Lipoteichoic Acid Synergizes with Glycosphingolipids To Potently Stimulate Secretion of Interleukin-6 from Human Blood Cells

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In the present study, we found that lipoteichoic acid (LTA) synergizes with glycosphingolipids to stimulate human blood cells to secrete cytokines. We employed globoside, kersarin, and lactosylceramide as representative neutral glycosphingolipids and mixed gangliosides GM2 and GM3 as representative acidic glycosphingolipids. LTA and the glycosphingolipids enhanced cytokine secretion by human whole blood, peripheral blood mononuclear cells, and purified monocytes in a dose-dependent manner. The level of synergy ranged up to 10-fold greater than the additive stimulation caused by LTA and glycosphingolipid alone. The greatest synergy was observed with GM3. We also found that LTA synergizes with the synthetic bacterial lipopeptide mimic Pam3CysK4. In contrast, the glycosphingolipids suppressed the stimulation caused by Pam3CysK4. The stimulation of human cells requires the simultaneous presence of LTA and the glycosphingolipids and probably requires their physical interactions, as shown by dot blotting and nondenaturing polyacrylamide gel electrophoresis experiments. We hypothesize that the enhanced stimulation is due to heterooligomers that form between LTA and glycosphingolipids at the subcritical micelle concentrations used in these experiments. Previous studies showed that LTA also synergizes with hemoglobin. The data taken together suggest that LTA may be a pathogen-associated molecular pattern, although its full activity requires the presence of a synergistic partner(s).

It has been established that septic shock caused by gram-negative pathogens is primarily due to the ability of lipopolysaccharide (LPS) to trigger the excessive secretion of proinflammatory cytokines by macrophages (1, 10). In contrast, the bacterial component(s) responsible for septic shock caused by gram-positive bacteria has not been confirmed. As a result, a number of constituents of gram-positive bacteria have been studied for their abilities to cause or contribute to shock. These include proteinaceous pyrogenic toxins, which act in vitro as superantigens; peptidoglycan oligomers (28); and lipoteichoic acid (LTA), a bacterial cell membrane component that has structural similarities to LPS. Over the last several years, highly purified LTA free of LPS contamination has been shown to induce monocyte and macrophage secretion of proinflammatory cytokines (7, 14, 18–20, 30, 31), and it shares many other functional features with LPS (12, 32). However, a major difference between LPS and LTA is that the injection of purified LPS alone can result in shock in an animal model, whereas the injection of purified LTA alone has never been shown to induce shock. Even when one examines the levels at which LPS and LTA induce cytokine release from macrophages/monocytes in vitro, it is clear that LPS is a more potent immunostimulant than purified LTA.

Recently, our laboratories and others have begun to define molecules of both human and bacterial origin that synergize with LTA and greatly enhance its ability to stimulate cells of the immune system. Muramyl dipeptide, the minimal structural unit of peptidoglycan that retains immunostimulating activity, synergizes with LTA to enhance the induction of inflammatory cytokines in human monocytes (34). In addition, we have shown that hemoglobin synergizes with both weakly active phenol-extracted LTA and the more highly active butanol-extracted LTA to markedly enhance cytokine secretion by both human blood monocytes and mouse macrophages (7, 14). Synergism with hemoglobin in activating macrophages was noted for LTAs from a variety of different gram-positive bacteria (14) and also for LPS (4, 25, 26). The mechanisms for the synergy between LTA and these other molecules have not yet been defined at the molecular level. However, we have shown that LTA and hemoglobin physically interact, so one possible hypothesis is that LTA forms a complex with hemoglobin in a way that facilitates the presentation of LTA to the macrophage Toll-like receptors (TLRs).

One of the reasons for the notable difference in potency between LTA and LPS may be related to the structural differences between their lipid moieties and their interactions with specific TLRs (TLR2 and TLR4, respectively) (3, 11). Even when one examines only the interaction between different species of LPS and the LPS receptor, TLR4, it is clear that differences in the lipid moiety (e.g., the numbers of fatty acid chains) can result in markedly different levels of cytokine secretion. LPS containing less than six fatty acids (e.g., penta- or tetra-acetylated LPS) exhibited less activity or was even nonactive, and in one case, signaling was even through a different...
receptor (1, 6). Interestingly, it has been shown that the mixing of the poorly active penta- and tetra-acylated LPS molecules at concentrations below their critical micelle concentrations (CMCs) before addition of the mixture to cells results in increased cytokine secretion (5). This result raises the possibility that the heterooligomers that formed between these two LPS structures were better able to interact with their receptor, subsequently resulting in enhanced cytokine release.

We show here that when LTA is combined with various glycosphingolipids and incubated with either human whole blood or isolated cells, cytokine release is markedly enhanced. We also show that LTA and some of the glycolipids physically interact and that enhanced cytokine release requires that both components be incubated simultaneously with the blood cells. On the basis of these observations, we hypothesize that mixtures of LTA and various glycosphingolipids at concentrations below their CMCs may form heterooligomers that are better able to interact with their receptors.

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**MATERIALS AND METHODS**

**Reagents.** Butanol-extracted *Streptococcus pyogenes* LTA was purified as described previously (14). The chain length of polyglycerolphosphate and the percent α-aniline content were analyzed by nuclear magnetic resonance (NMR) and were found to be 23 and 65%, respectively (14). The maximum level of LPS contamination of the LTA preparations was ≤0.004% (e.g., 400 pg/ml in 10 μg/ml LTA, as determined by the *Limulus* amoebocyte lysate assay) (14). Ten μg/ml polymyxin B (Sigma-Aldrich Co., St. Louis, MO) was added to all reaction mixtures to exclude stimulatory effects due to any residual LPS. The glycosphingolipids used in this study were purchased from Matreya LLC. Synthetic tri-palmitoylated bacterial lipopeptide Pam3CysK4 was purchased from InvivoGen (San Diego, CA).

**Preparation of human PBMCs and monocytes.** Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood with Ficoll-Hypaque (Amersham Biosciences), as described previously (17). Briefly, Ficoll-Hypaque was added to whole blood diluted 1:2 in Dulbecco’s phosphate-buffered saline (PBS), and the mixture was centrifuged at 700 × g for 30 min at room temperature (RT). The PBMC layer was collected, and the cells were washed three times with cold Dulbecco’s PBS. Monocytes were further isolated by allowing them to adhere to plastic. The PBMCs were resuspended in RPMI 1640 (supplemented with 10% FCS or donor plasma and exposed to stimuli by overnight incubation.

**Whole-blood assays.** Heparinized blood obtained from volunteers was diluted 1:5 in saline, as described by Morath et al. (19, 20). Diluted blood was incubated with LTA, glycosphingolipids, or mixtures of both components that had been preincubated at 37°C for 1 h. After overnight incubation (i.e., 19 to 22 h), the supernatants were removed and assayed for the levels of interleukin-6 (IL-6) that were found when LTA and GM3 were incubated with the PBMCs.

**IL-6 measurements.** Medium from the samples was collected and was assayed for the amount of IL-6 secreted by enzyme-linked immunosorbent assay, according to the manufacturer’s protocol (Pierce Epagen; Pierce Biotechnology, Inc.). This method is sensitive down to 300 pg/ml IL-6.

**RESULTS**

The ceramide-containing glycolipids (i.e., sphingolipids) are grouped according to the nature of the polar head group at position 1 of the ceramide backbone. One group, the glycosphingolipids, are further divided into two groups on the basis of the sugar composition of the head group. The neutral glycosphingolipids contain uncharged sugar residues, while the acidic glycosphingolipids, also known as gangliosides, contain charged sugar residues, such as sialic acid. In the present study, we employed galactosylceramide and lactosylceramide, which are the most predominant of the neutral glycosphingolipids, and globoside. As representatives of the acidic glycosphingolipids, we employed ganglioside GM2 or GM3 individually or as a mixture. The gangliosides vary by virtue of the overall size of their oligosaccharide chains, but they all contain only one sialic acid residue per molecule.

The addition of 15 μM of various neutral or acidic eukaryotic glycolipids markedly enhanced the level of IL-6 secretion by PBMCs in response to 0.062 μM LTA (Fig. 1). It should be noted that the magnitude of stimulation differed among the various sphingolipids, with the greatest level of synergy being found when LTA and GM3 were incubated with the PBMCs. This is of particular interest, since GM3 is the most prominent glycosphingolipid found in normal human serum (8, 22). By comparison, the level of IL-6 secretion in response to 0.062 μM LTA plus GM3 was equivalent to the level of IL-6 secretion in response to 50 ng LPS (114.53 ± 22 ng/ml and 149.23 ± 8.78 ng/ml, respectively). Results similar to those obtained with PBMCs were obtained in a separate set of experiments with LTA, GM3, and purified monocytes (Fig. 2). Human monocytes were also incubated with globoside mixed with LTA at concentrations ranging from 0 to 0.496 μM (which correspond to 0 to 2 μg/ml). This resulted in molar ratios of LTA to globoside that ranged from 1:480 to 1:60 (Fig. 3). The percent increases caused by the mixtures were the greatest at the lower ratios of LTA to globoside, and at a molar ratio of 1:60, there was no statistically significant increase in the level of stimulation.
Interactions of LTA and sphingolipids. Having determined the ability of glycosphingolipids to potentiate the activity of LTA, we next sought to examine whether this potentiation was the result of the separate activity of each component on the cells or was a shared activity. For this purpose, either the monocytes were incubated for 1 h with 0.062 μM LTA, followed by washing and overnight incubation with 15 μM globoside, or they were incubated for 1 h with 15 μM globoside, followed by washing and overnight incubation with 0.062 μM LTA. As a positive control, the monocytes were also incubated overnight with a mixture of both components (Fig. 4). The synergistic effects were achieved only when both components were simultaneously incubated with the cells, suggesting the

FIG. 1. Potentiation of PBMC response to LTA by glycosphingolipids. PBMCs at 1.5 × 10^6 to 2 × 10^6 cells/200 μl were exposed to 0.062 μM LTA, 15 μM of the indicated glycosphingolipids, and mixtures of both components that had been preincubated at 37°C for 1 h. After overnight incubation (19 to 22 h), the supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate or quadruplicate samples in a single representative experiment of five experiments performed. However, in all the experiments the magnitude of stimulation by the mixtures was significant compared to that by LTA or the glycosphingolipids alone (P ≤ 0.001). –LTA, without LTA; +LTA, with LTA.

FIG. 2. Stimulation of human monocytes by LTA is augmented by GM₁. Monocyte cultures were prepared as described in Materials and Methods. Monocytes at 2 × 10^6 cells/200 μl were exposed to medium alone; 0.062 μM LTA alone; GM₁ alone at the indicated concentrations; and mixtures of 0.062 μM LTA with 0 μM (i.e., LTA control), 7.5 μM, or 15 μM GM₁ that had been preincubated at 37°C for 1 h before being added to macrophages. After overnight incubation of the macrophages with these various stimuli (i.e., 19 to 22 h), the supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate samples in a single experiment. *, P ≤ 0.001.

FIG. 3. Stimulation of human monocytes by LTA is augmented by globoside. Monocyte cultures were prepared as described in Materials and Methods. Monocytes at 2 × 10^6 cells/200 μl were exposed to medium alone, LTA at the indicated concentrations (0.031 to 0.496 μM), 15 μM globoside, and mixtures of both components that had been preincubated at 37°C for 1 h. After overnight incubation (i.e., 19 to 22 h), the supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate samples in a single representative experiment of seven experiments performed. Differences between responses with and without globoside were statistically significant (*) (P ≈ 0.003) except when the LTA concentration was 0.496 μM, and at this concentration of LTA, no increase in the response over that achieved with LTA alone was observed.

followed by washing and overnight incubation with 15 μM globoside, or they were incubated for 1 h with 15 μM globoside, followed by washing and overnight incubation with 0.062 μM LTA. As a positive control, the monocytes were also incubated overnight with a mixture of both components (Fig. 4). The synergistic effects were achieved only when both components were simultaneously incubated with the cells, suggesting the

FIG. 4. Synergistic effect requires the simultaneous presence of stimuli. Monocytes were incubated either with 0.062 μM LTA alone for 1 h; with 15 μM globoside alone for 1 h; with LTA for 1 h, followed by washing and overnight incubation with 15 μM globoside (LTA→globoside); or with globoside for 1 h, followed by washing and overnight incubation with 0.062 μM LTA (globoside→LTA). The cells were also incubated overnight with a mixture of both components preincubated at 37°C for 1 h. The supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate samples in a single representative experiment of three experiments performed (*, P ≤ 0.003).
When the ratio of the host glycolipid was increased glycolipids had a dramatic effect on LTA migration. However, mixed gangliosides. At roughly equimolar ratios, neither of the ways, depending upon whether the glycolipid is globoside or ing acrylamide gel, the migration of LTA is affected in different solution (33). When LTA is incubated with two different cer- amide-containing glycolipids before it is run on a nondenatur- ing gel (Fig. 5). This migration pattern may be explained by the ability of LTA to form micelles in aqueous more slowly and, because of its microheterogeneous nature, in a much more diffuse manner on a nondenaturing gel (Fig. 5). This migration pattern may be explained by the ability of LTA to form micelles in aqueous solution (33). When LTA is incubated with two different cer- amide-containing glycolipids before it is run on a nondenatur- ing acrylamide gel, the migration of LTA is affected in different ways, depending upon whether the glycolipid is globoside or mixed gangliosides. At roughly equimolar ratios, neither of the glycolipids had a dramatic effect on LTA migration. However, when the ratio of the host glycolipid was increased ~10-fold, the use of globoside resulted in LTA migration that was slower and less diffuse, while the use of mixed gangliosides resulted in LTA migration that was more rapid (Fig. 5). The interaction with hemoglobin has also been shown to change the migration of LTA in a nondenaturating gel (14). LTA was also incubated with gelatin in the previous study to control for the nonspecific effect of the protein on LTA migration, and no alteration of migration was observed. In the dot blot assay, we spotted three glycosphingolipids on silica gel thin-layer chromatography plates, incubated them with LTA, and detected LTA binding by using an anti-LTA antiserum (Fig. 6). LTA bound strongly to lactosylceramide and kerasin, less so to GM2, and not at all to the negative control, polymyxin B. The negative control plate that was not incubated with LTA showed no binding of primary or secondary antibodies to kerasin, GM2, or polymyxin B. There appeared to be a weak cross-reaction with lactosyl- ceramide, but this may have been due to the low titers of antibody against ceramides that can be found in normal mammalian serum (15, 21). Nevertheless, there was such a marked difference between the two blots that we are convinced that it successfully makes the point that LTA interacts with lactosyl- ceramide.

Synergy of LTA preparations and glycosphingolipids is not due to lipoprotein contamination. Previous studies have sug- gested that the contamination of LTA preparations with small amounts of lipoproteins might be responsible for the ability of such LTA preparations to stimulate macrophages to secrete cytokines (13). Although NMR and amino acid analysis did not reveal any protein contamination in our LTA preparations (14), we examined the possibility that a trace amount of a lipoprotein or a lipopeptide other than LTA could be responsible for synergizing with glycolipids and enhancing monocyte-stimulating activity. We first examined whether the addition of very low concentrations of Pam3CysK4 to LTA would result in enhanced cytokine release (Fig. 7). The results showed that, indeed, low concentrations of Pam3CysK4 do synergize with LTA. However, when the same concentrations of Pam3CysK4 were added to the various glycosphingolipids, it was not able to synergize with any of the glycosphingolipids. Combinations of glycolipids with low concentrations of Pam3CysK4 give only an additive and not a synergistic effect, and this occurred only with globoside (Fig. 8). Furthermore, at higher concentrations of Pam3CysK4, glycolipids inhibited the secretion of IL-6 by the PBMCs. Taken together, these results suggest that the poten- tiation of stimulation by our LTA preparation and glycosphin- golipids is not due to a contaminating Pam3CysK4-like li- popeptide.

Potentiation of LTA activity by glycosphingolipids in human whole blood. We next wished to compare the abilities of different glycolipids to synergize with LTA to stimulate mono- cytes in whole blood. In these experiments, we used LTA at 0.062 μM (0.5 μg/ml), because at this concentration LTA alone stimulated the cells only weakly. The concentrations of glycosphingolipids that we added to the cells ranged from the lowest to the highest of the known glycosphingolipid concentra- tions in normal human serum (8, 22), and the molar ratio between LTA and glycosphingolipids was kept at ~1:120.
Several neutral and acidic glycosphingolipids in a human whole-blood system were examined, and all of them showed an increase in IL-6 secretion when they were mixed with LTA (Fig. 9). There were no differences in the levels of stimulation when 7.5/μM glycosphingolipid (the low concentration of glycosphingolipids in normal human serum) or 15/μM glycosphingolipid (the high concentration level of glycosphingolipids in normal human serum) was added. Similar results were obtained when deamylated LTA was used (data not shown).

Pam3CysK4 did not synergize with glycosphingolipids or LTA when whole blood from any of the four different human blood donors was used (data not shown).

In a separate set of experiments, we also assayed the culture supernatants for IL-1β secretion. In these experiments, similar synergies were seen when LTA was combined with ceramide containing glycolipids, with GM3 again giving the highest level of enhancement, in which the level of IL-1β secreted was roughly 10-fold higher than the level secreted in response to 50 ng/ml LPS (Fig. 10). In future experiments, it will be of interest to more completely define the profiles of the cytokines secreted and to assess their effects in vivo.

**DISCUSSION**

LTA is one of the cell wall components of gram-positive bacteria long considered to be a possible contributor to septic shock caused by gram-positive organisms, but its activity in stimulating cells of the immune system to secrete proinflamm-
amphiphiles physically interact with each other, as shown by based on the following results. First, synergy requires the si-
which may result in an enhanced response. This hypothesis is
amphiphilic molecules that more efficiently bind to a TLR,
this synergism is unclear at present. One possible hypothesis is
at an optimal molar ratio (Fig. 3). The mechanism underlying
synergism apparently requires the use of the two amphiphiles
by monocytes than the use of LTA or glycolipid alone. This
sphingolipids) resulted in a much stronger cytokine response
in this study, are normally present in the serum of uninfected
increased concentrations of several

We investigated the effects that combinations of glycosphingoli-
pids and LTA might have on the stimulation of immune
cells of the blood system for several reasons. Many ceramide-
containing molecules, including the glycosphingolipids tested
in this study, are normally present in the serum of uninfected
individuals at micromolar concentrations (8, 22). Sepsis has
been shown to result in increased concentrations of several
different ceramide species in serum (9), and interestingly,
α-galactosylceramide (i.e., kerasin) was recently shown to sen-
sitize mice to the development of lethal endotoxic shock (16).

We found that the use of mixtures of LTA and eukaryotic
amphiphiles (i.e., ceramide-containing glycolipids, or glyco-
sphingolipids) resulted in a much stronger cytokine response
by monocytes than the use of LTA or glycolipid alone. This
synergism apparently requires the use of the two amphiphiles
at an optimal molar ratio (Fig. 3). The mechanism underlying
this synergism is unclear at present. One possible hypothesis is
that oligomerization with certain amphiphiles, as shown here
for glycosphingolipids, may result in the formation of hetero-
oligomers (e.g., dimers and trimers) between LTA and other
amphiphilic molecules that more efficiently bind to a TLR,
which may result in an enhanced response. This hypothesis is
based on the following results. First, synergy requires the si-
multaneous presence of both amphiphiles. Second, the two
amphiphiles physically interact with each other, as shown by
their interaction on dot blots and by non-denaturing polyacryl-
amide gel electrophoresis followed by Western blotting. Fur-
thermore, all of these experiments were carried out at concentra-
tions below their CMCs. At the CMC, amphiphiles tend to
form dimers, tetramers, and oligomers until at the CMC they
form micelles (23, 24, 27). It has been shown that when mix-
tures of different lipids are used, the oligomers and micelles
always contain mixed lipids, with the degree of oligomerization
depending upon the concentration and the nature of the am-
phiphiles (23). This phenomenon may explain the recent ob-
servation in which two different LPS species expressed by Burk-
holderia mallei that are individually inactive due to suboptimal
numbers of acyl chains (i.e., tetra- and penta-acylated mole-
cules) became active when they were mixed together (5). It
should be noted, however, that the specific effects of different
glycosphingolipids on LTA migration in native gels do not
necessarily correlate directly with macrophage activation, nor
do they necessarily indicate that the mechanisms are the same
or different.

At present, there are basically two schools of thought with
regard to the proinflammatory properties of LTA: (i) LTA is a
gram-positive bacterial component that activates cells of the
immune system (e.g., monocytes and macrophages) to secrete
tumor necrosis factor alpha, IL-6, and other proinflammatory
molecules; or (ii) a copurifying lipoprotein is the component of
LTA preparations that stimulates cytokine secretion by mono-
cyes and macrophages and LTA is essentially inactive (13).
Since we had shown that our highly purified LTA preparations
could synergize with glycolipids to enhance cytokine secretion
from monocytes, we wanted to determine if the stimulation of
monocytes with the lipopeptide Pam3CysK4 and glycolipids
would also result in synergy. To the contrary, we found not only
that there was no synergy but also that glycosphingolipids
could actually inhibit the activity of Pam3CysK4. Furthermore,
we showed that in a similar range of molar ratios, Pam3CysK4
did synergize with LTA and resulted in enhanced cytokine
secretion. This result may also stem from the formation of
heterooligomers between these two amphiphiles.

In summary, we currently favor the concept that LTA will
exhibit potent stimulatory activity under certain conditions,
such as when LTA is released from the pathogen (2) in the
presence of hemoglobin (which is released from erythrocytes
by a hemolytic toxin, such as streptolysin O), muramyl dipep-
tide (which is released from bacterial cell walls by lysozyme
and amidases as part of the host response [29]), or glycosphin-
golipids (whose levels are known to be increased during sepsis
[9]). It is also quite possible that there are other bacterial or
host macromolecules that could synergize with LTA to in-
crease proinflammatory cytokine secretion. Future experi-
ments should focus on the synergistic effects of mixtures of
LTA and other compounds.

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FIG. 10. Potentiation of human whole-blood IL-1β response to
LTA by glycosphingolipids. Human whole blood diluted 1:5 in saline
was incubated with 0.062 µM LTA, glycosphingolipids alone at the
indicated concentrations, and mixtures of both components preincu-
bated at 37°C for 1 h. After overnight incubation (19 to 22 h), the
supernatants were removed and assayed for IL-1β secretion. The
data are given as the means ± standard deviations for triplicate samples in
a single representative experiment of seven experiments performed.
Compared to the magnitude of stimulation by LTA or glycosphingo-
lipids alone, the magnitude of stimulation by the mixtures was signif-
icant in each case (P ≤ 0.001).
REFERENCES

1. Alexander, C., and E. T. Reitschel. 2001. Bacterial lipopolysaccharides and innate immunity. J. Endotoxin Res. 7:167–202.

2. Alkan, M. L., and E. H. Beachy. 1978. Excretion of lipoteichoic acid by group A streptococci: influence of penicillin on excretion and loss of ability to adhere to human oral mucosal cells. J. Clin. Invest. 61:671–677.

3. Brandenburg, K., and A. Wiese. 2004. Endotoxin: relationships between structure, function, and activity. Curr. Top. Med. Chem. 4:1127–1146.

4. Brandenburg, K., P. Garidel, J. Andrand, G. Jürgens, M. Müller, A. Blume, M. H. J. Koch, and J. Levine. 2003. Cross-linked hemoglobin converts endotoxically inactive pentasaccharide endotoxins into a physiologically active form. J. Biol. Chem. 278:47660–47669.

5. Brett, P. J., M. N. Burntink, D. S. Snyder, J. G. Shannon, P. Azadi, and F. C. Gherardin. 2007. Burkholderia mallei expresses a unique lipopolysaccharide mixture that is a potent activator of human Toll-like receptor 4 complexes. Mol. Microbiol. 63:379–390.

6. Caroff, M., D. Caribian, J. M. Cavaillon, and N. Haeffner-Cavaillon. 2002. Structural and functional analyses of bacterial lipopolysaccharides. Microbes Infect. 4:915–926.

7. Cox, K. H., I. Ofek, and D. L. Hasty. 2007. Enhancement of macrophage stimulation by lipoteichoic acid and the co-stimulant, hemoglobin, is dependent on Toll-like receptors 2 and 4. Infect. Immun. 75:2638–2643.

8. Dawson, G., A. W. Kruski, and A. M. Scann. 1976. Distribution of glycosphingolipids in the serum lipoproteins of normal human subjects and patients with hypo- and hyperlipidemias. J. Lipid Res. 17:125–131.

9. Drobnik, W., G. Liebisch, F. X. Audebert, D. Frohlich, T. Gluck, P. Vogel, G. Rothe, and G. Schmitz. 1999. Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. J. Lipid Res. 44:754–761.

10. Galanos, C., and M. A. Freudenberg. 1993. Mechanisms of endotoxin shock and endotoxin hypersensitivity. Immunobiology 187:346–356.

11. Gay, N. J., and M. Gangloff. 2007. Structure and function of Toll receptors and their ligands. Annu. Rev. Biochem. 76:141–165.

12. Ginsburg, I. 2002. Role of lipoteichoic acid in infection and inflammation. Lancet Infect. Dis. 2:171–179.

13. Hashimoto, M., M. Furuyashiki, R. Kasaya, Y. Fukuda, M. Akimaru, K. Aoyama, T. Okuno, T. Tamura, T. Kirikea, F. Kirikea, N. Eiraku, H. Takada. 2004. Evidence of immunostimulating lipoprotein existing in the natural lipoteichoic acid fraction. Infect. Immun. 75:1926–1932.

14. Hasty, D. L., S. Meron-Sudai, K. H. Cox, T. Nagorna, E. Ruz-Bustos, E. Losi, H. S. Courtney, E. A. Mahrous, R. Lee, and I. Ofek. 2006. Monocyte and macrophage activation by lipoteichoic acid is independent of alanine and is potentiated by hemoglobin. J. Immunol. 176:5567–5576.

15. Ishii, N., and A. Watanabe. 1989. Analysis of autoantibodies to glycolipids on thymocytes in New Zealand Black mice. Immunology 68:215–220.

16. Ito, H., N. Koide, F. Hassan, S. Islam, G. Tumurkhuu, I. Mori, T. Yoshida, S. Kakamu, H. Moriwaki, and T. Yokochi. 2006. Lethal endotox shock using α-galactosyloceramide sensitization as a new experimental model of septic shock. Lab. Invest. 86:254–261.

17. Keisari, Y. 1996. Human mononuclear phagocytes in tissue culture, p. 153–160. In G. E. Jones (ed.), Methods in molecular medicine: human cell culture protocols. Humana Press Inc., Totowa, NJ.

18. Lotz, S., E. Agn, I. Wilde, G. van Zandbergen, T. Hartung, W. Solbach, and T. Laskay. 2004. Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. J. Immunol. 175:467–477.

19. Morath, S., A. Geyer, and T. Hartung. 2001. Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus. J. Exp. Med. 193:391–397.

20. Morath, S., A. Stadelmaier, A. Geyer, R. R. Schmidt, and T. Hartung. 2002. Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. J. Exp. Med. 195:1635–1640.

21. Schwarz, M., L. Spector, A. Gargini, A. Shesti, M. Gortler, R. T. Altstock, A. A. Dukler, and N. Dotan. 2003. A new kind of carbohydrate array, its use for profiling antiglycan antibodies, and the discovery of a novel human cellulose-binding antibody. Glycobiology 13:749–754.

22. Semm, H. J., M. Orth, E. Fütske, H. Wieland, and W. Gerek. 1989. Gangliosides in normal human serum. Concentration, pattern and transport by lipoproteins. Eur. J. Biochem. 181:657–662.

23. Small, D. M. 1986. The chemical physics of lipids from alkanes to phospholipids, p. 285. Plenum Publishing Corp, New York, NY.

24. Söll, R. V., and H. Fellermann. 2005. Lipid world. Molecular dynamics of lipid aggregates. http://complex.upf.es/~harold/lipid_world/index.html.

25. Su, D., R. I. Roth, and J. Levin. 1999. Hemoglobin infusion augments the tumor necrosis factor response to bacterial endotoxin (lipopolysaccharide) in mice. Crit. Care Med. 27:771–778.

26. Su, D., R. I. Roth, M. Yoshida, and J. Levin. 1997. Hemoglobin increases mortality from bacterial endotoxin. Infect. Immun. 65:1258–1266.

27. Tanford, C. 1980. The hydrophobic effect. Formation of micelles & biological membranes, 2nd ed. Wiley-Interscience, Hoboken, NJ.

28. Teti, G. 1999. Septic shock caused by gram-positive bacteria. Trends Microbiol. 7:100–101.

29. Traub, S., S. von Aulock, T. Hartung, and C. Hermann. 2006. MDP and other muropeptides—direct and synergistic effects on the immune system. J. Endotoxin Res. 12:69–85.

30. Triantafillou, M., M. Manukyan, A. Mackie, S. Morath, T. Hartung, H. Heine, and K. Triantafillou. 2004. Lipoteichoic acid and Toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. J. Biol. Chem. 279:40882–40889.

31. Von Aulock, S., T. Hartung, and C. Hermann. 2007. Comment on “Not lipoteichoic acid but lipoproteins appear to be the dominant immunobio logically active compounds in Staphylococcus aureus.” J. Immunol. 178:2610.

32. Wicken, A. J., and K. W. Knox. 1977. Biological properties of lipoteichoic acids, p. 360–365. In D. Schlesinger (ed.), Microbiology. ASM Press, Washington, DC.

33. Wicken, A. J., D. J. Evans, and K. W. Knox. 1986. Critical micelle concentrations of lipoteichoic acids. J. Bacteriol. 166:72–77.

34. Yang, S., R. Tamai, S. Akashi, O. Takeuchi, S. Akira, S. Sugawara, and H. Takada. 2001. Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to influence inflammatory cytokines in human monocytes in culture. Infect. Immun. 69:2045–2053.