Cellular Activation, Phagocytosis, and Bactericidal Activity Against Group B Streptococcus Involve Parallel Myeloid Differentiation Factor 88-Dependent and Independent Signaling Pathways[1]

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Group B streptococci (GBS) vigorously activate inflammatory responses. We reported previously that a secreted GBS "factor" activates phagocytes via Toll-like receptor (TLR)2 and TLR6, but that GBS cell walls activate cells independently of these receptors. We hypothesized that the phagocytic immune functions in response to GBS, such as inflammation, uptake, and elimination of bacteria, occur through a coordinated engagement of TLRs, along with the coreceptors CD14 and CD11b/CD18. Using various knockout mice we show that GBS-induced activation of p38 and NF-κB depends upon the expression of the cytoplasmic TLR adapter protein, myeloid differentiation factor 88 (MyD88), but not TLR2 and/or TLR4. Macrophages with deletions of CD14 and complement receptor 3 had a normal cytokine response to whole bacteria, although the response to GBS factor was abrogated in CD14-null cells. The intracellular formation of bactericidal oxygen species proved to be MyD88 dependent; however, uptake of GBS, a prerequisite for intracellular killing by O_2 radicals, occurred independently of MyD88. While deletion of complement receptor 3 greatly diminished the uptake of opsonized GBS, it did not affect the formation of bactericidal O_2 radicals or inflammatory signaling intermediates. We conclude that the inflammatory, bactericidal, and phagocytic responses to GBS occur via parallel but independent processes. *The Journal of Immunology*, **2002, 169: 3970–3977.**

Group B streptococcus (GBS)[3] remains the major cause of sepsis in newborn infants as well as a significant cause of sepsis in adults in the U.S. The estimated annual incidence of GBS sepsis in the U.S. is 17,400; ~10% of these patients will die (1). In addition, GBS is the third most frequent cause of bacterial meningitis (2).

The primary responsibility for limiting the invasion of bacteria that colonize mucosal surfaces belongs to the innate immune system. This is particularly true of GBS infections in preterm infants who lack a competent adaptive immune response (3). An effective first-line defense must be accomplished in the earliest stages of microbial invasion before the microbial sensing system is overwhelmed by large numbers of invading bacteria (4). Tissue macrophages are positioned at the interface between mucous membranes or skin and the vascular system. These macrophages have an especially rich diversity of receptor proteins complementing the diversity of microbial molecules that they are likely to encounter, often in the context of soluble opsonins such as complement or LPS-binding protein. Two membrane proteins, the β_2-integrin CD11b/CD18 (complement receptor 3 (CR3)) and the glycoprotein CD14, have been suggested to be integral parts of receptor complexes essential for proinflammatory signaling and have been implicated in the activation of the innate immune response by GBS (5–11). However, neither CD14, which is attached to the cell membrane by a glycosyl phosphatidylinositol anchor, nor CD11b/CD18, which has no known direct inflammatory signaling capabilities, can be expected to actually transfer the binding signal to a cytoplasmic signaling cascade (12, 13). Hence, an additional signaling protein for GBS can be predicted to exist, similar to that proposed for other bacterial products such as *Escherichia coli* LPS (14).

The discovery of mammalian orthologs of *Drosophila* Toll led to the identification of Toll-like receptor (TLR) 4 as the principal LPS signal transducer (15–19). We recently found that the cell walls of GBS induced secretion of TNF-α from macrophages, and that this stimulation was absolutely dependent upon the expression of myeloid differentiation factor 88 (MyD88) (8), a cytoplasmic Toll-IL-1R domain-containing protein that localizes to transmembrane TLRs. MyD88 expression appears to be obligatory for TLR-mediated cytokine responses (20–23), although some LPS-inducible responses (e.g., NF-κB translocation) seem to be mediated by an alternative adapter molecule: the MyD88 homolog Mal/TIRAP (24, 25). Mal/TIRAP may require the expression of MyD88 for optimal function, as it forms heterodimers with MyD88. It is not

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[3] Abbreviations used in this paper: GBS, group B streptococcus; GBS-F, GBS factor; MyD88, myeloid differentiation factor 88; TLR, Toll-like receptor; TIRAP, Toll-like IL-1R domain-containing adapter protein; CHO, Chinese hamster ovary; MAP, mitogen-activated protein; CR3, complement receptor 3; DHR, dihydrorhodamine.

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yet established whether other receptors besides TLR4 share Mal/TIRAP or whether all TLR4 ligands use it.

The finding that GBS cell wall-mediated activation was MyD88 dependent suggested that one or multiple TLRs were involved. In contrast to the GBS cell walls, a heat-labile extracellular factor of GBS (designated "GBS-F"), engaged both TLR2 and TLR6 as essential signaling molecules (8). The determination of the TLR(s) that recognizes GBS cell walls has proved more difficult. We did not observe an impaired cytokine response to GBS in mutant mice with targeted deletions of the individual TLRs 1, 2, 4, or 6, all of which seemed like potential participants in the response to GBS. While this was a surprising finding, particularly in view of the role that is commonly assigned to TLRs 2 and 4 in the recognition of microbial products, a functional reciprocal compensation of these TLRs remained a possibility that was not addressed.

TLRs and MyD88 are now widely believed to mediate a variety of signals in response to bacterial exposure, and their expression vastly improves immunologic outcome as measured by subsequent bacterial burden or survival after bacterial challenge (22). The response to bacteria includes the physical processes associated with bacterial clearance (e.g., phagocytosis) and the production of soluble host factors (e.g., cytokines and antibacterial molecules such as superoxide and NO) necessary to kill bacteria. Deficiencies of either of these important processes during infection may result in substantial morbidity or even death. We hypothesized that these apparently distinct events were tied together by the innate immune system through the use of TLRs and their associated signal transduction systems. This hypothesis proved, in fact, to greatly underestimate the complexity of these interrelated immune mechanisms.

In this study, we attempted to accomplish three goals that might elucidate the interrelationship of biological systems necessary to clear bacterial infection. First, we investigated the extent of MyD88 involvement in the generation of proinflammatory signals and toxic antibacterial molecules in response to whole GBS, testing whether the suggested signaling partners of TLRs, CD14 and CD11b/CD18, contribute to this response. Second, we tested whether the normal phenotypic response of cells from select mice with deletions of individual TLRs was due to redundancy and reciprocal compensation by other TLRs. Specifically, we analyzed the response of macrophages from mice with a combined deletion of TLRs 2 and 4, both of which have recognition capabilities for Gram-positive bacterial cell walls (26). Third, we investigated whether MyD88 and TLRs mediate the process of bacterial clearance by coordinating the uptake of microbial particles or inducing the formation of bactericidal oxygen species.

Materials and Methods

Reagents

PBS, Ham's F-12 medium, RPMI 1640, and trypsin-versene mixture (trypsin-EDTA) were purchased from BioWhittaker (Walkersville, MD) and low-endotoxin FBS was purchased from Summit Biotechnologies (Greeley, CO). Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). Puromycin was purchased from Sigma-Aldrich (St. Louis, MO), and G418 was purchased from Life Technologies (Gaithersburg, MD). Protein-free LPS from *E. coli* K 235 was a gift from S. Vogel (Uniformed Services University of the Health Sciences, Bethesda, MD) and soluble peptidoglycan from *Staphylococcus aureus* was a gift from R. Dziarski (Northwest Center for Medical Education, Indiana University School of Medicine, Gary, IN).

Cell lines

The Chinese hamster ovary (CHO)-K1 fibroblast-derived cell lines CHO/CR3 and CHO/TLR2 have been described (12, 28). The CHO/CR3-TLR2 cell line was derived from CHO/CR3 cells by stable transfection with human TLR2 in the pFLAG vector together with the puromycin^r-containing plasmid pRL/RSV/puro (gift of R. Kitchens, University of Texas Southwestern Medical Center, Dallas, TX) as described (28). CHO cell lines were grown in Ham's F12 medium containing FBS (10% v/v) and ciprofloxacin (10 μg/ml). CHO/CR3 cells were cultured with 0.5 mg/ml G418 and CHO/CR3/huTLR2 cells were cultured with G418 and 50 μg/ml puromycin.

Bacterial strains and the generation of bacterial supernatants and heat-inactivated GBS

GBS type III strain COH1, initially isolated from a newborn infant with sepsis, has been previously described (29, 30). Unless otherwise stated, experiments were performed with this strain. All strains were grown on blood agar plates (Remel, Lenexa, KS). Bacterial colonies were removed from the plates after overnight culture, washed three times in PBS, and then used to inoculate chemically defined medium (31) prepared from endotoxin-free water and grown to mid-log phase (ABS_{650} = 0.27–0.30). For the preparation of GBS-F, cultures were clarified of bacteria by centrifugation and filtered (0.2-μm pore diameter). Heat-inactivated GBS was prepared from mid-log phase cultures, washed once with PBS, resuspended in pyrogen-free PBS at a density of 3×10^{10} CFU/ml, and heat inactivated for 30 min at 80°C. The *Streptococcus pneumoniae* type 2 strain D39 (32) was grown to mid-log phase in chemically defined medium plus 0.03% choline chloride (Sigma-Aldrich) and heat inactivated. Cell-free supernatants and heat-killed GBS were stored at −80°C for further use. Endotoxin was not detectable in the above preparations according to a *Limulus* assay with a sensitivity of <0.6 EU/ml (BioWhittaker).

Peritoneal macrophages

MyD88$^{-/-}$, TLR2$^{-/-}$, TLR2$^{+/-}$, TLR9$^{-/-}$, CD14$^{-/-}$, and CR3$^{-/-}$ mice were engineered as described (22, 26, 33, 34). The generation of the TLR2$^{-/-}$TLR4$^{-/-}$ double knockout mice was achieved by interbreeding the respective single knockout animals. MyD88$^{-/-}$, TLR2$^{-/-}$, TLR2$^{+/-}$, TLR2$^{-/-}$TLR4$^{-/-}$, TLR9$^{-/-}$, CD14$^{-/-}$, CR3$^{-/-}$, and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were injected i.p. with 2.5 ml of a 3% thioglycolate solution (Remel). After 72–96 h, peritoneal exudate cells were harvested by lavage with RPMI 1640 medium containing 10% FBS and 10 μg/ml ciprofloxacin or autologous serum for CD14$^{-/-}$ mice. The cells were washed with medium, counted in a hemocytometer, and plated. After 24–72 h, nonadherent cells were removed by washing with medium and adherent cells were stimulated.

Measurement of proinflammatory activity of CHO cells and peritoneal macrophages

Nuclear translocation of NF-κB was determined as follows. CHO cells were seeded at a density of 5×10^5 cells/well in six-well dishes in Ham's F12 medium supplemented with 5% FBS plus 10 μg/ml ciprofloxacin and incubated overnight. On the following day, cells were incubated for 2 h as indicated in the figures. Peritoneal exudate macrophages (1×10^6/well) were stimulated in an identical manner. Nuclear extracts were isolated and analyzed for binding to a ^{32}P-labeled NF-κB-specific oligonucleotide by EMSA, exactly as described (35). For determination of TNF-α, peritoneal exudate cells were seeded at a density of 1×10^6 cells/ml in 96-well dishes in RPMI 1640 medium with 10% FBS plus 10 μg/ml ciprofloxacin and incubated over 16 h at 37°C in a 5% humidified CO_2 environment. Supernatants were processed directly for the determination of released TNF-α by ELISA (R&D Systems, Minneapolis, MN) per the manufacturer's protocols.

Flow cytometric analysis of surface expression of CD11b, CD18, and TLR2

Cells were plated at a density of 7×10^4/well in 24-well dishes overnight. The following day, the cells were harvested with trypsin-EDTA (BioWhittaker), centrifuged at 800 × *g* for 5 min, and labeled with FITC-labeled mAb to human CD11b or FITC human CD18 mAb (BD Biosciences, Mountain View, CA) in PBS/1% FBS for 30 min on ice as described (27). TLR2 expression was assessed by indirect immunofluorescence with a human TLR2 mAb (36). The cells were analyzed by flow cytometry on a FACScan microfluorometer. Data were analyzed with CellQuest software (BD Biosciences).

FITC labeling of GBS and determination of internalization

Heat-inactivated GBS (3×10^9/ml) were incubated with 0.3 mg/ml FITC for 60 min on a rotating platform. FITC-labeled GBS were washed four times in PBS and homogenous distribution of FITC labeling was confirmed by FACS analysis. Twelve-well dishes were plated with 1×10^5 CHO cells/well or 2×10^5 murine peritoneal macrophages/well. CHO cells were incubated on the following day, and peritoneal exudate macrophages were incubated after 72 h, with FITC-labeled GBS. CHO cells and peritoneal exudate macrophages were incubated in HAM's F12 or RPMI plus 10% FBS. If indicated, FBS was replaced by fresh human serum as a source of

complement. For opsonization, PBS-washed GBS were incubated in 10% serum from mice immunized with GBS type III capsular polysaccharide as a source of specific streptococcal Ab (37) plus 50% human serum as a source of complement for 30 min at 37°C. After incubation, cells were washed, incubated for 60 s with 0.2% trypan blue (to quench extracellular fluorescence) (38), washed again, scraped into suspension with a rubber policeman, and fixed with 2% paraformaldehyde. The number of cells positive for FITC-GBS was determined by FACS.

Formation of peroxynitrate in peritoneal macrophages

Peritoneal exudate macrophages (2×10^5/well) were plated in 12-well tissue culture dishes. After 72 h, cells were incubated simultaneously with GBS and 0.15 μg/ml dihydrorhodamine (DHR)123 (Molecular Probes, Eugene, OR) as indicated. In selected experiments, 2.5 or 25 μM of the proteasome inhibitor MG-132 (Calbiochem, San Diego, CA) were added together with GBS and DHR123 to assess the influence of NF-κB activation on the formation of peroxynitrate. After a 2-h incubation, the reaction was terminated by washing the cells with ice-cold PBS. The cells were detached with a rubber policeman, fixed with 2% paraformaldehyde, and analyzed by flow cytometry. The results are presented as the "fold induction" of peroxynitrate-induced reduction of DHR123, defined as the median fluorescence of the sample treated with the stimulus plus DHR123 divided by the median fluorescence of DHR123 treatment alone.

Results

Differential induction of NF-κB and TNF-α by GBS and S. pneumoniae *in murine macrophages*

GBS has been shown to induce significant amounts of proinflammatory cytokines from macrophages, including TNF-α (10, 36). Nuclear translocation of NF-κB is suggested to be a critical step in this process (10, 39) and is often used as an experimental surrogate for the measurement of cytokine production. We attempted to compare the relative potential of GBS to induce TNF-α with its potential to activate NF-κB. For comparison we chose *S. pneumoniae*, an encapsulated species that is the most common cause of bacterial pneumonia (40). GBS induced extremely high levels of TNF-α secretion at bacterial cell densities that were at least 100-fold lower than those required of *S. pneumoniae* (Fig. 1, *upper panel*). The maximal TNF-α concentrations induced by GBS were higher than those induced by *S. pneumoniae*. Furthermore, GBS induced nuclear translocation of NF-κB at lower concentrations (10^6 CFU/ml) than *S. pneumoniae* (10^7 CFU/ml); however, this difference was less pronounced than were the differences observed in induced cytokine release (Fig. 1, *lower panel*). Thus, the cytokine response to GBS was—in contrast to *S. pneumoniae*—poorly reflected by NF-κB activity, suggesting qualitatively different modes of transcriptional activation between these organisms.

Targeted deletion of MyD88 significantly impairs immune responses to GBS

The secretion of TNF-α in response to inactivated whole GBS depends on the expression of the cytoplasmic adapter protein, MyD88 (8). Despite this suggestive observation, we have yet to identify a specific TLR that transfers the GBS-triggered activation signal across the membrane to the cytoplasmic signaling cascade. Moreover, all of the microbial components that we have tested so far, including LPS, peptidoglycan, and lipoproteins, have also required the expression of MyD88 for the induction of measurable TNF-α secretion. In contrast, microbial ligands have been reported to vary significantly in their ability to activate other signaling events in MyD88$^{-/-}$ cells, including kinase activation and NF-κB translocation (20, 41). We wondered whether these differences were due to an isolated defect in the cytokine response or whether other GBS-induced signaling pathways are impaired in MyD88-deficient cells. Thus, we compared a variety of functions in peritoneal macrophages from normal and MyD88-deficient cells.

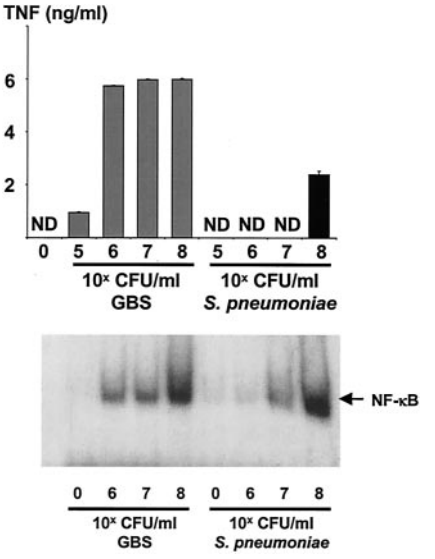

FIGURE 1. Differential induction of the transcription factor NF-κB and TNF-α by GBS and *S. pneumoniae* in mouse peritoneal macrophages. *Upper panel*, Peritoneal macrophages from C57BL/6 mice were incubated with graded concentrations of inactivated GBS or *S. pneumoniae* over 18 h, and TNF-α concentrations were determined in the supernatants. Depicted are the means ± SD of triplicate wells from one representative experiment of three. ND, Not detected. *Lower panel*, Macrophages were stimulated as indicated over 2 h, nuclear extracts were prepared, and EMSAs with ^{32}P-labeled oligonucleotides comprising the NF-κB binding site were performed. Depicted is one representative experiment of three.

First, we assessed the induction of nuclear translocation of NF-κB, a proinflammatory transcription factor that is activated in response to GBS in immune cells. Translocation of NF-κB was impaired in MyD88$^{-/-}$ cells, after both 1 and 2 h of stimulation (Fig. 2, *upper panel*), although longer exposure of the EMSA gels to film revealed a small amount of NF-κB translocation in MyD88-null cells following a 2-h stimulation with GBS (data not shown). Thus, GBS initiates NF-κB translocation and activation by a process that is primarily (although not entirely) MyD88 dependent. Next, we tested whether the mitogen-activated protein (MAP) kinase p38 was phosphorylated in response to GBS in MyD88$^{-/-}$ macrophages. Phosphorylation of p38

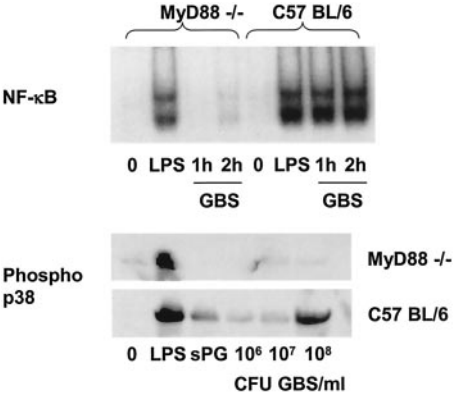

FIGURE 2. Impaired translocation of NF-κB and phosphorylation of the MAP kinase p38 in peritoneal macrophages from MyD88$^{-/-}$ mice in response to GBS. *Upper panel*, Peritoneal macrophages from MyD88$^{-/-}$ and C57BL/6 mice were incubated with GBS over 1 and 2 h and nuclear extracts were analyzed for content of NF-κB (EMSA) as outlined in Fig. 1. *Lower panel*, Macrophages were stimulated over 30 min, and cytosolic extracts were immunoblotted for activated p38 with pp38-HRP mAb. sPG, Soluble peptidoglycan from *S. aureus*.

was severely impaired in GBS-stimulated MyD88$^{-/-}$ cells, as with the induction of NF-κB translocation. However, LPS normally induced phosphorylation of p38 in these cells (Fig. 2, *lower panel*). This finding is in agreement with the reported finding that LPS activates p38 in a MyD88-independent fashion (20).

Deletion of TLR9 or the combined deletion of TLR2 and TLR4 does not significantly impair TNF responses to GBS

MyD88 constitutes an intermediate for the communication of transmembrane signals resulting from the binding of ligand to Toll/IL-1R family members (42). We have previously excluded individual TLRs 1, 2, 4, and 6 as essential for the inflammatory response to whole, heat-killed GBS by using macrophages from mice with targeted genetic deletions (8). We wondered whether TLR9 might serve as a receptor for GBS, because TLR9 has been shown to mediate the innate response to a bacterial component, CpG DNA motifs, in vitro (43). However, TLR9$^{-/-}$ macrophages exhibited a normal TNF-α response to GBS organisms (data not shown), suggesting that neither the bacterial DNA nor any other as-yet-unknown TLR9 ligand is responsible for GBS-induced immune stimulation.

In addition, we used a mouse with combined targeted mutations in both TLR2 and TLR4, because the overwhelming number of bacteria that have been tested appear to engage one of these two receptors. The list of specific bacterial ligands purified from bacteria that engage either TLR is extensive and includes peptidoglycan and bacterial lipoproteins for TLR2 (26, 44, 45) and lipoteichoic acid for TLR4 (26); more recent studies suggest that highly purified lipoteichoic signals primarily via TLR2 (46). In fact, all known Gram-positive and Gram-negative bacteria are thought to contain one or more of these common molecules. The purpose of this experiment was to determine whether GBS might activate redundant TLR pathways, such as a combination of TLR4/TLR2 heterodimers and TLR homodimers. In other words, we were concerned that the results of experiments using single knockout animals might be misleading due to the potential ability of one TLR to compensate for the lack of the other. However, the release of TNF-α from GBS-stimulated peritoneal macrophages harvested from mice carrying a combined null mutation for TLR2 and TLR4 was normal (Fig. 3), whereas these cells did not respond to LPS (Fig. 3) or bacterial lipoproteins (data not shown). Thus, while heat-treated GBS retain potent stimulatory activity and their cell walls contain molecules that are well-described ligands for TLR2 and TLR4, neither of these TLRs is essential for the stimulation by GBS.

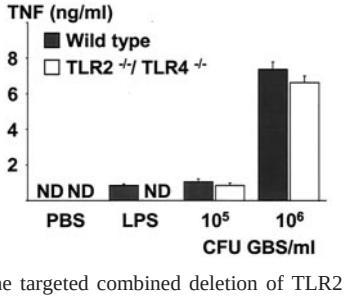

FIGURE 3. The targeted combined deletion of TLR2 and TLR4 does not affect the TNF-α response to GBS. Peritoneal macrophages from TLR2$^{-/-}$TLR4$^{-/-}$ and wild-type C57BL/6 mice were incubated with inactivated GBS over 18 h and TNF-α concentrations were determined in the supernatants. Depicted are means ± SD of triplicate wells from one representative experiment of three. ND, Not detected.

Neither CD11b/CD18 nor CD14 expression is required for proinflammatory response initiated by GBS

The glycoprotein CD14 exists in two forms, as a lipid-anchored membrane protein of 53 kDa and as an "anchor-free" 48-kDa soluble serum protein (47). Both forms have been shown to bind to diverse microbial components such as LPS and peptidoglycan of *S. aureus* and to greatly amplify proinflammatory signals in response to these ligands and to *S. pneumoniae* (28, 48, 49). We were interested in a potential role for CD14 on GBS-induced macrophage activation, because TLRs have been reported to colocalize with CD14 and CD14 has been suggested as part of the GBS-receptor complex (50, 51).

We cultured macrophages harvested from CD14 knockout mice in medium that contained autologous serum to avoid even brief exposure of the cells to soluble CD14. Experience with these cells has shown that limited exposure to soluble CD14 is sufficient to partially reconstitute CD14-dependent signal transduction (K. Moore, D. T. Golenbock, and M. Freeman, unpublished data). We found a normal cytokine response (TNF-α, NF-κB, and p38) from GBS-exposed macrophages compared with wild-type controls (Fig. 4 and data not shown). In sharp contrast to whole GBS organisms, the responses to soluble peptidoglycan from *S. aureus*, *E. coli* LPS, and GBS-F, a recently reported heat-labile soluble TLR2 ligand that is released by GBS (8), were entirely dependent on the expression of CD14 (Fig. 4).

CD11b/CD18, also known as CR3, is a member of the β_2 family of integrins and is highly expressed on phagocytic leukocytes. CR3 binds to numerous bacteria and bacterial products and has been suggested as a signaling molecule for diverse microbial structures, including GBS (7, 9–11). In fact, GBS-induced stimulation of cytokine release from human mononuclear cells was found to be diminished when these cells were treated with mAb to CR3 before GBS exposure (5).

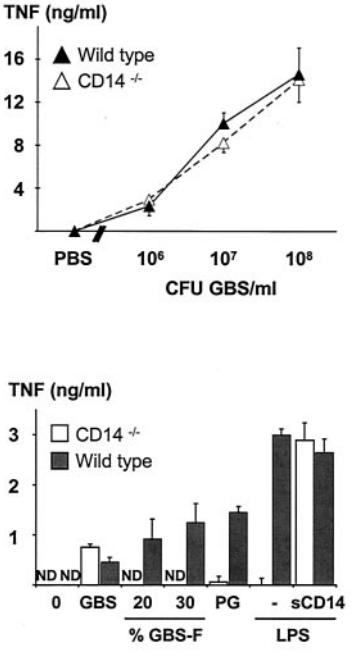

FIGURE 4. Expression of CD14 is essential for the inflammatory response to the soluble GBS-F, but not to whole heat-killed GBS. Peritoneal macrophages from CD14$^{-/-}$ and wild-type C57BL/6 mice were incubated with GBS (*upper panel*), GBS-F, 10^5 CFU GBS/ml, 10 μg/ml soluble peptidoglycan (PG), or 10 ng/ml LPS with or without 2 μg/ml soluble CD14 (*lower panel*) over 18 h, and TNF-α concentrations were determined in the supernatants. Depicted are means ± SD of triplicate wells from one representative experiment of three. ND, Not detected.

Accordingly, we tested whether the targeted deletion of CD11b would diminish the proinflammatory response to GBS. Peritoneal macrophages harvested from CD11b knockout animals have no detectable CD18, consistent with the observation that virtually the entire population of β_2 integrins expressed on wild-type peritoneal macrophages appears to be in the form of CR3. Thus, the CD11b knockout mouse has little or no CD11a, CD11b, CD11c, or CD11d on the cell surface (52). Despite the previous findings that Ab to CR3 inhibited GBS-induced activation of human monocytes, GBS-treated CR3-null macrophages exhibited normal proinflammatory responses, including induced translocation of NF-κB, phosphorylation of p38, and release of TNF-α (Fig. 5*A* and data not shown).

A complementary approach to the use of genetically modified animals is to heterologously express membrane receptors in cell lines that ordinarily lack both protein expression and function. Therefore, we performed a series of experiments designed to address whether heterologous expression of CR3 conferred responsiveness upon CHO cells exposed to whole bacteria. We stably transfected a previously reported CHO/CR3 cell line (28) with the cDNA for human TLR2 to be certain not to miss any heretofore-unappreciated cooperative effect of these receptors with TLR2. As a result, this cell line expressed high levels of three membrane proteins: CD11b, CD18, and TLR2 (Fig. 5*B*). Despite the coexpression of TLR2 and CR3, we did not observe translocation of NF-κB upon stimulation with as much as 10^8 CFU/ml heat-killed GBS, which corresponds to a dry weight of 180 μg/ml bacteria. In contrast, CHO/CR3-TLR2 cells responded to IL-1β and a control TLR2 ligand, nOspA from *Borrelia burgdorferi* (Fig. 5*C* and data not shown).

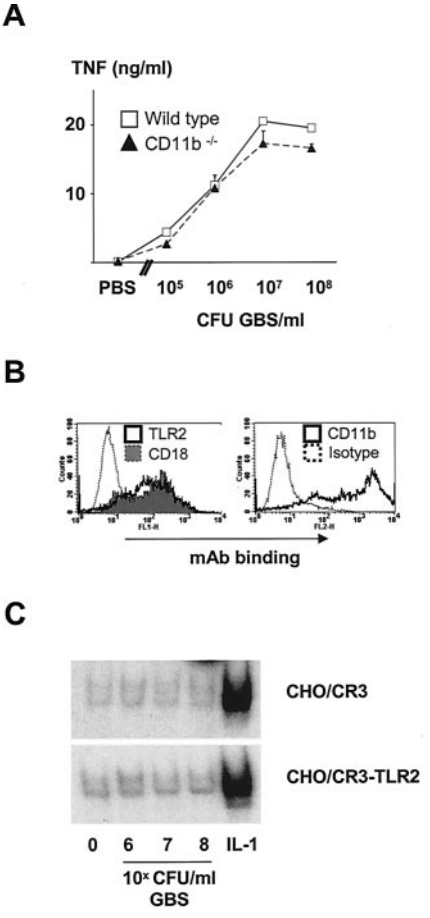

FIGURE 5. Expression of CD11b does not influence the activation of macrophages or heterologously transfected CHO cells by GBS organisms. *A*, Peritoneal macrophages from CD11b$^{-/-}$ and wild-type C57BL/6 mice were incubated with inactivated GBS, and TNF-α concentrations in the supernatants were determined as outlined in Fig. 2. *B*, CHO cells were stably transfected with human CD11b, CD18, and TLR2. Depicted is the surface expression of the respective proteins as assessed by indirect immunofluorescence. *C*, CHO/CR3 and CHO/CR3-TLR2 cells were stimulated with inactivated GBS or IL-1β (5 ng/ml), and nuclear extracts were analyzed for NF-κB (EMSA) as outlined in Fig. 1.

Internalization of GBS depends on CR3 expression but not on expression of TLRs or MyD88

Little is known about the role of TLRs and MyD88 in bacterial clearance. The removal of bacteria from soft tissues, as well as the bloodstream, depends upon phagocytosis, a process that involves a series of specialized signals beginning with the detection of bacteria on the cell surface. Localization of TLR2 to the phagosome upon uptake of zymosan particles (53) suggests that two major classes of innate immune receptors, i.e., those involved in particle uptake and those involved in signal transduction, cooperate to mediate host defense. However, it is still not known how phagosome localization relates to the signal transduction function of TLRs. In other words, it is still unclear whether TLRs mediate the formation and function of phagolysosomes or whether the redistribution of TLRs upon microbial uptake and the subsequent formation and function of phagosomes represent independent events.

The cytoplasmic adapter molecule MyD88 appears to interact with all transmembrane receptors that have Toll-like IL-1R domains via homophilic interactions (8, 20, 22). In view of the important role of MyD88 in GBS-induced cell activation, we hypothesized that it would also be important for bacterial internalization. We assessed whether macrophages that lack MyD88 expression differed from normal macrophages with respect to the internalization of GBS by using a FACS-based assay that determined the percentage of macrophages with internalized FITC-labeled bacteria. The extracellular fluorescence of FITC-labeled particles was quenched with trypan blue, a method that distinguishes intracellular from extracellular bacteria (12). Surprisingly, and in striking contrast to the dramatic phenotype of these cells with respect to the induced activation of proinflammatory mediators, MyD88 expression had no effect on the ability of macrophages to internalize GBS (Fig. 6*A*). Similarly, the deletion of TLR2, TLR4, or CD14 did not influence GBS uptake by peritoneal macrophages from knockout mice compared with wild-type controls (data not shown).

We then tested whether the deletion of CR3 impaired the uptake of GBS by macrophages. We found that nonopsonic uptake of GBS occurred independently of CR3 and was not FcR mediated, as it occurred under serum-free conditions (1% BSA; data not shown). In contrast, uptake was more than doubled in wild-type macrophages that were incubated with GBS that had been opsonized with specific GBS antiserum. This enhanced uptake of opsonized bacteria was not observed in CD11b$^{-/-}$ macrophages (Fig. 6*B*).

We determined next whether transfection with TLR2 and CR3 (CHO/TLR2, CHO/CR3, and CHO/CR3-TLR2) enabled nonprofessional phagocytes to internalize GBS. We observed rapid uptake of GBS by CR3-expressing CHO cells in the presence of complement-rich human serum, whereas the expression of TLR2 did not affect GBS uptake (Fig. 6*C*).

Opsonization of GBS appeared to be a prerequisite for the uptake of GBS by CHO/CR3 cells, as we observed no significant uptake of GBS in the absence of complement (data not shown). Thus, we hypothesized that opsonization and uptake of GBS would trigger proinflammatory signaling pathways in these cells. However, we failed to observe translocation of NF-κB under conditions that enabled these cells to dramatically internalize large numbers of bacteria (data not shown). In summary, nonopsonic uptake of

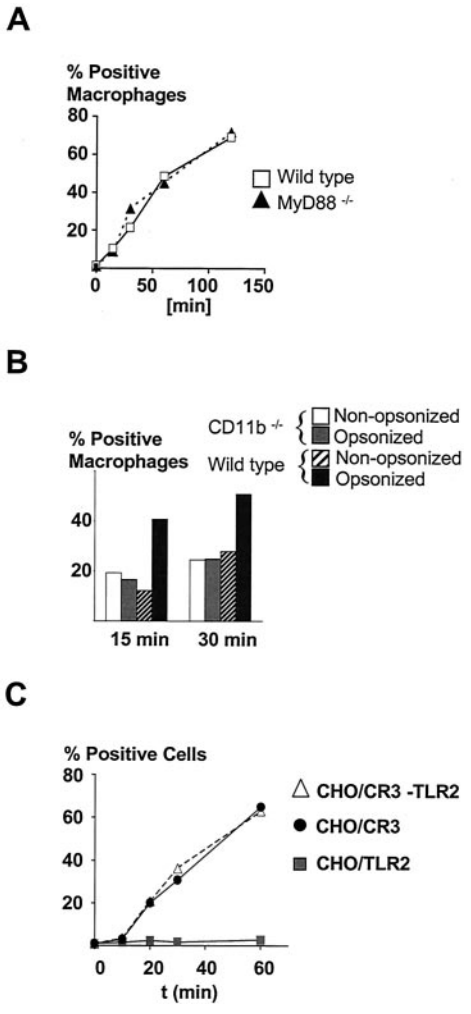

FIGURE 6. Expression of CD11b is critical for the opsonic uptake of GBS. Peritoneal macrophages from MyD88$^{-/-}$ mice (*A*, ▲) and C57BL/6 wild-type mice (*A*, □) or CD11b$^{-/-}$ mice and C57BL/6J wild-type mice (*B*) were incubated with FITC-labeled GBS (in *B*, 10^8 CFU/ml) over the indicated time periods. *B*, Inactivated GBS were preincubated for 30 min in the presence of mouse anti-GBS serum to fix complement on the bacterial surface. *C*, CHO/TLR2, CHO/CR3, and CHO/CR3-TLR2 were incubated with 10^8 CFU/ml FITC-labeled GBS over the indicated time periods. Cells were washed, extracellular fluorescence was quenched by incubation with 0.2% v/v trypan blue, and the number of cells positive for internalized FITC-GBS was determined by FACS. Depicted are representative results of three experiments for each condition described above.

GBS does not require CR3, MyD88, TLR2/4, or CD14. However, CR3 mediates the internalization of opsonized GBS. Finally, the phagocytic uptake of GBS appears to occur independently of MyD88-dependent proinflammatory signals.

Intracellular formation of toxic oxygen species, a bactericidal activity, in response to GBS is dependent on MyD88 but independent of NF-κB activation

Our previous results demonstrated that MyD88$^{-/-}$ cells internalize GBS normally but are severely impaired in proinflammatory signaling in response to GBS. In diametrical contrast, CR3-deficient cells exhibited a significant defect in GBS uptake but were activated by the presence of GBS normally. Accordingly, we investigated how the deletion of both receptors would affect intracellular bactericidal activity.

We used a surrogate marker of toxic oxygen radical generation, i.e., the oxidation of DHR to the fluorochrome rhodamine by oxygen species (mainly peroxynitrate, a product from NO and superoxide) (54). Synthesis of NO resulting from transcriptional activation of the inducible NO synthase has been shown to be an NF-κB-dependent process (55). However, inhibition of NF-κB with the proteasome inhibitor MG-132 exhibited no effect on the formation of GBS-induced peroxynitrate (data not shown). Hence, peroxynitrate formation at the time points studied (1 and 2 h) appears to be NF-κB independent. Although peroxynitrate formation is only a surrogate marker of bactericidal activity, a major advantage of this FACS-based assay is that the measurement of peroxynitrate can be assessed on a cell-by-cell basis and is not substantially influenced by experimental variations in the number of bacteria or phagocytes used in each testing condition.

Deletion of MyD88 (Fig. 7), but not of CR3 (data not shown), impaired the intracellular formation of toxic oxygen species. Furthermore, macrophages from CD14$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ knockout mice exhibited a normal phenotype when tested for inducible peroxynitrate production (data not shown). While it may seem logical a priori that the ingestion of bacteria by wild-type macrophages directly signals the production of bactericidal molecules, in fact this is not the case. Thus, GBS clearance via CR3-mediated phagocytosis and MyD88-dependent free radical production can be resolved experimentally as independent processes.

Discussion

The common route of a potentially life-threatening GBS infection in newborn infants is via perinatal aspiration of commensal bacteria that colonize the female genital tract. Shortly after the inoculation of bacteria into the parenchyma of the lung, GBS may achieve a density as high as 10^9–10^{11} CFU/g lung tissue, as confirmed in the primate model of neonatal GBS pneumonia (56). This enormous localized bacterial burden probably results from the relatively inefficient clearance of GBS by the incompletely developed neonatal immune system (57). Large numbers of bacteria both directly damage the lung parenchyma and indirectly exacerbate problems by triggering the overexuberant production of inflammatory mediators. The lung damage, the associated bacteremia, and the subsequent cytokine storm are often deadly.

The efficient removal of GBS from both the lung parenchyma and the bloodstream is a critical task for the infected neonate, particularly because GBS elicits a far greater proinflammatory response in phagocytes than other common bacterial causes of Gram-positive pneumonia and sepsis such as *S. pneumoniae* (Fig. 1). The survival of the infected child depends on the removal of free bacteria from the lung tissue and the bloodstream and on host bactericidal activity. All of these essential host functions are enhanced by the induction of cytokines and other immune mediators and facilitated by the generation of

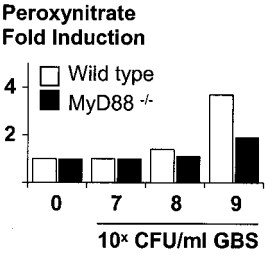

FIGURE 7. MyD88 mediates the generation of toxic oxygen species in response to GBS. Peritoneal macrophages from wild-type mice (open bars) or MyD88$^{-/-}$ mice (filled bars) were incubated with 10^7–10^9 CFU/ml GBS over 120 min in the presence of DHR123. The cells were washed, and the induced fluorescence was determined by FACS. Depicted is fold induction of DHR123 in presence of GBS compared with auto-oxidation of DHR123 alone. Shown are representative results of three experiments each.

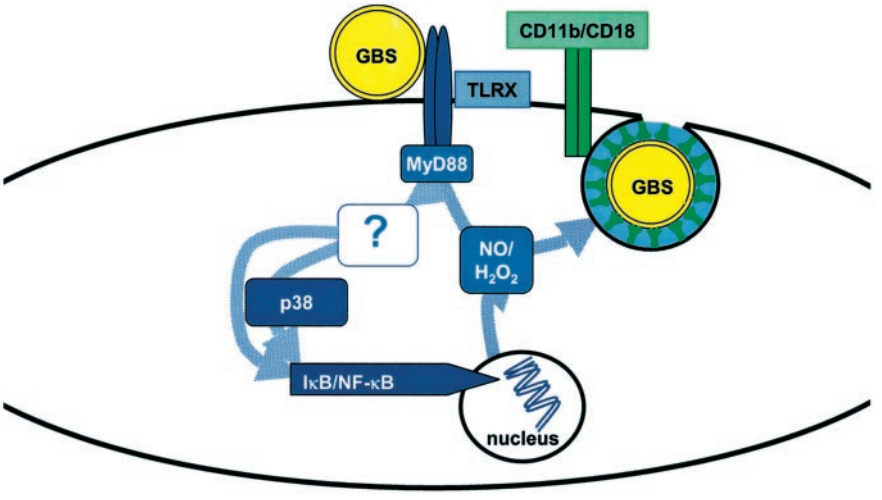

FIGURE 8. Model for the engagement of phagocytic surface proteins by GBS organisms resulting in discrete activation of interrelated pathways. GBS organisms activate at least two receptor entities to mediate inflammatory signaling and bacterial clearance. First, GBS organisms engage a TLR beyond TLRs 1, 2, 4, 6, and 9 for the activation of MAP kinases and transcription factors, as well as bactericidal oxygen species, as indicated by the dependence of these pathways on the expression of MyD88. Second, CD11b/CD18, but not MyD88, is involved in the uptake of opsonized GBS organisms without mediating proinflammatory or further bactericidal activity.

antimicrobial metabolites. This complicated process involves many unique but interrelated events. For example, the generation of intracellular oxygen radicals in this setting would not be beneficial to the infected host in the absence of phagocytosis. Thus, it seemed a safe assumption that the innate immune mechanisms responsible for bacterial recognition, phagocytosis, and bacterial killing are tightly coordinated via pattern recognition receptors and the associated downstream signaling molecules that have been shown individually to contribute to each of these processes.

Indeed, we have found that deletion of MyD88, an adapter protein for TLR, abrogates or severely reduces a host of activated signaling events and end points such as phosphorylation of MAP kinases and transcriptional activation of inflammatory genes. Furthermore, the formation of toxic oxygen species was impaired in MyD88$^{-/-}$ mice in response to GBS. These oxygen products are an essential intracellular weapon to kill internalized bacteria (58), and their reduced formation in MyD88$^{-/-}$ cells likely is reflected in the impaired ability of these cells to eliminate GBS. However, contrary to our expectations, the striking role of MyD88 in inflammatory signaling and antibacterial activity in response to GBS was not paralleled by a role in internalization. MyD88-deficient and wild-type macrophages internalized GBS similarly. Accordingly, our data do not suggest global coordination of these innate immune responses, but they do suggest that molecules known to be activated during phagocytosis, including protein kinase C and one of its major substrates, myristoylated alanine-rich C kinase substrate, as well as the cytoskeletal protein paxillin (as reviewed in Refs. 59 and 60), are unlikely to be impaired upon exposure to GBS by cells from MyD88 knockout mice.

TLRs share MyD88 as an essential adapter protein with IL-1R and IL-18R. This dual role of MyD88 raises the possibility that the effects that were observed in wild-type but not mutant mice were due to the blocking of the secondary autocrine IL-1/18 effects in response to GBS. However, it seems unlikely that the impressive phenotype of MyD88$^{-/-}$ cells in response to GBS was due to an interrupted feedback loop via cytokine receptors. First, several immediate events, including the phosphorylation of p38, were observed to be severely impaired long before released IL-1/18 would have been expected to exert autocrine function. Second, IL-1 and IL-18 are very poor activators of TNF in macrophages (Ref. 61 and P. Henneke and D. Golenbock, unpublished observations). Third, LPS induces a powerful IL-1 response in peritoneal macrophages but only weakly stimulates the formation of peroxynitrate when compared with the MyD88-dependent effect of GBS.

Despite all indications that TLRs mediate the responses of macrophages to GBS, we have not yet identified a specific TLR that mediates the proinflammatory response to the outer wall components of this organism. An important exception is the release by GBS of a heat-labile proteinaceous factor. This protein, designated GBS-F (8), induces NF-κB via CD14, TLR2, and TLR6. However, the cell wall preparations that were used for the studies reported in this work were subjected to heat treatment and washing. Thus, a significant effect of GBS-F on TLRs can be excluded. We report experiments using two newly available transgenic mice with targeted deletions of additional TLR genes, including mice lacking TLR9, the receptor for bacterial DNA (CpG motifs) (43), and mice lacking both TLR2 and TLR4. Macrophages from neither mouse showed a significantly decreased cytokine response to GBS. Thus, we have now excluded all of the TLRs that have been reported as potential receptors for subcellular components of Gram-positive bacteria (TLRs 1, 2, 4, 6, 9) as being responsible for the excessive proinflammatory response to GBS. These data also suggest that the inflammatory contribution of peptidoglycan within the cellular response to insoluble bacterial cell wall material may be overrated.

We conclude that MyD88 and an as-yet-unidentified TLR (or TLRs) mediate bactericidal activity in response to GBS without being involved in the uptake of whole bacteria (Fig. 8). Thus, while our original hypothesis was that TLRs were capable of integrating a variety of related antibacterial processes, in fact, activation of signaling cascades, bacterial uptake, and the generation of bactericidal molecules by immune cells under attack by GBS appear to be discrete and independent pathways.

References

1. Schuchat, A., T. Hilger, E. Zell, M. M. Farley, A. Reingold, L. Harrison, L. Lefkowitz, R. Danila, K. Stefonek, N. Barrett, et al. 2001. Active bacterial coresurveillance of the emerging infections program network. *Emerg. Infect. Dis. 7:92.*
2. Schuchat, A. 1999. Group B streptococcus. *Lancet 353:51.*
3. Lagergard, T., K. Thiringer, L. Wassen, R. Schneerson, and B. Trollfors. 1992. Isotype composition of antibodies to streptococcus group B type III polysaccharide and to tetanus toxoid in maternal, cord blood sera and in breast milk. *Eur. J. Pediatr. 151:98.*
4. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immunity. *N. Engl. J. Med. 343:338.*
5. Cuzzola, M., G. Mancuso, C. Beninati, C. Biondo, F. Genovese, F. Tomasello, T. H. Flo, T. Espevik, and G. Teti. 2000. β_2 integrins are involved in cytokine responses to whole Gram-positive bacteria. *J. Immunol. 164:5871.*
6. Cuzzola, M., G. Mancuso, C. Beninati, C. Biondo, C. von Hunolstein, G. Orefici, T. Espevik, T. H. Flo, and G. Teti. 2000. Human monocyte receptors involved in tumor necrosis factor responses to group B streptococcal products. *Infect. Immun. 68:994.*
7. Goodrum, K. J., L. L. McCormick, and B. Schneider. 1994. Group B streptococcus-induced nitric oxide production in murine macrophages is CR3 (CD11b/CD18) dependent. *Infect. Immun. 62:3102.*
8. Henneke, P., O. Takeuchi, J. A. van Strijp, H.-K. Guttormsen, J. A. Smith, A. B. Schromm, T. A. Espevik, S. Akira, V. Nizet, D. L. Kasper, and

D. T. Golenbock. 2001. Novel engagement of CD14 and multiple Toll-like receptors by group B streptococci. *J. Immmunol. 167:7069.*
9. Ingalls, R. R., B. G. Monks, R. Savedra, Jr., W. J. Christ, R. L. Delude, A. E. Medvedev, T. Espevik, and D. T. Golenbock. 1998. CD11/CD18 and CD14 share a common lipid A signaling pathway. *J. Immunol. 161:5413.*
10. Medvedev, A. E., T. Flo, R. R. Ingalls, D. T. Golenbock, G. Teti, S. N. Vogel, and T. Espevik. 1998. Involvement of CD14 and complement receptors CR3 and CR4 in nuclear factor-κB activation and TNF production induced by lipopolysaccharide and group B streptococcal cell walls. *J. Immunol. 160:4535.*
11. Perera, P. Y., T. N. Mayadas, O. Takeuchi, S. Akira, M. Zaks-Zilberman, S. M. Goyert, and S. N. Vogel. 2001. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR)4 to elicit full lipopolysaccharide and taxol-inducible gene expression. *J. Immunol. 166:574.*
12. Ingalls, R. R., M. A. Arnaout, and D. T. Golenbock. 1997. Outside-in signaling by lipopolysaccharide through a tailless integrin. *J. Immunol. 159:433.*
13. Ingalls, R. R., B. G. Monks, and D. T. Golenbock. 1999. Membrane expression of soluble endotoxin-binding proteins permits lipopolysaccharide signaling in Chinese hamster ovary fibroblasts independently of CD14. *J. Biol. Chem. 274: 13993.*
14. Fenton, M. J., and D. T. Golenbock. 1998. LPS-binding proteins and receptors. *J. Leukocyte Biol. 64:25.*
15. Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem. 274:10689.*
16. Golenbock, D. T., and M. J. Fenton. 2001. Extolling the diversity of bacterial endotoxins. *Nat. Immunol. 2:286.*
17. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol. 162:3749.*
18. Poltorak, A., X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085.*
19. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med. 189:615.*
20. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity 11:115.*
21. Schnare, M., A. C. Holtdagger, K. Takeda, S. Akira, and R. Medzhitov. 2000. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol. 10:1139.*
22. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol. 165:5392.*
23. Takeuchi, O., A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P. F. Muhlradt, and S. Akira. 2000. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol. 164:554.*
24. Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, et al. 2001. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature 413:78.*
25. Horng, T., G. M. Barton, and R. Medzhitov. 2001. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol. 2:835.*
26. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity 11:443.*
27. Delude, R. L., A. Yoshimura, R. R. Ingalls, and D. T. Golenbock. 1998. Construction of a lipopolysaccharide reporter cell line and its use in identifying mutants defective in endotoxin, but not TNF-α, signal transduction. *J. Immunol. 161:3001.*
28. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. 163:1.*
29. Rodewald, A. K., A. B. Onderdonk, H. B. Warren, and D. L. Kasper. 1992. Neonatal mouse model of group B streptococcal infection. *J. Infect. Dis. 166:635.*
30. Wessels, M. R., R. F. Haft, L. M. Heggen, and C. E. Rubens. 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. *Infect. Immun. 60:392.*
31. Paoletti, L. C., R. A. Ross, and K. D. Johnson. 1996. Cell growth rate regulates expression of group B *Streptococcus* type III capsular polysaccharide. *Infect. Immun. 64:1220.*
32. McDaniel, L. S., G. Scott, J. F. Kearney, and D. E. Briles. 1984. Monoclonal antibodies against protease-sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. *J. Exp. Med. 160:386.*
33. Coxon, A., P. Rieu, F. J. Barkalow, S. Askari, A. H. Sharpe, U. H. von Andrian, M. A. Arnaout, and T. N. Mayadas. 1996. A novel role for the β_2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity 5:653.*
34. Moore, K. J., L. P. Andersson, R. R. Ingalls, B. G. Monks, R. Li, M. A. Arnaout, D. T. Golenbock, and M. W. Freeman. 2000. Divergent response to LPS and bacteria in CD14-deficient murine macrophages. *J. Immunol. 165:4272.*
35. Delude, R. L., M. J. Fenton, R. Savedra, Jr., P. Y. Perera, S. N. Vogel, R. Thieringer, and D. T. Golenbock. 1994. CD14-mediated translocation of nuclear factor-κB induced by lipopolysaccharide does not require tyrosine kinase activity. *J. Biol. Chem. 269:22253.*
36. Flo, T. H., O. Halaas, E. Lien, L. Ryan, G. Teti, D. T. Golenbock, A. Sundan, and T. Espevik. 2000. Human Toll-like receptor 2 mediates monocyte activation by *Listeria monocytogenes*, but not by group B streptococci or lipopolysaccharide. *J. Immunol. 164:2064.*
37. Guttormsen, H. K., C. J. Baker, M. S. Edwards, L. C. Paoletti, and D. L. Kasper. 1996. Quantitative determination of antibodies to type III group B streptococcal polysaccharide. *J. Infect. Dis. 173:142.*
38. Sahlin, S., J. Hed, and I. Rundquist. 1983. Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. *J. Immunol. Methods 60:115.*
39. Vallejo, J. G., C. J. Baker, and M. S. Edwards. 1996. Roles of the bacterial cell wall and capsule in induction of tumor necrosis factor α by type III group B streptococci. *Infect. Immun. 64:5042.*
40. Eskola, J., A. K. Takala, E. Kela, E. Pekkanen, R. Kalliokoski, and M. Leinonen. 1992. Epidemiology of invasive pneumococcal infections in children in Finland. *J. Am. Med. Assoc. 268:3323.*
41. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature 413:732.*
42. Xu, Y., X. Tao, B. Shen, T. Horng, R. Medzhitov, J. L. Manley, and L. Tong. 2000. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature 408:111.*
43. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
44. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science 285:732.*
45. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem. 274:17406.*
46. Opitz, B., N. W. Schroder, I. Spreitzer, K. S. Michelsen, C. J. Kirschning, W. Hallatschek, U. Zahringer, T. Hartung, U. B. Gobel, and R. R. Schumann. 2001. Toll-like receptor-2 mediates *Treponema* glycolipid and lipoteichoic acid-induced NF-κB translocation. *J. Biol. Chem. 276:22041.*
47. Haziot, A., S. Chen, E. Ferrero, M. G. Low, R. Silber, and S. M. Goyert. 1988. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J. Immunol. 141:547.*
48. Weidemann, B., J. Schletter, R. Dziarski, S. Kusumoto, F. Stelter, E. T. Rietschel, H. D. Flad, and A. J. Ulmer. 1997. Specific binding of soluble peptidoglycan and muramyldipeptide to CD14 on human monocytes. *Infect. Immun. 65:858.*
49. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS-binding protein. *Science 249:1431.*
50. Jiang, Q., S. Akashi, K. Miyake, and H. R. Petty. 2000. Lipopolysaccharide induces physical proximity between CD14 and Toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-κB. *J. Immunol. 165:3541.*
51. Yang, R. B., M. R. Mark, A. L. Gurney, and P. J. Godowski. 1999. Signaling events induced by lipopolysaccharide-activated Toll-like receptor 2. *J. Immunol. 163:639.*
52. Bhat, N., P. Y. Perera, J. M. Carboni, J. Blanco, D. T. Golenbock, T. N. Mayadas, and S. N. Vogel. 1999. Use of a photoactivatable taxol analog to identify unique cellular targets in murine macrophages: identification of murine CD18 as a major taxol-binding protein and a role for Mac-1 in taxol-induced gene expression. *J. Immunol. 162:7335.*
53. Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature 401:811.*
54. Beckman, J. S., T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA 87:1620.*
55. Xie, Q. W., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem. 269:4705.*
56. Rubens, C. E., H. V. Raff, J. C. Jackson, E. Y. Chi, J. T. Bielitzki, and S. L. Hillier. 1991. Pathophysiology and histopathology of group B streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. *J. Infect. Dis. 164:320.*
57. Hall, S. L., and M. P. Sherman. 1992. Intrapulmonary bacterial clearance of type III group B streptococcus is reduced in preterm compared with term rabbits and occurs independent of antibody. *Am. Rev. Respir. Dis. 145:1172.*
58. Langermans, J. A., W. L. Hazenbos, and R. van Furth. 1994. Antimicrobial functions of mononuclear phagocytes. *J. Immunol. Methods 174:185.*
59. Abreu, M. T., P. Vora, E. Faure, L. S. Thomas, E. T. Arnold, and M. Arditi. 2001. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J. Immunol. 167:1609.*
60. Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol. 17:593.*
61. Netea, M. G., B. J. Kullberg, I. Verschueren, and J. W. Van Der Meer. 2000. Interleukin-18 induces production of proinflammatory cytokines in mice: no intermediate role for the cytokines of the tumor necrosis factor family and interleukin-1β. *Eur. J. Immunol. 30:3057.*