Peptidoglycan- and Lipoteichoic Acid-induced Cell Activation Is Mediated by Toll-like Receptor 2*

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The life-threatening complications of sepsis in humans are elicited by infection with Gram-negative as well as Gram-positive bacteria. Recently, lipopolysaccharide (LPS), a major biologically active agent of Gram-negative bacteria, was shown to mediate cellular activation by a member of the human Toll-like receptor family, Toll-like receptor (TLR) 2. Here we investigate the mechanism of cellular activation by soluble peptidoglycan (sPGN) and lipoteichoic acid (LTA), main stimulatory components of Gram-positive bacteria. Like LPS, sPGN and LTA bind to the glycosylphosphatidylinositol-anchored membrane protein CD14 and induce activation of the transcription factor NF-κB in host cells like macrophages. We show that whole Gram-positive bacteria, sPGN and LTA induce the activation of NF-κB in HEK293 cells expressing TLR2 but not in cells expressing TLR1 or TLR4. The sPGN- and LTA-induced NF-κB activation was not inhibited by polymyxin B, an antibiotic that binds and neutralizes LPS. Coexpression together with membrane CD14 enhances sPGN signal transmission through TLR2. In contrast to LPS signaling, activation of TLR2 by sPGN and LTA does not require serum. These findings identify TLR2 as a signal transducer for sPGN and LTA in addition to LPS.

Infection with bacteria may cause sepsis and lead to septic shock, characterized by refractory hypotension and eventually multiorgan failure and death (1–3). Gram-positive sepsis has been recognized as an important clinical condition (4–6). Its causative agents are cell wall components of Gram-positive bacteria, like peptidoglycan (PGN) and lipoteichoic acid (LTA). PGN, which predominates the cell wall of Gram-positive bacteria, is an alternating β(1, 4) linked N-acetylglucosaminyl and N-acetylmuramyl glycan whose residues are cross-linked by short peptides. LTA is anchored in the membrane by glycolipids found in many Gram-positive bacteria (7). Like lipopolysaccharide (LPS), a component of the outer cell membrane of Gram-negative bacteria, isolated PGN and LTA can elicit most of the clinical manifestation of bacterial infection. These bacterial agents stimulate the excessive release of pro-inflammatory cytokines like tumor necrosis factor, IL-1, and IL-6, and other inflammatory mediators from immune cells, including macrophages, which ultimately cause clinical symptoms (8–10).

The initial event in LPS recognition is its complexation with LPS-binding protein (LBP) in the plasma. This LPS-LBP complex subsequently binds to CD14, which triggers cell activation (1, 11). Because anti-CD14 monoclonal antibodies and LPS antagonists were found to inhibit activation of CD14 positive monocytes by PGN and LPS, CD14 was proposed to be the receptor for both PGN and LPS (8). Consequently, it has been demonstrated that the binding of PGN (12, 13) and LTA (9, 10) to CD14 lead to CD14-dependent cell activation.

Because CD14 is present either as a glycosylphosphatidylinositol-linked membrane receptor lacking an intracellular signaling domain or in soluble form in serum (14), a transmembrane co-receptor has been postulated in LPS responsive cells, which transduces the LPS signal into the cytoplasm (1). Recently, two candidate signal transducing receptors have been identified which are members of the Toll-like receptor (TLR) family, TLR2 and TLR4 (15–19).

Toll controls dorsoventral patterning during embryogenesis in Drosophila melanogaster (20) and is involved in the host defense reactions upon fungal infection in the adult fly (21). Data base searches have identified a family of human Toll homologues, termed Toll-like receptors (22–25). The intracellular domain of TLRs resembles that of the mammalian IL-1 receptor (IL-1R), thereby defining the Toll/IL-1R receptor family. Consistent with this, Drosophila Toll signaling is similar to mammalian IL-1R signaling (26). Several members of the Toll/IL-1 receptor family have been shown to activate NF-κB (15, 19, 22, 24, 27). Overexpression of a constitutively active mutant of TLR4 (also termed human Toll) has been shown to induce the activation of NF-κB in mammalian cells, leading to induction of NF-κB-dependent genes including those encoding inflammatory cytokines and the T cell costimulatory protein B7.1 (24). Although the extracellular domains of the IL-1 receptors identify them as members of the IgG superfamily, the extracellular domains of human Toll-like receptors and Drosophila Toll lack IgG domains and share characteristic repeated leucine-rich motives (25).

Because Drosophila Toll participates in innate immune responses in flies, previous studies examined whether members of this family are involved in these responses in mammalian systems. These studies implicated TLR family members TLR2 and TLR4 as mediators of responses to LPS (15–17, 19). In this study we demonstrate that PGN and LTA from Gram-positive bacteria can activate cells in a TLR2-dependent manner.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Agents—A subclone of the human embryonic kidney HEK293 cell line has been maintained at Tularik, Inc., since 1993 as described (28). Tissue culture experiments were performed in the presence of 10% certified fetal bovine serum (Life Technologies, Inc.) unless stated otherwise. The serum was not heat-inactiv-
vated and contained endotoxin at <10 enzyme units/ml as specified by the manufacturer. Purified preparations of soluble PGN (sPGN) from *Staphylococcus aureus* Rb were used to stimulate cultured cells (4). sPGN was isolated by vancomycin affinity chromatography and the purity of sPGN was established by quantitative chemical analysis, silver stain analysis after polyacrylamide gel electrophoresis, and fluorooautoradiography of sPGN biosynthetically labeled with [14C]alanine. The purity of the sPGN was estimated at ≥98.5%. The average molecular mass was 125,000 Da (4, 29, 30). PGN from *Streptococcus pyogenes* Group A was purchased from Lee Chemicals Inc. (Grayson, GA). LTA preparations from *Bacillus subtilis*, ATCC 6051, *S. pyogenes*, strain IID 698, and *Streptococcus sanguis*, Type I, ATCC10556 were purchased from Tularik Inc. All LTAs applied were prepared using a phenolic extraction procedure (31) and tested for LPS contamination by incubation with polymyxin B sulfate (Calbiochem-Novabiochem Co., La Jolla, CA).

All different PGN and LTA preparations behaved similarly in cell activation experiments. For control stimulation, LPS from *Escherichia coli* serotype O111:B4 prepared by phenol extraction (Sigma) was used. Gram-positive bacteria of the species *S. aureus* ATCC 29213 and *B. subtilis* pV79 (prototroph SPβ) (kindly provided by Dr. Simon Lynch, Tularik Inc.) were plated on Penassay broth agar culture plates, transferred to Luria broth, and cultured by shaking at 37°C overnight. Bacteria were pelleted and washed three times with ice-cold phosphate-buffered saline. Aliquots of 15-fold concentrated bacterial suspensions were frozen at −80°C. The cell titer of bacterial suspensions was 6 × 10^8 cells/ml for *S. aureus* and 9 × 10^6 cells/ml for *B. subtilis*.

**Expression Plasmids**—Expression plasmids for CD14, TLRs, and the TLR truncation mutant were generated as described earlier (15).

**NF-κB Reporter Assays**—Human embryonic kidney HEK293 cells (3 × 10^5) were plated in 3.5-cm dishes and transfected on the following day by the calcium phosphate precipitation method (32) with the indicated amounts of expression vectors, 0.1 μg of ELAM-1 luciferase reporter plasmid (33), and 1 μg of RSV β-galactosidase plasmid for normalization. The cells were lysed, and luciferase activity was determined using protocols and reagents from Promega Co. (Madison, WI). Luciferase activities were calculated as fold induction compared with unstimulated vector control. All bar diagrams are shown as the means ± S.E. for one representative experiment in which each transfection was performed in duplicate. Each experiment was repeated at least twice in all cases.

**RESULTS AND DISCUSSION**

LPS, a component of the outer membrane of the envelope of Gram-negative bacteria, is capable of eliciting immune stimulatory effects similar to whole bacteria in the host organism. Activation of NF-κB by LPS in mammalian HEK293 was shown to depend on the expression of TLR2. This finding prompted us to investigate whether Gram-positive bacteria and their immunologically active components PGN and LTA utilize members of the TLR family to signal cellular activation. Human embryonic kidney HEK293 cells were transiently transfected with a NF-κB-dependent E-selectin promoter luciferase reporter gene. NF-κB-driven reporter gene activation was observed in cells stimulated with whole Gram-positive bacteria of the species *S. aureus* and *B. subtilis* in cells expressing TLR2 but not in cells expressing TLR1 or TLR4 (Fig. 1). To investigate whether PGN and LTA were responsible for NF-κB activation induced by whole Gram-positive bacteria, purified sPGN and LTA were used for subsequent experiments. As previously observed for LPS, no significant induction of reporter gene activity was detected after stimulation with sPGN or LTA in cells transfected with control vector alone (Fig. 2). In addition, expression of CD14 did not mediate responsiveness to sPGN and LTA in the absence of TLRs (Fig. 2). Transient overexpression of TLR4 constitutively activated the reporter gene, and no significant increase in activity was observed upon stimulation with sPGN or LTA (Fig. 2). Transient overexpression of TLR1 and TLR2 caused only marginal reporter gene activation, which was strongly induced by stimulation with sPGN or LTA only in cells expressing TLR2 but not TLR1 (Fig. 2). Again, this is similar to results obtained for LPS stimulation (15). Cells expressing a TLR2 mutant lacking the intracellular domain (TLR2 1–720) did not respond to sPGN or LTA (Fig. 2). Thus, the intracellular domain of TLR2 is essential for signal transduction.

sPGN and LTA induced NF-κB-dependent reporter gene activation in TLR2-expressing cells occurred in a dose-dependent manner (Fig. 3). Coexpression of CD14 with TLR2 led to a slight increase in reporter gene activation upon sPGN stimulation. The effect was more pronounced at lower sPGN concentrations (Fig. 5B). Coexpression of CD14 had no significant effect on LTA stimulation (Fig. 5C). Cellular activation by PGN is CD14-dependent like activation by LPS, whereas LTA induction might be CD14-independent. On the other hand, dependence of cell activation by PGN and LTA on CD14 has been shown for hematopoietic cells on the basis of genetic evidence and CD14 blocking experiments (8–10). HEK293 cells might lack a component of a receptor complex required for full CD14 function. Alternatively the model system in HEK293 cells may resemble a receptor complex for detection of microbial components by cells of nonhematopoietic origin. Although the molecular basis for responsiveness to microbial agents may be sim-
ilar in both cell types (34), differences in recruitment of CD14 and other components may exist for specific stimuli.

It has been demonstrated that LPS signaling is dependent on serum as a source for LBP and/or soluble CD14 (sCD14). Consistent with this, LPS signaling via TLR2 in HEK293 cells is serum-dependent (15, 19) (Fig. 4A). In contrast, removal of serum before stimulation with sPGN or LTA did not inhibit reporter gene activation (Fig. 4, B and C). Under serum-free conditions sPGN and LTA stimulation were even markedly increased compared with serum-containing conditions (Fig. 4, B and C). Serum components might compete for sPGN and LTA binding with cellular receptors and thereby neutralize immunostimulatory activity.

In Drosophila, genes encoding antimicrobial peptides are activated by Toll upon fungal infection (21), whereas the Toll homologue 18-wheeler regulates antimicrobial peptide synthesis in response to bacterial infection (35). Evidence for the existence of mammalian signal transducers with similar function was reported (1). The present study suggests the involvement of TLR2 in the activation of NF-κB by Gram-positive bacteria based on overexpression of TLRs in mammalian HEK293 cells. Our results indicate that TLR2 may not be exclusively a pattern recognition receptor for LPS from Gram-negative bacteria as proposed by others (19) but may also act as a receptor for PGN and LTA from Gram-positive bacteria. This does not exclude differential composition of a receptor complex upon stimulation with different microbial components. Genetic evidence from two different mouse strains, LPS hyporesponsive
C3H/HeJ and C57BL/10ScCr mice, has recently implicated TLR4 (human Toll) to be crucial for immune responses toward Gram-negative infection (16, 17). Interestingly, differences in the cellular response to LPS and components of Gram-positive bacteria were reported for one of these mouse strains. C3H/HeJ mice harbor a single inactivating point mutation of TLR4, which results in hyporesponsiveness to LPS. However, C3H/HeJ mice respond normally to stimulation with preparations from Gram-positive group B streptococci type III as evaluated in vivo, whereas C3H/HeJ mice are not (37). Reduced responsiveness to bacterial dextran B512 in C57BL/10ScCr mice may be due to an additional genetic defect as indicated earlier (38). Therefore the contribution of individual members of the TLR family to signaling by bacterial or other microbial stimuli in vivo remains to be elucidated. Nevertheless, the implication of TLR2 in detection and signal transduction of PGN and LTA by host cells provides further insight into the mechanisms of innate immune response to Gram-positive infection.

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