Lipoteichoic Acid Is Important in Innate Immune Responses to Gram-Positive Bacteria[▽]

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To define the role of lipoteichoic acid (LTA) in innate immunity to gram-positive bacteria, we investigated the production of tumor necrosis factor alpha (TNF- α) by macrophages stimulated with gram-positive bacterial culture supernatants (GPCSs) after their LTA was removed or inactivated. GPCSs were obtained from three gram-positive species (pneumococci, staphylococci, and group B streptococci) during the exponential growth phase (designated early GPCSs) or at the senescent stage (designated late GPCSs). LTA was removed using an anti-LTA antibody or was inactivated by alkaline hydrolysis or platelet-activating factor acetylhydrolase (PAF-AH) treatment. Both early and late GPCSs from the three gram-positive bacteria stimulated macrophages to produce TNF- α primarily via Toll-like receptor 2 (TLR2), although late pneumococcal supernatant could stimulate macrophages via TLR4 as well. Following LTA inactivation by both methods, early GPCS lost about 85 to 100% of its activity and late GPCS lost about 50 to 90%. Both early and late culture supernatants from *Escherichia coli* could be inactivated by alkali hydrolysis but not by PAF-AH. In addition, removal of LTA from an early staphylococcal culture supernatant with a monoclonal antibody reduced about 70 to 85% of its potency. Reconstitution of inactivated early GPCS with a highly purified LTA restored its inflammatory activity, but the restored GPCS had higher activity than the pure LTA alone. These findings indicate that LTA is the primary TLR2 ligand in the early phase of gram-positive bacterial infection and remains a major ligand in the late phase when another TLR2 and TLR4 ligand(s) appears. In addition, our findings suggest that another gram-positive bacterial factor(s) synergizes with LTA in inducing inflammatory responses.

Innate immunity to bacteria provides the first line of defense against bacterial invasion by triggering the host's initial inflammatory responses (7, 11, 40). Central to innate immunity are Toll-like receptors (TLRs), which recognize the conserved pathogen-associated molecular patterns and trigger the innate immune response (1, 2, 53). In the case of gram-negative bacteria, TLR4 is the primary receptor and mainly senses lipopolysaccharide (LPS), which is a major component of the outer membrane (27, 30, 48). For gram-positive bacteria, TLR2 is the primary host receptor involved in inflammatory responses to the bacteria (14, 34, 47), but the nature of TLR2 ligands is unclear.

Several components of the gram-positive bacterial cell wall have been proposed to be TLR2 ligands. Although there are controversies (17, 28, 31, 51), peptidoglycan (PGN) may be a TLR2 ligand (13). Many studies have suggested that lipoteichoic acid (LTA) is the key TLR2 ligand (12, 42, 43, 52). Indeed, highly purified LTA, as well as chemically synthesized LTA analogs, can stimulate TLR2 (39). Despite the body of evidence, a recent study reported evidence that a contaminating lipoprotein, not LTA, may have been the TLR2 ligand even when LTA was purified using an updated method (21, 23). This controversy has arisen primarily because of difficulties in obtaining LTA without structural damage and/or biologically ac-

tive contaminants, although methods of purifying LTA have been greatly improved (15, 16, 33).

Therefore, to evaluate the role of LTA in innate immune responses to gram-positive bacteria, we investigated the effects of inactivating LTA on the inflammatory properties of bacterial culture supernatants, which contain molecules that are shed or released from bacteria (24, 26, 32, 46, 54, 57). Selective inactivation was possible since we have shown that platelet-activating factor acetylhydrolase (PAF-AH), a phospholipase A₂, selectively inactivates staphylococcal and pneumococcal LTAs by removing their acyl-2 chain (44) but does not affect other acylated bacterial molecules, such as LPS and phosphatidylcholine (44). We report here the effects of LTA inactivation on the inflammatory properties of gram-positive bacterial culture supernatants (GPCS).

MATERIALS AND METHODS

Reagents. Recombinant human plasma PAF-AH was kindly provided by ICOS Corporation (Bothell, WA). This enzyme was prepared by expressing the full-length cDNA of human plasma PAF-AH in *Escherichia coli* (50), which is as active as the native plasma PAF-AH enzyme and has been used in clinical studies (25, 29). Pefabloc SC (a serine protease inhibitor), PGN of *Staphylococcus aureus*, and an anti-phosphorylcholine antibody (TEPC-15) were obtained from Sigma-Aldrich (St. Louis, MO). A mouse anti-LTA immunoglobulin G1 monoclonal antibody (MAb) (BD1701) and a rabbit anti-staphylococcal LTA polyclonal antibody were obtained from BioDesign (Saco, ME). An MAb to pneumolysin was purchased from NovoCastra (Newcastle, United Kingdom). An MAb to pneumococcal surface protein A (PspA) (clone Xir126) (10) and a rabbit polyclonal antibody to pneumococcal surface protein C (PspC) (9) were obtained from D. Briles (University of Alabama at Birmingham, Birmingham, AL) and L. McDaniel (University of Mississippi Medical Center, Jackson, MS), respectively. A synthetic triacylated lipoprotein (Pam3CSK4) and synthetic muramyl dipeptide (MDP) were obtained from Calbiochem (San Diego, CA). The TLR ligands

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synthetic double-stranded RNA [poly(I-C)], a synthetic diacylated lipoprotein (FSL-1), an oligonucleotide with unmethylated CpG (ODN1826), and a TLR7/8 ligand (Imiquimod) were purchased from InvivoGen (San Diego, CA). Protein G-conjugated agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells and culture conditions. Mouse macrophage cell line RAW 264.7 (= ATCC TIB-71) was obtained from the American Type Culture Collection (Manassas, VA) and was cultured in Dulbecco's modified Eagle's medium (Cellgro Mediatech, Herndon, VA) supplemented with 10% defined fetal bovine serum (FBS) (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. Two Chinese hamster ovary (CHO) cell lines, 3E10-TLR2 and 3E10-TLR4, which constitutively express human CD14 as well as human TLR2 and TLR4, respectively, were obtained from D. Golenbock (Boston Medical Center, Boston, MA) (37). All the cell lines can express CD25 upon stimulation since they have the gene encoding membrane CD25 with the human E-selectin (ELAM-1) promoter, which has NF-κB binding sites. The cells were grown in Ham's F-12 medium (GIBCO-BRL, Rockville, MD) supplemented with 10% defined FBS (HyClone, Logan, UT), 1 mg/ml of G418 (Calbiochem, La Jolla, CA), and 400 U/ml of hygromycin B (Calbiochem, La Jolla, CA) at 37°C in a humidified incubator with 5% CO₂. To determine the responses of the CHO cell lines to bacterial products, CHO cells (1 × 10⁵ cells/ml) were incubated with various bacterial culture supernatants for 16 h, harvested using 10 mM EDTA, stained for CD25 expression with phycoerythrin-labeled anti-CD25 (Becton Dickinson, San Diego, CA), and analyzed by flow cytometry.

Bacterial strains and generation of bacterial supernatants. *Streptococcus pneumoniae* strain R36A (= ATCC 12214), *Staphylococcus aureus* ATCC 6538, group B streptococcus (GBS) strain COH1, and *E. coli* ER2357 were cultured in 100 ml of Dulbecco's modified Eagle's medium with 5% FBS, and supernatants were harvested at mid-log phase (optical density at 600 nm, ~0.4 to 0.47) and late stationary phase (at 16 h).

Neutralizing LTA activity in bacterial culture supernatants. Three different approaches were used to inactivate or remove LTA in bacterial culture supernatants. In the first approach, 9 volumes of bacterial supernatant was mixed with 1 volume of 2 N NaOH, incubated for 2 h at 37°C, and neutralized with 6 N HCl to obtain a pH of 6.5 to 7.5. In the second approach, the supernatants were incubated at 37°C for 2 h with various concentrations of PAF-AH and then mixed with Pefabloc SC (final concentration, 100 µM), as we have described previously (44). In the third approach, LTA was removed from staphylococcal GPCS with an anti-LTA MAb (BD1701). Briefly, the MAb was first immobilized on protein G-coupled agarose beads by incubating the two reagents together (final concentrations, 40% beads and 10 µg/ml of MAb) with gentle shaking for 6 h at 4°C. After the agarose beads were washed twice with phosphate-buffered saline, the agarose bead pellets were mixed with the same volume of staphylococcal GPCS and the mixture was gently shaken for 2 h at 4°C. LTA-depleted GPCS was obtained by removing the agarose beads by centrifugation.

Purification of LTA. Pneumococcal and staphylococcal LTAs were prepared using organic solvent extraction, octyl-Sepharose, and an ion-exchange chromatography method, as previously described (15, 16, 33). The level of endotoxin contamination was determined using the QCL-1000 quantitative chromogenic *Limulus* amoebocyte lysate assay according to the manufacturer's directions (Bio-Whittaker, Walkersville, MD). The resulting LTA preparations contained less than 10 pg of endotoxin per µg of LTA.

TNF-α measurement. RAW 264.7 cells (2 × 10⁵ cells/well) and mouse peritoneal macrophages were cultured in 96-well plates (Costar, Corning, NY) and stimulated with bacterial culture supernatants for 24 h. The amount of mouse tumor necrosis factor alpha (TNF-α) in the culture supernatant was determined with a commercially available sandwich-type enzyme-linked immunosorbent assay (eBioscience, San Diego, CA), using the manufacturer's protocol.

Western blot analysis. Bacterial culture supernatants and purified staphylococcal LTA were loaded onto a 15% native polyacrylamide gel electrophoresis gel. After 100 min of electrophoresis at 60 to 100 V/cm, the gel was blotted onto a nitrocellulose membrane. The membrane was blocked with 1.5% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 and incubated with anti-LTA MAb BD1701 (1:5,000), TEPC-15 (1:5,000), antipneumolysin MAb (1:4,000), anti-PspA hybridoma culture supernatant (1:50), or polyclonal anti-PspC rabbit antibodies (1:8,000). The membrane was then washed and incubated with a 1:5,000 dilution of peroxidase conjugates of a rabbit anti-mouse immunoglobulin (Southern Biotech, Birmingham, AL) or goat anti-rabbit immunoglobulin (Southern Biotech) reagent. After washing, the membrane-bound peroxidase was detected with the Lumiglo reagent (Cell Signaling, Danvers, MA).

Data analysis. All of the experiments in this study were conducted at least three times. The data shown below are representative results. Experimental values are expressed as means ± standard deviations. The statistical significance of a difference between two means was evaluated with Student's unpaired *t* test.

RESULTS

Early and late GPCSs have different inflammatory properties and molecular compositions. To establish whether the GPCS primarily stimulates cells via TLR2, we determined if early or late GPCS could activate CHO cell lines 3E10-TLR2 and 3E10-TLR4. All early GPCSs induced up-regulation of CD25 expression on 3E10-TLR2 cells but not on 3E10-TLR4 cells (Fig. 1A). In contrast, the early and late culture supernatants of *E. coli* activated both 3E10-TLR2 and 3E10-TLR4 cells. The response with the 3E10-TLR2 cell line was not unexpected since it is known that the CHO cells express hamster TLR4 (19, 42).

When we examined the ability of late GPCS to stimulate the CHO cell lines expressing human TLRs, we found that staphylococcal and GBS GPCSs stimulated 3E10-TLR2 cells and did not stimulate 3E10-TLR4 cells (Fig. 1A). In contrast, late pneumococcal GPCS stimulated both 3E10-TLR2 and 3E10-TLR4 cells (Fig. 1A). Late pneumococcal GPCS should contain pneumolysin that is a TLR4 ligand (36) and is released from dead pneumococci (6). Thus, we examined a late culture supernatant of a pneumococcal strain without a pneumolysin gene and found that the supernatant did not stimulate 3E10-TLR4 cells (Fig. 1B), although it stimulated 3E10-TLR2 cells. These results suggest that the main inflammatory molecules in late GPCSs are likely TLR2 ligands, although pneumolysin in a late pneumococcal GPCS may activate cells via TLR4 as well.

To determine if pneumolysin was present only in the late pneumococcal culture supernatants, we directly examined the presence of LTA and pneumolysin in GPCSs harvested at various times. Pneumolysin and other proteins, like PspA, were easily detected in late culture supernatants; however, pneumolysin was not detected in early pneumococcal culture supernatants (Fig. 1C). In contrast, LTA was detected in both early and late GPCSs (Fig. 1D). When pneumococcal LTA was visualized with an MAb (clone TEPC-15) in Western blots, bands were found in both early and late pneumococcal culture supernatants. Similarly, all staphylococcal and GBS supernatants showed LTA bands, which were visualized as 10-kb bands binding to an LTA-specific MAb (clone BD1701) (20, 58) (Fig. 1D). No LTA was detected in the *E. coli* culture supernatants with either the TEPC-15 or BD1701 MAb (data not shown). These Western blot analyses suggested that about 0.2 to 1 µg/ml of LTA was present in early GPCSs and about 1 to 5 µg/ml of LTA was present in late GPCSs (Fig. 1D). Since 0.1 µg/ml of staphylococcal LTA is known to be inflammatory (39, 42), our results indicate that both early and late GPCSs contain biologically relevant amounts of LTA. Furthermore, the molecular compositions of early and late culture supernatants are different and consistent with their inflammatory properties.

Inflammatory capacity of GPCS is significantly reduced (almost eliminated) by LTA inactivation. We have previously reported (44) that PAF-AH can monodeacylate pneumococcal and staphylococcal LTAs, which makes them inactive in stim-

FIG. 1. (A) Percentages of CD25⁺ 3E10-TLR2 (open bars) or 3E10-TLR4 (filled bars) cells in response to early and late bacterial culture supernatants. Cells were stimulated with the bacterial culture medium alone (Med), pneumococcal GPCS (SP), staphylococcal GPCS (SA), GBS GPCS (GBS), or *E. coli* culture supernatant (EC). Based on preliminary studies, early GPCSs were used at a concentration of 10%, late GPCSs were used at a concentration of 5%, early *E. coli* supernatant was used at a concentration of 0.1%, and late *E. coli* supernatant was used at a concentration of 0.01%. (B) Percentages of CD25⁺ 3E10-TLR4 cells in response to different concentrations of early and late culture supernatants collected from *S. pneumoniae* strain D39 and a pneumolysin-deficient isogenic mutant of this strain (PLN-A). (C) Presence of pneumolysin (Ply) (53 kDa), PspA (90 kDa), and PspC (92 kDa) in various pneumococcal culture supernatants. Truncated PspC (85 kDa) was detected only in the late supernatant (overnight culture [O/N]). The times of harvest for the culture supernatants are indicated above the lanes. The two lanes on the left were loaded with lysates of 10⁸ and 10⁷ CFU of pneumococcal strain R36A. OD₆₀₀, optical density at 600 nm. (D) LTA in GPCSs of *S. pneumoniae*, *S. aureus*, and GBS. Twenty microliters of purified LTA was loaded in the left three lanes, 20 μl of early GPCS was loaded in the middle three lanes, and 20 μl of late GPCS was loaded in the right three lanes. The concentrations of purified LTA and GPCS are indicated above the lanes. The molecules in the samples were separated by polyacrylamide gel electrophoresis and visualized with an anti-phosphorylcholine antibody (TEPC-15) for pneumococcal LTA or with a mouse anti-LTA MAb (BD1701) for the other preparations.

ulation of RAW 264.7 cells. Although it is unlikely that PAF-AH, a phospholipase A₂, affects other pathogen-associated molecular patterns, we next investigated the effect of PAF-AH on other TLR ligands, including poly(I-C) (a TLR3 ligand), LPS (a TLR4 ligand), R837 (a TLR7 or TLR8 ligand), ODN1826 (a TLR9 ligand), and MDP (a NOD2 ligand) (Fig. 2). We found that PAF-AH treatment did inactivate LTA, as we previously reported (44), but all other ligands were unaffected by treatment with 10 μg/ml of PAF-AH (Fig. 2). Included in the ligands tested were PGN and two synthetic lipoproteins, Pam3CSK4 (a TLR2/1 ligand) and FSC-1 (a TLR2/6 ligand) (Fig. 2), which represent other TLR2 ligands of gram-positive bacteria (13, 23). Therefore, PAF-AH is a highly specific LTA inhibitor.

To determine the role of LTA in early GPCSs, we next investigated the inflammatory properties of early (Fig. 3A) and late (Fig. 3B) GPCSs after treatment with PAF-AH or alkaline hydrolysis (0.2 N NaOH). For these experiments, we used 10% of early GPCS and 5% of late GPCS. Untreated early and late culture supernatants dramatically increased TNF-α production (Fig. 3A and 3B). When culture supernatants were treated with

0.2 N NaOH and neutralized with HCl, early culture supernatants from the three gram-positive bacterial species, as well as *E. coli*, exhibited less than 10% of the original activity (Fig. 3A). Hydrolysis also reduced the activity of late culture supernatants by about 50 to 90% (Fig. 3B). Alkaline hydrolysis could inactivate LTA, synthetic lipopeptides, and LPS, but it did not inactivate PGN or MDP (data not shown). Thus, LTA, lipoprotein, or LPS may be an important inflammatory factor in these culture supernatants.

To selectively inactivate LTA, bacterial culture supernatants were treated with PAF-AH. When early supernatants of pneumococci and staphylococci were treated with PAF-AH, 1 μg/ml PAF-AH reduced TNF-α production by 70 to 90% and 10 μg/ml of PAF-AH eliminated TNF-α production almost completely (>90%) (Fig. 3A). When the late culture supernatants of these organisms were investigated, PAF-AH treatment (10 μg/ml) reduced their ability to induce TNF-α production by 50 and 70%, respectively (Fig. 3B). The inactivation procedure itself did not affect GPCS activity since inactivation without PAF-AH did not alter GPCS activity (Fig. 3A and 3B) and addition of 10 μg/ml of PAF-AH alone did not affect the

FIG. 2. TNF- α production by RAW 264.7 cells in response to stimulation by pneumococcal LTA (PnLTA), staphylococcal LTA (StLTA), staphylococcal PGN (PGN), Pam3CSK4 (P3Cys), FSL-1, poly(I:C) [P(I:C)], LPS, Imiquimod (R837), ODN1826 (ODN), or MDP before (open bars) or after (filled bars) treatment with 10 μ g/ml PAF-AH. The ligand concentrations and receptor specificities are indicated at the bottom. The bars indicate the means of the results for triplicate wells in a representative experiment. The error bars indicate standard deviations. *P* values are indicated above the bars.

activities of RAW 264.7 cells (data not shown) (44). In contrast to treatment of GPCSs, treatment of early and late *E. coli* culture supernatants with 10 μ g/ml of PAF-AH had no effect on the inflammatory activity (Fig. 3A and 3B). These results strongly suggest that LTA is the dominant inflammatory factor in early GPCSs and is still a major factor in late GPCSs.

To further confirm the inflammatory role of LTA in GPCSs, we depleted LTA in the staphylococcal GPCS with anti-LTA antibody and examined the ability of the antibody-treated GPCS to induce RAW 264.7 cells to produce TNF- α (Fig. 4). Treatment with Mab BD1701 reduced the inflammatory properties of early staphylococcal supernatants by 80% but reduced the inflammatory properties of late supernatants by only 65%. The control mouse immunoglobulin G1 antibody had no effect on the inflammatory properties of either GPCS (Fig. 4). Also, Mab BD1701 did not reduce the inflammatory capacity of *E. coli* culture supernatant and LPS (Fig. 4). Thus, the results obtained with all three independent approaches for removing LTA supported the contention that LTA is essential for the ability of early GPCS to stimulate RAW 264.7 cells and is important for the parallel ability of late GPCS.

Addition of purified LTA restores the inflammatory capacity of LTA-inactivated early GPCS. To investigate whether the treatments used to inactivate LTA described above also removed critical molecules other than LTA, we examined the effect of restoring the activity of deactivated early GPCS with purified LTA. Ten percent of alkali-hydrolysis-inactivated early staphylococcal supernatant, which contained about 0.02 to 0.1 μ g/ml LTA prior to inactivation (Fig. 1D), exhibited the original activity when 0.1 μ g/ml of staphylococcal LTA was added (Fig. 5A). In contrast, LTA by itself induced several times less TNF- α than the same amount of LTA mixed with inactivated early GPCS (Fig. 5A). Almost identical results were obtained with PAF-AH-inactivated staphylococcal supernatants (Fig. 5B). These findings demonstrate the inflama-

tory activity of LTA and suggest that early GPCS contains a factor(s) that synergizes with LTA for inflammatory activity.

When the pneumococcal culture supernatants were investigated in a similar experiment, we found that these supernatants could also be reconstituted with purified LTA. However, the restoration of activity required about 3 to 10 μ g/ml of LTA (Fig. 5C and D), even though 10% of early pneumococcal supernatant contained only about 0.02 to 0.1 μ g/ml LTA. Thus, the restoration of activity of inactivated culture supernatants required about 10 times more purified LTA. In addition, the activity of purified pneumococcal LTA was enhanced several-fold with the inactivated pneumococcal culture supernatants (Fig. 5C and D). This suggests that pneumococcal culture supernatants also contain factors that synergize with LTA for inflammatory activity.

DISCUSSION

The significance of LTA during gram-positive bacterial infection is controversial (55). Several studies (12, 42, 43, 52), including a study using chemically synthesized LTA analogs (39), have reported that LTA is a highly potent TLR2 ligand. In contrast, other workers have proposed that the factor critical for gram-positive bacterial inflammation is not LTA but lipoproteins, which contaminate LTA preparations (22, 23). The controversy arises mainly because "purified" LTA is often contaminated (13, 18, 23) or structurally damaged (38). To avoid these limitations, we examined bacterial culture supernatants after selectively removing or inactivating LTA. Our LTA inactivation and removal methods involved the use of PAF-AH and Mab BD1701, which do not inactivate two synthetic lipoproteins (Pam3CSK4 and FSC-1), PGN, or various model ligands for other TLRs (Fig. 3) (44). PAF-AH is a phospholipase A_2 and selectively monoacylates and inactivates LTA (44). We found that inactivation of LTA almost

FIG. 3. TNF- α production by RAW 264.7 cells in response to culture supernatants of *S. pneumoniae*, *S. aureus*, GBS, or *E. coli*. The two bars on the left in each graph indicate the results for controls, including an unstimulated preparation (first bar) and a preparation stimulated with untreated bacterial culture supernatants (second bar). The third bar in each graph indicates the results for the cells stimulated with culture supernatant treated with 0.2 N NaOH, and the remaining bars (bars 4 to 7) indicate the results for the cells stimulated with supernatants treated with PAF-AH at the concentrations indicated (in μ g/ml). PAF-AH was inactivated with 100 μ M Pefabloc SC after the reaction. Early GPCSs were used at a concentration of 10%, late GPCSs were used at a concentration of 5%, early *E. coli* supernatant was used at a concentration of 0.1%, and late *E. coli* supernatant was used at a concentration of 0.01%. The bars indicate the means of the results for triplicate wells in a representative experiment. The error bars indicate standard deviations. *P* values are indicated above the bars.

completely eliminated the inflammatory properties of early GPCS (Fig. 4 and 5), whereas the inflammatory activity of the LTA-depleted early GPCS could be quantitatively restored with purified LTA (Fig. 5). These findings clearly demonstrate that LTA is the dominant inflammatory agent in early GPCS.

On their own, studies of purified LTA cannot demonstrate

changes in the functional potency of LTA during its purification. This situation has been a serious limitation in the study of the inflammatory properties of LTA. However, the selective inactivation approach used here permits workers to investigate the functional potency of LTA. When we restored the level of LTA in the early GPCS with LTA inactivated to the natural

FIG. 4. TNF- α production by RAW 264.7 cells in response to bacterial culture medium (Med), staphylococcal early GPCS, staphylococcal late GPCS, purified staphylococcal LTA, *E. coli* early culture supernatant, or *E. coli* LPS. Each stimulant was used unabsorbed (None), after absorption with an irrelevant isotype-matched control MAb (mouse immunoglobulin G1 [mIgG1]), or after absorption with an anti-LTA MAb (α -LTA). The bars indicate the means of the results for triplicate wells in a representative experiment. The error bars indicate standard deviations. *P* values are indicated above the bars.

early GPCS level (about 0.2 to 1 μ g/ml) (Fig. 1), the inflammatory potency of the staphylococcal early GPCS was quantitatively restored (Fig. 5). This finding strongly suggests that staphylococcal LTA was not damaged by the LTA isolation procedure used in this study. In contrast to the results for staphylococcal LTA, we needed about 10-fold more pneumococcal LTA to restore the inflammatory capacity of pneumococcal early GPCS (Fig. 5). One possible explanation for this finding is that the widely used procedure for purifying pneumococcal LTA yields pneumococcal LTA with reduced activity. Consistent with our conclusion, Draing et al. reported, during preparation of this paper, that the classical purification

procedure removes the alanyl group from pneumococcal LTA and thus reduces its inflammatory potency (12). LTA inactivation also made the late GPCSs of several gram-positive bacteria less inflammatory (Fig. 4 and 5). However, unlike the results for the early supernatants, LTA inactivation left the late GPCSs with significant amounts of residual inflammatory activity. Thus, late GPCS must have a TLR2 ligand(s) other than LTA, which may be PGN or a lipoprotein. Lipoproteins should be present in late GPCS because many bacteria begin to die in the late phase of culture and because a lipoprotein(s) accounts for the majority of inflammatory properties of dead gram-positive bacteria (4, 35, 41, 45). Thus, our data

FIG. 5. TNF- α production by RAW 264.7 cells in response to early GPCS from staphylococci (Early SA) (A and B) or from pneumococci (Early SP) (C and D). Early GPCS was inactivated by alkali hydrolysis (0.2 N NaOH) or with 10 μ g/ml PAF-AH (PAF-AH). Then purified pneumococcal LTA (PnLTA) or staphylococcal LTA (StLTA) at the concentrations indicated (in μ g/ml) was added to inactivated culture supernatants (10%). The bars indicate the means of the results for triplicate wells in a representative experiment. The error bars indicate standard deviations.

suggest that the dominant TLR2 ligands may vary during the different phases of infection; LTA may be a critically important TLR2 ligand in the early phase of infection, but other molecules, such as lipoproteins, may become significant TLR2 ligands in the late phase of infection.

Currently, there is a debate about whether lipoprotein or LTA is the dominant inflammatory molecule of gram-positive bacteria or their lysates. Staphylococci with a Δ lgtA mutation cannot produce lipoproteins, and their lysates are less stimulatory than the wild-type staphylococcal lysates (45). Yet a chemically synthesized staphylococcal LTA analog is strongly stimulatory (39). There is also disagreement concerning the ability of LTA extracted from Δ lgtA mutant bacteria to stimulate human blood cells (55). Since we did not investigate gram-positive bacteria themselves, our study did not directly address this question. However, our data do show that LTA is the dominant TLR2 ligand in early GPCs and is still a significant ligand in late GPCs. Thus, LTA should be considered an important TLR2 ligand. LTA is readily released into culture supernatants and is abundant in them (32, 57), whereas lipoproteins are primarily associated with bacteria and are not released into supernatants (45). In fact, consistent with our conclusions is the finding that the culture supernatant of the lipoprotein-deficient bacteria (Δ lgtA) is as inflammatory as wild-type bacterial culture supernatant (45).

In the present study, we provide evidence that the role of LTA in innate immunity changes with the bacterial culture stage. For instance, LTA is responsible for almost all the inflammatory activity of early GPCs, but late culture supernatants contain other important inflammatory molecules, including pneumococcal pneumolysin (Fig. 1). This suggests that the role of LTA during infections should be assessed in the context of a specific pathophysiological process. For instance, pneumolysin may be more prominent than LTA in pneumococcal pneumonia when a large number of dead pneumococci are present. Indeed, deletion or neutralization of pneumolysin makes pneumococci less effective for causing pneumonia but does not significantly affect their ability to mediate sepsis (3, 8). In contrast, LTA would be relatively more important than other inflammatory molecules when the pneumococcal density is low, such as in nasopharyngeal carriage or at the beginning of a pathological process. Such a bacterium-limiting step may be seen during the invasion of the endothelial barrier by a single live bacterium or early sepsis. Consistent with this, anti-LTA antibodies were shown to protect animals in a sepsis model (49, 56). Also, a recent study on pneumococcal invasion of endothelium showed that LTA could replicate the morphological changes of the endothelium that are observed with pneumococci (5).

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