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### Lipoproteins Are Critical TLR2 Activating Toxins in Group B Streptococcal Sepsis<sup>1</sup> ✓

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*J Immunol* (2008) 180 (9): 6149–6158.

<https://doi.org/10.4049/jimmunol.180.9.6149>

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# Lipoproteins Are Critical TLR2 Activating Toxins in Group B Streptococcal Sepsis<sup>1</sup>

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Group B streptococcus (GBS) is the most important cause of neonatal sepsis, which is mediated in part by TLR2. However, GBS components that potently induce cytokines via TLR2 are largely unknown. We found that GBS strains of the same serotype differ in released factors that activate TLR2. Several lines of genetic and biochemical evidence indicated that lipoteichoic acid (LTA), the most widely studied TLR2 agonist in Gram-positive bacteria, was not essential for TLR2 activation. We thus examined the role of GBS lipoproteins in this process by inactivating two genes essential for bacterial lipoprotein (BLP) maturation: the prolipoprotein diacylglyceryl transferase gene (*lgt*) and the lipoprotein signal peptidase gene (*lsp*). We found that Lgt modification of the N-terminal sequence called lipobox was not critical for Lsp cleavage of BLPs. In the absence of *lgt* and *lsp*, lipoprotein signal peptides were processed by the type I signal peptidase. Importantly, both the  $\Delta$ *lgt* and the  $\Delta$ *lsp* mutant were impaired in TLR2 activation. In contrast to released factors, fixed  $\Delta$ *lgt* and  $\Delta$ *lsp* GBS cells exhibited normal inflammatory activity indicating that extracellular toxins and cell wall components activate phagocytes through independent pathways. In addition, the  $\Delta$ *lgt* mutant exhibited increased lethality in a model of neonatal GBS sepsis. Notably, LTA comprised little, if any, inflammatory potency when extracted from  $\Delta$ *lgt* GBS. In conclusion, mature BLPs, and not LTA, are the major TLR2 activating factors from GBS and significantly contribute to GBS sepsis. *The Journal of Immunology*, 2008, 180: 6149–6158.

Group B streptococcus (GBS)<sup>4</sup> (*Streptococcus agalactiae*), is the leading cause of sepsis and meningitis in newborn infants (1, 2). GBS sepsis is a highly inflammatory disease that carries a mortality rate of ~10% and is clinically indistinguishable from *Escherichia coli* sepsis. Furthermore, GBS potently induces a host of inflammatory signaling intermediates, cytokines and NO in phagocytes in vitro (3).

We previously identified TLR2 as an essential signaling molecule in a mouse model of neonatal GBS sepsis (4). Importantly, interaction of GBS with TLR2 was beneficial in a low dose GBS sepsis model, whereas it was detrimental in the high dose model of septic shock. However, in vitro TLR2 was, in concert with TLR6 and the coreceptor CD14, only essential for the recognition of released factors from GBS, whereas whole GBS organisms as fixed particles potently induced cytokines in a TLR2-, TLR6-, and CD14-independent, but MyD88-dependent fashion. Furthermore, we found lipoteichoic acid (LTA), a diacylated glycolipid from the GBS cell wall, to be released during growth of the organism and to stimulate TLR2 and TLR6 (5). The observation that LTA engaged TLR2 was in line with reports on other Gram-positive organisms such as staphylococci (6, 7). Over 200 publications make LTA the best established inflammatory molecule of Gram-positive bacteria. LTA is an attractive candidate for a Gram-positive equivalent of LPS, the classical endotoxin from Gram-negative bacteria. Both are anchored via their glycolipids to the membrane and carry a polysaccharide chain extending into the peptidoglycan (PGN) layer of the cell wall (3). LPS has been instrumental for the understanding of the role of TLRs in antibacterial immunity because it was the first TLR agonist in humans, TLR4 being the long sought after LPS receptor (8). However, GBS-LTA prepared by the most widely accepted method (butanol extraction at room temperature under stringent acidic conditions) was a relatively weak stimulus of TLR2, effective only at concentrations of  $\geq 1$   $\mu$ g/ml. Hence the potency of LTA is exceeded by that of LPS by at least 10,000-fold. Besides LTA, other molecules of GBS and further streptococci that have been reported to induce cytokines in phagocytes are of low potency (e.g., capsular polysaccharide, PGN,  $\beta$ -hemolysin, (5, 9, 10)). The observation of synergism between LTA and the macromolecular cell wall constituent PGN from

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Received for publication February 13, 2008. Accepted for publication February 13, 2008.

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<sup>1</sup> This work was supported in part by Grants He 3127/2-3 and He 3127/3-1 from Deutsche Forschungsgemeinschaft (to P.H.), Grant GPH 09 from the Institut Pasteur, Grant ANR-06-PATHO-001-01 from the Agence Nationale de la Recherche "ERANET PathoGenoMics," Grant LSHB-CT-2005-512061 from the Network of Excellence "Europathogenomics" (to P.T.-C.), and by Grants AI52455 and GM54060 from the National Institutes of Health (to D.T.G.).

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<sup>4</sup> Abbreviations used in this paper: GBS, group B streptococcus; BLP, bacterial lipoprotein; LTA, lipoteichoic acid; Lgt, prolipoprotein diacylglyceryl transferase; Lsp, lipoprotein signal peptidase; Lmb, laminin-binding protein; MDP, muramyl dipeptide; PGN, peptidoglycan.

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*Staphylococcus aureus* constituted a plausible solution to the discrepancy between the low potency of isolated streptococcal substructures and the remarkable inflammatory “toxicity” of the organism in vivo (11, 12). Indeed, we previously observed an amplification of the LTA response by addition of PGN. However, the LTA response was amplified only by muramyl dipeptides (MDP), the minimal PGN fragments that interact with the intracellular NOD receptors, and not by macromolecular PGN (5, 13, 14). In disagreement with this model, however, recent work from Hashimoto and colleagues (15–17) challenged LTA from *S. aureus* as a TLR2 agonist altogether. Hence, we were left with the puzzle of whether LTA might act in concert with other GBS substructures (e.g., MDP) in vitro and in vivo or whether molecules from GBS other than LTA were more potent stimulants of TLR2.

The aim of this study was to identify inflammatory molecules that are released by GBS and that stimulate TLR2. Contrary to our expectations, supernatant of GBS synthesizing LTA devoid of D-alanine residues, which have been repeatedly reported as structural prerequisites for the TLR2 agonistic effect of LTA (18–20), induced increased activation of TLR2 when compared with isogenic wild-type GBS. In contrast to these D-alanine-deficient mutants, GBS mutants deficient in the maturation of bacterial lipoproteins (BLPs) were greatly impaired in TLR2 activation. Specifically, prolipoprotein diacylglyceryl transferase (*lgt*)-mediated N-terminal acylation of prelipoproteins and subsequent lipoprotein signal peptidase (*lsp*)-mediated cleavage of the signal peptide were essential to the ability of released factors of GBS to activate TLR2 both in vitro and in vivo.

## Materials and Methods

### Reagents

Unless stated otherwise, reagents were obtained from Sigma-Aldrich. PBS, DMEM, G418, and trypsin-versene mixture were from Cambrex. Low endotoxin FBS was from HyClone Laboratories. LPS derived from *E. coli* strain O111:B4 was purchased from Sigma-Aldrich and re-extracted twice by phenol/chloroform.

### Bacterial strains, growth conditions, and medium

GBS strain NEM316 was originally isolated from an infant with fatal septicemia. NEM316 belongs to the capsular serotype III and its genome has been entirely sequenced. *E. coli* DH5 $\alpha$  (Invitrogen Life Technologies) was used in cloning experiments. GBS was cultured in Todd-Hewitt broth (Difco) or on sheep blood agar plates (REMEL) and *E. coli* in trypticase soy medium. RPMI 1640 (Merck Eurolab), DMEM, and a chemically defined medium (21) prepared from endotoxin-free water were also used to study the growth of GBS strains. Unless otherwise specified, antibiotics were used at the following concentrations: for *E. coli*, 100  $\mu$ g/ml ampicillin; 150  $\mu$ g/ml erythromycin; 50  $\mu$ g/ml kanamycin; 50  $\mu$ g/ml spectinomycin; 50  $\mu$ g/ml streptomycin; for GBS, 10  $\mu$ g/ml erythromycin; 1000  $\mu$ g/ml kanamycin; 500  $\mu$ g/ml streptomycin; and 250  $\mu$ g/ml spectinomycin. GBS liquid cultures were grown in standing filled flasks. All incubations were performed at 37°C.

### General DNA techniques

Genomic DNA from GBS was isolated as previously described (22). Standard recombinant DNA techniques were used for nucleic acid preparation and analysis. Plasmid DNA was isolated with Nucleospin Plasmid kit (Macherey Nagel). PCRs were conducted with Ampli Taq Gold polymerase as described by the manufacturer (Applied Biosystems). Amplification products were purified on Sephadex S-400 columns (Pharmacia) and sequenced with an ABI 310 automated DNA sequencer, using the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems). Electrocompetent cells of GBS were prepared as described (23).

### Construction of bacterial strains

In NEM316, *lgt*, and *lsp* genes are referred to as *gbs0758* and *gbs1436*, respectively (<http://genolist.pasteur.fr>). To construct GBS  $\Delta$ *lgt* mutant (NEM2188), we inserted the promoterless and terminatorless kanamycin resistance cassette *aphA-3* (22) within DNA segments encompassing the 5'

and 3' ends of *lgt*. This insertion was done by ligation, after digestion with the appropriate enzymes, of the three amplicons: O1-O2 (5' end of *lgt*), KanK-KanB (*aphA-3* gene), and O3-O4 (3' end of *lgt*). The corresponding *EcoRI-PstI* fragment was cloned into the thermosensitive shuttle plasmid pG+host5, and the resulting recombinant vector, pG+host5 $\Delta$ *lgt*, was introduced by electroporation into NEM316. Erythromycin-sensitive/kanamycin-resistant mutants that carried the expected *lgt* insertion-deletion were selected as described (22). For construction of GBS  $\Delta$ *lsp* mutant, the promoterless and terminatorless streptomycin resistance cassette *aad6* was used in a similar strategy. In this case, the amplicons O5-O6 (5' end of *lsp*), SmK-SmB (*aad6* gene), and O7-O8 (3' end of *lsp*) were ligated and cloned into the thermosensitive shuttle plasmid pG+host5. The vector pG+host5 $\Delta$ *lsp* was introduced by electroporation into NEM316 and NEM2188 to generate NEM2189 (NEM316 $\Delta$ *lsp*) and NEM2194 (NEM316 $\Delta$ *lgt*/ $\Delta$ *lsp*), respectively. All mutations were verified by sequencing of the inactivated *lgt* and *lsp* genes. The  $\Delta$ *lta* mutant strain in the NEM316 background has been previously described in detail (22).

### Preparation of intact GBS cells and LTA

Overnight GBS colonies were used to inoculate chemically defined medium prepared from endotoxin-free water. Then, bacterial cultures were grown to mid-log phase (OD<sub>650</sub> = 0.27–0.30). Alternatively, overnight cultures in Todd-Hewitt broth were diluted 1/20 in DMEM (Cambrex) and grown to mid-log phase. In both cases, GBS were washed once with PBS, resuspended in 70% ethanol in pyrogen-free H<sub>2</sub>O at a density of  $\approx 3 \times 10^{10}$  CFU/ml and inactivated for 30 min on ice. Bacteria were washed again in PBS and stored at –80°C. All procedures were performed under pyrogen-free conditions, resulting in preparations that were essentially free of endotoxin, as determined by a highly LPS-sensitive reporter system (CHO-CD14 with endothelial cell-leukocyte adhesion molecule ELAM promoter driven CD25, lower limit of detection 10–100 pg/ml, data not shown). GBS were cultured under permanent agitation in chemically defined medium at 37°C. Bacteria were harvested at an OD<sub>650</sub> of 0.6 by centrifugation, washed in endotoxin-free water, and lyophilized. LTA was subsequently extracted with *N*-butanol (Merck) under stirring for 30 min at room temperature exactly as described (18, 24). The concentration of LTA in culture supernatant was measured semiquantitatively by Western blot analysis using a commercial mAb against LTA (Hycult). LTA from strain COH1 was prepared exactly as described (5).

### Preparation of GBS supernatant and proteins

GBS supernatant was produced as described (9). Briefly, GBS was grown in chemically defined medium to mid-log phase (OD<sub>650</sub> ~0.3), bacteria were pelleted by centrifugation, the resulting supernatant was filter sterilized with a 0.2- $\mu$ m membrane, dialyzed extensively against endotoxin-free water, and lyophilized. Concentration of the supernatant is depicted either in wt/vol or as fold concentration, which indicates the concentration of the GBS supernatant in the cell culture medium as compared with the original bacterial culture. Total protein of GBS was prepared as follows. A 50-ml overnight GBS culture was spun down, washed twice with 5 ml of PBS, and resuspended in 1 ml of PBS. The bacterial suspension was poured into a Fastprotein Blue tube placed in a FastPrep instrument (Qbiogene). Bacterial lysis was obtained at 4°C using three 30-s maximum speed cycles with 1-min rest intervals. After lysis, the reaction mixture was centrifuged twice (21,500 relative centrifugal force, 4°C, 15 min) to remove cell debris and remaining bacteria. The supernatant containing total GBS protein was kept frozen at –20°C for subsequent analysis. The corresponding culture medium was filter sterilized and the proteins present in the filtrate were precipitated with 2% TCA for 2 h at 4°C. Following centrifugation, the pellet was washed twice for 30 min in acetone at –20°C, solubilized in 1 ml of Tris-HCl buffer (10 mM (pH 8.0)), and kept frozen at –20°C.

### [<sup>3</sup>H]palmitic acid labeling of BLPs

Bacteria were grown in 5 ml of Todd-Hewitt broth containing [<sup>3</sup>H]palmitic acid at a final concentration of 20  $\mu$ Ci/ml. Following 5 h of incubation at 37°C, cells were harvested by centrifugation, washed twice with 500  $\mu$ l of PBS, and resuspended in 500  $\mu$ l of this buffer. Total protein was extracted as described and analyzed by PAGE under denaturing conditions and autoradiography.

### Immunoblots of proteins and LTA and immunofluorescence staining

A 999-bp DNA fragment containing the laminin-binding (*lmb*) gene was amplified by using the primers O13 and O14 and cloned into pET28a+ (Novagen) that had been digested with *EcoRI* and *XhoI*. The corresponding



recombinant Lmb protein containing a C-terminal 6-histidine tag was expressed in *E. coli* BL21 and purified by affinity chromatography on Ni-NTA columns, according to the manufacturers' recommendations (Novagen). The purified Lmb protein was injected to a rabbit to produce polyclonal Lmb Abs. Rabbit antiserum raised against the coaggregation adhesin A protein ScaA was provided by Dr. P. E. Kolenbrander (National Institutes of Health, Bethesda, MD). This ScaA Ab was raised against ScaA from *Streptococcus gordonii*, which displays 73.5% of sequence identity with ScaA (GBS1589) from GBS NEM316. Following electrophoresis under denaturing conditions, the proteins of GBS were transferred to a nylon membrane and detection of Lmb and ScaA was performed with rabbit anti-recombinant Lmb or anti-ScaA sera (diluted 1/1000 in PBS) as described (23). For the LTA dot blot, an LTA Ab that binds to the polyglycerophosphate backbone of LTA was used (Hycult).

**Neonatal mouse models of GBS sepsis.** Neonatal (24-hour-old) BALB/c and C57BL6/J mice (both from Charles River Breeding Laboratories) or TLR2<sup>-/-</sup> mice pups (C57BL6/J background), a gift of S. Akira (Osaka, Japan) (4), were used for virulence studies. Randomized groups of two to four mice pups were s.c. inoculated with serial log dilutions of mid-log-phase bacteria (0.03 ml of each strain in 0.9% NaCl). Mice were observed daily for up to 6 days after infection because, in this model, deaths are rarely observed after 5 days.

All of the procedures were in agreement with the guidelines of the European Commission for the handling of laboratory animals, and the studies presented in this work were approved by the relevant national and institutional committees. Statistical analysis of the mortality data was performed with the Mann-Whitney *U* test as calculated with GraphPad Instant v.3.0 software.

#### Measurement of proinflammatory activity

RAW 264.7 mouse macrophages were cultured in DMEM containing 10% FBS and 10  $\mu$ g/ml ciprofloxacin. Cultures were kept at 37°C in a 5% CO<sub>2</sub> atmosphere. For determination of cytokine formation, RAW macrophages were seeded at a density of 1  $\times$  10<sup>6</sup> cells/ml in 96-well dishes in RPMI 1640 with 10% FBS and incubated over 16 h at 37°C in a 5% humidified CO<sub>2</sub> environment. Supernatants were processed directly for the determination of released cytokines by ELISA (R&D Systems) per the manufacturer's protocols.

Human PBMC 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. After addition of the indicated stimuli, PBMC were incubated for 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).

#### Reporter gene analysis

HEK293 cells and the stable cell line HEK-TLR2YFP were used in the reporter gene studies as described (5). Cells were plated into 96-well tissue-culture plates at a density of 5  $\times$  10<sup>3</sup> cells/well. The following day, cells were transiently transfected with TransIT-LTI Transfection Reagent (Mirus Bio) following the manufacturer's protocol. Plasmid pCDNA was used to assure equal amounts of transfected DNA. The following day, cells were incubated for 6 h with the indicated stimulants. Cells were then lysed in passive lysis buffer (Promega) and reporter gene activity was measured using a plate reader luminometer (MicroLumat Plus; Berthold). In all cases, the depicted data represent one of at least three separate but similar experiments and are presented as the mean value  $\pm$  SD of triplicate samples.

#### Oligonucleotides

The sequences (5' to 3') of the relevant oligonucleotides used in this study include the following: O1, 5'-CATGAATTCTGGTATGTTGTTAGCAG-3'; O2, 5'-GAGAGGTACCCCGTAATTAACCCTCCG-3'; O3, 5'-GATGTGGATCCTTCATTATTGAAGGGATG-3'; O4, 5'-AAGCTGCAGCTGCACCTGATGCAGCACTG-3'; O5, 5'-GAAAGAATTCAGCTTATCGCAGGGCTC-3'; O6, 5'-TFAAGGTACCATAACTTAGATGTTGATC-3'; O7, 5'-GTAGCAGGATCCTATCTGACAATCGG-3'; O8, 5'-GTGGCCTGCAGAAGGGTGAACAACC-3'; O9, 5'-TTGAGGATCCTTAAACACATTTGATTAGTCAG-3'; O10, 5'-AACAACTGCAGTGGCAATAATTAATACAGCA-3'; O11, 5'-CTTTAGGATCCGTTGCTGTTATGGAAGAGTA-3'; O12, 5'-GCTAACTGCAGATCTAGCCTAACTCTGCTA-3'; O13, 5'-GATGGTAGAATTCATTGACAAAGCATTG-3'; O14, 5'-AATCTCCTCTCGAGCAACTGTTGATAGAGCACTTCC-3'; KanK, 5'-GGGGTACCTTTAAACTACTGTAG-3'; KanB, 5'-TCTGGAT

CCTAAAACAATTCATCC-3'; SmK, 5'-TCGGTACCGAAGAAGATGTAATAATATAG-3'; SmB, 5'-TTGGATCCCTGTAATCACTGTCCCGCCT-3'.

#### Protein sequencing

Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and stained with AmidoBlack. Selected stained polyvinylidene difluoride bands were excised and subjected to Edman degradation on an Applied Biosystems 473A or 494 sequencer (performed by J. d'Alayer, Institut Pasteur, Paris, France).

## Results

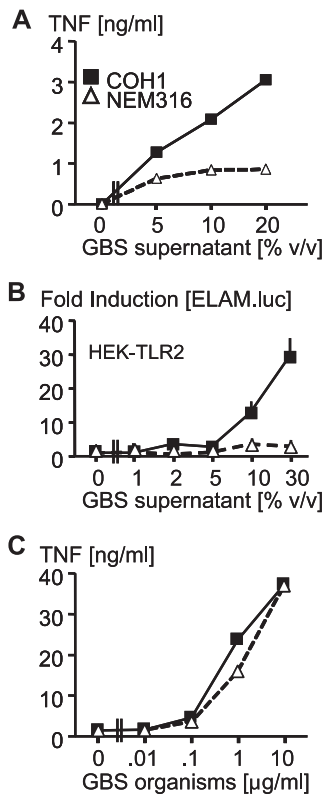
### *GBS type III strains differ with respect to the release of cytokine inducing factors but not cell wall-induced cytokine formation*

Most of the published work on the interaction of GBS with TLRs is based on COH1, a serotype III strain originally isolated from a septic newborn infant. COH1 belongs to the so-called hypervirulent multi-locus sequence typing MLST cluster ST-17. We previously found that GBS COH1 releases factors that potently activate TLR2 in vitro (9). However, it was unclear whether the release of TLR2 activating molecules was a general feature of GBS. Hence, we tested a number of GBS strains for the release of factors that activate TLR2. Surprisingly, we found that supernatants from other serotype III strains that belong to the same multi-locus sequence typing cluster, like BM110, or to a different cluster, like the fully sequenced and well-characterized strain NEM316 (ST-23), were substantially less effective than COH1 in stimulating TLR2 and cytokine production, as assessed in RAW 264.7 macrophages and TLR2-expressing epithelial cells (Fig. 1, *A* and *B*). It seems noteworthy that GBS supernatant induces neither cytokines in TLR2-deficient macrophages nor NF- $\kappa$ B activation in HEK cells that do not express TLR2 (5, 9, 10 and data not shown). In contrast, the inflammatory potency of ethanol- or heat-fixed GBS particles did not differ between strains (Fig. 1C).

### *LTA does not constitute the major TLR2 activating factor in GBS supernatant*

We previously reported that GBS LTA activates TLR2 and TLR6. Because we found that LTA is released into the extracellular medium during bacterial replication (our unpublished observation), LTA was the putative molecular identity of the soluble GBS factor that interacts with TLR2. Hence, we enriched GBS supernatant for TLR2 activating factors (designated in this study as GBS-F) by size exclusion chromatography and compared the resulting fractions to butanol extracted LTA from the same strain in a TLR2-dependent reporter cell system (HEK TLR2) and in Western blot analysis for LTA. We found that GBS-F was over 100-fold more potent in inducing the NF- $\kappa$ B-dependent reporter than purified LTA when they were compared on a weight basis (Fig. 2, *A* and *B*). We previously reported on a super additive effect of LTA and MDP in PBMC (5). Accordingly, we wondered whether MDP-mediated amplification of the response to LTA accounted for differences between GBS-F and LTA in a TLR2-specific reporter assay. However, combination of LTA and MDP resulted in only a very modest increase in reporter activation as compared with LTA alone. Hence, the inflammatory potency of GBS-F could not be explained by effects of LTA and MDP (Fig. 2C). Moreover, Western blot analysis of the same preparations depicted in Fig. 2, *A* and *B*, revealed that only ~1% of GBS-F was LTA (~20 ng of LTA in 2  $\mu$ g of lyophilized supernatant) (Fig. 2D). Accordingly, GBS-F was  $\approx$ 10,000 fold more active than its LTA content.

Screening of our library of GBS mutants revealed that GBS NEM1636, which carries a targeted mutation in the gene encoding the cytoplasmic D-alanine-D-alanyl carrier protein ligase DltA (22),

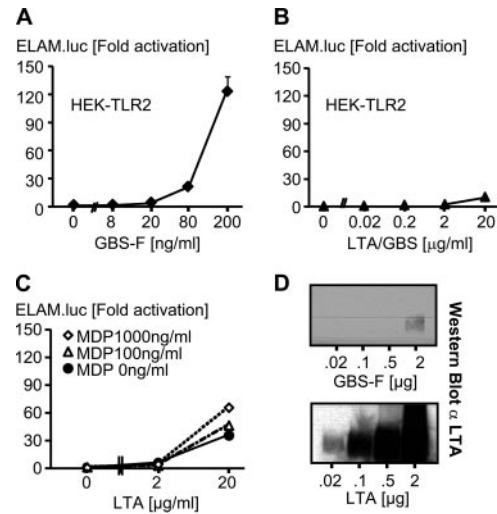


**FIGURE 1.** GBS type III laboratory strains differ with respect to the release of cytokine-inducing factors but not cell wall-induced cytokine formation. *A* and *B*, RAW 264.7 macrophages (*A*) and HEK-TLR2 cells transfected with an NF- $\kappa$ B dependent ELAM-luciferase reporter gene (*B*) were incubated with escalating concentrations of cell-free GBS supernatant. After 16 h, the macrophage supernatant was analyzed for TNF formation (*A*), or cells were lysed after 5 h and luciferase activity was determined by luminometry (*B*). *C*, Ethanol-fixed GBS III strains COH1 and NEM316 were analyzed for induction of TNF in RAW macrophages (16 h of incubation). TNF in the supernatants was determined by ELISA. Data depicted are mean + SD of triplicate wells from one representative experiment of three or more conducted. SD bars are in part hidden by the symbol indicating the mean.

exhibited increased activation of TLR2 through extracellular factors when compared with isogenic wild-type GBS (Fig. 3*A*). This was an unexpected finding because DltA-mediated D-alanyl esterification had been repeatedly shown to be an essential structural prerequisite for the TLR2 agonistic effect of LTA (18–20). Importantly, NEM1636 retained normal cell wall-mediated inflammatory activation of mouse macrophages and human PBMC, as compared with the isogenic parental GBS NEM316 (Fig. 3*B*). Because these data provided strong evidence that LTA was not the main TLR2/6 activating factor released by GBS, we subsequently focused on the role of BLPs as putative TLR2/6 agonists in GBS.

#### Lipoprotein acylation in $\Delta$ lgt and $\Delta$ lsp GBS mutants

Complete maturation of BLPs in many Gram-positive bacteria involves two enzymes, Lgt that catalyzes acylation of the signal peptide lipobox, and the signal peptidase Lsp that cleaves the modified signal peptide upstream of the acyl ester. To better understand the role of protein acylation in the inflammatory potency of GBS, we insertionally inactivated the corresponding genes *lgt* and *lsp* in the GBS NEM316 genetic background as described in *Materials and Methods*. To analyze protein acylation in wild-type (NEM316) and mutant ( $\Delta$ lsp NEM2189 and  $\Delta$ lgt NEM2188) GBS strains, we cultured GBS in the presence of [ $^3$ H]palmitate for incorporation of

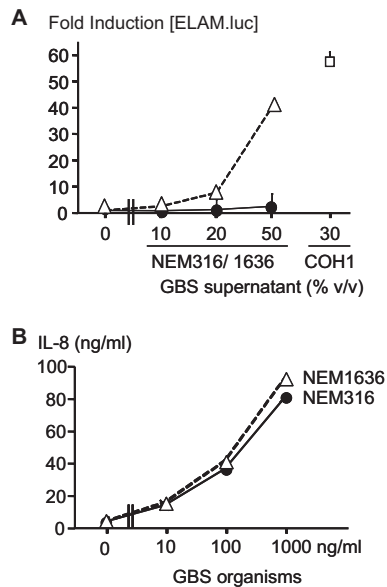


**FIGURE 2.** Supernatant of GBS enriched for activity is 100 times more potent in activating TLR2 than LTA. *A* and *B*, HEK-TLR2 cells transfected with an ELAM-luciferase reporter gene were incubated with escalating concentrations of cell-free GBS (COH1) supernatant that had been enriched for TLR2 activation by size exclusion chromatography (*A*), with butanol extracted LTA from the same strain (*B*), or with combination of LTA with MDP as indicated in *C*. After 5 h, cells were lysed and luciferase activity was determined by luminometry. *D*, Preparations tested in *A* and *B* were subjected to SDS-PAGE and analyzed by Western blot with a mAb that was raised against the polyglycerophosphate backbone of LTA.

labeled acyl anchors into the lipoprotein N terminus. The autoradiogram of total bacterial extracts separated by SDS-PAGE showed numerous bands in NEM316 with apparent molecular mass ranging from 20 to 98 kDa (Fig. 4*A*, lanes 1 and 3). This observation is consistent with the molecular mass spectrum of the 41 predicted BLPs in the NEM316 strain that range from 7.2 kDa (Gbs0086) to 97.5 kDa (Gbs0918) (<http://genolist.pasteur.fr/SagaList/>). The pattern of the  $\Delta$ lsp mutant (Fig. 4*A*, lane 2) was similar, but not identical with that of the parental strain NEM316. In particular, some bands exhibited a slight increase in m.w. This observation was consistent with the fact that, in the absence of Lsp, BLPs possess both a signal peptide and an acyl anchor. As expected, due to the absence of protein acylation, no labeled bands were detectable in the  $\Delta$ lgt mutant NEM2188 (Fig. 4*A*, lane 4).

#### Lipobox processing by Lsp in the absence of Lgt

To further characterize the NEM316 derivatives altered in BLPs biosynthesis, we performed Western blot analysis of total cellular and culture supernatant proteins from NEM316, NEM2188 ( $\Delta$ lgt), NEM2189 ( $\Delta$ lsp), and NEM2194 ( $\Delta$ lgt/ $\Delta$ lsp) with polyclonal Abs raised against the best-characterized lipoprotein from GBS, Lmb (25) and the putative BLP ScaA, a streptococcal adhesin (21). This analysis revealed that ScaA was retained in the bacterial membrane of wild-type (Fig. 4*B*, lanes 1 and 4) and  $\Delta$ lsp mutant strains, whereas it was not detected in the culture supernatant of these strains (Fig. 4*B*, lane 3). On the contrary, a substantial amount of ScaA was detected in the culture supernatant of  $\Delta$ lgt and  $\Delta$ lgt/ $\Delta$ lsp mutants (Fig. 4*B*, lanes 2 and 5). Hence N-terminal acylation appeared to be a prerequisite for effective lipoprotein anchoring to the cell membrane. The same results were observed with anti-Lmb antisera (Fig. 4*B*). In NEM316 and the  $\Delta$ lsp mutant, the secreted non-BLPs GBS0153 and CAMP were the two most abundant proteins detected in culture supernatants, as identified by NH<sub>2</sub> sequencing (Fig. 4*C*). One additional band at around 34 kDa was

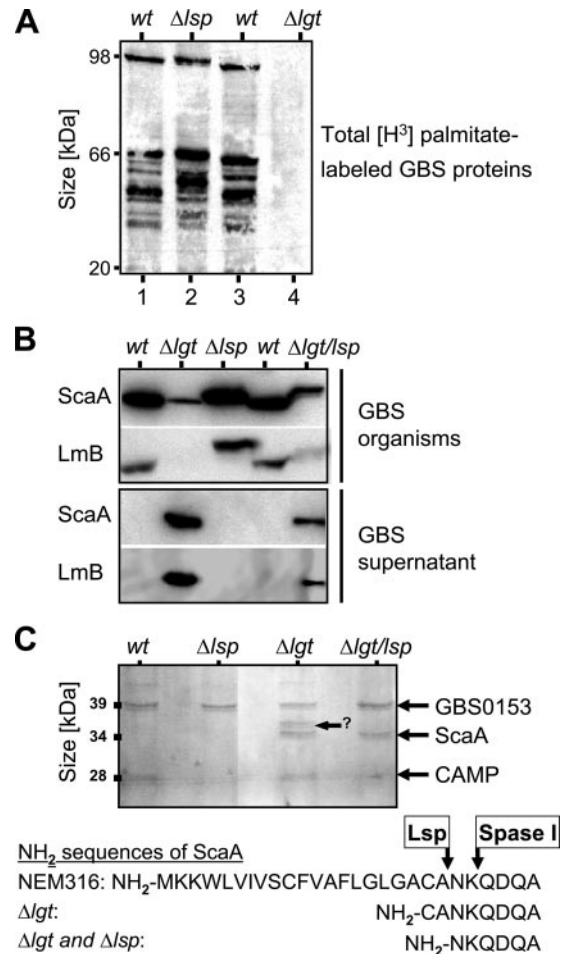


**FIGURE 3.** GBS deficient in the D-alanine transferase *dltA* gene (NEM1636) exhibit increased release of TLR2 activating factors while retaining a normal inflammatory phenotype of the cell wall. **A**, HEK-TLR2 cells were transfected with an ELAM-luciferase reporter gene and incubated with escalating concentrations of cell-free GBS supernatant from *dltA*-deficient GBS (NEM1636) ( $\Delta$ ), isogenic wild-type GBS (NEM316) ( $\bullet$ ), or GBS COH1 ( $\square$ ). After 6 h, cells were lysed and luciferase activity was determined by luminometry. **B**, Ethanol-fixed GBS III strains NEM1636 and NEM316 were analyzed for induction of IL-8 in human PBMC from normal donors (16 h of incubation). IL-8 in the supernatants was determined by ELISA. Data depicted are mean + SD of triplicate wells from one representative experiment of three performed.

detected in the supernatants of both the  $\Delta lgt$  and  $\Delta lgt/\Delta lsp$  mutants (Fig. 4C). Edman degradation revealed that the additional band present in both strains was ScaA. Interestingly, the NH<sub>2</sub> sequence of the mature protein differed in the  $\Delta lgt$  and  $\Delta lgt/\Delta lsp$  strains (Fig. 4C, bottom). In the  $\Delta lgt$  mutant, the first amino acid residue was the signature cysteyle of the lipobox, a feature expected with a protein processed by Lsp. In the  $\Delta lgt/\Delta lsp$  mutant, the sequence started with an asparagyl residue, a feature that was expected for a protein processed by the type I signal peptidase. We thus concluded that Lgt modification of the lipobox was not critical for Lsp cleavage and that, in the absence of both enzymes, the type I signal peptidase could process the signal peptide. This interpretation is consistent with the finding that ScaA and Lmb are both found in the supernatant of the  $\Delta lgt/\Delta lsp$  mutant, albeit in lower amounts than in the  $\Delta lgt$  mutant (Fig. 4B). Details of the biosynthetic pathway of BLPs in GBS are summarized in Fig. 5.

#### GBS releases BLPs into the extracellular medium, which essentially interact with TLR2

The interaction of streptococcal BLPs with TLRs has not been assessed and there is very incomplete evidence on the interaction of BLPs from other Gram-positive organisms with this receptor. On the functional level, supernatants of  $\Delta lgt$  GBS exhibited dramatically reduced inflammatory activation of RAW macrophages as assessed by TNF release (Fig. 6A). This lipoprotein-dependent host cell activation corresponded to the NF- $\kappa$ B-dependent transcriptional activation in a TLR2-specific assay (NF- $\kappa$ B-dependent reporter activation in HEK-TLR2 cells) (Fig. 6B). Importantly, ethanol-fixed  $\Delta lgt$  GBS normally stimulated cytokine formation in macrophages and PBMCs (Fig. 6C). It seems important to note

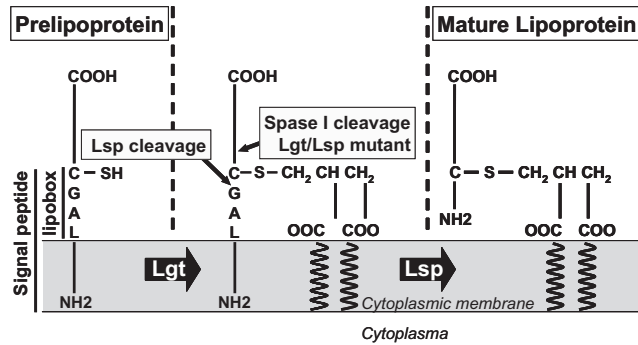


**FIGURE 4.** Lsp and Lgt are essential for lipoprotein acylation and maturation in GBS. **A**, Analysis of GBS protein acylation in wild-type GBS and isogenic strains with targeted deletions of *lsp* and *lgt*. Wild-type GBS and GBS with targeted deletions in the Lgt or the Lsp were cultivated in the presence of [<sup>3</sup>H]palmitic acid. Total protein was extracted, separated by PAGE under denaturing conditions, and autoradiographed. **B**, Expression and localization of the lipoproteins ScaA and Lmb in *lgt*- and *lsp*-deficient strains. Cell bound and culture supernatant proteins were purified from the wild-type strain NEM316, the  $\Delta lgt$  mutant NEM2188, the  $\Delta lsp$  mutant NEM2189, and the  $\Delta lgt/\Delta lsp$  mutant NEM2194. The resulting extracts were analyzed for ScaA and Lmb by Western blot with polyclonal sera. **C**, Protein sequencing of the lipoprotein ScaA in  $\Delta lsp$ ,  $\Delta lgt$ , and  $\Delta lgt/\Delta lsp$  GBS strains. Culture supernatant proteins were purified from the wild-type GBS or  $\Delta lsp$ ,  $\Delta lgt$ , or  $\Delta lgt/\Delta lsp$  mutant GBS. The N-terminal sequence of ScaA was determined by microsequencing after separation by SDS page.

that transfer of the same  $\Delta lgt$  mutation into the GBS COH1 background generated a mutant strain that exhibited the same phenotype as the  $\Delta lgt$  mutant derived from NEM316 (near complete loss of TLR2 activation by GBS COH1 supernatant, data not shown). Hence, differences between these two strains in TLR2 activation are due to differences in the formation of mature BLPs.

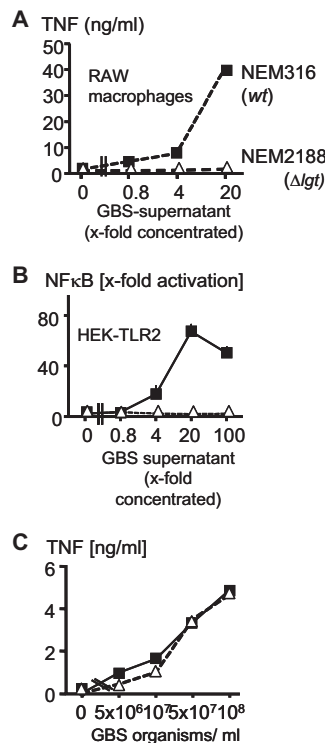
In contrast to our expectations, the inflammatory phenotype of  $\Delta lsp$  mutant in vitro mimicked that of  $\Delta lgt$  strain. The supernatant of  $\Delta lsp$  GBS exhibited largely impaired activation of NF- $\kappa$ B and IL-8 (data not shown) in HEK293 cells stably transfected with TLR2 (Fig. 7A). According to the data depicted in Fig. 4, proteins are acylated in the  $\Delta lsp$  strain. Furthermore, Western blot analysis of this mutant suggested that BLPs were retained in the membrane (Fig. 4B). However, similar to the effects observed with GBS supernatant, the TLR2-dependent activation by fixed GBS organisms



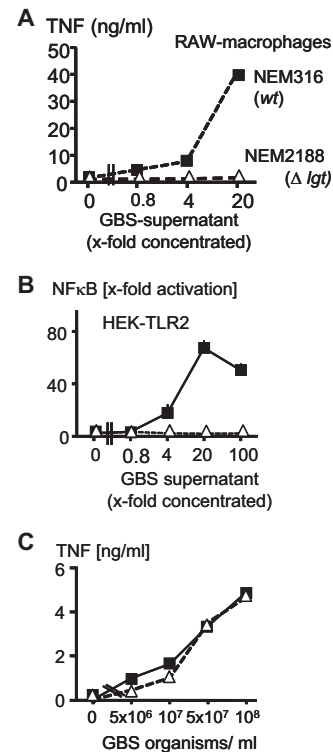


**FIGURE 5.** Model of biosynthesis and localization of lipoproteins in GBS. Prelipoproteins are translocated across the cytoplasmic membrane through the Sec pathway. Then, Lgt catalyzes acylation of the signal peptide lipobox. Finally, the signal peptidase Lsp (signal peptidase II) cleaves between the glycine and the lipid-modified cysteine residue. In the absence of *lgt* and *lsp*, lipoprotein signal peptides could be processed by the type I signal peptidase.

was abrogated in  $\Delta lsp$  GBS (Fig. 7B). As described earlier, the modest interaction of fixed GBS organisms with TLR2 is not critical for cytokine induction in macrophages, i.e., TLR2-deficient macrophages mount a normal cytokine response to fixed GBS organisms. However, analysis of the interaction at the cell-to-cell interface seemed important because it indicated that protein acylation by Lgt is required, but not sufficient, for the interaction of GBS-BLPs with TLR2. Rather the signal peptidase Lsp provides a



**FIGURE 6.** Genetic deletion of protein acylation in GBS abrogates activation of TLR2 by extracellular factors of GBS, but does not impair cytokine induction by fixed GBS organisms. Supernatant from wild-type GBS NEM316 (A and B) or ethanol-fixed (C) wild-type GBS NEM316 (■) and equal preparations from the isogenic  $\Delta lgt$  mutant NEM2188 (△) were analyzed for induction of TNF in RAW macrophages (16 h of incubation in A and C) or activation of an NF- $\kappa$ B reporter gene in HEK-TLR2 cells (B). Data depicted are mean + SD of triplicate wells. SD bars are in part hidden by the symbols indicating the mean.

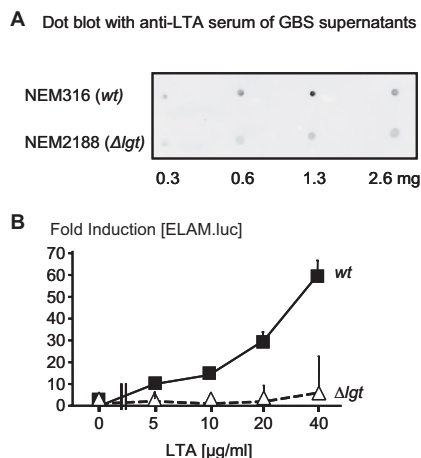


**FIGURE 7.** The signal peptidase Lsp mediates inflammatory signaling induced by extracellular GBS factors but does not essentially mediate cytokine formation by fixed GBS organisms. HEK-TLR2 cells transfected with an NF- $\kappa$ B dependent ELAM-luciferase reporter gene (A and B) or RAW 264.7 macrophages (C) were incubated with escalating concentrations of cell-free GBS supernatants from wild-type GBS (■) or  $\Delta lsp$  GBS (△) (A) or with ethanol-fixed GBS of the same strains (B and C). ELAM-luciferase activity was measured in HEK cell lysates by luminometry and is depicted as fold activation over background (medium control). TNF in the RAW 264.7 supernatants was determined by ELISA. Data depicted are mean + SD of triplicate wells from one representative experiment of three or more performed.

second essential modification. In contrast to the TLR2-restricted transcriptional activation in the HEK-TLR2 model, the global cytokine formation induced by fixed GBS organisms in macrophages was similar between  $\Delta lsp$  and wild-type GBS (Fig. 7C). Hence, lipoprotein maturation requires both Lgt and Lsp for interaction with TLR2, and BLPs are the essential TLR2 partners both in fixed GBS organisms and the extracellular medium. However, fixed GBS organisms potentially initiate cytokine formation in macrophages in a lipoprotein and TLR2 independent manner.

#### *Butanol-extracted LTA from $\Delta lgt$ GBS does not activate TLR2 at concentrations as high as 20 $\mu$ g/ml*

LTA is widely regarded as an important TLR2 agonist in Gram-positive bacteria (26–28). However, GBS supernatant containing LTA seemed to activate TLR2 largely through BLPs (Figs. 6 and 7). These findings were consistent with those of Hashimoto et al. (17) who provided strong evidence that BLPs from *S. aureus* stimulate host cells via TLR2. Hence, we extracted LTA from the  $\Delta lgt$  GBS strain NEM2188 and compared its activity to that of LTA extracted from the wild-type isogenic parental strain NEM316. As depicted in Fig. 8A, *lgt* inactivation did not essentially interfere with the formation and release of LTA into the supernatant. With respect to the TLR2 stimulation, LTA from the  $\Delta lgt$  mutant retained only little, if any, potency as compared with LTA from wild-type GBS NEM316 (Fig. 8B). This finding indicated that



**FIGURE 8.** Butanol extracted lipoteichoic acid from  $\Delta lgt$  GBS does not activate TLR2 in concentrations as high as 20  $\mu\text{g/ml}$ . *A*, Concentrated and lyophilized supernatants in the indicated quantities from the wild-type GBS strain NEM316 (■) or from the isogenic *lgt*-deficient mutant NEM2188 ( $\Delta$ ) were subjected to dot blot analysis with a mAb directed against the polyglycerol backbone of LTA. This Ab did not react with diacylated proteins because it did not stain MALP-2 used as a control (data not shown). *B*, LTA from the same GBS strains and analyzed for NF- $\kappa$ B activation by ELAM-luciferase reporter gene analysis in HEK-TLR2 cells. Data depicted are mean + SD of triplicate wells from one representative experiment of three conducted.

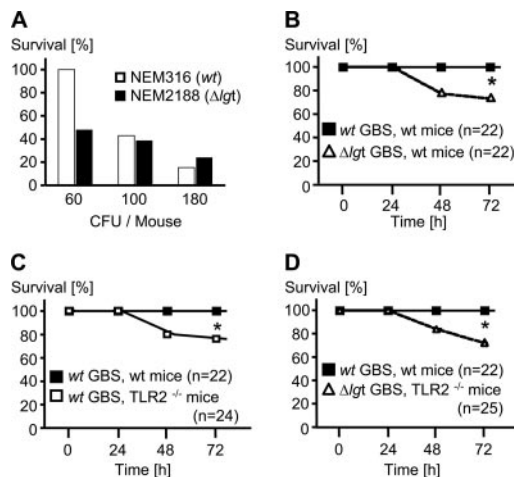
LTA purified by the most widely accepted method (butanol extraction) contained traces of diacylated BLPs that carry most of the activity for TLR2.

#### Growth of $\Delta lgt$ and $\Delta lsp$ mutants

In rich Todd-Hewitt broth, the wild-type NEM316 strain and the mutants NEM2188 ( $\Delta lgt$ ), NEM2189 ( $\Delta lsp$ ), and NEM2194 ( $\Delta lgt/\Delta lsp$ ) exhibited similar growth rates (data not shown). However, following several subcultures in RPMI 1640 or chemically defined medium used as a minimal medium, we noticed a 1.5- to 3-fold increase of the doubling generation time of all mutant strains, as compared with the parental strain. However, the CFU numbers of NEM316 and the mutant strains were similar after an overnight incubation in minimal medium reaching  $3 \times 10^8$  CFU/ml. We thus concluded that *Lgt*, *Lsp*, or both were dispensable for GBS growth in rich and minimal medium although they contributed to the bacterial fitness under nutrient limitation (data not shown).

#### Role of BLPs in GBS sepsis in vivo

BLPs appeared to be the main GBS product that interacted with TLR2 in vitro. In previous studies, we had found that TLR2 substantially contributed to the course of GBS sepsis in mice (4). Hence, it seemed important to assess the phenotype of the  $\Delta lgt$  mutant in a neonatal mouse model of GBS sepsis. To this end, we infected neonatal mice s.c. with an escalating doses of wild-type and  $\Delta lgt$  strains. We found that a sublethal dose of wild-type NEM316 (60 CFU) corresponded to the LD<sub>50</sub> of  $\Delta lgt$  mutant in BALB/c mice (Fig. 9A). The phenotype of *lgt*-deficient strain in wild-type C57BL6/J mice resembled that of wild-type NEM316 in TLR2-deficient mice (Fig. 9, B and C). The combination of TLR2-deficient mice and *lgt*-deficient GBS did not increase lethality any further (Fig. 9D). Hence, BLPs are the main substructures from GBS that interact with TLR2 during the early stage of sepsis in vivo. In contrast to the sublethal model, BLPs were not essential for GBS sepsis models with high rates of mortality, i.e., models of septic shock (Fig. 9A). In this model, s.c. injection of 90 CFU



**FIGURE 9.** Sensing of lipoproteins by TLR2 is essential for the protection of neonatal mice in a low dose model of GBS sepsis. *A*, Neonatal wild-type mice (BALB/c) were infected with NEM316 or the isogenic  $\Delta lgt$  strain NEM2188 in escalating concentrations equivalent to the LD<sub>0</sub> (60 CFU,  $n = 24$  for NEM316 and  $n = 25$  for NEM2188), LD<sub>50</sub> (100 CFU,  $n = 28$  for NEM316,  $n = 26$  for NEM2188), or LD<sub>90</sub> (180 CFU,  $n = 13$  for each group). *B–D*, Neonatal TLR2<sup>-/-</sup> mice or isogenic C57BL6/J wild-type controls were s.c. infected with 60 CFU of NEM316 or the isogenic  $\Delta lgt$  strain NEM2188 as indicated. The 60 CFU correspond to the LD<sub>0</sub> in C57BL6/J wild-type mice (sublethal dose). The lethality was monitored until 6 days postinfection but no additional death was recorded after day 3.  $p < 0.05$  indicates that the differences in lethality between wild-type and *lgt*-deficient strains after 72 h are significant, as determined by Mann-Whitney *U* test.

resulted for  $\Delta lgt$  GBS in 16 deaths of 28 total animals (57% lethality) after 72 h, as compared with 16 deaths of 26 total (61.5%) for wild-type GBS strain NEM316 mice. The respective numbers for the LD<sub>90</sub> model (180 CFU) were 11 deaths of 13 animals (lethality 85%) for NEM2188 and 10 deaths of 13 total (77%) for NEM316. Accordingly, the interaction of BLPs with TLR2 seems to be especially important for the early recognition of GBS and therefore timely clearance of GBS during sepsis.

## Discussion

Sepsis and meningitis are the typical manifestations of invasive GBS disease. The activation of TLR2 contributes substantially to the course of invasive GBS disease. In this study, we provide evidence that BLPs, and not LTA, constitute the primary GBS substructures that interact with TLR2. BLPs are released by GBS during growth, and interact with TLR2 on phagocytes and probably many other cell types.

Bacterial BLPs are involved in a large variety of processes, which range from uptake of nutrients, resistance against antibiotics, protein secretion, cell-wall biogenesis, and adhesion to extracellular matrix and host tissues. BLPs are synthesized as prelipoproteins with a distinct signal sequence containing a conserved C-terminal lipobox (-Leu<sub>-3</sub>-Ser/Ala<sub>-2</sub>-Ala/Gly<sub>-1</sub>-Cys<sub>+1</sub>). The first step in the biosynthesis of BLPs is the transfer of a diacylglycerol moiety from phosphatidylglycerol to the sulfhydryl group of the invariant cysteyle residue. This reaction is catalyzed by the product of *Lgt* encoded by the *lgt* gene. When BLPs are translocated across the cytoplasmic membrane through the Sec pathway, the specific *Lsp*, also known as signal peptidase II, cleaves between the amino acid at position -1 and the lipid-modified cysteine residue (29) (Fig. 5). *Lgt* and *Lsp* are highly conserved enzymes in bacteria. Interestingly, both enzymes appear to be essential for growth in Gram-negative bacteria but dispensable in



Gram-positive bacteria. BLPs are further processed in Gram-negative bacteria by a third enzyme designated lipoprotein *N*-acyl transferase Lnt that catalyzes the addition of an *N*-acyl group to the diacylglyceryl cysteine. This modification is necessary for efficient recognition of outer membrane BLPs by the Lol system, which transports them from the plasma membrane to the outer membrane (30).

In contrast to *lgt* and *lsp*, we did not find a gene encoding a lipoprotein *N*-acyltransferase Lnt homolog in the published genome sequences of GBS, which suggests that BLPs in GBS are only diacylated. Our previous observation that recognition of secreted factors from GBS requires TLR6 is in full agreement with the notion that BLPs from GBS are diacylated similar to the putative TLR6 ligands LTA and BLPs from *Mycoplasma fermentans* and mycobacteria. In contrast, TLR1 is required as a TLR2 coreceptor for a full response to triacylated proteins from Gram-negative bacteria, but is dispensable for the response to extracellular factors from GBS (9, 31). The absolute requirement of *lgt* for the TLR2 activation by extracellular factors of GBS provides strong evidence that di-*O*-acetylation of *S*-cysteyl residues in bacterial proteins is essential for lipoprotein-TLR2 interaction. Unexpectedly, the  $\Delta$ *lsp* mutant was as defective as the  $\Delta$ *lgt* mutant in TLR2 stimulation, suggesting that proper processing of BLPs is important for full TLR2 stimulatory activity. Hence di-*O*-acetylation of the protein N terminus is necessary, but not sufficient for TLR2 activation. The genetic evidence provided in this study is in line with biochemical evidence generated with synthetic lipopeptides, where di-*O*-acetylation was an insufficient prerequisite for TLR2 activation (32).

As outlined in this study, the protein product of Lgt/Lsp processing is devoid of a signal peptide and is retained in the membrane by its lipidated NH<sub>2</sub> extremity (Fig. 5). However, whereas the biochemical pathway leading to proper maturation of BLPs has been deciphered in several bacterial species, information on how BLPs are released into the extrabacterial medium remains as yet unknown. Both passive release during bacterial fission and active cleavage by yet to be identified mechanisms are conceivable. It seems likely that the quantity of BLPs released by  $\Delta$ *lsp* GBS is reduced compared with wild-type GBS due to the presence of an additional anchor. However, because the TLR2-stimulating capacity of fixed bacteria, i.e., cell wall, was abrogated in this mutant as well, retention of BLPs in the cell wall alone cannot explain the reduced stimulation of TLR2 by the *lsp* mutant. In contrast, proper maturation through Lsp peptidase modification appears to be a necessary requirement for recognition of BLPs by the TLR2/6 multimer.

As shown in other Gram-positive bacteria, Lgt and Lsp are dispensable for bacterial growth in vitro. Moreover, phenotypic characterization of the mutants (colony morphology, hemolytic activity, sensitivity to various antibiotics, and detergents) did not show significant differences between the wild-type strain NEM316 and the isogenic  $\Delta$ *lgt*,  $\Delta$ *lsp*, and  $\Delta$ *lgt/\Delta**lsp* mutants (data not shown). Detection of BLPs with [<sup>3</sup>H]palmitate clearly showed that the  $\Delta$ *lgt* mutant was completely deficient in lipid modification of prelipoproteins. Thus, there is no additional functional Lgt homolog in the GBS genome. Analysis of the processing of two previously described BLPs, Lmb (33) and ScaA (34) by Western blotting showed that first, in the absence of lipid modification by Lgt most of the BLPs are found in the supernatant, and second, Lsp is indeed involved in cleavage of the prelipoprotein signal peptide. Modification of the cysteine residue by Lgt is conventionally thought to be a prerequisite for specific processing by Lsp. Our data provide strong evidence that Lsp processing can occur in the absence of lipidation of the cysteine residue. Indeed, N-terminal sequencing

of proteins found in the supernatant of the  $\Delta$ *lgt* mutant showed the ScaA lipoprotein was correctly processed by Lsp even in the absence of cysteine modification (Figs. 4C and 5). Our observation is also consistent with the fact that overexpression of the *sitC* gene encoding a lipoprotein of 32 kDa in *S. aureus* results in the secretion of a correctly processed protein in the supernatant of  $\Delta$ *lgt* mutant. Because SitC does not comprise a typical Ala-X-Ala motif for signal peptidase I cleavage, SitC from  $\Delta$ *lgt* *S. aureus* was most likely processed by Lsp (signal peptidase II), despite the lack of SitC lipid modification (35). The fact that lipidation by Lgt is not a prerequisite for Lsp cleavage was very recently confirmed in *Listeria monocytogenes* (36).

The role of lipoprotein biosynthesis in bacterial virulence has been studied in other Gram-positive bacteria through the characterization of Lgt or Lsp mutants. In particular, Lgt was found to contribute to virulence of *Streptococcus pneumoniae* (37) and *S. aureus* (35) in mouse infection models. Lsp is required for full virulence of *L. monocytogenes*, *Mycobacterium tuberculosis*, *S. aureus*, and *Streptococcus equi* (38, 39). Most importantly, Lgt-mediated acylation was shown to be essential to induce the inflammatory response in *S. aureus* sepsis (35). In addition, two signature-tagged mutagenesis screens revealed that *lsp* contributes to the virulence of *S. aureus* (40, 41). In contrast, inactivation of *lsp* in *Streptococcus suis* did not appear to alter virulence in a piglet infection model (42). Until now, the attenuated virulence of  $\Delta$ *lgt* or  $\Delta$ *lsp* mutants in Gram-positive bacteria were considered to result from the reduced expression of specific BLPs. However, the discovery that BLPs are potent inducers of the host inflammatory responses adds a novel dimension into their role in pathogenesis. Two groups have evaluated the interaction of BLPs from Gram-positive bacteria with TLR2 (16, 43). However, to our knowledge, no study has evaluated the virulence of *lgt*-deficient strains in wild-type and TLR2 knockout mice, although the analysis of lipoprotein mediated virulence in combination with TLR2, the cognate receptor for BLPs, seems essential. BLPs represent ~2% of the predicted proteomes and are as described involved in many unrelated functions potentially important for bacterial fitness and thus full virulence. Consistently, the growth characteristics of our GBS mutants suggest that Lgt and Lsp facilitate growth in poor medium, although they are dispensable for bacterial growth in rich medium. However, differences in bacterial growth should not substantially influence the outcome of the infection in our model of GBS sepsis because, first, the more fastidious *lgt*-deficient strain is more virulent and, second, the phenotype of  $\Delta$ *lgt* GBS in wild-type mice mimics that of the wild-type parental strain in TLR2-deficient mice.

Our data are in support of those of Hashimoto et al. (15–17) who reported that contaminating BLPs carry the immunostimulatory activity commonly assigned to LTA from *S. aureus*. Thus, in contrast to LTA, GBS BLPs qualify as highly potent bacterial toxins. When adequately purified, LTA is >99% pure (18). Based on these results, we estimate that <1% of LTA from the GBS wild-type NEM316 are contaminating BLPs but that this spurious contamination carries most of the activity. Because the LTA preparation activates phagocytes at concentrations of 1  $\mu$ g/ml, BLPs should be active at concentrations <10 ng/ml. This corresponds well to the data depicted in Fig. 2, where 80 ng/ml of a relatively crude GBS supernatant elicited a potent response in HEK-TLR2 cells. GBS strain NEM316 encodes for 41 putative BLPs, but with the exception of Lmb that mediates adherence to fibronectin (33, 44, 45), the exact role of these proteins for GBS growth and virulence remains essentially speculative. In addition, no specific lipoprotein from Gram-positive bacteria that interacts with TLR2 has been reported on up to now. Deletion of individual BLPs from

Gram-positive bacteria seems important to resolve the ongoing dispute on the relative contribution of LTA and BLPs to Gram-positive sepsis (17, 46).

Several other open questions remain. It is currently unclear why two commonly used laboratory serotype III strains (COH1 and NEM316) that were originally isolated from newborn infants with GBS sepsis differ substantially with respect to the release of BLPs. Beyond the strains described in this study, we have analyzed additional laboratory and clinical GBS isolates. We found that the overwhelming number of strains resembles the “low in vitro” TLR2 phenotype NEM316 rather than the “high in vitro” TLR2 phenotype of COH1. We demonstrated that the ability of COH1 to highly stimulate TLR2 and cytokine production is related to acylation of BLPs as inactivation of *lgt* in this genetic background yield a mutant strain that no longer interacts with TLR2 (data not shown). The molecular basis of this remarkable COH1 phenotype is currently unclear but might be consecutive to the acquisition, by this strain, of mutations in regulatory systems controlling the expression of one or more BLPs. In support of this hypothesis, we observed that inactivation of the two-component regulatory system CovS/CovR in NEM316 resulted in a mutant with an increased activation of TLR2 (our unpublished observation). This mutant overexpresses several BLPs (47). It is thus conceivable that during decades of laboratory culture, GBS COH1 has lost a factor like CovR that negatively regulates lipoprotein biosynthesis. Another open question is why TLR2 contributes to ~50% of the lethality in a high dose neonatal GBS COH1 sepsis model, whereas deletion of protein acylation that nearly abrogates activation of TLR2 by NEM316 does not substantially influence the lethality in the same high dose model (4).

TLR2 contributes 40–50% of the total lethality in a lethal dose 90 model of GBS sepsis in neonatal mice. However, as described in this study, fixed GBS organisms engage TLR2 only at very high concentrations >100 µg/ml (dry weight) (Fig. 6B). Moreover, TLR2 is redundant with other MyD88-dependent receptors for TNF induction by whole GBS organisms, although BLPs are integral part of the GBS cell wall and thus form part of the interface between GBS and host cells (9, 48). Hence, albeit important, the interaction of BLPs with TLR2 is one among several possible mechanisms for alerting the innate immune system during GBS sepsis. Furthermore, besides TLRs, other innate signaling mechanisms such as complement component C3 and the complement receptor CR3 determine the monocytic cytokine response to GBS in a mixed leukocyte environment, although both are not essential for the inflammatory activation of isolated macrophages (48, 49). It is conceivable that opsonization by complement factors and subsequent phagocytosis positively regulate GBS-induced cytokine formation under some circumstances. The role of BLPs in this context has not been evaluated.

In conclusion, the integration of data obtained with lipoprotein-deficient GBS and TLR2-deficient mice suggests that BLPs are the dominant TLR2 activating molecules from GBS. As a model, we propose that during sublethal infection, mature BLPs activate local defense to ensure immediate elimination of GBS. If this rapid TLR2-mediated response to BLPs is insufficient, GBS will further disseminate and generalized inflammation, multiorgan failure, and even death potentially ensue.

## Acknowledgments

We are grateful to Inga Möller and Sybille Kenzel for expert technical assistance.

## Disclosures

The authors have no financial conflict of interest.

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