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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 diacylglycerol 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 Δlgt and the Δlsp mutant were impaired in TLR2 activation. In contrast to released factors, fixed Δlgt and Δlsp GBS cells exhibited normal inflammatory activity indicating that extracellular toxins and cell wall components activate phagocytes through independent pathways. In addition, the Δlgt mutant exhibited increased lethality in a model of neonatal GBS sepsis. Notably, LTA comprised little, if any, inflammatory potency when extracted from Δlgt 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.

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3 Abbreviations used in this paper: GBS, group B streptococcus; BLP, bacterial lipoprotein; LTA, lipoteichoic acid; Lgt, prolipoprotein diacylglycerol 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 GBS 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 β-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 β-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 PBS 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* DH5α (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 tryptose soy medium. RPMI 1640 (Merck Eurolab), DMEM, and a chemically defined medium prepared from endotoxin-free water. Then, bacterial cultures were grown to mid-log phase. Overnight GBS colonies were used to inoculate chemically defined medium to mid-log phase (OD650 ≈ 0.6) and remaining bacteria. The supernatant containing total GBS protein was prepared as follows. A 50-ml of this buffer. Total protein was extracted by guest on January 21, 2018 http://www.jimmunol.org/ Downloaded from

**Genomic 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). Electrococponent 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 Δlgt mutant (NEM2188), the inserted 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 ampiclons: 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-hoSt5Δlgt, was introduced by electroporation into NEM316. Erhythmicin-sensitive/kanamycin-resistant mutants that carried the expected lgt insertion-deletion were selected as described (22). For construction of GBS Δlsp mutant, the promoterless and terminatorless streptomycin resistance cassette *aadB* was used in a similar strategy. In this case, the amplicons O5-O6 (5′ end of lsp), SmkSmB (aadB gene), and O7-O8 (3′ end of lsp) were ligated and cloned into the thermosensitive shuttle plasmid pG+host5. The vector pG-hoSt5Δlsp was introduced by electroporation into NEM316 and NEM2188 to generate NEM2189 (NEM316Δlsp) and NEM2194 (NEM163Δlsp), respectively. All mutations were verified by sequencing of the inactivated lgt and lsp genes. The Δlsp 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 (OD650 = 0.2–0.3). 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 H2O at a density of ≈3 × 10^8 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 OD650 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 (OD650 = 0.3), bacteria were pelleted by centrifugation, the resulting supernatant was filter sterilized with a 0.2-µm membrane, dialyzed extensively against endotoxin-free water, and lyophilized. Concentration of the supernatant is depicted either in wet/odor 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 Fastprep Blue tube placed in a Fastprep instrument (Qiogene). 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.

**1Hpalmitic acid labeling of BLPs**

Bacteria were grown in 5 ml of Todd-Hewitt broth containing [1^H]palmitic acid at a final concentration of 20 μCi/ml. Following 5 h of incubation at 37°C, cells were harvested by centrifugation, washed twice with 500 µl of PBS, and resuspended in 500 µl of this buffer. Total protein was extracted as described and analyzed by PAGE under denaturing conditions and autoradiography.

**Immunoblotts 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 Xhol. 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 was provided by Dr. P. E. Kolenbrander (National Institutes of Health, Bethesda, MD). This ScA Ab was raised against ScA from Streptococcus gordoni, which displays 73.5% of sequence identity with ScA (GBS NEM316) from GBS NEM316. Following electrophoresis under denaturing conditions, the proteins of GBS were transferred to a nylon membrane and detection of Lmb and ScA was performed with rabbit anti-recombinant Lmb or anti-ScA sera (diluted 1/1000 in PBS) as described (23). For the LTA dot blot, an LTA Ab that binds to the poly-glycrophosphate backbone of LTA was used (Hycult).

Neonatal mouse models of GBS sepsis. Neonatal (24-hour-old) BALB/c and C3H/HeN mice (both from Charles River Breeding Laboratories) or TLR2−/− mice (C57BL/6j 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 the protocols 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% human AB serum. After addition of the indicated stimuli, RAW 264.7 cells were incubated at 37°C in a 5% CO2 atmosphere. For determination of cytokine formation, RAW macrophages were seeded at a density of 1 × 105 cells/ml in 96-well dishes in RPMI 1640 with 10% FBS and incubated for 16 h at 37°C in a 5% humidified CO2 environment. Supernatants were processed directly for the determination of released cytokines by ELISA (R&D Systems) per the manufacturer’s protocols.

Purified LTA was added to live or heat-killed GBS type III strains at different concentrations with a commercial ELISA (Hycult) v.3.0 software.

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-κB 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 (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-κB-dependent reporter than purified LTA when they were compared on a weight basis (Fig. 2, A and B). We previously reported on a superadditive 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 μg of lyophilized supernatant) (Fig. 2D). Accordingly, GBS-F was ~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),...
exhibited increased activation of TLR2 through extracellular factors when compared with isogenic wild-type GBS (Fig. 3A). This was an unexpected finding because DltA-mediated D-alanyl ester-tors when compared with isogenic wild-type GBS (Fig. 3A) exhibited increased activation of TLR2 through extracellular factor release of cytokine-inducing factors but not cell wall-induced cytokine formation.

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-κB 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 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.

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 and 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.

Lipoprotein acylation in Δlgt and Δlsp 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 (Δlsp NEM2189 and Δlgt NEM2188) GBS strains, we cultured GBS in the presence of [3H]palmitate for incorporation of 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. 4A, 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 Δlsp mutant (Fig. 4A, 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 Δlgt mutant NEM2188 (Fig. 4A, 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 (Δlgt), NEM2189 (Δlsp), and NEM2194 (ΔlgtΔlsp) 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. 4B, lanes 1 and 4) and Δlsp mutant strains, whereas it was not detected in the culture supernatant of these strains (Fig. 4B, lane 3). On the contrary, a substantial amount of ScaA was detected in the culture supernatant of Δlgt and ΔlgtΔlsp mutants (Fig. 4B, 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. 4B). In NEM316 and the Δlsp mutant, the secreted non-BLPs GBS0153 and CAMP were the two most abundant proteins detected in culture supernatants, as identified by NH2 sequencing (Fig. 4C). One additional band at around 34 kDa was...
detected in the supernatants of both the Δlgt and Δlgt/Δlsp mutants (Fig. 4C). Edman degradation revealed that the additional band present in both strains was ScaA. Interestingly, the NH2 sequence of the mature protein differed in the Δlgt and Δlgt/Δlsp strains (Fig. 4C, bottom). In the Δlgt mutant, the first amino acid residue was the signature cysteyl of the lipobox, a feature expected with a protein processed by Lsp. In the Δlgt/Δlsp 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 Δlgt/Δlsp mutant, albeit in lower amounts than in the Δlgt 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 Δlgt 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-κB-dependent transcriptional activation in a TLR2-specific assay (NF-κB-dependent reporter activation in HEK-TLR2 cells) (Fig. 6B). Importantly, ethanol-fixed Δlgt GBS normally stimulated cytokine formation in macrophages and PBMCs (Fig. 6C). It seems important to note that transfer of the same Δlgt mutation into the GBS COH1 background generated a mutant strain that exhibited the same phenotype as the Δlgt 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 Δlsp mutant in vitro mimicked that of Δlgt strain. The supernatant of Δlsp GBS exhibited largely impaired activation of NF-κB 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 Δlsp 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.
was abrogated in Δlsp 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 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 Δlsp 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 potently initiate cytokine formation in a lipoprotein and TLR2 independent manner.

Butanol-extracted LTA from Δlgt GBS does not activate TLR2 at concentrations as high as 20 μg/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 Δlgt 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 Δlgt mutant retained only little, if any, potency as compared with LTA from wild-type GBS NEM316 (Fig. 8B). This finding indicated that
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 Δlgt and Δlsp mutants

In rich Todd-Hewitt broth, the wild-type NEM316 strain and the mutants NEM2188 (Δlgt), NEM2189 (Δlsp), and NEM2194 (Δlgt/Δlsp) 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 Δlgt 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 GBS does not activate TLR2 in concentrations as high as 20 μg/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 (△) 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-κB 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.

FIGURE 8. Butanol extracted lipoteichoic acid from Δlgt GBS does not activate TLR2 in concentrations as high as 20 μg/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 (△) 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-κB 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.

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 Δlgt strain NEM2188 in escalating concentrations equivalent to the LD₉₀ (60 CFU, \(n = 24\) for NEM316 and \(n = 25\) for NEM2188), LD₅₀ (100 CFU, \(n = 28\) for NEM316, \(n = 26\) for NEM2188), or LD₉₀ (180 CFU, \(n = 13\) for each group). B–D, Neonatal TLR2−/− mice or isogenic C57BL/6/j wild-type controls were s.c. infected with 60 CFU of NEM316 or the isogenic Δlgt strain NEM2188 as indicated. The 60 CFU correspond to the LD₅₀, in C57BL/6/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 Δlgt 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 LD₉₀ 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 cysteine 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 diacylglycerol cysteine. This modification is necessary for efficient recognition of outer membrane BLPs by the L1 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 N-cysteyl residues in bacterial proteins is essential for lipoprotein-TLR2 interaction. Unexpectedly, the Δlsp mutant was as defective as the Δlgt 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 TLR6 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 NH2 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 extracellular 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 Δlsp 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 Δlgt, Δlsp, and Δlgt/Δlsp mutants (data not shown). Detection of BLPs with [3H]palmitate clearly showed that the Δlgt 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 Δlgt 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 Δlgt mutant. Because SitC does not comprise a typical Ala-X-Ala motif for signal peptidase I cleavage, SitC from Δlgt 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 Δlgt or Δlsp 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 Δlgt 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 μg/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 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 yields 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 monocyte 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.

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Disclosures

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References


