

# Novel Engagement of CD14 and Multiple Toll-Like Receptors by Group B Streptococci<sup>1</sup>

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Group B streptococcus (GBS) imposes a major health threat to newborn infants. Little is known about the molecular basis of GBS-induced sepsis. Both heat-inactivated whole GBS bacteria and a heat-labile soluble factor released by GBS during growth (GBS-F) induce nuclear translocation of NF- $\kappa$ B, the secretion of TNF- $\alpha$ , and the formation of NO in mouse macrophages. Macrophages from mice with a targeted disruption of MyD88 failed to secrete TNF- $\alpha$  in response to both heat-inactivated whole bacteria and GBS-F, suggesting that Toll-like receptors (TLRs) are involved in different aspects of GBS recognition. Immune cell activation by whole bacteria differed profoundly from that by secreted GBS-F. Whole GBS activated macrophages independently of TLR2 and TLR6, whereas a response to the secreted GBS-F was not observed in macrophages from TLR2-deficient animals. In addition to TLR2, TLR6 and CD14 expression were essential for GBS-F responses, whereas TLR1 and TLR4 or MD-2 did not appear to be involved. Heat lability distinguished GBS-F from peptidoglycan and lipoproteins. GBS mutants deficient in capsular polysaccharide or  $\beta$ -hemolysin had GBS-F activity comparable to that of wild-type streptococci. We suggest that CD14 and TLR2 and TLR6 function as coreceptors for secreted microbial products derived from GBS and that cell wall components of GBS are recognized by TLRs distinct from TLR1, 2, 4, or 6. *The Journal of Immunology*, 2001, 167: 7069–7076.

Group B streptococcus (GBS)<sup>3</sup> inhabits a unique niche among infectious pathogens. GBS is a harmless commensal of the genitourinary tract in 20% of women but is the leading infectious threat to newborn infants in the western world (1, 2). Up to 70% of newborn infants from colonized women acquire GBS. About 1% of these infants develop the sepsis syndrome and, depending on the gestational age, up to 42% of affected infants may die. Approximately one-half of infants that survive GBS meningitis suffer permanent neurological impairment (3).

The expeditious elimination of microorganisms like GBS that invade from mucosal surfaces is a primary goal of the innate immune system. APCs recognize traces of microbial components and subsequently orchestrate the antibacterial defense. The crucial sensory function is assigned to pattern recognition receptors that distinguish self from conserved microbial structures shared by differ-

ent pathogens (4, 5). Mammalian Toll-like receptors (TLRs) lately have been identified as type I transmembrane signaling receptors with pattern recognition capabilities (6). Recent observations attributing the dependence of mammalian immune responses to LPS, the major cell wall component of Gram-negative bacteria, to the expression of functional TLR4 (7–10) serve as a paradigm for our understanding of the biology of TLRs. Previously enigmatic observations that the inability to respond to LPS correlated to reduced resistance to Gram-negative bacterial infections can now be understood in this context.

Although Gram-positive and Gram-negative bacterial sepsis are due to distinct microorganisms, the similarities in the physiological consequences of invasive infection with the bacteria are extraordinary. There is extensive evidence that the immediate immune response to Gram-negative bacterial invasion is mediated by LPS, but no surface equivalent of LPS in Gram-positive bacteria has ever been identified. GBS subcellular cell wall components include its capsular polysaccharide, peptidoglycan, and lipoteichoic acid and have all been reported to have proinflammatory activities in vitro (11, 12). TLR2 has been identified as a receptor that is central to the innate immune response to several whole Gram-positive bacteria, as well as a receptor for peptidoglycan and lipoteichoic acid (13–16). Although it is an attractive hypothesis that capsular polysaccharide, peptidoglycan, and lipoteichoic acid are responsible for the immune activity associated with GBS infection, none of these bacterial products is a potent inducer of cytokine production. Moreover, we failed to observe a role for TLR2 in the innate immune response to killed GBS, despite its content of peptidoglycan and lipoteichoic acid (17). This suggests that the ability of peptidoglycan and lipoteichoic acid to activate cells via TLR2 and the ability of whole organisms to activate the innate immune system are not causally related.

To further delineate the capabilities of different TLRs to discriminate microbial products, we hypothesized that GBS would be

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<sup>3</sup> Abbreviations used in this paper: GBS, group B streptococcus; TLR, Toll-like receptor; CHO, Chinese hamster ovary; huTLR2, human TLR2; GBS-F, soluble factor released by GBS; PEM, peritoneal exudate macrophage.

recognized by both known and novel Toll receptors. We specifically examined the role of the TLR adapter molecule MyD88 and the receptors TLR1, 2, 4, and 6 using the companion approaches of cellular transfection with cDNA constructs (gain of function) and the examination of macrophages from genetically deficient animals (loss of function). We found that GBS engagement of TLR2 and TLR6 is essential for cells to respond to the presence of bacteria, but that this recognition is due to a novel secreted (or shed) bacterial product. We hypothesize that this secreted factor might prove to be a toxin that can systemically activate immune cells in vivo and contribute to the sepsis syndrome in newborn infants. In contrast to the effects of this soluble product, GBS cell walls appear to engage TLRs different from TLR2 and TLR6, although the precise bacterial ligand, as well as the TLR that is activated, have not yet been determined.

## Materials and Methods

### Reagents

PBS, Ham's F-12 medium, RPMI 1640, and trypsin-versene mixture (trypsin-EDTA) were from BioWhittaker (Walkersville, MD). Low endotoxin FBS was from Summit Biotechnology (Greeley, CO). Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). Hygromycin B was purchased from Calbiochem (San Diego, CA), puromycin and polymyxin B were purchased from Sigma-Aldrich (St. Louis, MO), and G418 was from Life Technologies (Gaithersburg, MD). Protein-free LPS from *Escherichia coli* K 235 was a gift from S. Vogel (Uniformed Services University of the Health Sciences, Bethesda, MD), immunoaffinity purified native OspA from *Borrelia burgdorferi* was a gift from J. Radolf (University of Connecticut Health Center, Farmington, CT), and soluble peptidoglycan from *Staphylococcus aureus* was donated by R. Dziarski (Indiana University, Gary, IN). Microcentrifuge filters were purchased from Sigma-Aldrich. The LPS antagonist B1287 was a gift from the Eisai Research Institute (Andover, MA).

### Cell lines

The Chinese hamster ovary (CHO)/CD14.ELAM.tac reporter cell line (clone 3E10) is stably transfected with human CD14 and expresses inducible membrane CD25 (tac Ag) under the transcriptional control of the NF- $\kappa$ B-dependent human E-selectin promoter (18). The CHO/CD14/huTLR2.ELAM.tac cells were constructed by stable cotransfection of CHO/CD14.ELAM.tac with the cDNA for human TLR2 (huTLR2), together with the neo<sup>r</sup>-containing plasmid pcDNA3 (Invitrogen, San Diego, CA) as described (14). CHO cell lines were grown in Ham's F-12 medium containing 10% FBS and 10  $\mu$ g/ml ciprofloxacin. CHO/CD14.ELAM.tac were cultured with 400 U/ml hygromycin B; CHO/CD14/huTLR2.ELAM.tac were cultured with hygromycin plus 0.5 mg/ml G418. The LPS-resistant nonresponder CHO/CD14 cell line, clone 7.19 (as described in Ref. 18) was transfected with huTLR2 (7.19/TLR2). Clone 7.19 carries a point mutation resulting in a C95Y amino acid exchange for the TLR4 coreceptor, MD-2, that renders it unresponsive to LPS (19). The 7.19/TLR2.ELAM.tac was cultured with hygromycin and G418.

### Bacterial strains, the generation of bacterial supernatant, and inactivated GBS

The  $\beta$ -hemolytic GBS type III strains COH1 and M781, initially isolated from newborn infants with sepsis, have been previously described (20, 21). Unless otherwise stated, experiments were performed with strain COH1. The nonhemolytic mutant COH1-20, an isogenic derivative of strain COH1, was generated by a single insertion of Tn916 $\Delta$ E into its chromosome (22). All strains were grown overnight on blood agar plates (REMEL, Lenexa, KS). Bacterial colonies were removed from the plates in stationary phase, washed three times with PBS, and then used to inoculate chemically defined medium (23) prepared from endotoxin-free water or, where indicated, RPMI 1640 with 10% FBS to ABS<sub>650</sub> = 0.03 and grown to midlog phase (ABS<sub>650</sub> = 0.27–0.30). For the culture of strain COH1-20, the medium was supplemented with chloramphenicol (5  $\mu$ g/ml) and the nonhemolytic phenotype was confirmed in each experiment (blood agar). Subsequently, cultures were clarified of bacteria by centrifugation and were filtered (0.2- $\mu$ m pore diameter). For some experiments, the GBS supernatant was size fractionated by spin filters using molecular mass cutoff at 30, 100, and 300 kDa. Heat-inactivated GBS was prepared from midlog phase cultures, washed three times with PBS, resuspended at a density of 3  $\times$  10<sup>10</sup> CFU/ml, and heat-inactivated (30 min, 80°C). Cell-free supernatants

and heat-inactivated bacteria were stored at –80°C for further use. Endotoxin was not detectable in the above preparations using a *Limulus* assay with a sensitivity of <0.6 EU/ml (BioWhittaker). Quantitative protein determination in GBS supernatants was performed using the Bio-Rad Protein Assay per the manufacturer's protocol (Bio-Rad, Hercules, CA).

### Purification of the GBS supernatant by anion exchange column chromatography

Filtered GBS supernatant was concentrated 20-fold using a PREP/SCALE Cartridge (Millipore, Bedford, MA) diluted 1/3 in 10 mmol Tris/EDTA (pH 8.0) and loaded on a MonoQ anion exchange column (Pharmacia, Peapack, NJ) using fast protein liquid chromatography (Äkta) and Unicorn 3.00 software (both from Amersham Pharmacia Biotech, Uppsala, Sweden). Bound proteins were eluted in a total volume of 80 ml with a linear NaCl gradient up to 1 M. Fractions were screened for soluble factor released by GBS (GBS-F) activity with 7.19/TLR2 cells for induction of CD25 expression.

### Transient transfection

Transient transfection was performed using the SuperFect reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. HEK 293 cells were plated at a density of 0.5  $\times$  10<sup>6</sup> cells/well in six-well dishes and incubated for 18 h. Plasmid DNA was added to 100  $\mu$ l of serum-free DMEM per well. For each transfection, a total of 2.5  $\mu$ g of plasmid DNA per well, consisting of 1  $\mu$ g of a previously described reporter plasmid (pELAM-luc) (10), together with 1  $\mu$ g of huTLR2 in pcDNA3 and 0.5  $\mu$ g of human MD2 in the mammalian expression plasmid pEFBOS (24) were used. The next day, the cells were stimulated for 5 h and cytoplasmic extracts were prepared as described by the manufacturer (Promega, Madison, WI). The response to stimulation was measured by assessing luciferase activity using luciferase substrate (Promega) and a Monolight 3010 luminometer (BD Pharmingen, San Diego, CA), per the manufacturer's protocol. Depicted in Fig. 6 are the mean values  $\pm$  SD expressed as fold induction above background. Each point was assayed in triplicate; one representative of three experiments is shown.

### Peritoneal macrophages

MyD88<sup>-/-</sup> (25), TLR2<sup>-/-</sup>, TLR2<sup>+/-</sup> (16, 26), and TLR6<sup>-/-</sup> (27) mice were engineered as described. The generation of the TLR1<sup>-/-</sup> mice will be described in an independent manuscript. MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup>, TLR2<sup>+/-</sup>, and TLR6<sup>-/-</sup> mice, Chinese hamsters (Cytogen, Boston, MA), Golden Syrian hamsters (Charles River Breeding Laboratories, Wilmington, MA), C57BL/6J, C3H/HeOuJ, and C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were injected i.p. with 2.5 ml (mice and Chinese hamsters) or 10 ml (Syrian hamsters) of 3% thioglycolate (REMEL). After 3 days, peritoneal exudate cells were harvested by lavage with RPMI 1640 medium containing 10% FBS and 10  $\mu$ g/ml ciprofloxacin. The cells were washed with medium, counted in a hemocytometer, and plated at a density of 1  $\times$  10<sup>6</sup> cells/well in six-well dishes (for NF- $\kappa$ B assays) or 1  $\times$  10<sup>5</sup> cells/well in 96-well dishes (for the determination of cytokine release and NO). After 24–72 h, nonadherent cells were removed by washing with medium and adherent cells were stimulated.

### Isolation of PBMCs and measurement of TNF- $\alpha$

Human PBMCs were isolated by gradient centrifugation of heparinized blood from healthy donors on Histopaque 1077 (Sigma-Aldrich) according to the manufacturer's protocol. The cells were resuspended in RPMI 1640 medium containing 10% FBS and plated at a density of 2  $\times$  10<sup>6</sup>/ml in a 96-well dish. For blocking experiments, PBMCs were preincubated with 10  $\mu$ g/ml of the human CD14 mAb 3C10 (American Type Culture Collection, Manassas, VA) or the control mAb 6H8, which recognizes a widely distributed 180-kDa glycoprotein (T. A. Espevik and B. Naume, unpublished observations), for 30 min at room temperature before further stimulation. After addition of the indicated preparations, incubation proceeded for an additional 16 h at 37°C and 5% CO<sub>2</sub>. Supernatants were collected and stored at –80°C until assayed for TNF- $\alpha$  concentrations with a commercial ELISA for human TNF- $\alpha$  (R&D Systems, Minneapolis, MN).

### Measurement of proinflammatory activity of peritoneal macrophages

Nuclear translocation of NF- $\kappa$ B was determined as follows. Peritoneal exudate macrophages (PEMs; 1  $\times$  10<sup>6</sup>/well in six-well dishes) were incubated for 2 h as indicated in the figures. For coculture experiments, macrophages (1  $\times$  10<sup>6</sup>/well) were seeded in the lower compartment and midlog GBS in the upper compartment of a Transwell dish (Costar, Cambridge,

MA), in which compartments are separated by a 0.4- $\mu$ m membrane. Macrophages/bacteria were cultured in a total of 3 ml of RPMI 1640 plus 10% FBS/well for 16 h. Nuclear extracts were subsequently isolated and analyzed for binding to a  $^{32}$ P-labeled NF- $\kappa$ B-specific oligonucleotide by EMSA, exactly as described (28). Depicted in Figs. 1 and 3 is one representative experiment of three. For determination of TNF- $\alpha$  and NO, RAW 267.4 cells or peritoneal exudate cells ( $1 \times 10^6$  cells/ml in 96-well dishes) were incubated for 16 h at 37°C in a 5% humidified CO<sub>2</sub> environment. Supernatants were processed directly for the determination of released TNF- $\alpha$  by ELISA (R&D Systems) and NO by Griess reaction (naphthylethylenediamine, sulfanilamide, and sodium nitrite were all from Sigma-Aldrich), per the manufacturers' protocols. Depicted are the means  $\pm$  SD of triplicates from one representative experiment of three.

#### Flow cytometry analysis

Cells were plated at a density of  $7 \times 10^4$ /well in 24-well dishes. On the next day, the cells were stimulated as indicated in Ham's F-12 medium containing 10% FBS (total volume of 0.5 ml/well) and incubated for 18 h. Subsequently, the cells were harvested with trypsin-EDTA, spun down by centrifugation at  $800 \times g$  for 5 min, and labeled with FITC-labeled anti-human CD25 mAb (BD Biosciences, Mountain View, CA) in PBS/1% FBS for 30 min on ice, as described (18). The cells were analyzed by flow cytometry using a FACScan microfluorometer. Data were analyzed with CellQuest software (BD Biosciences). Depicted are representative results from three or more experiments.

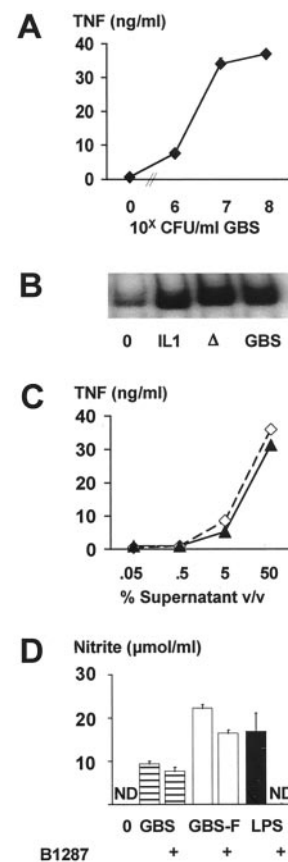
#### Sequencing of TLR2 from Syrian hamsters

Genomic DNA was extracted from the livers of Chinese and Syrian hamsters. Extirpated tissue was homogenized in Trizol, and DNA was separated from RNA by a 1-bromo-3-chloropropane (Molecular Research Center, Cincinnati, OH) gradient. A 2- $\mu$ l portion of the resulting DNA was used as a template in a 25- $\mu$ l PCR as described (29). The PCR was conducted in an automatic thermal cycler (Thermo Hybaid, Franklin, MA) using primers for hamster TLR2 (5'-ATCTCATTTCGTTTTCTTTG and 3'-GATCTTCATGACCAGTAACAC) that flank the previously described mutation (30). The resulting product was extracted from a 2% agarose gel using a QIAEX II gel extraction kit (Qiagen) and sequenced at the Boston University Core Facility (Boston, MA) using an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA). The result was confirmed in an independent PCR experiment.

## Results

### GBS release a proinflammatory factor (GBS-F) in vitro

To address the question of whether the proinflammatory effect of GBS was restricted to a physical interaction between bacteria and host cells, murine macrophages (RAW 267.4 and peritoneal macrophages) were stimulated with heat-inactivated GBS, with live GBS (harvested during logarithmic growth) separated from the macrophages by a 0.4- $\mu$ m membrane, or with filtered supernatants of GBS harvested during log growth. Heat-inactivated whole GBS induced secretion of TNF- $\alpha$  and NO in a dose-dependent manner (Fig. 1, A and D). Interestingly, coculture of live GBS with macrophages in a Transwell dish system that prevents direct interaction between bacteria and macrophages induced nuclear translocation of NF- $\kappa$ B (Fig. 1B). This effect could be reproduced by using cell-free bacterial supernatants of GBS that induced the secretion of both NO and TNF- $\alpha$  at concentrations as low as 0.5% (v/v) (bacterial supernatant/total volume; Fig. 1, C and D). This finding strongly suggested that GBS secrete one or more proinflammatory factors. The activation observed was not due to the production of  $\beta$ -hemolysin, because the nonhemolytic GBS strain COH1-20 induced the translocation of NF- $\kappa$ B and formation of TNF- $\alpha$  similarly, in comparison with the  $\beta$ -hemolytic parental strain COH1 (Fig. 1, B and C). The proinflammatory effect of both bacterial preparations was designated GBS-F. GBS-F was not significantly inhibited by addition of the TLR4 antagonist B1287, a lipid A analog that completely blocked activation by LPS (Fig. 1C). Supernatants of GBSIII serotype M781 induced a quantitatively similar proinflammatory response in RAW 267.4 cells when

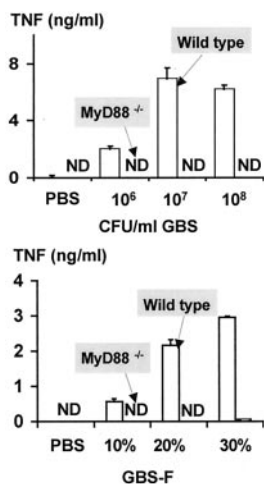


**FIGURE 1.** Supernatant from GBS (GBS-F) induces proinflammatory activity by murine macrophages. RAW 267.4 (A, C, and D,  $0.2 \times 10^6$ /ml) or normal mouse peritoneal macrophages (B, C3H/HeOuJ,  $10^6$ /ml) were incubated for 18 h with heat-inactivated GBS (A); viable normal GBS (strain COH1) or hemolysin-deficient isogenic mutant GBS ( $\Delta$ , COH1-20,  $10^8$  CFU/ml), separated from the macrophages by a 0.4- $\mu$ m membrane, or recombinant human IL-1 $\beta$  (5 ng/ml for 1 h) (B); or filtered supernatant (GBS-F) from midlog hemolysin-deficient GBS (COH1-20, open symbols) or hemolysin-positive GBS (closed symbols) grown in tissue culture medium (C). D, Synthetic LPS inhibitor, B1287 (10  $\mu$ g/ml), was added 30 min before heat-inactivated GBS ( $10^8$  CFU/ml), GBS-F (50%), or LPS (10 ng/ml) as indicated. Supernatants were collected after 18 h and TNF- $\alpha$  was determined by ELISA (A and C), NF- $\kappa$ B translocation by EMSA (B), or NO by Griess reaction (D). ND, Not detectable.

compared with strain COH1 (data not shown). Thus, GBS release a proinflammatory factor that is not  $\beta$ -hemolysin.

### The secretion of TNF- $\alpha$ in response to GBS and GBS-F was abrogated in peritoneal macrophages from mice with a targeted deletion of the TLR adapter protein MyD88

The adapter protein MyD88 has been identified as a signal transduction molecule linking TLRs to NF- $\kappa$ B and/or protein kinase activation. To test whether MyD88 was involved in the cellular response to heat-inactivated GBS and GBS-F, PEMs from normal C57BL/6J mice and MyD88 $^{-/-}$  mice were exposed to either whole heat-inactivated GBS or soluble GBS-F. Whereas whole GBS and GBS-F induced secretion of TNF- $\alpha$  in normal PEMs, both GBS and GBS-F failed to induce significant TNF- $\alpha$  secretion by MyD88 $^{-/-}$  macrophages, suggesting that one or multiple TLRs are necessary for activation by whole bacteria as well as soluble GBS-F (Fig. 2). Macrophages from MyD88 $^{-/-}$  and C57BL/6J mice did not differ in their ability to internalize whole streptococcal organisms (data not shown).



**FIGURE 2.** Targeted deletion of the gene encoding MyD88 abrogated secretion of TNF- $\alpha$  by peritoneal macrophages in response to GBS-F and GBS. Peritoneal macrophages from MyD88<sup>-/-</sup> and C57BL/6 wild-type mice were seeded into RPMI 1640/10% FBS ( $10^6$  peritoneal macrophages/ml). Peritoneal macrophages were kept in culture for 1–3 days and washed twice. Then cells were stimulated with heat-inactivated GBS (upper panel) or GBS-F (lower panel) over 18 h, and TNF concentrations in the supernatants were determined by ELISA. ND, Not detectable.

*TLR2 and TLR6 are essential receptors for GBS-F, but not for whole, heat-inactivated organisms*

The finding that responses to GBS-F and GBS were MyD88 dependent prompted an effort to define which Toll-like receptors were involved in GBS-induced activation. Our laboratory has previously reported that macrophages from Chinese hamsters exhibit a frameshift mutation at bp 1758 in the TLR2 gene that results in a premature stop codon in the predicted extracellular portion of TLR2. The resultant TLR2 transcript fails to produce functional TLR2 (30). We functionally characterized TLR2 of elicited peritoneal exudative macrophages from Golden Syrian hamsters. Macrophages from Golden Syrian hamsters, unlike Chinese hamster cells, responded to TLR2 ligands including soluble peptidoglycan from *S. aureus* and nOspA, a lipoprotein from *B. burgdorferi*, as indicated by the inducible translocation of NF- $\kappa$ B (data not shown). Sequence analysis of PCR products that were amplified using flanking primers and genomic DNA from hamster liver as template revealed a normal sequence for Syrian hamster TLR2. The results contrast to the previously reported frameshift mutation in Chinese hamster DNA (30).

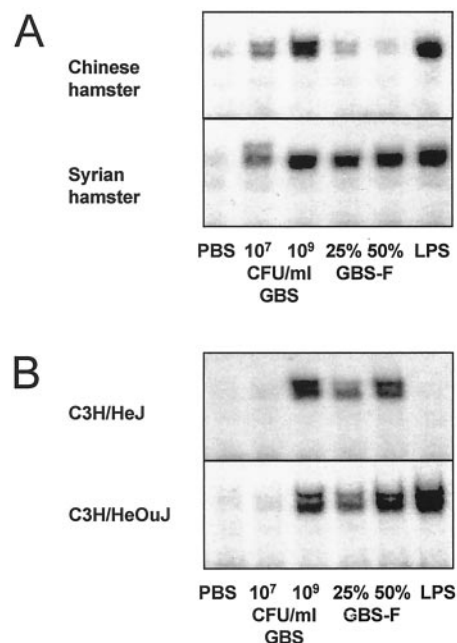
Macrophages from Chinese and Golden Syrian hamsters were exposed to whole GBS and soluble GBS-F. Macrophages from Golden Syrian hamsters, but not from the TLR2-negative Chinese hamsters, translocated NF- $\kappa$ B in response to GBS-F. Both types of macrophages responded similarly to whole, heat-inactivated GBS as well as to LPS (Fig. 3A). We next tested peritoneal macrophages from C3H/HeJ mice, an LPS hyporesponder strain that expresses a dominant-negative TLR4 (8). Peritoneal macrophages from C3H/HeJ and normal C3H/HeOuJ mice responded equally to GBS and GBS-F (Fig. 3B). These data expanded our previous findings that macrophages from C3H/HeJ and normal C3H/HeOuJ mice are equally activated by lipoteichoic acid and protein-free GBS cell walls (31). Thus, expression of TLR2, but not TLR4, appeared to be essential for responses to GBS-F. However, expression of neither TLR2 nor TLR4 was critical for NF- $\kappa$ B translocation in response to whole, heat-inactivated streptococcal organisms.

We confirmed the TLR2 dependence of the soluble GBS-F, but not whole, heat-inactivated GBS, using peritoneal macrophages

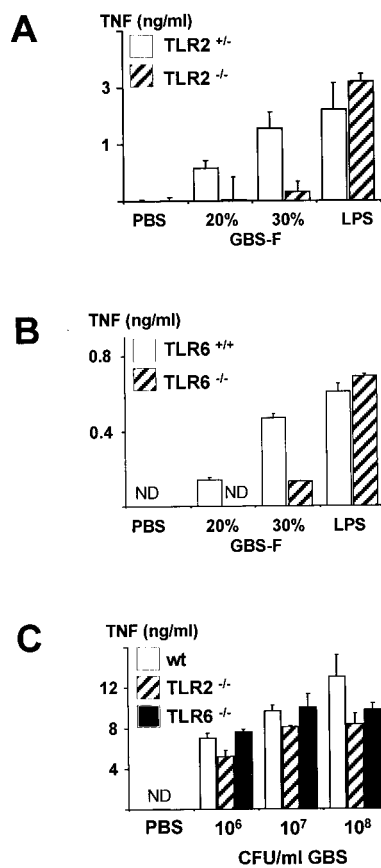
from TLR2<sup>-/-</sup> mice by assessing the induced secretion of TNF- $\alpha$  as a marker of proinflammatory activity (Fig. 4, A and C). TLR2 expression was essential for nuclear translocation of transcription factors and secretion of proinflammatory cytokines in response to GBS-F, but not to whole, heat-killed GBS. The lack of a significant TLR2 engagement by whole GBS appeared not to be simply due to heat treatment of GBS, in that ethanol-inactivated and washed GBS (70% ethanol, 45 min) activated TLR2<sup>-/-</sup> and TLR2<sup>+/-</sup> peritoneal macrophages similarly (data not shown).

TLR2 has been reported to colocalize with TLR1 and TLR6 (32) and may function as a heterodimeric receptor in response to ligation with the appropriate bacterial product, such as bacterial lipoprotein. Thus, we measured the cytokine secretion of peritoneal macrophages from TLR1<sup>-/-</sup> and TLR6<sup>-/-</sup> mice in response to soluble GBS-F. Macrophages that were harvested from TLR1<sup>-/-</sup> mice responded normally to GBS-F (data not shown). However, peritoneal macrophages from TLR6 knockout mice did not respond to GBS-F, in contrast to the stimulatory effects of whole, heat-inactivated GBS in both knockout animals (Fig. 4, B and C). These data are consistent with the concept that TLR2, TLR6, and MyD88 are portions of a single receptor complex that recognizes GBS-F.

To complement these studies in macrophages from genetically engineered animals, TLR2 was tested as a signaling molecule in heterologously transfected cell lines. GBS-F failed to induce translocation of NF- $\kappa$ B in CD14-transfected CHO cells (which are null for TLR2) (30). In contrast, these cells responded to concentrations as low as 10 pg/ml LPS (data not shown). However, stable transfection into a CHO/CD14 background of huTLR2, but not huTLR4, conferred responsiveness to GBS-F (Fig. 5). We PCR amplified and partially sequenced TLR6 from a CHO-K1 cDNA library (data not shown) by using cross-species primers derived

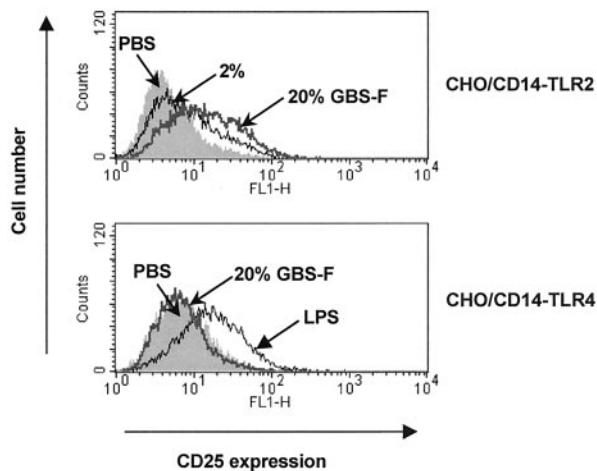


**FIGURE 3.** Peritoneal macrophages from TLR2-deficient Chinese hamsters are activated by GBS, but not by GBS-F. Peritoneal macrophages from Chinese hamsters (TLR2 mutant) and Syrian hamsters (normal TLR2) (A), or C3H/HeOuJ mice and C3H/HeJ mice (B) were seeded into RPMI 1640/10% FBS ( $10^6$  PEM/ml). PEMs were kept in culture for 1–3 days and were repeatedly washed. Then cells were stimulated with heat-inactivated GBS, GBS-F, or LPS (10 ng/ml) over 2 h, and nuclear extracts were prepared as described in *Materials and Methods*.



**FIGURE 4.** TLR2 and TLR6 are essential coreceptors for GBS-F, but not GBS. Peritoneal macrophages from TLR2<sup>+/+</sup> and TLR2<sup>-/-</sup>, TLR6<sup>-/-</sup>, and C57BL/6J (wild type) mice were seeded into RPMI 1640/10% FBS (10<sup>6</sup> PEM/ml). After 1–3 days, PEMs were stimulated with GBS-F, LPS (10 ng/ml) (A and B), or heat-inactivated GBS (C) over 18 h, and TNF concentrations in the supernatants were determined by ELISA. ND, Not detectable.

from the known sequences of mice and humans. Hence, CHO-K1 cells transcribe endogenous TLR6 that may serve as part of a signaling complex if coexpressed with exogenous TLR2.

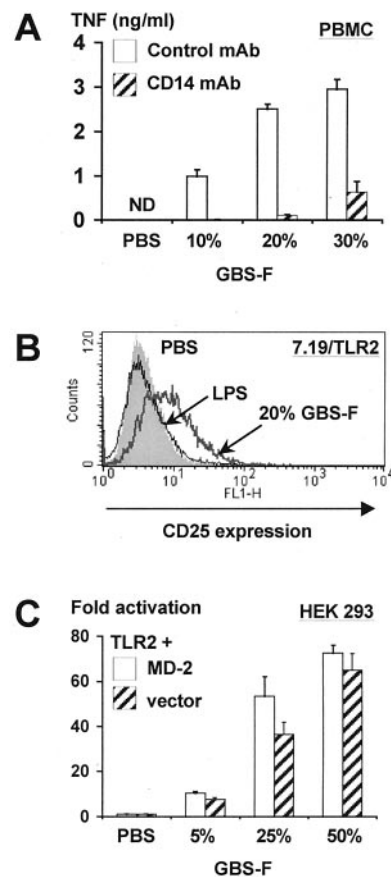


**FIGURE 5.** GBS-F activates CHO/CD14-TLR2 but not CHO/CD14-TLR4 cells. CHO/CD14-TLR2.ELAM.tac (upper panel) and CHO/CD14-TLR4.ELAM.tac (lower panel) were incubated over 18 h with 2 and 20% GBS-F (v/v of PEM supernatant) or 10 ng/ml LPS. After stimulation, CHO cells were stained with anti-CD25 mAb and analyzed by flow microfluorometry.

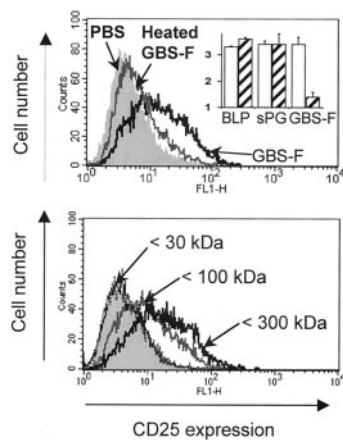
*CD14, but not MD2, serves as a coreceptor for GBS-F*

CD14 is a 55-kDa glycosylphosphatidylinositol-linked protein that functions as a coreceptor for numerous bacterial products, including LPS, peptidoglycan, and bacterial lipoprotein. Hence, we tested whether signaling in response to GBS-F was inhibited by the CD14 mAb 3C10. We found a significant reduction of the GBS-F-induced TNF secretion from human PBMCs by preincubation with 3C10, but not with the control, mAb 6H8 (Fig. 6, A).

Another potential coreceptor both for TLR2 and TLR4 in response to peptidoglycan and LPS is the secreted protein MD2 (33, 34). We investigated the role of MD2 in GBS-F-induced signaling using two experimental approaches. First, we tested an LPS non-responder cell line, clone 7.19 (18), which we have recently determined to contain a mutation in MD-2 (19). This cell line was further engineered to express huTLR2 and is referred to as 7.19/TLR2. The 7.19/TLR2 cell line was found to be activated by GBS-F, but not by LPS (Fig. 6, B). Thus, a mutation in MD2 that abrogated LPS-induced signaling did not significantly interfere



**FIGURE 6.** CD14 but not MD2 amplifies the cellular response to GBS-F. A, PBMCs were stimulated over 18 h with 10–30% (v/v) GBS-F in the presence of a control mAb, 6H8, or the human CD14 mAb, 3C10. TNF- $\alpha$  concentrations in the supernatants were determined by ELISA. B, Clone 7.19, a CHO/CD14 cell line that contains an ELAM.tac (CD25) reporter plasmid and is LPS-nonresponsive (18), was stably transfected with huTLR2. Clone 7.19 does not respond to LPS due to a point mutation in MD2 (C95Y) (19). The 7.19/TLR2 were stimulated over 18 h with 20% GBS-F (v/v) or 10 ng/ml LPS. After stimulation, CHO/CD14-TLR2 were stained with anti-CD25 mAb and analyzed by flow microfluorometry. C, HEK 293 cells ( $5 \times 10^5$  cells/well) were transfected with huTLR2 and the ELAM.luc reporter plasmid, (▨) or with TLR2 plus human MD2 and the ELAM.luc reporter plasmid, (□). After 24 h, cells were stimulated over 5 h with GBS-F, and luciferase activity was determined as outlined in *Materials and Methods*. ND, Not detectable.



**FIGURE 7.** The activity of GBS-F is heat labile. *Upper panel*, CHO/CD14-TLR2.ELAM.tac ( $1 \times 10^6$ /ml) were incubated over 18 h with 10% crude GBS-F or GBS-F that had been heat treated for 15 min. The bacterial lipoprotein nOspA from *B. burgdorferi* (BLP, 0.3  $\mu$ g/ml) and soluble peptidoglycan from *S. aureus* (sPG, 5  $\mu$ g/ml) with (▨) and without (□) heat treatment for 15 min served as controls (*inset*, fold induction of ELAM.tac reporter assay compared with unstimulated controls). *Lower panel*, Supernatants from GBS were subjected to spin filters that separate fractions of different molecular mass. CHO/CD14-TLR2.ELAM.tac ( $1 \times 10^6$ /ml) were incubated over 18 h with 10% (v/v) of the resulting fractions of an approximate molecular mass <30, <100, and <300 kDa.

with NF- $\kappa$ B translocation in response to soluble GBS-F. Second, we tested whether expression in HEK 293 cells of MD2 together with TLR2 would amplify the response to GBS-F when compared with expression of TLR2 alone. Whereas cotransfection of MD2 and TLR4 dramatically enhanced the response to LPS when compared with TLR4 alone (data not shown), MD2 expression did not significantly affect the response of HEK 293 cells to GBS-F (Fig. 6, C).

#### *GBS-F is heat labile with a molecular mass that exceeds 30 kDa*

Certain cell wall components of Gram-positive bacteria, including peptidoglycan and lipoproteins, have been demonstrated to activate cells in a TLR2-restricted manner. To exclude these cell wall components as immunologically active substances in GBS supernatant and to further investigate the nature of GBS-F, we subjected the filtered GBS supernatant to heat treatment (100°C, 15 min).

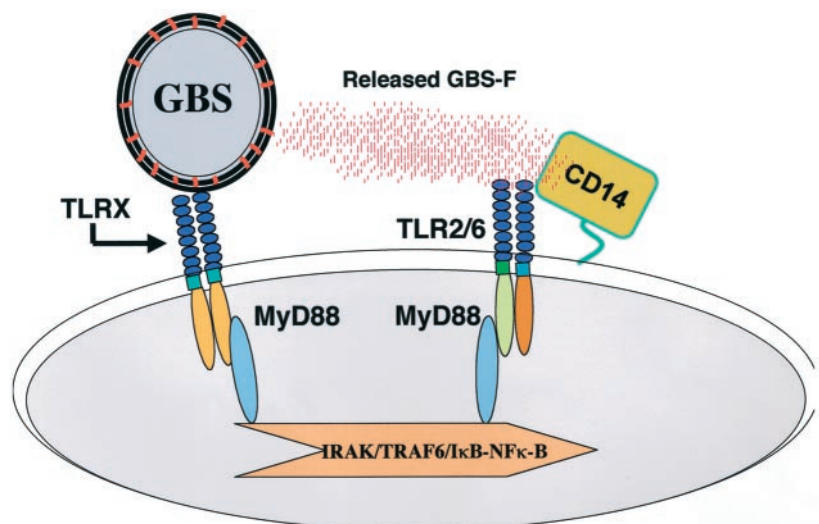
Heat treatment abrogated the response of CHO/CD14-TLR2 to GBS-F, but not to soluble staphylococcal peptidoglycan or the lipoprotein nOsp A from *B. burgdorferi* (Fig. 7, *upper panel, inset*).

Determination of the protein content of GBS supernatant harvested from early log-phase cultures ( $OD_{595} = 0.3$ ) revealed a concentration of  $\sim 5$   $\mu$ g/ml total protein. Biological activity was generally observed at a 1/100 dilution of such cultures, suggesting that GBS-F is capable of activating cells at concentrations as low as 50 ng/ml or less. Furthermore, we subjected the supernatant to spin filters that separate fractions of different molecular mass. Fractions >30 kDa, but not those <30 kDa, activated the CHO/CD14-TLR2 cells, and most of the activity was observed at a molecular mass cutoff of <100 kDa (Fig. 7, *lower panel*). Similarly, these fractions were far more potent than other fractions with respect to activation of RAW 267.4 cells tested in a TNF- $\alpha$  release assay (data not shown). Anion exchange chromatography also suggested that GBS-F is negatively charged, although the active column fractions consisted of more than a single band on an SDS-PAGE gel (data not shown).

## Discussion

Immune evasion and pathogen recognition are diametrically opposed survival strategies of microbes and their respective hosts (35). The discovery of the Toll family of receptors has suggested that the innate immune system uses a limited but sufficiently diverse combination of receptors that recognize the major microbial patterns likely to be encountered in the earliest phases of infectious illnesses (36). The efficacy of the TLR system is evidenced by their remarkable conservation throughout evolution (6, 37, 38). These transmembrane signaling molecules mediate a wide variety of cellular functions including ventral-dorsal patterning in the fruit fly, apoptosis in mammalian cells, and innate immune activation across species (14, 37, 39, 40). This multiplicity of functions suggests that these seemingly unrelated events have more in common than might otherwise be presumed. The diversity of known TLRs and the high number of recognized microbial patterns contrast with a striking similarity of downstream signaling events. For example, all the TLRs that have been examined are believed to transduce the binding signal of microbial products via the scaffolding protein MyD88, finally resulting in the transcription of cytokine genes (26, 41, 42). Indeed, one can reasonably conclude that the similarities in clinical syndromes associated with different types of infectious illnesses are due to the similarity in the signal transduction systems

**FIGURE 8.** Model for the engagement of macrophage receptors by GBS cell wall products. This report supports the hypothesis that GBS engages at least two distinct signaling complexes to initiate inflammation. Cell-bound bacterial components activated a MyD88-dependent (and thus TLR-dependent) signal transduction pathway. This pathway is probably initiated by a TLR distinct from TLR1, 2, 4, and 6, because GBS cell wall fragments activated cells normally from each of the respective knockouts. Furthermore, GBS release a heat-labile, immunologically active factor, designated GBS-F. Recognition of GBS-F required the expression of TLR2, TLR6, MyD88, and CD14. GBS-F was found to be distinct from peptidoglycan,  $\beta$ -hemolysin, and capsular polysaccharide.



that are activated by Gram-positive and Gram-negative bacteria, fungi and viruses through the engagement of TLRs. In accord with this hypothesis, we report in this work that the induction of proinflammatory cytokines by whole GBS cell walls, as well as secreted streptococcal products such as GBS-F, is entirely dependent on MyD88. This result suggests that the proinflammatory response to GBS occurs in its entirety as a result of a Toll receptor-dependent signaling mechanism.

The currently accepted paradigm regards TLR2 as an essential receptor for many eubacterial cell wall components, including lipoproteins and peptidoglycan. Bacterial species as diverse as mycobacteria, spirochetes, mycoplasma, *S. aureus*, and *Streptococcus pneumoniae* have all been shown to mediate cellular activation via TLR2 (14, 39, 43). In contrast to expectations, we found here that the deletion of TLR2 did not significantly alter the cellular response to whole GBS as compared with normal cells, suggesting a lesser role of peptidoglycan in streptococcal pathogenesis than might otherwise have been predicted. An alternative hypothesis that is currently the subject of investigation is that peptidoglycan from GBS activates TLRs that are different from those engaged by peptidoglycan of *S. aureus* (14). The requirement for MyD88 expression, but not TLR2, suggests that GBS is recognized by other TLRs. As documented here, using macrophages from mutant mice carrying targeted deletions or spontaneous mutations, these TLRs are not TLR1, TLR4, or TLR6.

In addition to narrowing our understanding of TLR use by the innate immune system in response to GBS, the present study has identified the existence of a novel proinflammatory factor from GBS. This factor, GBS-F, is secreted or shed in bacterial culture, and this activity is already present in early log phase of bacterial growth. GBS-F appears to have considerable inflammatory potency as GBS supernatant, with a total protein content as low as 5  $\mu\text{g/ml}$  activated macrophages and TLR2-transfected CHO cells in dilutions of up to 1/100. The total bacterial mass removed from the supernatant that produced this activity was only 180  $\mu\text{g/ml}$  (dry weight per volume). GBS-F engages both TLR2 and TLR6 and hence is distinctly different from whole GBS, whose cell wall constituents induce signaling in the absence of TLR2 or TLR6 expression. The complexity of GBS recognition by cells of the innate immune system is summarized in Fig. 8.

The uniqueness of the Toll receptor-mediated proinflammatory effects of GBS are most clearly understood in the context of several decades of research that have identified numerous biologically active products from this organism. Several partially purified and sequenced secretion products of GBS have been described, including hyaluronate lyase, group B streptococcal cocytolysin, and GBS extracellular toxin (44–46). Filtered extracts of GBS have been reported to modify the arachidonic acid metabolism of cultured human amnion cells, favoring production of PG E<sub>2</sub> (47). However, among the known “extracellular toxins,” only  $\beta$ -hemolysin from GBS has been assigned a proinflammatory function (48). None of these known molecules is likely to be the GBS-F described herein. GBS-F clearly differs from  $\beta$ -hemolysin in that GBS-F activity was produced similarly by  $\beta$ -hemolytic and nonhemolytic GBS strains. Similarly, GBS-F is not a capsular polysaccharide, in that GBS-F activity was detected from mutant GBS deficient in the production of capsular polysaccharide (data not shown). GBS-F can be discriminated from the cell wall components lipoteichoic acid, peptidoglycan, and bacterial lipoproteins by its heat lability and its release starting at early log phase, before cell wall lysis is observed. In addition, no proinflammatory lipoproteins from Gram-positive bacteria have been identified yet. When GBS was compared with a pneumolysin-deficient strain of *S. pneumoniae*, a bacterial species that exhibits extensive autolysis, only GBS re-

leased a proinflammatory factor (data not shown). These results challenge the current dogma that TLR2 is a critical mediator for cell wall components common to all bacteria (36). Instead, they support the argument that species-specific molecules from different genera of Gram-positive bacteria are selectively recognized by this important receptor.

We conclude that GBS-F is a novel proinflammatory factor secreted by GBS. GBS-F, but not whole bacteria, engages TLR2 and TLR6 as essential receptors. Currently we do not know whether GBS-F is secreted and immunologically relevant in vivo. However, it is tempting to speculate that the spillage of GBS-F from the lung or other sites of infection into the blood stream drives systemic hyperinflammation in GBS sepsis. We believe that the discovery of GBS-F will prove to lend significant insight into the mechanisms by which the invasion of human tissue by GBS results in sepsis, excessive morbidity, and even death.

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