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Role of Lipoteichoic Acid in the Phagocyte Response to Group B *Streptococcus*¹

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Group B *Streptococcus* (GBS) cell walls potently activate phagocytes by a largely TLR2-independent mechanism. In contrast, the cell wall component lipoteichoic acid (LTA) from diverse Gram-positive bacterial species has been shown to engage TLR2. In this study we examined the role of LTA from GBS in phagocyte activation and the requirements for TLR-LTA interaction. Using cells from knockout mice and genetic complementation in epithelial cells we found that highly pure LTA from both GBS and *Staphylococcus aureus* interact with TLR2 and TLR6, but not TLR1, in contrast to previous reports. Furthermore, NF- κ B activation by LTA required the integrity of two putative PI3K binding domains within TLR2 and was inhibited by wortmannin, indicating an essential role for PI3K in cellular activation by LTA. However, LTA from GBS proved to be a relatively weak stimulus of phagocytes containing ~20% of the activity observed with LTA from *Staphylococcus aureus*. Structural analysis by nuclear magnetic resonance spectrometry revealed important differences between LTA from GBS and *S. aureus*, specifically differences in glycosyl linkage, in the glycolipid anchor and a lack of *N*-acetylglucosamine substituents of the glycerophosphate backbone. Furthermore, GBS expressing LTA devoid of α -alanine residues, that are essential within immune activation by LTA, exhibited similar inflammatory potency as GBS with alanylated LTA. In conclusion, LTA from GBS is a TLR2/TLR6 ligand that might contribute to secreted GBS activity, but does not contribute significantly to GBS cell wall mediated macrophage activation. *The Journal of Immunology*, 2005, 174: 6449–6455.

Group B *Streptococcus* (GBS)⁴ is a common commensal of the human genital and intestinal tract. Yet GBS is a potent inflammatory stimulus once it becomes invasive. GBS causes fulminant sepsis in newborn infants and constitutes the third most common cause of bacterial meningitis in countries that have implemented immunization with *Haemophilus influenzae* type b conjugate vaccines (1).

Because 15% of healthy human beings are fecal carriers of GBS, the invasion of small numbers of GBS can be assumed to be a

frequent event (2). Early recognition of GBS structures by phagocyte receptors must ensure immediate and nonphlogistic removal of GBS from blood and tissue to prevent the host from succumbing to bacterial spread and subsequent generalized inflammation. We have previously shown that the evolutionary conserved TLR constitute a highly sensitive receptor system for GBS that determine the cause of invasive GBS disease in vivo (3–5). However, whereas LPS from Gram-negative bacteria carries most activities of the whole bacteria, the identity of toxins from GBS that mediate the inflammatory response to infection, as with many other Gram-positive bacteria, is still unclear.

The cell wall of Gram-positive bacteria constitutes a macromolecular assembly of surface proteins, cross-linked peptidoglycan (PGN), polyanionic teichoic acid, and lipoteichoic acid (LTA) (6). Because PGN and LTA, teichoic acid or similar anionic molecules are shared by virtually all Gram-positive bacteria, these molecules are candidates as conserved toxins or “pathogen-associated molecular patterns” that are widely believed to be recognized by TLRs (7). Indeed, LTA and PGN from *Staphylococcus aureus* have been demonstrated to interact with TLR2 and the TLR coreceptor CD14 (8, 9). LTA is particularly interesting as a Gram-positive “endotoxin” because it resembles LPS in some respects. LTA and LPS are both anchored via their glycolipids to the membrane and carry a polysaccharide chain extending into the PGN layer of the cell wall. Whereas glycolipids like LTA are widely recognized TLR ligands, PGN preparations, which are a heterogeneous mixture of repeating disaccharide (*N*-acetylmuramic acid-(b1–4)-*N*-acetylglucosamine) containing cell wall fragments, have no known TLR2 binding domain and have recently been questioned as a TLR2 ligand (11). Within the cell wall network LTA is thought to provide elasticity, porosity and tensile strength of the

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⁴ Abbreviations used in this paper: GBS, group B streptococcus; LTA, lipoteichoic acid; HEK, human epithelial kidney; PGN, peptidoglycan; NMR, nuclear magnetic resonance; MALP-2, macrophage-activating lipopeptide of 2 kDa; MDP, muramyl dipeptides.

bacterial envelope. However, its contribution to the interaction between bacteria and cells of the mammalian immune system within invasive infections remains less well understood. As an example, we previously demonstrated that cell wall preparations from GBS activate immune cells in the absence of both TLR2 and CD14. These preparations were likely to be rich in LTA, thus raising questions about the cognate receptor of LTA from GBS and the contribution of LTA to phagocyte activation by GBS (3, 4, 12).

To answer the question about the significance of PGN and LTA in GBS infection, we defined several issues: 1) whether LTA from GBS differs from LTA from *S. aureus* with respect to chemical structure and phagocyte activation; 2) whether LTA engages TLR2 and TLR1 on phagocytes, as has been suggested previously (10, 11); 3) whether the presence of alanine in LTA affects the inflammatory potency of GBS cell walls; and finally 4) whether LTA synergizes with PGN.

Materials and Methods

Reagents

Reagents were obtained from Sigma-Aldrich unless stated otherwise. PBS, DMEM, G418, and trypsin/versene mixture were from Cambrex. Low endotoxin FBS was from HyClone Laboratories. LPS derived from *Escherichia coli* strain O111:B4 was purchased from Sigma-Aldrich and twice re-extracted by phenol chloroform as described (19). Clone 55, a monoclonal LTA Ab that recognizes the glycerophosphate epitope, was from Hycult Biotechnology.

Primary cell culture and cell lines

Human PBMC were isolated by gradient centrifugation of heparinized blood from healthy donors on Histopaque 1077 (Sigma-Aldrich), resuspended in RPMI 1640 medium containing 10% FBS. Cells were seeded at a density of 2×10^6 /ml stimulated and analyzed for TNF formation as described (3).

Bacterial strains and cell wall preparations

GBS type III strains COH1, NEM 316, and the isogenic NEM 316 mutant deficient in D-alanine-D-alanyl carrier protein ligase NEM1636 have been previously described (13, 14). All strains were grown on blood agar plates (Remel). Bacterial colonies were removed from the plates after overnight culture, washed three times in PBS, and then used to inoculate chemically defined medium (15) prepared from endotoxin-free water. Subsequently, bacterial cultures were grown to mid-log phase ($OD_{650} = 0.27-0.30$). Alternatively, bacteria were grown overnight in brain heart infusion. The next morning, the culture was diluted 1/20 in DMEM (Cambrex) and grown to mid-log phase ($OD_{650} = 0.27-0.30$). Then, GBS were washed once with PBS, resuspended in 70% ethanol in pyrogen-free H₂O at a density of 3×10^{10} CFU/ml and inactivated for 30 min on ice. Bacteria were washed again in PBS and stored at -80°C .

Preparation of LTA

GBS (strain COH1) was cultured under permanent agitation in chemically defined medium (15) prepared from endotoxin-free water in a 20 L fermentor (37°C). Bacteria were harvested at an OD_{650} of 0.6 by centrifugation, washed in endotoxin-free water and lyophilized. Subsequently, LTA was extracted with *n*-butanol (Merck) under stirring for 30 min at room temperature as exactly as described (16). LTA from *S. aureus* was extracted from strain DSM 20233 as described (16).

Structural resolution of GBS LTA by nuclear magnetic resonance (NMR) spectrometry

NMR spectra were determined using a Bruker Avance 600 MHz spectrometer and standard acquisition software. Spectra were recorded at 300 K using 3-(trimethylsilyl) 3,3,2,2-tetradeuteropropionic acid Na salt (*d*₄-TSPA) as the shift reference ($\delta = 0.00$ parts per million). Homonuclear assignments were taken from double-quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), rotating frame Overhauser enhancement spectroscopy (ROESY). ¹³C assignments were based on heteronuclear multiple-quantum correlation (HMQC).

Luciferase reporter assays for NF-κB activation

Human epithelial kidney (HEK)293 cells that stably expressed human TLR2 or wild-type HEK cells were seeded into 96-well tissue culture

plates at a density of 2×10^4 cells/well. The following day, cells were transiently transfected with luciferase reporter genes using Fugene (Roche) per the manufacturer's recommendations. To assess NF-κB activation, a reporter gene consisting of an E-selectin promoter driving a firefly luciferase gene was used. In individual experiments, cells were cotransfected with a constitutively active Renilla luciferase reporter gene (Promega) to normalize between conditions for transfection efficacy. The following day, the cells were stimulated as indicated. When indicated, HEK293 cells were transfected with human TLR2^{FLAG}, TLR2-A616^{FLAG}, TLR2-A761^{FLAG}, TLR2-A616/761^{FLAG}, or with empty vector. Expression vectors for TLR2-A616^{FLAG}, TLR2-A761^{FLAG}, and TLR2-A616/761^{FLAG} were kind gifts of U. Knaus (The Scripps Research Institute, La Jolla, CA). These TLR2 constructs carry an N-terminal FLAG-tag. After 4–6 h of stimulation, the cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured using a plate reader luminometer (MicroLumat Plus; Berthold Detection Systems). In all cases, the data shown represent one of three separate but similar experiments and are presented as the mean values \pm SD of triplicate samples.

Peritoneal macrophages

The generation of TLR1^{-/-}, TLR2^{-/-}, and TLR6^{-/-} mice was previously described (17–21). Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. Mice were injected i.p. with 2.5 ml of a 3% thioglycolate solution (Remel). After 72–96 h, peritoneal exudate cells were harvested by lavage with RPMI 1640 medium containing 10% FBS and 10 μg/ml ciprofloxacin. The cells were washed with medium, counted in a hemocytometer, and plated at a density of 1×10^6 cells/ml in 96-well dishes in RPMI 1640 medium with 10% FBS plus 10 μg/ml ciprofloxacin and incubated for 16 h at 37°C in a 5% humidified CO₂ environment. Supernatants were assayed directly for released TNF-α by ELISA (R&D Systems) per the manufacturer's protocols.

Results

Tyrosine residues at positions 616 and 761 of TLR2 are essential for LTA-induced activation of NF-κB

GBS LTA engages TLR2. LTA from GBS activated an NF-κB dependent reporter (ELAM.luc) in HEK293 cells, transfected with human TLR2, but not in cells transfected with the empty expression vector (Fig. 1A). NF-κB activation was abrogated when tyrosine residues at positions 616 or 761 were substituted by alanine. These tyrosine residues are parts of putative binding domains (YxxM and YxxW) for the PI3K subunit p85 (22). LTA from GBS required the domains around Tyr⁷⁶¹ and Tyr⁶¹⁶, as essential signaling domains of TLR2 (Fig. 1A). A similar dependency on the respective TLR2 tyrosine residues was observed with Pam₃CSK₄, a TLR 2 ligating lipopeptide from *E. coli* (data not shown). Of note, the proximal Tyr⁶¹⁶ is located right downstream of the transmembrane TLR2 domain and outside the signaling Toll/IL-1R domain. In contrast to purified TLR2 ligands, mutation of either tyrosine-containing motif resulted in an intermediate phenotype in response to whole heat inactivated *S. aureus*. However, mutation of both motifs abolished activation altogether (Fig. 1B). Finally, we found that wortmannin, a PI3K inhibitor, inhibited the LTA-induced activation of the ELAM.luc reporter in HEK TLR2 cells (>60% inhibition of 20 μg/ml LTA by 100 nM wortmannin as compared with vehicle control). Hence we conclude that PI3K significantly contributes to the response to LTA via TLR2.

TLR6, not TLR1, is required as a TLR2 coreceptor for responses to LTA

Like the well studied macrophage-activating lipopeptide of 2 kDa (MALP-2), LTA comprises a diacylated moiety (16). In contrast, the predominant lipoprotein from *E. coli* (Pam₃CysK₄) is triacylated. In both MALP-2 and Pam₃CysK₄, the fatty acids are believed to constitute the activation domains of these lipoproteins. MALP-2 has been reported to activate a heterodimer consisting of TLR2 and TLR6, Pam₃CysK₄ and a triacylated lipoprotein from *Borrelia burgdorferi* (OspA) activate TLR1 in concert with TLR2 (17, 23, 24). Furthermore, converting Pam₃CysK₄ into a diacylated

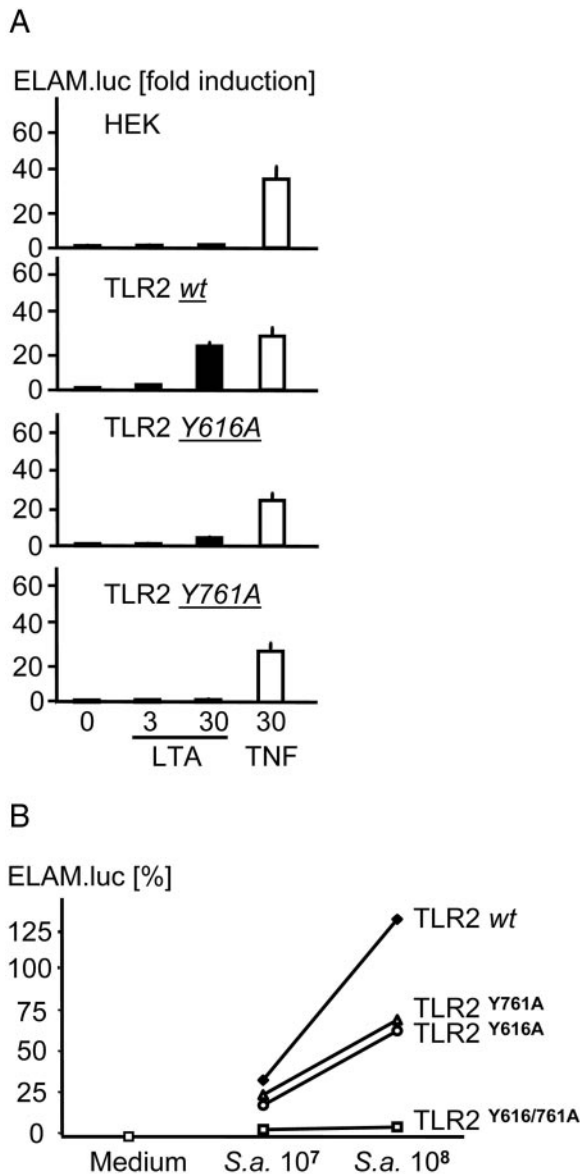


FIGURE 1. LTA from GBS engages TLR2. Tyr⁶¹⁶ and Tyr⁷⁶¹ of TLR2 are essential for LTA-induced activation of NF- κ B. *A*, HEK293 were transfected with an ELAM-luciferase reporter and either empty vector, human TLR2^{FLAG}, TLR2-Y616A^{FLAG}, TLR2-Y761A^{FLAG}, or TLR2-Y761,616A^{FLAG}. After 24 h, transfected cells were incubated with LTA from GBS and human recombinant TNF (nanogram per milliliter) over 5 h and luciferase activity was determined in the cell lysates by luminometry. Depicted are fold activation as compared with unstimulated controls. *B*, HEK293 were transfected as outlined in *A* and stimulated with heat-inactivated *S. aureus* in concentrations corresponding to CFU per milliliters shown. Depicted is the activation of an ELAM-luciferase reporter normalized to TNF control as means \pm SD of triplicate wells from one representative out of three experiments.

protein converts the TLR1/2 ligand into a TLR2/6 ligand (17). In apparent contrast to the effects of these ligands, the diacylated LTA has recently been reported to engage TLR1 plus TLR2 for cellular activation (10, 11). Hence, we addressed this question by testing the induced responses in cells from genetically modified mice that lack TLR1, TLR2, or TLR6. Both LTA from GBS and LTA from *S. aureus* similarly required cellular expression of TLR2 and TLR6 to mount an inflammatory response (Fig. 2, *B* and *C*). In contrast, macrophages deficient in TLR1 responded normally to LTA, whereas the response to Pam₃CysK₄ was markedly

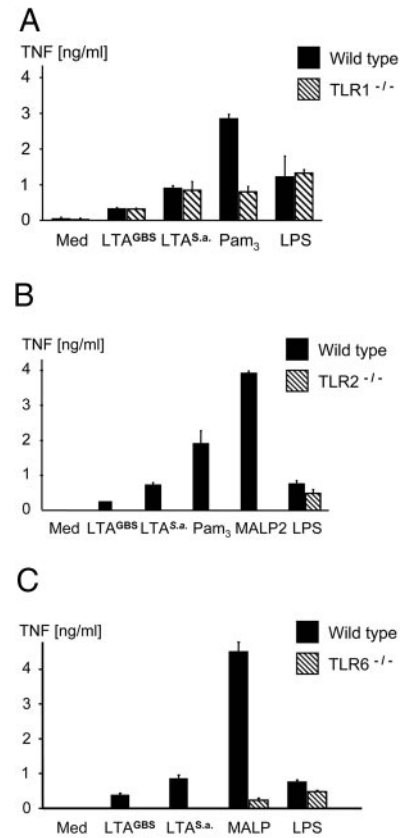


FIGURE 2. TLR2 and TLR6, but not TLR1, are essential for macrophage activation by LTA. Peritoneal macrophages from TLR1^{-/-} mice (▨) and C57BL/6 wild-type mice (■) (*A*), TLR2^{-/-} mice (▨) and C57B/6J wild-type mice (■) (*B*), or TLR6^{-/-} mice (▨) and C57B/6J wild-type mice (■) (*C*) were incubated as indicated with 10 μ g/ml LTA from GBS (LTA^{GBS}), 10 μ g/ml LTA from *S. aureus* (LTA^{S.a.}), 100 ng/ml Pam₃CysK₄, 30 ng/ml MALP-2, or 100 ng/ml LPS over 18 h, and TNF- α concentrations were determined in the supernatants. Depicted are the mean \pm SD of triplicate wells from one representative experiment of three.

impaired in cells from these mice (Fig. 2*A*). These results imply that LTA is recognized by a receptor complex consisting of TLR2 and TLR6.

LTA from S. aureus exceeds LTA from GBS in activation of inflammatory cytokines

LTA that was extracted from GBS serotype III strain COH1 stimulated PBMC from normal human volunteers to secretion of IL-8 and TNF. However, GBS-LTA was >5-fold less potent, as assessed by the approximate shift in the midrange of the dose-response curve, than LTA extracted by the same method from *S. aureus* (Fig. 3). With respect to the release of inflammatory cytokines from PBMC LTA was consistently \sim 1000-fold less potent than another TLR2 ligand, lipopeptide from *Mycoplasma fermentas* (MALP-2, data not shown).

The structure of GBS LTA

Because LTA from GBS and LTA from *S. aureus* differed substantially with respect to the extent of phagocyte activation but not the composition of the cognate receptor, we wondered whether both LTA were structurally different. To address this question we performed NMR analysis of LTA from GBS (type III, strain COH1). GBS were grown under pH control to preserve pH labile structural details. Several structural differences between GBS LTA and *S. aureus* LTA could be characterized by this approach (Fig. 4): 1) The average polyglycerophosphate backbone of GBS LTA

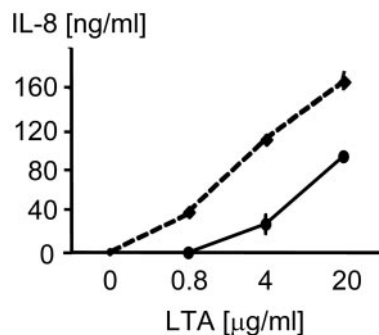


FIGURE 3. LTA from *S. aureus* exceeds LTA from GBS in activation of inflammatory cytokines. Human PBMC were incubated with butanol extracted LTA from GBS (solid line) or LTA from *S. aureus* (dashed line) over 18 h, and IL-8 concentrations were determined in the supernatants. Depicted are the mean \pm SD of triplicate wells from one representative experiment of three.

was relatively short with an average of $n = 19$ glycerophosphate repeats; 2) In GBS LTA a kojibiose linked the polyglycerophosphate chain to the membrane anchor resulting in a Glc- α -1,2Glc- α -1-3-diacylglycerol, whereas gentiobiose was found at this position in *S. aureus* LTA with a different glycolipid linkage leading to a Glc- β -1-6Glc- β -1-3-diacylglycerol structure; 3) In GBS LTA the polyglycerophosphate backbone was substituted only with D-alanine whereas in *S. aureus* and many other *Streptococcus* species D-alanine and N-acetyl-glucosamine can be found. Nevertheless, the percentage of D-alanine substituents was higher in *S. aureus* LTA (70%) than in GBS LTA (46%); 4) The average chain length of the fatty acids was 14 carbons in GBS LTA.

Alanylated LTA is not essential for GBS-induced inflammatory activation of phagocytes

To establish the role of LTA to GBS-induced inflammatory activation of phagocytes, we used a GBS targeted deletion mutant of

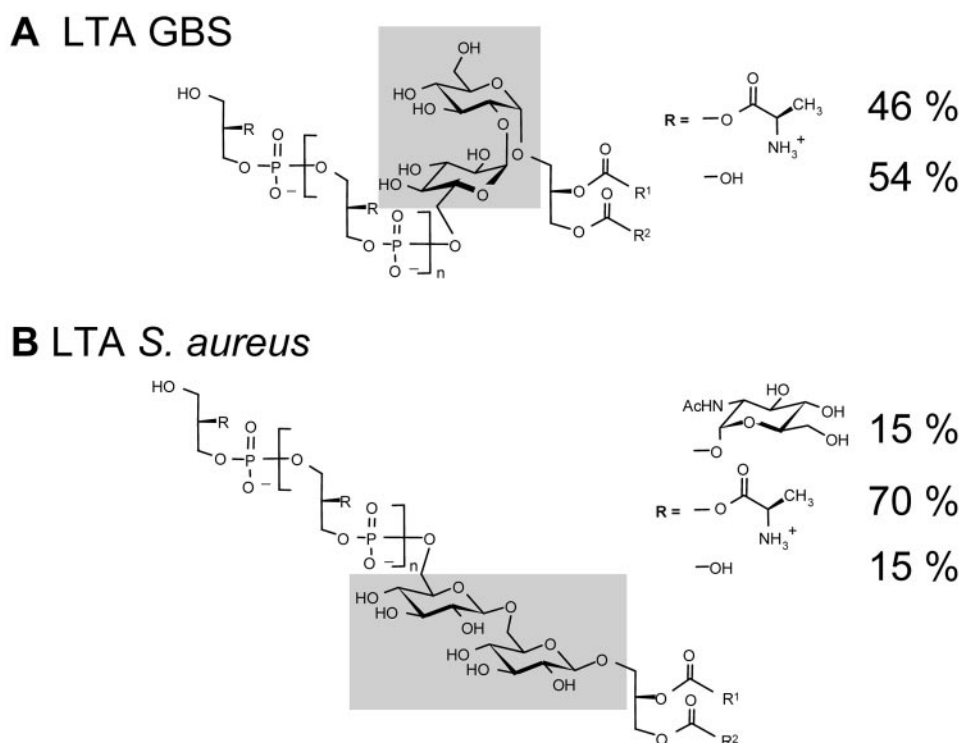
the *dlt* operon ($DltA^-$) that completely lacks D-alanine esters of the polyglycerophosphate backbone (13). First, we analyzed the LTA content of GBS cells by Western blot using an LTA-specific glycerophosphate Ab. The $DltA^-$ mutant NEM1636 that exhibited normal growth in tissue culture medium (DMEM) contained approximately twice as much LTA as the wild-type parental control GBS (strain NEM 316, Fig. 5A). This slight increase in cell wall associated LTA in the $DltA^-$ mutant might indicate that deprivation of D-alanyl esters of LTA stimulate LTA biosynthesis by an as yet unknown mechanism.

Careful titration experiments in PBMC and RAW cells did not reveal any difference in the inflammatory activity (formation of TNF and IL-8) of wild type (NEM 316) and $DltA^-$ mutant (NEM1636) strains despite a considerably higher D-alanine content in NEM 316 (20 vs 0% in $DltA^-$, Fig. 5, B and C) (13). Accordingly, because D-alanine esters are critical for LTA activity (16), LTA appears not to contribute essentially to the inflammatory activity of GBS cell wall.

LTA and PGN do not synergize

Because GBS cell walls are a very potent inflammatory stimulus to macrophages (nanogram per milliliter quantities are sufficient to induce substantial cytokine production), and GBS LTA proved to be only moderately effective in inducing inflammatory signals, we wondered, whether LTA synergized with other cell wall components such as PGN to elicit a more powerful inflammatory response. In light of the close structural relationship of PGN from different species of Gram-positive bacteria, we used that of *S. aureus* and combined it with LTA from GBS in both human PBMC and transfected HEK293 cells. LTA and PGN did not synergistically activate immune cells or epithelial cells overexpressing TLR2 (HEK/TLR2) disproving our hypothesis (Fig. 6A). However, a combination of LPS and TLR2 ligands results in a strong synergistic response, demonstrating that synergistic responses to bacterial products can occur (data not shown). Further, LTA synergized with muramyl dipeptides (MDP), disaccharide cleavage products of PGN, to result in increased IL-8 production

FIGURE 4. The structure of GBS LTA as determined by NMR spectroscopic analysis. A, LTA from GBS (serotype III, strain COH1) was structurally resolved by NMR. B, The published structure of *S. aureus* LTA (16). The structural differences of the membrane anchor between the two LTA species are highlighted in gray and the different substitution of the glycerophosphate backbone in each LTA is given as a percentage.



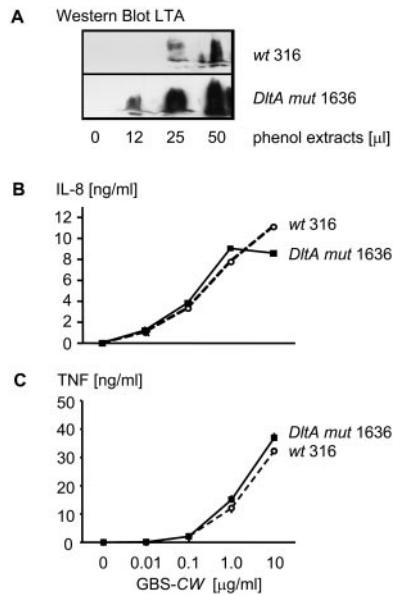


FIGURE 5. Alanylation of LTA does not affect GBS-induced inflammatory activity. *A*, Phenol extracts of cells from GBS wild-type strain NEM 316 and isogenic *DltA*⁻ mutant NEM1636 were subjected to immunoblots with monoclonal LTA Abs. *B* and *C*, PBMC from normal adult volunteers were incubated over 18 h with the indicated concentrations of ethanol-inactivated wild-type (NEM 316) or isogenic *DltA*⁻ mutant (NEM1636) GBS and IL-8 (*B*) or TNF (*C*) concentrations were determined in the supernatants. Depicted are the mean \pm SD of triplicate wells from one representative experiment of three.

in a dose-dependent fashion. In contrast to the strong synergistic response, both MDP and LTA did not induce IL-8 by themselves at the concentrations chosen (Fig. 6*B*). Hence, TLR2 ligands appear not to synergize with each other but synergize with TLR4 and with NOD2, the intracellular receptor for MDP.

Discussion

In a primate model of neonatal GBS infection GBS accumulates to vast bacterial counts in the neonatal lung (10^9 – 10^{11} CFU/g of lung tissue) surrounded by an intense inflammatory infiltrate. This suggests that phagocytes interacting with bacterial cell walls initiate an inflammatory response without sufficiently eliminating the bacteria in newborn species (25). In vitro, GBS cells are powerful inflammatory stimuli for phagocytes from neonates and adults (3, 4, 26). In contrast, the expression of adhesion molecules and the intracellular killing of GBS both appear to be impaired in newborn infants (27, 28). Of note, a similar imbalance of antibacterial and inflammatory responses to GBS can be observed in diabetic mice and overcome by the addition of insulin (29). Thus, a preponderance of proinflammation is likely to accord for the particular susceptibility of both newborn infants and diabetic patients to invasive GBS disease. Accordingly, the identification of GBS substructures that trigger a potentially detrimental systemic inflammation is of apparent interest.

Among several structural bacterial components LTA appeared to be a most attractive candidate toxin of GBS. LTA of *S. aureus* (16) and *Streptococcus pneumoniae* (30) had recently been shown to potently induce cytokines in phagocytes, thus clarifying a long standing controversy on the inflammatory potency of LTA, including synthetic preparations (31, 32). Because both functional and structural data on highly pure GBS LTA were missing we embarked on both biological and chemical analysis of this molecule.

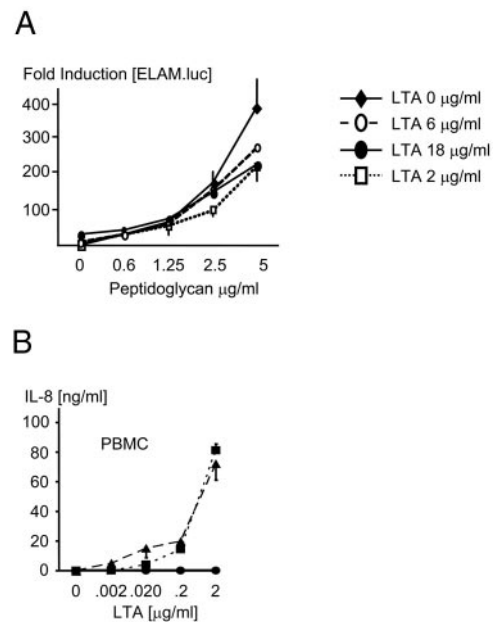


FIGURE 6. LTA and peptidoglycan do not synergize. *A*, HEK293 that expressed human TLR2 were transfected with an ELAM-luciferase reporter. Subsequently, cells were stimulated with combinations of increasing doses of GBS LTA and *S. aureus* PGN over 5 h and luciferase activity was determined in the cell lysates by luminometry. Depicted are fold activation as compared with unstimulated controls as mean \pm SD of triplicate wells from one representative experiment of three. *B*, Human PBMC from normal adult volunteers were stimulated for 16 h with LTA alone (●), with LTA plus 10 ng/ml MDP (■), or with LTA plus 100 ng/ml MDP (▲). Depicted are IL-8 concentrations in the supernatants as mean \pm SD of triplicate wells from one representative experiment of three.

TLR2, the principal signal transducer for LTA, is believed to function only in concert with other TLRs because homodimerization of the cytoplasmic domain of TLR2 does not induce inflammatory cytokines in macrophages, whereas similar dimerization of TLR4 is believed to do so (33). We found TLR6, but not TLR1, to be an essential component of the receptor for LTA from both GBS and *S. aureus*. This observation clearly supports the notion that diacylated ligands interact with the TLR2/6 heteromer (34). Currently the differences of our data to recent reports on TLR1 as part of the LTA receptor are unclear (10, 11). Differences related to the species, mouse macrophages in our study vs human cells in previous studies, cannot be ruled out at this stage. Further, because previous reports were based entirely on data generated with TLR1 Abs or heterologous expression of TLR1 in the context of endogenous TLR1 and TLR6 (HEK cells), interference with formation of the entire TLR1/2/6 complex might have affected the specificity of these approaches (10, 11). A disruption of this multimer by a TLR1 blocking Ab might further explain the reported complete abrogation of an LTA response in PBMC, which seemed surprising in view of the established intermediate phenotype of TLR1 deficiency in mice (17). In addition to TLR1 we identified PI3K as a critical TLR2 signaling partner for LTA induced activation of NF- κ B. Purified TLR2 ligands appear to be exquisitely dependent on both putative PI3K binding domains of TLR2 whereas both motifs are capable of partially compensating for each other in response to *S. aureus* cells (22). This indicates the engagement of further receptor or adapter molecules by these organisms.

Whereas GBS LTA and *S. aureus* LTA engaged a similar receptor heteromer we observed considerable interspecies variation in inflammatory potency of equally prepared LTA. To unravel the

molecular basis of the impaired potency, albeit similar receptor engagement of GBS and *S. aureus* LTA, we chemically analyzed GBS LTA. NMR analysis revealed GBS cells to contain type I LTA: In type I LTA a polyglycerophosphate is attached to the C-6 of the nonreducing glucosyl of the glycolipid anchor (6, 35). GBS shares the basic configuration of LTA with other streptococci, staphylococci, and bacilli (6, 35). However, beyond these similarities, we found interesting and as yet unknown features of the GBS LTA structure. The average length of the hydrophilic LTA chain was 19 glycerophosphate units and is clearly exceeded by the length of *S. aureus* LTA ($n = 45-50$) (16). Furthermore, the structure of the glycolipid Glc- α -1,2Glc- α -1-3-diaclylglycerol was different from those revealed in *S. aureus* and *S. pneumoniae* but similar to those in *Enterococcus*, *Leuconostoc*, and *Lactococcus* spp. (36). In addition, in GBS LTA the polyglycerophosphate backbone was substituted with D-alanine only whereas in *S. aureus* and many other species D-alanine substituents are alternating with N-acetyl-glucosamine. The individual contributions of gentiobiose, N-acetyl-glucosamine and the length of the polyglycerophosphate backbone to the substantial functional differences between GBS and *S. aureus* LTA remain to be elucidated.

Although partially substituted with N-acetyl-glucosamine, *S. aureus* LTA contains a higher percentage of D-alanine substituents (70%) than LTA GBS (46%) (13, 16). These differences in D-alanyl ester content might be relevant for the inflammatory properties of the molecule. Structural analysis of native and synthetic LTA from *S. aureus* revealed, that next to the lipid anchor itself, alanine substituents are required for proper inflammatory activity of the molecule (16, 31, 37). Replacing D-alanine substituents with L-alanine reduces the activity of the molecule at least 10-fold, indicating stereoselectivity (31). Both with respect to conservation of D-alanine residues and inflammatory potency, the butanol extraction procedure of LTA that was used in this study is far superior to the conventional phenol extraction (16). Moreover, LTA from *S. aureus* and *Lactobacillus rhamnosus* are better inducers of NO in macrophages than LTA from *Bacillus subtilis* that exhibits a low percentage of alanylated LTA (38, 39).

We used the apparent dependency of LTA D-alanylation on cytokine induction to further explore the contribution of LTA to the inflammatory activity of GBS cell wall. In GBS, the incorporation of D-alanine residues in LTA biosynthesis requires the *dlt* operon that encodes the four proteins DltA, DltB, DltC, and DltD. DltA is a cytoplasmic D-alanine-D-alanyl carrier protein ligase that catalyzes the D-alanylation of the D-alanyl carrier protein DltC (40). We found cell wall material from DltA⁻ GBS that have been previously shown to completely lack alanylated LTA, to exhibit similar inflammatory activity as the isogenic parental strain (20% alanylated LTA) (13). Further, whereas purified LTA from GBS was considerably weaker with respect to inflammatory phagocyte activation as compared with LTA from *S. aureus*, whole GBS and *S. aureus* cells were similarly potent stimuli (data not shown). These observations add some insight to in vivo studies that used DltA⁻ Gram-positive bacteria in mouse sepsis models. DltA⁻ GBS display a severely decreased virulence in mouse and rat infection models. This loss in virulence correlates to an increased susceptibility to killing by phagocytes (14). Deletion of D-alanine increases the net anionic charge of the bacterial cell wall and therefore alters binding of cationic peptides. Our data suggest that in contrast to the important contribution of alanylated LTA to bacterial survival in the host, additional and as yet unidentified GBS components are the dominant inflammatory stimuli of the cell wall. However, because expression of TLR2 is critical for the course of disease in a neonatal GBS sepsis model, secreted LTA might contribute to phagocyte activation in vivo (5).

In this study, we found LTA from GBS to be an ~100-fold weaker inflammatory stimulus for mouse macrophages as compared with cells from the same strain (comparison on a dry weight basis, P. Henneke and D. T. Golenbock, unpublished observations). Corresponding to this observation and in line with several reports we hypothesized that a synergism between PGN and LTA accounts for the "endotoxic" properties of Gram-positive bacteria both in vivo and in vitro (38, 41-43). LTA and PGN apparently share the cognate receptor TLR2, although considerable concern has been raised about an as yet to be identified contaminant carrying the TLR2 activity of PGN preparations. However, LTA and PGN preparations appear to differ with respect to engagement of the coreceptor because PGN appears to activate immune cells independently of TLR6 and TLR1 (44). Bearing this in mind, one could envision that both ligands engage different and independent domains of TLR2, hence resulting in synergism. In fact, a synergistic effect for PGN and LTA has been described (38, 45). To our surprise we did not observe any synergism between PGN and the highly pure LTA in vitro, whereas we and others found TLR4 ligands to synergize with TLR2 ligands (46, 47). Further, LTA synergized with MDP, disaccharide cleavage products of PGN, presumably via its intracellular receptor NOD2. These results are not entirely consistent with previously published reports. However, as has been reported elsewhere, commercial preparations of LTA that were used in previous studies were notoriously contaminated with LPS (37, 48). This contamination might have accounted for a synergism between TLR2 and TLR4 rather than between various TLR2 heteromers.

In conclusion, highly pure LTA extracted from GBS is an inflammatory TLR2/6 ligand with substantial structural and functional differences to LTA from *S. aureus*. LTA from GBS requires tyrosine phosphorylation of TLR2 for inflammatory activation. LTA synergizes with MDP but not with PGN in vitro. However, whereas synergism between LTA and MDP might contribute to secreted activity of GBS, it is unlikely to explain the strong inflammatory activity of GBS cell wall because TLR2 is nonessential for GBS activation by macrophages (3, 4, 12) and, because GBS expressing LTA without alanine substitutions that are crucial to LTA activity, elicit full inflammatory activity.

Disclosures

The authors have no financial conflict of interest.

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