Palo Alto, CA), respectively. Stable transfectants were selected by cell sorting and limiting dilution cloning. All transfectants were maintained in RPMI 1640 medium (Life Technologies, Paisley, U.K.) with 0.01% l-glutamine, 40 μg/ml gentamicin (referred to as RPMI), 10% heat-inactivated FCS (HyClone, Logan, UT), and 0.5 mg/ml G418 (Sigma, St. Louis, MO) at 37°C and 5% CO2.

Generation of nuclear extracts and EMSA

Transfected CHO cells were seeded in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. Monolayers were then washed twice in HBSS (Life Technologies) and treated with different stimuli in 1 ml RPMI/2% human serum (HS) A *®* (University Hospital, Trondheim, Norway) for the indicated period of time. Nuclear extracts were prepared and analyzed for NF-κB-binding activity with a 32P end-labeled NF-κB-specific oligonucleotide probe as described previously (24), except that autoradiography was performed with a PhosphorImager SF system (Molecular Dynamics, Sunnyvale, CA). The intensity of the bands was quantitated by the PhosphorImager SF system (Molecular Dynamics, Sunnyvale, CA). The intensity of the bands was quantitated by the PhosphorImager SF system (Molecular Dynamics, Sunnyvale, CA).

Flow cytometry analysis

All steps were performed at 0–4°C. Adherent CHO transfectants were detached by trypsin/0.02% EDTA/PBS, washed twice in PBS/1% heat-inactivated FCS, and incubated with anti-FLAG M2 mAb (Sigma) or PBS/ FCS for 45 min. After two washes, the cells were labeled for 30 min with either FITC-conjugated goat anti-mouse mAbs (FITC-GAM; Becton Dickinson, Lincoln Park, NJ), a CD14 mAb (FITC-LeuM3; Becton Dickinson), or the TLR2-associated mAb (TL2.1) conjugated to Alexa 488 fluorochrome (Molecular Probes, Eugene, OR). PBMC were isolated from human A® buffy coats (The Bloodbank, University Hospital, Trondheim, Norway) by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation, which yielded PBMC with <5% contaminating granulocytes. Cells were then washed four times in HBSS and once in PBS/FCS and labeled with Alexa 488-conjugated TL2.1, PE-conjugated CD14 mAb LeuM3 (Becton Dickinson), or both for 30 min. Cells were then washed twice and analyzed with a FACscan flow cytometer (Becton Dickinson).

Metabolic labeling

CHO cells were seeded in 6-well plates and grown to 50–90% confluence. The adherent cells were washed twice with PBS, and labeled with 80–100 μCi TRAN35S-LABEL/Well (ICN Pharmaceuticals, Costa Mesa, CA) in methionine- and cysteine-free DMEM (ICN) containing 10% dialyzed FCS for 45 min. After two washes, the cells were labeled for 16 h at 37°C in 5% CO2. All of the subsequent steps were performed at 0–4°C. Cells were washed once in ice-cold PBS and lysed for 10 min with 400 μl lysis buffer/well (50 mM Tris, 150 mM NaCl, 0.1% BSA, 1% Igepal CA630 (Sigma), 0.5 mM PMSF, pH 7.5). Cells lysates were transfected into Eppendorf tubes and spun down at 10,000 × g for 10 min to remove cell debris. The lysates were preclarified by incubation with 100 μl Sepharose goat anti-mouse IgG for 30 min, and the supernatants were split into two and immunoprecipitated overnight with 30 μl of either TLR2-associated mAb (TL2.1) or control CD14 mAb (3C10) attached to Sepharose goat anti-mouse IgG (5 μg mAb/1 ml Sepharose). Precipitated samples were washed three times in 50 mM Tris, 0.5 M NaCl, 0.1% BSA, and 0.2% Igepal CA630 (pH 7.5), twice in 50 mM Tris and 0.2% Igepal CA630 (pH 7.5), and separated by SDS gel electrophoresis on an 8% gel. Gels were fixed, incubated in Amplify solution (Amersham, Buckinghamshire, U.K.) for 30 min, and dried before exposure to Kodak MR film (Kodak, Rochester, NY) at −70°C.

Stimulation of monocytes

Adherent cell monolayers (1×10^5 monocytes/well/24 well dishes) were prepared from PBMC as described previously (31). The cells were washed three times in HBSS before addition of 10 μg/ml of TLR2-associated mAb (TL2.1), CD14 mAb (3C10), a combination of TL2.1 and 3C10, or a control mAb (6H8) for 30 min at room temperature in RPMI. After addition of the indicated stimuli, incubation proceeded for 8 h at 37°C before supernatants were collected and stored at −20°C until assayed for TNF activity in the WEHI 164 clone 13 bioassay as described (32). Results from one representative experiment are presented as means ± SD of triplicate TNF measurements.

Results

HKLM induce IL-6 production from CHO-TLR2 cells, but not CHO-CD14 cells

Recently, CHO-K1 cells were shown to express mRNA encoding a truncated and nonfunctional TLR2 (33). Based on this and recent studies that suggest TLR2 involvement in cellular responses to Gram-positive bacteria (28, 34), we examined the ability of HKLM and GBS to activate CHO cells transfected with human
TLR2, CHO-neo, CHO-CD14, and CHO-TLR2 cells were exposed to HKLM, GBS, or LPS for 14 h in the presence of 5% HS, and IL-6 production was measured in supernatants. As shown in Fig. 1, LPS induced IL-6 production from CHO-CD14 and CHO-TLR2 cells with similar potency. Addition of HKLM resulted in IL-6 production from CHO-TLR2 cells, but not CHO-CD14 cells (Fig. 1). GBS did not induce IL-6 production in either CHO-TLR2 or in CHO-CD14 cells (Fig. 1). The results indicate that expression of TLR2, but not CD14, is sufficient for activation of CHO cells by HKLM, whereas neither TLR2 nor CD14 make CHO cells responsive to GBS. LPS activates cells in a CD14-dependent manner (4, 27, 35), and CHO-CD14 cells respond to LPS in the absence of TLR2 (33). Thus, CD14 or TLR2 are sufficient, but not necessary, for LPS-induced activation of CHO cells.

**HKLM induce a dose-dependent translocation of NFκB in CHO-TLR2 cells**

In the next series of experiments, CHO-neo and CHO-TLR2 cells were incubated together with increasing amounts of GBS, HKLM, or LPS for 1 h in the presence of 2% HS before nuclear extracts were isolated and analyzed for translocation of the transcription factor NF-κB. As shown in Fig. 2, both HKLM and LPS induced a dose-dependent NF-κB activation of CHO-TLR2 cells. GBS failed to induce NF-κB activation in CHO-TLR2 cells, even at a concentration of 100 μg GBS/ml (Fig. 2). As we have shown that the NF-κB response to GBS cell wall fragments is delayed compared with LPS (24), we confirmed that GBS did not activate CHO-TLR2 cells at any time points measured during a 3-h incubation (data not shown). Control CHO-neo cells were unresponsive to HKLM and GBS, and were hyporesponsive to LPS, comparable to the data presented in Fig. 1 (data not shown). These data suggest that TLR2 differs in the way of recognizing these two types of Gram-positive bacteria.

**Generation of a mAb, TL2.1, that recognizes a TLR2-associated epitope**

To study the biological significance of TLR2 in responses to HKLM, LPS, and GBS, hybridomas producing mAbs recognizing TLR2-associated epitopes were generated. The mAb TL2.1 showed profound binding to CHO-TLR2 cells, but not to CHO-neo, CHO-CD14, or CHO-TLR4 cells (Fig. 3A) suggesting that this mAb specifically recognizes a TLR2-associated epitope. Controls with mAbs to the FLAG-tag or to CD14 confirmed that the cells expressed FLAG-TLR2, FLAG-TLR4, or CD14, respectively, and the lack of binding of TL2.1 to CHO-TLR4 cells further indicate that TL2.1 is not directed toward the FLAG epitope.

To further establish the specificity of TL2.1, lysates from 35S-labeled CHO-neo, CHO-TLR2, and CHO-TLR4 cells were immunoprecipitated with TL2.1 or a CD14 control mAb, 3C10, and subjected to SDS gel electrophoresis. As shown in Fig. 4, one protein band of ~98 kDa appeared only in the CHO-TLR2 lysate immunoprecipitated with TL2.1. The same ~98-kDa band was obtained from CHO-TLR2 cells, but not CHO-neo or CHO-TLR4 cells, when TL2.1 immunoprecipitates were subjected to Western

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**FIGURE 1.** HKLM induce IL-6 production from CHO-TLR2, but not from CHO-CD14 cells. CHO-TLR2, CHO-CD14, and CHO-neo cells were incubated with increasing concentrations of HKLM, LPS, or GBS for 14 h. Supernatants were collected and assayed for IL-6 bioactivity. Background levels of IL-6 for each cell transfectant are subtracted from the data presented. One representative of four experiments is shown (mean ± SD of triplicate wells).

**FIGURE 2.** HKLM, but not GBS, induce NFκB translocation in CHO-TLR2 cells. CHO-TLR2 and CHO-neo cells were exposed to increasing concentrations of HKLM, LPS, or GBS for 1 h before nuclear extracts were prepared and analyzed for NF-κB translocation by a PhosphorImager system (Molecular Dynamics). The intensity of the bands was quantified, and results are presented as relative units.
blot and detection with biotinylated anti-FLAG mAb (data not shown). We further compared the expression of the TL2.1-defined epitope and CD14 on PBMC by incubating PBMC with Alexa 488-labeled TL2.1 and a PE-labeled CD14 mAb (LeuM3). As shown in Fig. 3B, TL2.1 colocalized with LeuM3, and 2% of the PBMC were TL2.1+/LeuM3-. Thus, in PBMC the expression of the TLR2-associated epitope defined by TL2.1 is mainly restricted to monocytes, reinforcing the assumptions that TLR2 serves a role in human innate immunity.

**TL2.1 inhibits HKLM- and LPS-induced IL-6 production from CHO-TLR cells**

To examine the ability of TL2.1 to specifically block TLR2-mediated cell activation, CHO-TLR2 and CHO-CD14 cells were pre-treated with TL2.1 or a control mAb (6H8) before addition of HKLM or LPS. Results in Fig. 5 show that TL2.1 inhibited both HKLM- and LPS-induced IL-6 production from CHO-TLR2 cells with about 60%, but had no inhibiting effect on the LPS-induced activation of CHO-CD14 cells. The control mAb, 6H8, did not affect the IL-6 production induced in either cell transfectants. These data show that the TL2.1 mAb interacts with a TLR2 epitope that is involved in HKLM and LPS signaling in CHO-TLR2 cells.

**HKLM activate human monocytes through a TLR2- and CD14-dependent pathway**

In the next experiments, we studied the inhibitory action of the TLR2-associated mAb (TL2.1) and a CD14 mAb (3C10) on TNF production from human monocytes stimulated with HKLM, GBS, or LPS at serum-free conditions. Both TL2.1 and, to a lesser extent, 3C10, independently inhibited HKLM-induced TNF production, but combining the two mAbs was more effective in blocking the response than adding them individually (Fig. 6). The data indicate that both CD14 and TLR2 are involved in mediating HKLM-induced TNF production from monocytes. Neither TL2.1 nor 3C10 inhibited the GBS-induced TNF production from monocytes (data not shown), which confirms the earlier observations that different mechanisms mediate cell activation by HKLM and GBS.

As previously reported (4), the CD14 mAb 3C10 completely blocked LPS-induced TNF production from monocytes, but the
TLR2-associated mAb TL2.1 did not inhibit TNF production induced by the smooth LPS from *S. minnesota* used in this study (Fig. 6).

**Discussion**

The recognition of invading bacteria or bacterial products by innate immune mechanisms is the first step in a sequence of events leading to a host response intended to eradicate the pathogen. Evolutionary conserved structures on cells of the innate immune system are thought to perform this function, and by now the Toll proteins in *Drosophila* and their homologues in other species, like humans, are good examples in this respect. In *Drosophila*, the Toll protein plays an important role in antifungal defenses (14) while the homologue receptor, 18-Wheeler, serves in antibacterial responses (15). In mammals, a role for TLRs in Gram-negative LPS responses has been demonstrated (10, 11, 13). The present study shows that TLR2 can mediate cell activation by one Gram-positive pathogen, *L. monocytogenes*, but not by another, GBS. Thus, human TLR2 discriminates between similar classes of pathogens. Furthermore, by making a blocking mAb to a TLR2-associated epitope, we found that although TLR2 mediates LPS activation in transfected cell lines, this does not seem to be the main pathway of generating a TNF response in human monocytes stimulated with smooth LPS from *S. minnesota*.

The Gram-positive bacterial surface is composed of similar structures like teichoic acids, lipoteichoic acids (LTA), and the cell wall peptidoglycan, but also dissimilar polysaccharides and proteins (36). Thus, even if common Gram-positive structures activate cells through the same pathways, as the CD14-mediated stimulation by LTA (37) and peptidoglycan (38–40), the signaling mechanism used by whole bacteria can differ according to the complex surface expression of proteins and carbohydrates. This is different from most Gram-negative bacteria, where LPS clearly is the dominant surface component.

TLR2 or CD14 did not mediate GBS-induced activation of either CHO cells or human monocytes. These results are in sharp contrast to TLR2-mediated cellular activation demonstrated in response to HKLM and other Gram-positive organisms, such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (28). We and others have found that GBS use a CR3-dependent pathway to stimulate TNF production from monocytes (M. Cuzzola, et al., manuscript in preparation) (24, 41). Thus, CR3 may serve to bring GBS in closer contact with putative signal transducers similar to CD14 in responses to LPS. The results from this study indicate that TLR2 is not the GBS signal transducer, and as the LPS hyporesponsive and TLR4-defective C3H/HeJ mice respond normally to GBS (24), the identity of the GBS-signalizing component(s) remains to be found.

By use of blocking mAbs to a TLR2-associated epitope and CD14, we demonstrated that HKLM used CD14 and TLR2 to induce TNF production from human monocytes. HKLM differ from GBS in cellular composition. Whereas group- and type-specific polysaccharides cover the encapsulated GBS surface, it has been proposed that HKLM adhere to mammalian cells by proteins called internalins and/or by LTA (25). Abs against CD14 may inhibit the subsequent interaction of LTA on HKLM with TLR2 on the monocytes. The results that expression of CD14 on CHO cells was insufficient to signal cell activation by HKLM could be due to the inability of TLR2 to induce cell activation in these cells (33). Recently, the MD-2 molecule was shown to confer LPS responsiveness by physical association with TLR4 (42). Both MD-2 and TLR4 contain extracellular LRRs, and the possibility exists that CD14 interacts with TLR2 through their LRRs to mediate HKLM signaling. On the other hand, we cannot exclude that TL2.1 and the anti-CD14 mAb inhibited separate pathways in monocytes activated by HKLM.

Both HKLM- and LPS-induced activation of CHO-TLR2 cells was inhibited by the TL2.1 mAb. In contrast to this, TL2.1 failed to inhibit LPS-induced TNF production from human monocytes. However, it cannot be ruled out that other types of LPS than the one used in this study may activate monocytes through TLR2. Recently, Brightbill et al. (43) showed that a TLR2 mAb could inhibit IL-12 production from human monocytes stimulated with LPS from *Salmonella typhosa*. LPS activation through TLR2 has mostly been studied in transfected cell lines (10, 11), and our results indicate that this is not necessarily the main pathway of LPS-induced TNF production in monocytes. TLR4 is a strong candidate as a LPS signal transducer in normal phagocytes, as *Thr4*-mutant or knockout mice show hyporesponsiveness to LPS (9, 13, 22). In addition, TLR4-expressing cell lines become LPS responsive by MD-2 transfection (42).

Our study shows that human TLR2 discriminates between different pathogens. It remains to be shown, however, whether microbial components are true TLR ligands, whether they bind accessory receptor molecules like MD-2 (42), or whether the ligands are generated by some proteolytic cascade initiated by the pathogen (14).

**References**

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