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Cutting Edge: Recognition of Gram-Positive Bacterial Cell Wall Components by the Innate Immune System Occurs Via Toll-Like Receptor 2[1]

Atsutoshi Yoshimura,* Egil Lien,* Robin R. Ingalls,* Elaine Tuomanen,† Roman Dziarski,‡ and Douglas Golenbock[2]*

Invasive infection with Gram-positive and Gram-negative bacteria often results in septic shock and death. The basis for the earliest steps in innate immune response to Gram-positive bacterial infection is poorly understood. The LPS component of the Gram-negative bacterial cell wall appears to activate cells via CD14 and Toll-like receptor (TLR) 2 and TLR4. We hypothesized that Gram-positive bacteria might also be recognized by TLRs. Heterologous expression of human TLR2, but not TLR4, in fibroblasts conferred responsiveness to *Staphylococcus aureus* and *Streptococcus pneumoniae* as evidenced by inducible translocation of NF-κB. CD14 coexpression synergistically enhanced TLR2-mediated activation. To determine which components of Gram-positive cell walls activate Toll proteins, we tested a soluble preparation of peptidoglycan prepared from *S. aureus*. Soluble peptidoglycan substituted for whole organisms. These data suggest that the similarity of clinical response to invasive infection by Gram-positive and Gram-negative bacteria is due to bacterial recognition via similar TLRs. *The Journal of Immunology*, 1999, 163: 1–5.

Bacterial infection typically results in activation of the innate immune system. Although bacteria differ in the composition of their cell walls, the host reaction to invasion is remarkably similar regardless of the species or type of bacterium. Invasion of the bloodstream by both Gram-positive bacteria and Gram-negative bacteria cause the sepsis syndrome in humans. This syndrome results from the induction of cytokines and other inflammatory mediators and is characterized by alterations in temperature, pulse, hemodynamic instability, and end organ damage. Conservative estimates suggest that nearly 400,000 Americans develop bacteremia, and that 70,000 of these individuals will directly die of the sepsis syndrome (1, 2).

The outermost leaflet of the outer membrane of the Gram-negative bacterial cell wall consists of LPS, a toxic moiety that appears to be the cause of immune activation. Gram-positive bacteria, in contrast, do not contain a single constituent that is as clearly linked to the sepsis syndrome. Nevertheless, the interest in how Gram-positive bacteria activate the immune system is intense, fueled in large part by the enormous clinical significance of Gram-positive infections. The pneumococcus, for example, is a leading cause of death with a mortality rate in otherwise healthy elderly individuals of 40% (3). Staphylococcal infection is the major cause of bacteremia in US hospitals today (4). Together, these two species of bacteria account for nearly 75% of all antibiotic usage in the United States.

Although the exact mechanism of immune activation by Gram-positive bacteria remains unknown, recent studies of immune activation by bacterial LPS provide a clue. The family of Toll proteins appears to be responsible for specific immune recognition in *Drosophila melanogaster*. For example, the Toll homologue known as 18-wheeler is responsible for responses to Gram-negative bacteria (5), whereas Toll regulates antifungal responses (6). Yang et al. (7), and Kirschning et al. (8) recently demonstrated that a human homologue of Toll, known as Toll-like receptor 2 (TLR2),[3] apparently functions as an LPS signal transducer when transfected into LPS nonresponder cell lines. This activity of TLR2 was potentiated by CD14, the LPS-binding receptor. Additional evidence that TLRs function as LPS signal transducers comes from positional cloning of *Lps*, the genetic locus for LPS sensitivity that is abnormal in C3H/HeJ mice. *Lps* mapped to the same region as TLR4 (9, 10). TLR4 cloned from the C3H/HeJ mouse proved to harbor a point mutation that rendered it nonfunctional (9–11), consistent with the concept the mutant TLR4 might function as a dominant-negative mutation accounting for LPS hyporesponsiveness in the C3H/HeJ mouse. Indeed, the LPS hyporesponder phenotype of C3H/HeJ mice is so profound that, despite the LPS signaling capability of TLR2, it seems likely that TLR4 is the major

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; PGN, peptidoglycan; sPGN, soluble PGN; CHO, Chinese hamster ovary fibroblasts.

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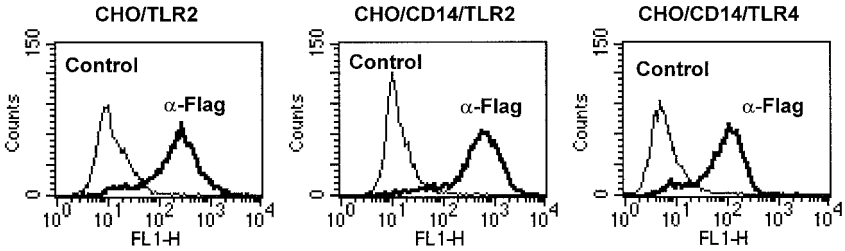

FIGURE 1. Expression of human Toll receptors in CHO cells. Clonal CHO cell lines transfected with FLAG-epitope-tagged cDNA were stained by indirect immunofluorescence using anti-Flag mAb and analyzed by flow cytometry. "Control" represents the same cells stained only with FITC-anti-IgG^{mu}.

mammalian LPS signal transducer. This suggests the hypothesis that the true role of TLR2 is the recognition of other bacterial ligands that in some way are similar to LPS.

Like Gram-negative bacteria, major components of the Gram-positive bacterial cell wall employ CD14 for immune recognition. Both peptidoglycan (PGN) and lipoteichoic acid have been demonstrated to activate macrophages in a CD14-dependent manner (12, 13). Given the similarity in responses to exposure to Gram-positive bacteria and Gram-negative bacteria, and the common dependence on many of their cell wall products upon CD14, we hypothesized that the downstream elements of the signal transduction system might consist of common genetic elements. We report here that the coexpression of CD14 and human TLR2 resulted in the recognition of two distinct and clinically important genuses of Gram-positive bacteria. In contrast, TLR4 appears to be excluded as a component of a receptor system involved in the recognition of these types of bacteria. Furthermore, the recognition of these bacteria, at least in part, occurs via the PGN skeleton. The use of common receptor systems suggests that the often observed clinical parallels between Gram-positive and Gram-negative bacterial infection result from the activation of similar signal transduction systems.

Materials and Methods

Reagents

PBS, α-MEM, and Ham's F-12 were obtained from BioWhittaker (Walkersville, MD). Heat-inactivated FBS (LPS < 10 pg/ml) was obtained from Summit Biotechnology (Fort Collins, CO). Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). G418 was obtained from Life Technologies (Gaithersburg, MD). Hygromycin B was obtained from Calbiochem (San Diego, CA). Anti-CD25 mAb conjugated with FITC was obtained from Becton Dickinson (Bedford, MA). Recombinant human IL-1β was purchased from Genzyme (Cambridge, MA). The soluble PGN (sPGN) released by *Staphylococcus aureus* Rb in the presence of penicillin was purified as described previously (14), prepared at 2 mg/ml in PBS, and stored at −20°C. Before use, the suspensions were thawed and sonicated in an 80-W sonicator bath (Lab Supply, Hicksville, NY) for 1 min. All other reagents were obtained from Sigma (St. Louis, MO).

Cell lines

All cell lines were grown as adherent monolayers at 37°C in a 5% saturated CO_2 atmosphere, and were passaged at least twice weekly to maintain logarithmic growth. The engineering of the CD14-expressing Chinese hamster ovary (CHO)-K1 reporter fibroblast cell line CHO/CD14.elam.tac, also known as clone 3E10, has been previously described in detail (15). This clonal line has been cotransfected with CD14 and a NF-κB-dependent reporter plasmid that drives the expression of surface CD25 Ag resulting from LPS-, TNFα-, or IL-1β-induced NF-κB translocation. The cDNAs for human TLRs 2 and 4 were the gifts of Carsten Kirschning and Mike Rothe (Tularik, South San Francisco, CA), and were cloned into the vector pFLAG as described (8). Stable expression of TLRs was obtained by cotransfection of these epitope-tagged plasmids with pcDNA3 (Invitrogen, San Diego, CA) or pRL/RSV/puro (gift of R. Kitchens, University of Texas Southwestern Medical Center, Dallas, TX) into CHO-K1 wild-type cells or CHO/CD14 reporter cells (16). After selection in G418 (1 mg/ml) or puromycin (50 μg/ml), clonal cell lines expressing high levels of human TLR2 or TLR4 were derived using fluorescent-activated cell sorting combined with limiting dilution cloning. In addition, CHO-K1 or 3E10 (CHO/CD14.elam.tac) reporter cells were transfected with pcDNA3 as a control.

Bacterial strains, growth, and preparation

S. aureus (ATCC 25923) was grown in LPS-free α-MEM. *Streptococcus pneumoniae* (D39) and its pneumolysin-defective derivative (17) were grown in Brain Heart Infusion Broth (Remel, Lenexa, KS) supplemented with horse blood (3.3%, Remel) and β-diphosphopyridine nucleotide (2 μg/ml) (Anderson's broth). The cells were grown to mid-logarythmic phase (OD_{620} = 0.4) and washed twice with PBS (BioWhittaker). The determination of cell density was made by limiting dilution of washed bacteria. Bacteria were resuspended in PBS, killed by incubation at 95°C for 20 min, and stored at −20°C until use.

Flow cytometry analysis of CHO transfectants

Adherent monolayers of CHO cells were plated in 12-well tissue culture dishes at a density of 2.5×10^5 cells per well. After overnight incubation, the cells were stimulated for 18 h with various ligands. Cells were detached from the surface with trypsin/EDTA and assessed by flow microfluorometry for the presence of surface CD25 exactly as described (15).

Analysis of NF-κB translocation

Cells were plated in six-well tissue culture dishes at a density of 5×10^5 per well. After overnight incubation at 37°C in 5% CO_2, the cells were stimulated for 45 min. Cells were washed in PBS, and nuclear extracts were prepared and analyzed using the EMSA exactly as described (18).

Results and Discussion

To examine the potential role of TLRs in responses to bacteria, clonal cell lines were engineered in which epitope-tagged chimeric proteins were expressed at high levels. Although little is known about endogenous TLR expression in CHO cells, we have recently discovered that these cells do not express a functional TLR2 transcript; in contrast, CHO cells express a functional mRNA encoding TLR4 (19). As shown in Fig. 1, the levels of tagged TLRs in CHO/CD14 cells and CHO-K1 cells were comparable.

We exposed CHO transfectants to heat-killed *S. aureus* for 45 min and subsequently subjected nuclear extracts from these cells to the EMSA to assess for the presence of induced NF-κB, a transcription factor that is involved in cytokine regulation. No nuclear translocation of NF-κB was observed in either CHO/Neo or CHO/CD14. In contrast, CHO/TLR2 cells were activated with heat-killed *S. aureus* at the highest concentration tested (Fig. 2*A*). In view of the known role of CD14 in potentiating the effects of the Gram-positive cell wall constituents lipoteichoic acid and PGN (13, 20), we compared the responses of these cell lines to a clonal line expressing both TLR2 and CD14. A highly synergistic response was observed, as demonstrated in the gel-shift mobility assays on bacteria-exposed cells shown in Fig. 2*A*. Although no response to staphylococcus was observed at 10^8 cfu/ml in CHO/TLR2 cells, and only a modest response was observed at 10^9 cfu/ml, coexpression of CD14 with TLR2 resulted in a strong response at the lowest inoculum tested. These data suggest that at least some components of Gram-positive bacteria that are recognized by TLR2 are also ligands for CD14.

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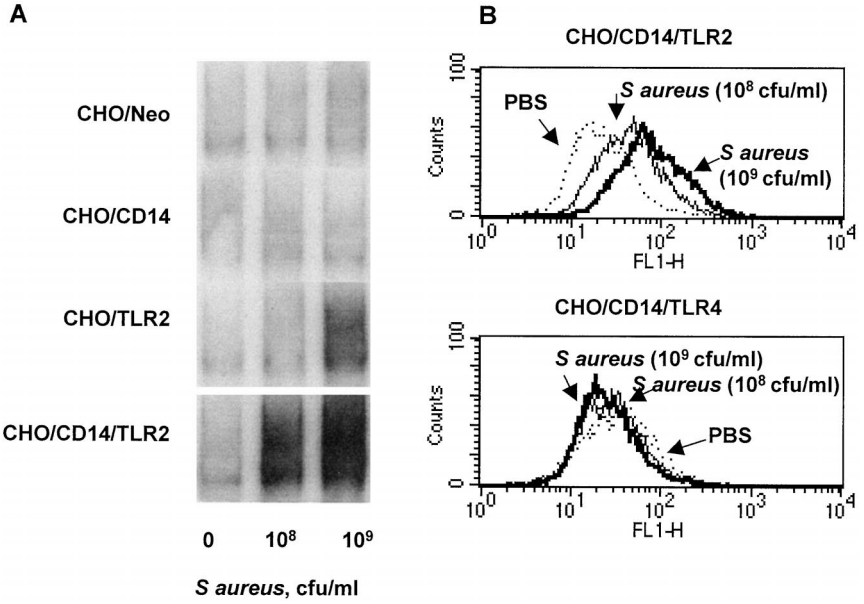

FIGURE 2. TLR2, but not TLR4, expression imparts responsiveness to *S. aureus* synergistically with CD14. *A*, CHO/Neo, CHO/TLR2, CHO/CD14, and CHO/CD14/TLR2 were treated with PBS or stimulated with heat-killed *S. aureus* (10^8 or 10^9 CFU) for 45 min. Nuclear extracts from these cells were assessed for the presence of NF-κB using the EMSA. Shown are the NF-κB/^{32}P-labeled probe complexes. *B*, CHO/CD14/TLR2 and CHO/CD14/TLR4 reporter cell lines that express surface CD25 as a result of NF-κB translocation (15) were exposed to either PBS or heat-killed *S. aureus* for 18 h. The cells were stained with FITC-labeled anti-CD25 mAb and subjected to flow cytometric analysis for transgene expression. Not shown are all cell lines responded equivalently to TNF-α (10 ng/ml) and IL-1β (5 ng/ml).

In *D. melanogaster*, Toll may subserve different functions from its homologues. Proper expression of Toll results in normal antibacterial responses to fungal challenge (6), whereas the homologous receptor, 18-wheeler, is necessary for responses to Gram-negative bacteria (5). Therefore, we sought to determine whether other TLRs might have defined functions that differed with TLR2 with respect to Staphylococcal recognition. To date, five TLRs have been cloned; TLRs 2 and 4 form a cluster of highly homologous genes (21). Therefore, we examined several clonal cell lines that expressed human TLR4 with CD14. Heat-killed *Staphylococcus aureus* did not activate the NF-κB reporter construct in any of the CHO/CD14/TLR4 cells (e.g., Fig. 2*B*). Similar results were observed when the same cells were tested and analyzed by gel-shift assay (data not shown). The failure of TLR4 to mediate responses to Gram-positive cell wall products might have been predicted based upon the prior observation that C3H/HeJ mice, which express a mutant form of TLR4 (9), responded to cell wall preparations from *S. aureus* (22). Next, we tested the responses of the CHO/TLR cell lines using a second Gram-positive bacterium for challenge. We chose heat-treated *S. pneumoniae* as a stimulus because of the important role this pathogen plays in human disease. Unlike heat-killed *S. aureus*, 10^8 CFU of heat-killed *S. pneumoniae* partially stimulated CHO/CD14 cells (Fig. 3*A*, *top panel*) in the absence of TLR overexpression. However, careful experiments where increasing concentrations of pneumococcus were used as a stimulant demonstrated that the dose necessary for a 50%

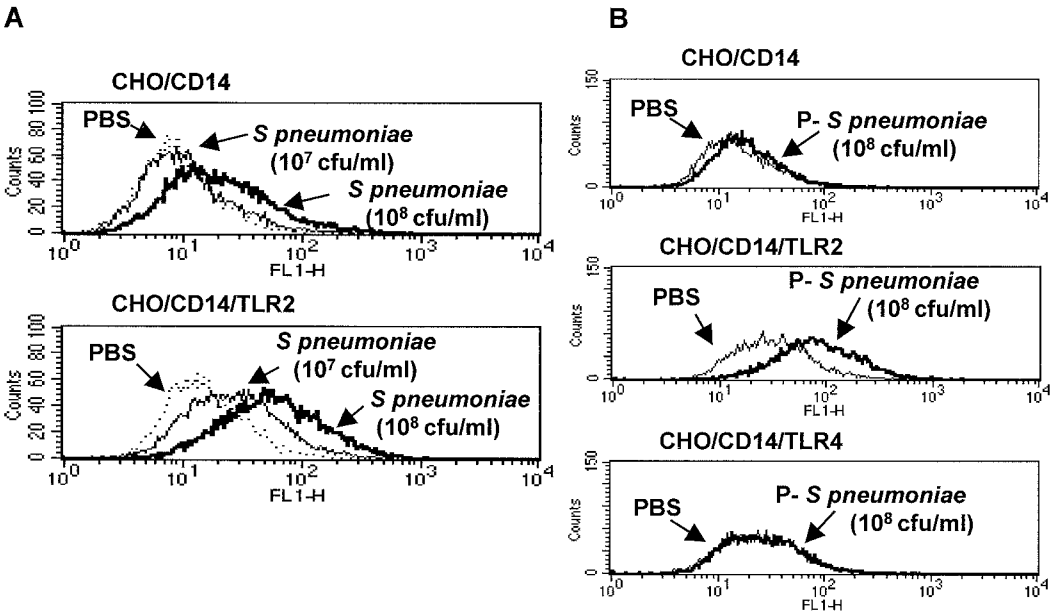

FIGURE 3. TLR2, but not TLR4, mediates cellular activation by heat-killed *S. pneumoniae*. *A*, CHO/CD14 and CHO/CD14/TLR2 reporter cells were treated with PBS or with a clinical strain of heat-killed *S. pneumoniae* for 18 h. The cells were stained with FITC-labeled anti-CD25 mAb and subjected to flow cytometric analysis for the expression of the NF-κB-dependent transgene (CD25). *B*, Because of the potential role of pneumolysin as a nonspecific activator of cells, a *ply* mutant of *S. pneumoniae* (P-) was used to activate CHO/CD14 reporter cells and its derivative human TLR-expressing cell lines. After 18 h, cells were again analyzed for CD25 expression by flow microfluorometry.

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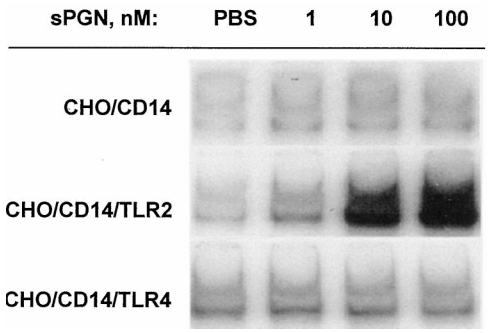

FIGURE 4. Soluble PGN from *S. aureus* activates TLR2 but not TLR4 transfected CHO/CD14 cells. Clonal cell lines expressing CD14 and human TLRs were stimulated with PBS or Staphylococcal sPGN (1, 10, or 100 nM) for 45 min. Nuclear extracts were prepared, and the nuclear levels of NF-κB were determined by the gel shift assay.

maximal response was reduced by 30- to 100-fold in cell lines that coexpressed CD14 and TLR2 (Fig. 3*A*, *bottom panel*), suggesting that *S. pneumoniae* stimulated both a TLR2-dependent pathway and a TLR2-independent pathway.

The pneumococcus has been reported to secrete a toxin known as pneumolysin, a pore-forming cytolysin (23) that nonspecifically activates cytokine production from immune cells (24). To test the hypothesis that TLR2-independent activation of the reporter cell line was due to pneumolysin, we tested a mutant strain of *S. pneumoniae* in which the *ply* gene was knocked out. Exposure of the CHO/CD14/TLR2 cell lines to *ply* mutants of the pneumococcus resulted in NF-κB translocation (data not shown) and reporter cell transgene activation as measured by surface CD25 expression (Fig. 3*B*). CHO/CD14/TLR4 cells, in contrast, did not respond to this mutant *S. pneumoniae*. Indeed, in all respects that we can measure, the *ply* mutant strain of *S. pneumoniae* are immunologically identical to *S. aureus* in that recognition requires TLR2.

To investigate which cell wall components of Gram-positive bacteria are responsible for the activation of the transfectants, we exposed the cells to purified sPGN from Staphylococcal cell walls. This cell wall preparation was released from *S. aureus* Rb by penicillin (average M_r = 125,000) and affinity purified on a vancomycin column (14). Quantitative analysis of the PGN demonstrated that ≥98.5% of the mass was accounted for by amino acids and amino sugars. LPS content by *Limulus* assay was ≤90 pg/mg. We observed the same pattern of recognition of PGN as was observed with the whole organisms: while CHO/CD14 cells had no response, expression of TLR2, but not TLR4, rendered these cells responsive to PGN (Fig. 4). Furthermore, we have observed strong TLR2-dependent responses to a separately prepared PGN preparation (gift of W. Fischer, Universitat Erlangen-Nurnberg, Erlangen, Germany) from a strain of *S. pneumoniae* (25).

LPS is a ubiquitous contaminant of aqueous solutions, and one possibility that would explain the response of CHO/CD14 in the absence of TLR overexpression was that LPS contaminated the cell suspension. This seemed unlikely, because CHO/CD14 cells respond to concentrations of LPS as low as 10 pg/ml (D. Golenbock, unpublished data). The lack of response in CHO/CD14 cells to *S. aureus*, *S. pneumoniae*, or sPGN is strong evidence that these preparations of whole bacteria are not contaminated with LPS.

The discovery that *Drosophila* Toll, a primitive receptor with IL-1-receptor homology (26), imparts some degree of pathogen specificity was a clue that similar molecules in mammals might account for the ability of the host to recognize and respond to so many dissimilar organisms. Like 18-wheeler in the fly, TLR4 may be more specific for Gram-negative bacteria and their LPS, whereas other TLRs might have other patterns of ligand recognition. There are currently at least four TLRs that have been identified in flies; given the relative complexity of the *Drosophila* genome compared with human, there might prove to be several dozen mammalian TLRs.

It is tempting to speculate that like the IL-1R (27), TLRs might form heterodimeric complexes upon ligand binding. The specificity for one bacterial product over another might then be best accounted for by which TLRs comprise the signaling receptor. With dozens of potentially available TLRs, the ability of immune cells to recognize a diverse array of stimuli would be very large. Coexpression of TLRs with more specific binding receptors such as CD14, might further define and expand the repertoire of the innate immune system.

Although the evidence that TLRs actually bind bacterial products remains to be convincingly elucidated, it seems likely that these receptors directly interact with their pathogenic targets. Despite our efforts, we were unable to observe the direct binding of bacteria to TLRs. Although there are numerous technical reasons why such an experiment might not produce a predicted result, other possibilities need to be explored. One prominent possibility is that Ag exposure results in the processing of an endogenous ligand that activates TLRs, much like the proteolytic peptide spatzle is thought to be the true ligand for Toll receptors in *Drosophila* (28). Whatever the picture that ultimately emerges for how TLRs function to provide specificity of recognition for diverse types of bacteria, the use of a common family of signaling receptors by seemingly diverse bacteria may explain why the clinical picture of sepsis caused by Gram-negative bacteria and Gram-positive bacteria is nearly identical.

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