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Use of Synthetic Derivatives To Determine the Minimal Active Structure of Cytokine-Inducing Lipoteichoic Acid[▽]

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Lipoteichoic acid (LTA) from gram-positive bacteria is the counterpart to lipopolysaccharide from gram-negative bacteria. LTA, which activates Toll-like receptor 2 (TLR2), induces a unique cytokine and chemokine pattern. The chemical synthesis of LTA proved its immunostimulatory properties. To determine the minimal active structure of LTA, we reduced synthetic LTA in a number of steps down to the synthetic anchor and employed these molecules to stimulate interleukin-8 (IL-8) release in human whole blood. Ten times more of the synthetic structures with four to six D-alanine-substituted polyglycerophosphate units (50 nM) than of the native LTA preparation was required to induce IL-8 release. A further reduction to three backbone units with two or no D-alanine residues resulted in cytokine induction only from 500 nM. The synthetic anchor was not able to induce IL-8 release even at 5 μM. When the LTA derivatives were used at 500 nM, they induced increasing levels of IL-8 and tumor necrosis factor alpha with increasing elongation of the backbone. Peritoneal macrophages were less responsive than human blood to the synthetic structures. Therefore, TLR2 dependency could be shown only with cells from TLR2-deficient mice for the two largest synthetic structures. This was confirmed by using TLR2-transfected HEK 293 cells. Taken together, these data indicate that although the synthetic anchor (which, unlike the native anchor, contains only myristic acid) cannot induce cytokine release, the addition of three backbone units, even without D-alanine substituents, confers this ability. Lengthening of the chain with D-alanine-substituted backbone units results in increased cytokine-inducing potency and a more sensitive response.

Recognition of conserved bacterial structures called pathogen-associated molecular patterns occurs via pattern recognition receptors on immune cells and leads to activation of the innate immune system and the induction of a variety of cytokines. Lipopolysaccharide (LPS) has been known as the most important pathogen-associated molecular pattern of gram-negative bacteria for more than 50 years (16) and has been well examined in detail over the decades. Immune recognition takes place by the binding of LPS to Toll-like receptor 4 (TLR4) and also involves the cofactors CD14 (17) and MD-2 (12, 14).

The immunostimulatory component of gram-positive bacteria was not clear for a long time, although a structural counterpart to LPS, called lipoteichoic acid (LTA), was found in the bacterial membrane. Like LPS, LTA is an amphiphilic molecule with a lipid anchor and a negatively charged backbone. Inefficient preparation methods on the basis of hot phenol, which resulted in the decomposition and the subsequent loss of activity, or LPS contamination during preparation led to inconsistent findings (9). Meanwhile, an improved preparation method based on *n*-butanol extraction at an ambient temperature was developed to purify the biologically active LTA of *Staphylococcus aureus* (8) and other organisms (3, 4). Structural analysis by nuclear magnetic resonance imaging and gas chromatography/mass spectrometry showed that the LTA molecules from *S. aureus* consist of up to 50 repeating glycerophosphate units linked to a lipid anchor with two fatty acids consisting of C_{14} to C_{19} acyl chains that partly contain methyl groups. The pattern recognition receptor required for LTA recognition was TLR2 (7), accompanied by the cofactors TLR6 (4), CD14 (5, 13), and CD36 (6).

Modifications of the LTA structure gave information about the prerequisites for LTA induction of cytokine release. When native LTA was deacylated by alkaline hydrolysis, the remaining backbone fragment failed to induce cytokine release. This result indicated that the lipid anchor is essential for the immunostimulatory potency of LTA. In line with this finding, the lipid anchor alone was much less potent than native LTA in inducing cytokine release in human whole blood and became inactive after the loss of one of the two fatty acids. The hydrolysis of D-alanine backbone substituents significantly reduced the immunostimulatory potency of LTA, resulting in a shift of the concentration response curve by 1 log order (8, 9).

The results of studies with a chemically synthesized simplified LTA structure, based on the native LTA structure of *Staphylococcus aureus*, confirmed these results (10). To determine the key components of the LTA molecule necessary to activate immune cells, several LTA derivatives were synthesized and examined for their abilities to induce cytokine release (1), starting from a molecule with an anchor with two C_{14} acyl chains and a backbone with six glycerophosphate units replaced with four D-alanines and one *N*-acetyl-D-glucosamine. The replacement of D-alanine with L-alanine blunted the cytokine-inducing potency, indicating stereoselectivity, and neither

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the absence of gentiobiose nor the loss of *N*-acetyl-D-glucosamine altered the ability of the molecule to induce cytokine release (1). These results approximated the crucial pattern required for the immune recognition of LTA and prompted us to synthesize further LTA derivatives with a step-by-step reduced structure down to the synthetic anchor to determine the minimal immunostimulatory structure of LTA.

MATERIALS AND METHODS

Stimuli. LTA from *Staphylococcus aureus* (DSM 20233) was prepared by *n*-butanol extraction, as described previously (8). The general strategy of *Staphylococcus aureus* LTA synthesis has been published elsewhere (15), and details on the synthesis of the structural analogs investigated in this paper are also reported elsewhere (2). LPS from *Salmonella enterica* serovar Abortus Equi was purchased from Sigma (Deisenhofen, Germany), and recombinant human tumor necrosis factor alpha (TNF-α) was obtained from Innogenetics (Heiden, Germany).

All non-LPS stimuli had an endotoxin contamination level below 1 endotoxin unit per mg, as shown by the kinetic chromogenic *Limulus* amoebocyte lysate assay (Charles River Endosafe, Charleston, NC).

Whole-blood incubation. Heparinized venous blood was obtained from healthy volunteers. Differential blood cell counts were routinely determined with a Pentra 60 apparatus (ABX Diagnostics, Montpellier, France) to exclude acute infections. Blood was diluted fivefold with RPMI 1640 (Cambrex, Verviers, Belgium) and incubated in the presence of the different stimuli in polypropylene vials (Eppendorf, Hamburg, Germany). After incubation at 5% CO_2 and 37°C overnight, the supernatants were collected after centrifugation and were stored at −80°C for cytokine determination.

Peritoneal macrophage populations. TLR2-knockout mice were provided by Tularik (South San Francisco, CA) and bred in the animal house of the University of Konstanz. The TLR2-knockout mice and the corresponding wild-type mice (strain 129Sv/B57BL6/6) were kept at 25°C with 55% humidity and a 12-h day and 12-h night rhythm and were given a diet of Altromin C 1310 (Altromin, Lage, Germany). All animals received humane care in accordance with the guidelines of the National Institutes of Health and legal requirements in Germany.

The mice were killed by terminal pentobarbital anesthesia (Narcoren; Merial, Hallbergmoos, Germany) by intravenous injection into the tail vein. Peritoneal cells were isolated by peritoneal lavage with 10 ml ice-cold phosphate-buffered saline. After centrifugation, the cells were resuspended in RPMI 1640 containing 10% fetal calf serum (Biochrom, Berlin, Germany), and 10^5 cells/well were transferred to 96-well cell culture plates (Greiner, Frickenhausen, Germany). After stimulation overnight, the culture supernatants were stored at −80°C until measurement by an enzyme-linked immunosorbent assay (ELISA).

Stimulation of TLR2-transfected HEK 293 cells. Stably murine TLR2-transfected human embryonic kidney cells (HEK 293) and wild-type HEK 293 cells were kindly provided by A. Dalpke (Heidelberg, Germany). The transfected cells were selected for neomycin resistance and the ability to release interleukin-8 (IL-8). The cells were seeded at 2×10^5 cells per well in six-well cell culture plates (Greiner) in Dulbecco's modified Eagle medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum, 1% polysaccharide (PAA Laboratories), and 0.8 mg/ml G418 (Merck, Darmstadt, Germany) and were stimulated overnight at 37°C in 5% CO_2. The amount of IL-8 released in the supernatant was measured by ELISA.

Cytokine ELISA. Cytokine levels were determined by a sandwich ELISA based on pairs of antibodies against human IL-8 and TNF-α (Endogen, Eching, Germany). Recombinant IL-8 (PeproTech; Tebu, Frankfurt, Germany) and TNF-α (National Institute for Biological Standards and Controls, South Mimms, United Kingdom) were used as standards. Murine TNF-α was determined by use of a mouse TNF-α DuoSet ELISA kit (R&D). Assays were carried out in flat-bottom 96-well immunoplates (MaxiSorp; Nunc, Wiesbaden, Germany). The binding of secondary biotinylated antibody was quantified by using streptavidin-peroxidase (Biosource, Camarillo, CA) and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, Steinheim, Germany).

Statistics. Data are presented as means ± standard errors of the means (SEMs). Statistical significance was determined with Prism software (version 4.0; GraphPad Software, San Diego, CA). Cytokine levels are given as the amount per ml blood, i.e., corrected for a dilution factor of 5 in the 20% blood incubation. In cases of two groups, data were analyzed by an unpaired *t* test (mouse experiments) or a paired *t* test. Data for three or more groups were compared by repeated-measures analysis of variance, followed by Dunnett's multiple-comparison test.

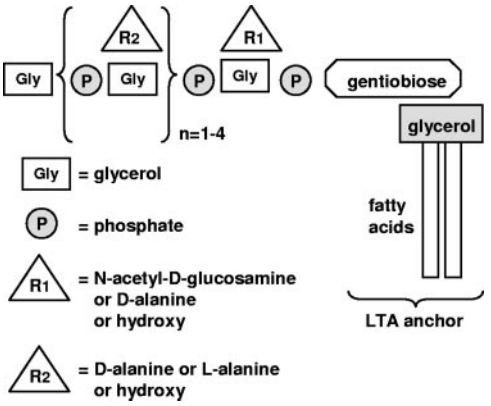

FIG. 1. Configuration of synthetic LTA and derivatives. The synthetic LTA and its derivatives consist of a backbone and an anchor with two fatty acids ($C_{13}H_{27}$) linked across a glycerol and a gentiobiose. The backbone is composed of three to six glycerophosphate units carrying either no subunits (LTA without D-alanine), two to five D-alanines (R = D-alanine), or four D-alanines or L-alanines (D-Ala–LTA or L-Ala–LTA) plus one *N*-acetyl-D-glucosamine (R = *N*-acetyl-D-glucosamine); or the backbone is linked to the anchor in the absence of gentiobiose.

RESULTS

The immunostimulatory activities of the LTA derivatives depend on the backbone substituents and the backbone length. Recently, we described that a synthetic LTA made up of an anchor with two fatty acids and a backbone with six polyglycerophosphate units and five D-alanine substituents can induce cytokine release (1). In this study, we examined at what point a further reduction of the synthetic LTA molecule abrogates its ability to induce cytokine release in human whole blood. For comparison with the new derivatives, we employed native LTA and the synthetic derivatives D-Ala–LTA, L-Ala–LTA, LTA without gentiobiose, and LTA without *N*-acetyl-D-glucosamine, as described previously (1). New LTA derivatives were synthesized with a further truncated backbone, resulting in LTAs with two, three, or four D-alanine residues and three, four, or five backbone units, respectively. Furthermore, a structure consisting of a lipid anchor with three backbone subunits without any substituents and the bare synthetic anchor were tested. A graphic description of all the synthetic compounds used is given in Fig. 1. Codes which describe the composition of the LTA and the derivatives used are shown in Table 1. Because of the heterogeneity of native LTA, the average numbers of the components are indicated in this case. The native LTA of *Staphylococcus aureus* differs from the synthetic LTA in that its backbone has, on average, 50 units, 70% of which are substituted with D-alanine and 15% with *N*-acetyl-D-glucosamine; and it may contain fatty acids of various chain lengths, and these may also be partially methylated (11).

The immunostimulatory potencies of the derivatives and native LTA were determined by measurement of the amount of cytokine released in whole blood. We incubated human whole blood with equimolar concentrations of stimulus from 5 nM to 5 μM overnight and measured the release of the che-

TABLE 1. Concentrations of LTA derivatives required to induce significant IL-8 release in human whole blood

LTA structure code[a]	Synonym	Stimulus concn (nM)
50B35D8N1G2F	Native LTA	5
6B4D1N1G2F	D-Ala–LTA	50
6B4L1N1G2F	L-Ala–LTA	500
6B4D1N0G2F	LTA without gentiobiose	50
6B5D0N1G2F	LTA with five alanines	50
5B4D0N1G2F	LTA with four alanines	50
4B3D0N1G2F	LTA with three alanines	50
3B2D0N1G2F	LTA with two alanines	500
3B0D0N1G2F	LTA without alanine	500
0B0D0N1G2F	Synthetic anchor	>5,000

[a] B, backbone subunit glycerophosphate; D, D-alanine; L, L-alanine; N, *N*-acetyl-D-glucosamine; G, gentiobiose; F, fatty acid (C_{13} for synthetic LTA and C_{14} to C_{19} for native LTA). For native LTA, the structure code expresses the mean number of units per molecule ($n \geq 11$).

mokine IL-8 and the proinflammatory cytokine TNF-α in the supernatants by ELISA. We determined the stimulus concentration that was sufficient to induce statistically significant levels of IL-8 (Table 1) or TNF-α. Native LTA induced IL-8 release at a stimulus concentration as low as 5 nM, whereas D-Ala–LTA (6B4D1N1G2F) first induced cytokine release at a 10-fold higher concentration. TNF-α secretion commenced only at a stimulus concentration of 50 nM for both native LTA and D-Ala–LTA. LTA without gentiobiose (6B4D1N0G2F) and LTA without *N*-acetyl-D-glucosamine (6B5D0N1G2F) showed the same immunostimulatory potencies as D-Ala–LTA, and replacement of D-alanine with L-alanine reduced the sensitivity 10-fold, confirming our previous results (1). The reduction of the backbone from six units by one (5B4D0N1G2F) or two (4B3D0N1G2F) glycerophosphate subunits with D-alanine substituents had no influence on significant IL-8 induction starting at a stimulus concentration of 50 nM. A loss of sensitivity occurred after reduction of the LTA molecule to three backbone subunits and two D-alanines (3B2D0N1G2F) or no D-alanine (3B0D0N1G2F). In this case, a concentration of 500 nM was required to stimulate the immune cells to release significant levels of IL-8. A further loss of sensitivity occurred upon stimulation with only the synthetic anchor (0B0D0N1G2F). No IL-8 or TNF-α release was measured in whole blood in the concentration range of 5 nM to 5 μM.

Additionally, we compared the IL-8 and TNF-α levels induced in human whole blood after stimulation with 500 nM synthetic anchor (0B0D0N1G2F) or LTA without D-alanine (3B0D0N1G2F) or with two (3B2D0N1G2F), three (4B3D0N1G2F), four (5B4D0N1G2F), or five (6B5D0N1G2F) D-alanines and native LTA (Fig. 2). The IL-8 and TNF-α induction levels increased with an increasing backbone length and an increasing D-alanine content, respectively. Thus, not only the thresholds at which cytokine induction commences but also the cytokine-inducing capacities differ between the LTA derivatives.

LTA derivatives activate cytokine release via TLR2. Peritoneal macrophages were isolated from TLR2$^{-/-}$ and wild-type mice by peritoneal lavage and were stimulated with 10 μM LTA with five D-alanines (6B5D0N1G2F), LTA without gentiobiose (6B4D1N0G2F), native LTA, and LPS overnight. Murine TNF-α levels were determined in the cell supernatants by ELISA (Fig. 3). Like native LTA, both LTA derivatives induced significant release of TNF-α in wild-type peritoneal cells but not in TLR2-deficient peritoneal cells. The amounts of TNF-α released in response to stimulation with LPS, a TLR4 agonist used as a positive control, were equivalent in TLR2$^{-/-}$ and wild-type macrophages. Stimulations of macrophages from TLR2$^{-/-}$ and wild-type mice were also performed with the other derivatives of synthetic LTA given in Table 1; however, these elicited only a very weak TNF response in the wild-type cells, making it impossible to determine the TLR2 dependency.

Furthermore, we examined whether the TLR2 receptor confers cytokine responsiveness to LTA derivatives in HEK 293 cells stably transfected with murine TLR2 by measuring IL-8 induction (Fig. 4). The stimuli TNF-α (1.5 nM) and LPS (1 nM) were used as positive and negative controls, respectively. Cells responded with significant IL-8 release after activation with the TLR-independent stimulus, TNF-α, whereas the TLR4-dependent stimulus, LPS, did not induce IL-8 release. Native LTA (500 nM) with five D-alanines (6B5D0N1G2F; 5

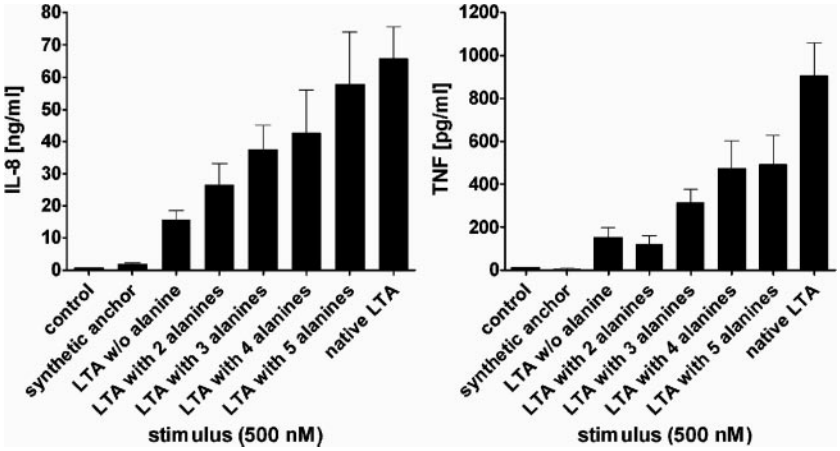

FIG. 2. The cytokine-inducing potency of LTA derivatives depends on the alanine content. ELISA was used to measure the amounts of IL-8 and TNF-α released in human whole blood in response to 500 nM native LTA isolated from *S. aureus*; the synthetic anchor (0B0D0N1G2F); LTA without (w/o) alanine (3B0D0N1G2F); and LTA with two (3B2D0N1G2F), three (4B3D0N1G2F), four (5B4D0N1G2F), or five (6B5D0N1G2F) alanines. Data are presented as the means ± SEMs (n = 11 to 43).

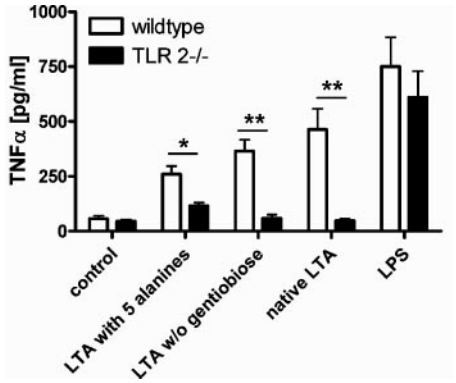

FIG. 3. TNF-α release in response to synthetic LTA derivatives requires TLR2. Peritoneal macrophages (10^5/well) from TLR2-knockout mice ($TLR2^{-/-}$) and wild-type mice ($n = 7$) were stimulated with 10 μM LTA with five alanines (6B5D0N1G2F), LTA without (w/o) gentiobiose (6B4D1N0G2F), native LTA, LPS, or saline (control) overnight. Murine TNF-α release was determined by a sandwich ELISA. Data are presented as the means ± SEMs of pooled data from two independent experiments. The statistical difference between the wild-type and the knockout mouse response was calculated by a two-tailed paired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

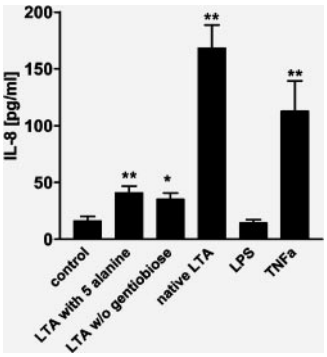

FIG. 4. Induction of IL-8 release in TLR2-transfected HEK 293 cells by synthetic LTA derivatives. HEK 293 cells stably transfected with TLR2 were stimulated with 5 μM LTA with five alanines (6B5D0N1G2F), LTA without (w/o) gentiobiose (6B4D1N0G2F), 500 nM native LTA, 1.5 nM TNF-α, 1 nM LPS, or saline (control) overnight. The amount of IL-8 released in the supernatants was determined by a sandwich ELISA. Data are presented as the means ± SEMs. Statistics were calculated by a one-way analysis of variance and Dunnett's multiple-comparison test (on log-transformed data) (*, $P < 0.05$ versus the results for the control; **, $P < 0.01$ versus the results for the control; $n = 6$; pooled data from two independent experiments). Wild-type HEK 293 cells lacking TLR2 expression showed no IL-8 release upon stimulation with native and synthetic LTA and LPS, while stimulation with TNF-α induced IL-8 release.

μM) and LTA without gentiobiose (6B4D1N0G2F; 5 μM) elicited significant IL-8 release. Wild-type HEK 293 cells lacking TLR2 expression showed no IL-8 release upon stimulation with native LTA, the synthetic structure, or LPS, while stimulation with TNF-α induced IL-8 release, as expected. As was the case with the murine macrophages, the other, shorter LTA derivatives listed in Table 1 did not induce significant IL-8 release in the TLR2-transfected HEK 293 cells, even at the high concentration of 500 nM. In conclusion, immune stimulation by the larger synthetic molecules is clearly TLR2 dependent.

DISCUSSION

On the basis of the structural analysis of native, biologically active LTA of *Staphylococcus aureus*, a complete LTA molecule was synthesized (10). This molecule, with a backbone five times shorter than that of native LTA, induced cytokine release but was about 10 times less potent than the native LTA. Neither the loss of *N*-acetyl-D-glucosamine nor the loss of gentiobiose altered its potency (1).

Here, we reduced the backbone length of the synthetic structure step by step down to the synthetic anchor. We found that at two stages we required 10-fold more LTA to induce significant IL-8 release. The synthetic anchor induced no IL-8 release, even at the highest concentration tested (5 μM). Furthermore, we compared the potencies of the LTA derivatives by testing them at an equimolar stimulus concentration (500 nM) and found that IL-8 and TNF-α induction increased with the number of glycerophosphate units replaced with D-alanine.

The differences in the IL-8-inducing potencies between native LTA and the LTA derivatives as well as between the anchor generated from native LTA and the synthetic anchor may be due to the difference in the compositions of the anchors. The fatty acids of native LTA differ in their chain lengths and contain additional methyl groups (11), whereas the synthetic anchor contains only unmethylated myristic acids.

The TLR2 dependency of the two largest synthetic LTA derivatives was proven in peritoneal macrophages of $TLR2^{-/-}$ and wild-type mice. Murine peritoneal macrophages are less sensitive to LTA stimulation than human cells (5, 7). Therefore, higher LTA concentrations were required to induce cytokine release. In line with this, the smaller LTA derivates were unable to induce significant TNF-α release in the wild-type cells, thus precluding determination of TLR2 dependency in this experimental approach. While untransfected HEK 293 cells were unresponsive to LTA stimulation, TLR2-transfected HEK 293 cells responded to native LTA and the two largest LTA derivatives with IL-8 release.

Taken together, these data indicate that LTA has to be composed of an anchor with two fatty acids and at least a short backbone of three glycerophosphate subunits to facilitate immune recognition, and lengthening of the backbone results in higher levels of TLR2-dependent cytokine induction at lower concentrations. This study defines for the first time the minimal structural requirements for the pattern recognition of LTA by immune cells.

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