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Presentation of lipoteichoic acid potentiates its inflammatory activity

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Abstract

Lipoteichoic acid (LTA) is a major immunostimulatory molecule in the cell wall of Gram-positive bacteria. Adhesion of LTA to a polystyrene surface drastically increased its immunostimulatory potency in human whole blood in comparison to soluble LTA, although only 1% of the LTA had bound, as determined using rhodamine-labelled LTA. The release of the proinflammatory cytokines IL-1β, TNF and IL-6 and the chemokines IL-8 and G-CSF was increased 2- to 10-fold, but IL-10 release was unaltered. This presentation effect was not shared by lipopolysaccharide (LPS) or other toll-like receptor 2 agonists and was less pronounced in polypropylene vessels. LTA did not induce cytokine release in silicone-coated borosilicate vessels, but covalent coupling of LTA to polystyrene beads restored cytokine induction in these vessels, indicating that presentation of LTA on a surface is in fact essential for its immunostimulatory potency.

This novel aspect of presentation as a factor in the recognition of LTA may reflect the physiological situation in the bacterial cell wall, where LTA is anchored in the bacterial membrane and projects through the peptidoglycan. In practical terms, contamination of medical devices with components of Gram-positive bacteria may pose an underestimated inflammatory risk.

Keywords: Bacteria; Cytokines; Inflammation; Lipoteichoic acid; Potency

Introduction

The clinical symptoms of sepsis caused by Gram-positive or Gram-negative bacteria are practically identical. Lipopolysaccharide (LPS) has been known as the main immunostimulatory component of Gram-negative bacteria since the 1950s and is sufficient to cause lethal septic shock (Opal and Gluck 2003). There was contention whether lipoteichoic acid (LTA) represented its counterpart in Gram-positive bacteria until it was shown that inappropriate preparation methods as

Abbreviations: G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PGN, peptidoglycan; PP, polypropylene; PS, polystyrene; SBS, silicone borosilicate; TLR, toll-like receptor; TNF, tumour necrosis factor.

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well as heterogenic and endotoxin-contaminated commercial preparations had been the cause of the conflicting results (Gao et al. 2001; Kusunoki et al. 1995; Morath et al. 2002a). An optimized preparation procedure based on butanol extraction at ambient temperatures yielded highly pure and potent cytokine inducing LTA from *Staphylococcus aureus* (Morath et al. 2001; von Aulock et al. 2003). Chemical synthesis of LTA confirmed its immunostimulatory potency and enabled structure–function studies to identify the components essential for immune recognition (Deininger et al. 2003; Morath et al. 2002b).

Like LPS, LTA is an amphiphilic molecule. It is made up of a hydrophilic backbone of repetitive glycerophosphate units with D-alanine or N-acetyl glucosamine substituents and a lipophilic anchor. The latter is integrated into the bacterial membrane while the backbone projects through the murein to the bacterial surface (Aasjord and Grov 1980). While LPS signals via lipopolysaccharide-binding protein (LBP), CD14 and toll-like receptor 4 (TLR4), LTA induces cytokine release via the concerted action of CD36, CD14, LBP and TLR2 (Hermann et al. 2002; Hoebe et al. 2005; Lehner et al. 2001; Schröder et al. 2003). The platelet-activating factor receptor may be involved in inducing NO production (Han et al. 2006).

LPS and LTA induce different patterns of cytokine release. LPS is a potent inducer of proinflammatory cytokines and IL-10, whereas LTA is a weaker inducer of proinflammatory cytokines and does not induce IL-12 and subsequent IFNγ formation (Hermann et al. 2002). Instead, LTA induces a stronger release of chemoattractants, such as IL-8, macrophage chemoattractant protein-1 (MCP-1), leukotriene B_4 (LTB_4) as well as the growth factor G-CSF, than LPS (von Aulock et al. 2003). LTA protects neutrophils from spontaneous apoptosis (Lotz et al. 2004) but does not prime them for oxidative burst or degranulation (von Aulock et al. 2003). LTA induces tissue factor expression and procoagulant activity in human monocytes (Mattsson et al. 2004) and activates the complement system via L-ficolin (Lynch et al. 2004).

In the present study, we made the observation that LTA immobilized on a polystyrene surface induces a far stronger release of cytokines than LTA in solution. This suggested that the presentation of LTA to immune cells represents a new dimension of immune recognition and raised the following questions: How are the kinetics and cytokine patterns affected by presentation of LTA, and does this effect also occur on other surface materials, and is it specific to LTA?

Materials and methods

Incubation vessels

Ninety-six-well polystyrene cell culture plates and polypropylene plates (Greiner, Frickenhausen, Germany), polypropylene tubes (1.5 ml, Eppendorf, Hamburg, Germany) and silicone-coated borosilicate tubes (5 ml, BD Biosciences, Heidelberg, Germany) were used.

Stimuli

All non-endotoxins were tested in the kinetic chromogenic limulus amoebocyte lysate (LAL) assay (Charles-River/Endosafe, Charleston, USA) and were found to contain less than 1.5 EU endotoxin/mg stimulus. LTA from *S. aureus* (DSM 20233) was isolated in-house by *n*-butanol extraction at room temperature as described (Morath et al. 2001). LPS from *Salmonella abortus equi* was purchased from Sigma (Deisenhofen, Germany) and LPS from *Porphyromonas gingivalis* (MCCM 3199) was isolated in-house, in analogy to LTA, by *n*-butanol extraction. Peptidoglycan (PGN) from *S. aureus* was obtained from Fluka (Buchs, Switzerland) and Pam_3Cys from EMC microcollections (Tuebingen, Germany).

Rhodamine-labelled LTA was prepared by sonifying 3 mg LTA from *S. aureus*, 4.5 mg sulphorhodamine Q 5-acid fluoride (Fluka, Buchs, Switzerland), 2.5 ml dimethyl sulphoxide (pH < 7, Wak-Chemie-Medical GmbH, Steinbach, Germany) and 25 µl triethylamine (pH < 7, Acros Organics, Leicestershire, UK) for 10 min, and then shaking the mixture (pH < 8) overnight at 37 °C. For separation of unbound rhodamine molecules, it was spun at 7000 × *g* for 90 min at room temperature four times in a pyrogen-free centrifugal ultrafilter unit (cut-off 3 kDa, Microsep 3 K Centricons, Pall, USA) and additionally filtered through a pyrogen-free sepharose column (PD-10 desalting column, Amersham Biosciences, Freiburg, Germany). The yield of labelled LTA was determined by phosphate content, which was measured by the molybdenum blue method (Vogel and Bassett 1978): 200 µl ashing solution [H_2SO_4:$HClO_4$:-H_2O (556:105:3339, v:v:v)] were mixed with 50 µl LTA solution and incubated at 145 °C for 2 h in open polypropylene tubes (Eppendorf, Hamburg, Germany). One millilitre reducing solution [ascorbic acid:ammoniumheptamolybdenum sodium acetate (1:9, v:v)] was added and incubated at 50 °C for 2 h. Absorption was measured at 700 nm. Labelling efficiency, calculated as fluorescence (560 nm/620 nm) per phosphate content, was about 1 molecule rhodamine per LTA.

The rhodamine-labelled LTA was negative in the Limulus test for Gram-negative endotoxin (i.e. < 1.5 pg LPS/mg LTA; Endosafe Limulus amoebocyte lysate (LAL) Endochrome; Charles River Laboratories).

To prepare LTA-beads, LTA was covalently coupled to polystyrene microspheres with functional carboxylate surface groups and a diameter of 4 µm (Bangs Laboratories, Fishers, IN, USA). One millilitre beads (100 mg/ml) was washed twice with 10 ml MES buffer (100 mM

morpholino-ethansulfonic acid, pH 7.2, Sigma-Aldrich, Seelze, Germany) by centrifugation at 1200*g* for 15 min at room temperature and resuspended in 10 ml MES buffer. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (Fluka) was used as activator and cross-linking reagent. Beads were incubated with 100 mg EDAC for 15 min at room temperature under continuous mixing. After activation of the microspheres, they were washed twice and resuspended in 5 ml MES buffer. Five hundred micrograms LTA was combined with the bead suspension (100 mg beads) and incubated for 2 h at room temperature with constant shaking. After centrifugation, the pellet was resuspended in 10 ml quenching solution with 30 mM hydroxylamine (Merck-Schuchardt, Hohenbrunn, Germany) and mixed gently for 30 min. The beads were washed again and stored at 4 °C in 10 ml MES buffer until further use.

Fluorescence measurements

For quantification of LTA bound to polystyrene or polypropylene, 96-well plates were coated with 50 µl of the given concentrations of rhodamine-labelled LTA for 2 h at 37 °C, washed twice and refilled with 50 µl saline. The absorption at 560 nm (reference wavelength 620 nm) was measured in a fluorometer (Victor^2 Fluorometer, Wallac, Turku, Finland). For calibration, the fluorescence signal of a dilution series of rhodamine-labelled LTA in 50 µl saline was determined.

Cultivation and pretreatment of *S. aureus*

S. aureus bacteria (DSMZ 20233) were grown in 100 ml shaking flasks in tryptic soy broth medium (BD Biosciences, Heidelberg, Germany) supplemented with 25 g/l beef extract (BD Biosciences) and 5 g/l glucose solution, for 14 h at 37 °C. Only materials having no or low pyrogen content and highly purified water (Milli-Q, EASYpure, Barnstead, Wilhelm Werner GmbH, Leverkusen, Germany) were used. After harvest, bacteria were washed three times with PBS (PAA Laboratories, Pasching, Austria) by centrifugation at 1100*g* for 10 min at room temperature and resuspended with physiological saline (Berlin-Chemie AG, Berlin, Germany). An optical density of 1.0 at 600 nm corresponded to 10^9 bacteria/ml. Both live and UV-inactivated bacteria were used for stimulation of blood cells. For the UV-inactivation, bacteria were adjusted to 10^8 bacteria/ml and 2 ml/well of the bacterial suspension were irradiated (UV-Stratalinker 1800, Stratagene, La Jolla, CA, USA) with an energy density of 1 kN/cm^2 (3 mW/cm^2 × 300 s) in a 6-well cell culture plate (Greiner Bio-One, Frickenhausen, Germany). To control the effectiveness of UV-inactivation, bacteria were plated on blood agar plates (Columia-blood agar, Heipa Diagnostika, Eppelheim, Germany) after irradiation and cultured for 24 h at 37 °C and 5% CO_2. No colonies grew on the plates.

Whole blood incubation

Blood was drawn from healthy human volunteers. Differential blood cell counts were performed with a Pentra 60 (ABX Diagnostics, Montpellier, France) to exclude acute infections. Heparinized venous blood was diluted five-fold with RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 2.5 IU heparin (Liquemin®, Hoffmann La Roche, Reinach, Switzerland), and incubated in the presence of different stimuli in various reaction vessels. After incubation in the presence of 5% CO_2 at 37 °C overnight, cell-free supernatants, obtained by gentle mixing and then centrifugation at 16,000*g* for 2 min, were stored at −80 °C for cytokine determination.

Cytokine ELISA

Cytokines were measured by in-house sandwich ELISA (enzyme-linked immunosorbent assay) based on antibody pairs against human IL-8, TNF (Endogen, Eching, Germany), IL-6, G-CSF, IL-1β (R&D, Wiesbaden, Germany) and IL-10 (BD Pharmingen, Heidelberg, Germany). Recombinant IL-1β, TNF, IL-6 (NIBSC, South Mimms, UK), IL-8 (PeproTech, Tebu, Frankfurt, Germany), IL-10 (BD Pharmingen) and G-CSF (Amgen, Munich, Germany) were used as standards. Murine TNF was measured with the murine TNFα DuoSet ELISA kit (R&D). Assays were carried out in flat-bottom 96-well immunoplates (MaxiSorp, Nunc, Wiesbaden, Germany). Binding of secondary biotinylated antibody was quantified using streptavidin-peroxidase (Biosource, Camarillo, CA, USA) and the substrate TMB (3,3′,5,5′-tetramethylbenzidine, Sigma).

Quantification of mRNA by real-time PCR

After 6 h of human whole blood incubation, RNA was isolated with a QIAamp© RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Possible contaminating DNA was digested using an RNase-free DNase set (Qiagen). Reverse transcription was performed using a Perkin-Elmer Geneamp PCR system 9600 with 5 mM $MgCl_2$, 2 µl 10 × -PCR-buffer II, 1 mM dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 µM Oligo d(T)16 (Thermo Hybaid, Ulm, Germany), 20 U/µl RNase-inhibitor (Roche), 50 U/µl MuLV-RT (Perkin-Elmer) and 4.2 µl RNase-free water using the following program: 21 °C, 10 min; 42 °C, 15 min; 99 °C, 5 min; 5 °C, 5 min; then kept at 4 °C. Real-Time PCR was

performed on a LightCycler® (Roche, Grenzach-Whylen, Germany) using LightCycler FastStart DNA Master SYBR Green (Roche). $MgCl_2$-concentrations were adjusted to 3 mM. Primers were purchased from ThermoHybaid: TNF forward primer 5′-GAG CAC TGA AAG CAT GAT CCG G-3′, reverse primer 5′-AAA GTA GAC CTG CCC AGA CTC GG-3′, cyclophilin forward primer 5′-CTC CTT TGA GCT GTT TGC AG-3′ and reverse primer 5′-CAC CAC ATG CTT GCC ATC C-3′. Amplification products had a length of 682 bp for TNF and 325 bp for cyclophilin. Amplification (50 cycles) was performed with an annealing temperature of 68 °C and 28 s for TNFα and 56 °C and 13 s for cyclophilin. The product-specific melting points were 93 °C for TNF and 87 °C for cyclophilin. mRNA levels given for TNF were normalized to mRNA levels of cyclophilin.

Statistics

Data are presented as means ± S.E.M. Statistical significance was determined using GraphPad Prism 4.0 (GraphPad software, San Diego, USA). Cytokine levels are given per millilitre of blood, i.e. corrected for a dilution factor of 5 in the 20% blood incubation. In case of two groups, data were analysed by unpaired (mouse experiments) or paired *t*-test. Three or more groups were compared by one-way ANOVA followed by Dunn's multiple comparison test.

Results

Binding of LTA to polystyrene amplifies cytokine release

We investigated whether LTA presented to immune cells by immobilization on a surface has a different inflammatory potency than LTA in solution. This could resemble the physiological situation in the bacterial cell wall, where LTA is integrated into the murein sacculus. We preincubated 2 µg LTA or LPS in 96-well polystyrene cell culture plates for 2 h. Then the supernatant containing unbound molecules was transferred to new wells. The preincubated wells were washed twice with physiological saline before incubation with human blood. In parallel, blood was stimulated with the same amount of the stimulus in solution without preincubation. After overnight incubation with blood, IL-1β release was determined in the supernatants. The cytokine release induced by bound LTA was about four times greater than that induced by LTA in solution (Fig. 1). Regarding LPS, both incubation conditions induced equal cytokine amounts.

We hypothesized that this presentation effect cannot occur upon stimulation with the soluble stimulus because the polystyrene surface is quickly occluded with plasma proteins. To test this hypothesis, we preincubated the polystyrene wells with autologous plasma for 1 h at 37 °C before allowing LTA or LPS to bind to the surface. Again, unbound molecules were removed by washing before addition of whole blood. Under these conditions, the preincubated LTA was not able to induce a stronger cytokine release in comparison to the soluble stimulus. LPS potency was not significantly decreased by preincubation with plasma.

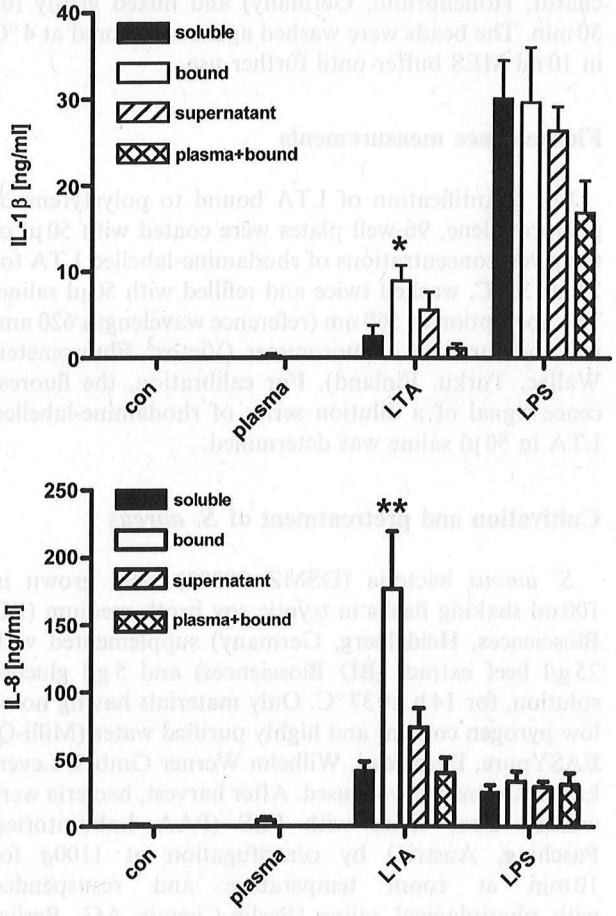

Fig. 1. Binding of LTA to polystyrene increases its immunostimulatory potency. 2 µg/well LTA or LPS were either coated for 2 h at 37 °C before transfer of the supernatant to a new well (supernatant) and subsequent rinsing to remove further unbound stimulus (bound), or coated after preincubation and subsequent rinsing with human plasma for 1 h (plasma + - bound), or employed directly as soluble stimulus (soluble). IL-1β and IL-8 release in human whole blood diluted 1:5 in RPMI was measured by ELISA after overnight incubation. Statistics were calculated using one-way ANOVA and Dunn's multiple comparison test, $^{*}p<0.05$, $^{***}p<0.001$ versus soluble; $n = 8$–16, pooled data from four independent experiments.

This demonstrates that LTA presented on a polystyrene surface more potently induces cytokine release and that this effect cannot occur if the surface is occluded with plasma proteins.

Rhodamine labelling of LTA

After testing a large number of different labelling substances unsuccessfully, it was found that LTA could be labelled with sulphorhodamine Q 5-acid fluoride. Fluorescence experiments showed a labelling efficiency of one molecule rhodamine per molecule LTA.

NMR analysis could not detect the rhodamine label to identify where it had bound to the molecule but revealed that much of the D-alanine substitution of the LTA was lost in the labelling procedure. We have shown in previous work (Morath et al. 2001; Draing et al. 2006) that the concentration response curve of LTA shifts to the right by a factor of 10 when the D-alanine is lost, but that the pattern of cytokine induction remains the same. To investigate whether the rhodamine label caused further changes in the activity of LTA, we performed concentration response curves in human whole blood and measured the release of TNF, IL-8 (Fig. 2) and IFNγ (not shown). As expected, the concentration response curve was shifted by a factor of 10 by the labelling procedure and the cytokine inducing potency of the rhodamine-labelled LTA was comparable to that of the mock-labelled LTA.

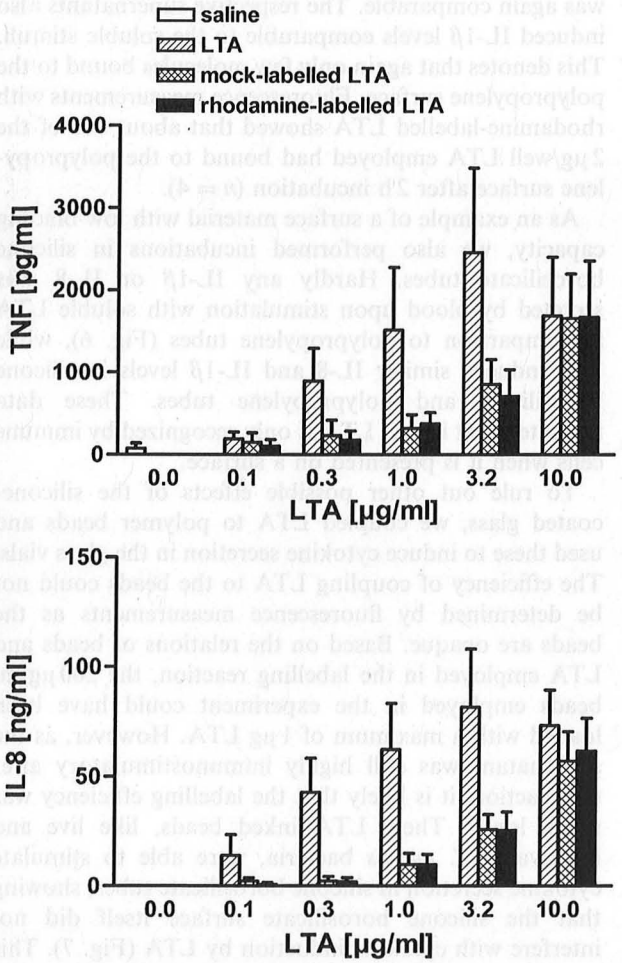

Fig. 2. Cytokine-inducing activity of rhodamine-labelled LTA. Saline, LTA, mock-labelled LTA and rhodamine-labelled LTA were incubated with 20% human whole blood overnight in the indicated concentrations. TNF and IL-8 release were measured by ELISA, $n = 4$.

Quantification of LTA binding to polystyrene

Stimulation of blood with the supernatants from the coated wells had shown that these still contained a large portion of the LTA molecules employed, as they still induced cytokine release (Fig. 1). Fluorescence measurements with rhodamine-labelled LTA showed that only about 1% (after 2 h incubation, $n = 9$) of the 2 µg/well LTA employed remained bound to the polystyrene surface, whether the surface was preincubated with plasma or not. Further increasing the amount of LTA resulted only in minor increases of deposition.

Thus, the IL-1β-inducing activity of LTA was amplified by at least a factor of 400, i.e. 1% of the LTA was deposited, but that was more than four times as potent as soluble LTA with regard to IL-1β induction.

Kinetics of LTA coating and presentation on a polystyrene surface

To study the progression and saturation of LTA binding to the surface, we added 2 µg LTA/well to 96-well polystyrene cell culture plates and incubated for different durations at 37 °C before washing twice with physiological saline to remove unbound LTA molecules. Cytokine levels induced by the surface-bound LTA increased with the coating duration (Fig. 3). IL-1β induction was maximal after 24 h coating duration, though the increase from 2 to 24 h was not statistically significant. In further experiments, coating durations of at least 2 h were chosen throughout.

Cytokine pattern induced by bound LTA

The presentation of LTA on the polystyrene surface increased its cytokine-inducing activity significantly with regard to the release of TNF, IL-1β, IL-6, G-CSF and IL-8 (Fig. 4) in comparison to soluble LTA, while the release of IL-10 was not altered. The 10-fold increase of TNF release was the greatest effect observed; IL-6 release was only augmented by a factor of 2.5. This was also reflected on the mRNA level measured by real-time

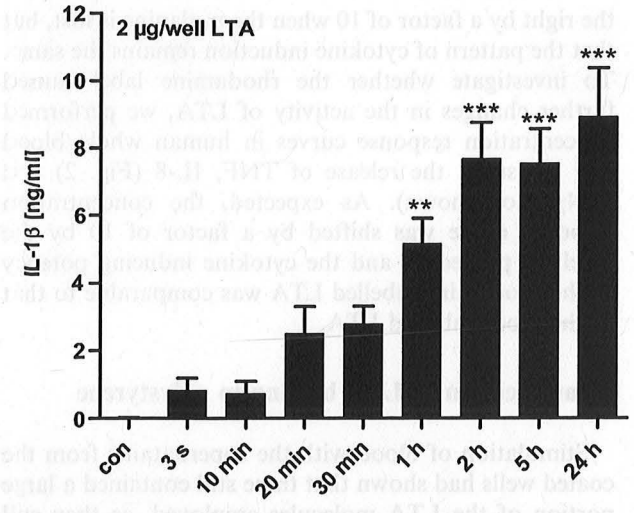

Fig. 3. IL-1β induction after LTA binding to polystyrene for the times indicated. The wells of a polystyrene cell-culture plate were coated with 2 µg/well LTA for different durations between 3 s and 24 h and then rinsed. Twenty percent whole blood was added and incubated overnight. IL-1β release was measured by ELISA. Statistics were calculated using one-way ANOVA and Dunn's multiple comparison test, $^{**}p<0.01$, $^{***}p<0.001$ versus control, $n=7$, pooled data from two independent experiments.

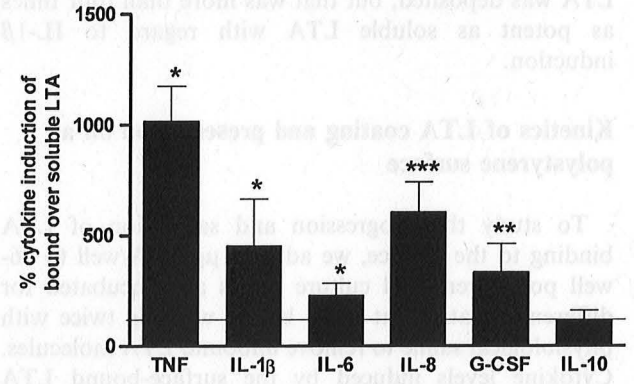

Fig. 4. Binding of LTA to polystyrene modifies cytokine release in a proinflammatory manner. Ratio of cytokine response of whole blood to 2 µg/well LTA coated to polystyrene over soluble LTA (set to 100%). LTA was either coated overnight at 37 °C before subsequent rinsing to remove further unbound stimulus or employed directly. Release of TNF, IL-1β, IL-6, G-CSF, IL-10 and IL-8 by human whole blood diluted 1:5 in RPMI was measured by ELISA. Statistics were calculated with raw data using paired *t*-test, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $n=11$, pooled data from three independent experiments.

PCR (LTA-induced TNF mRNA at 6 h: soluble LTA 3.6-fold $\pm$ 0.6 over control; bound LTA 8-fold $\pm$ 0.9 over control, all normalized to the housekeeping gene cyclophilin, $n=3$, $p<0.05$).

Role of the surface material in the presentation of LTA

Next to polystyrene, polypropylene is also a material commonly used in the laboratory for incubation experiments. Both materials are uncharged polymers, however polystyrene surfaces for cell culture experiments are commonly treated by physical methods to include polar groups, such as carboxyl and hydroxyl groups, which makes them more hydrophilic. This increases the binding of proteins and cells to the plastic surface. Polypropylene vessels are not treated in this way.

The immunostimulatory potency of bound versus soluble LTA and LPS was compared in polypropylene reaction vessels. Bound LTA induced more IL-1β release in polypropylene cups than soluble LTA, but the difference was not as large as seen in polystyrene wells (Fig. 5). IL-1β release of bound and soluble LPS was again comparable. The respective supernatants also induced IL-1β levels comparable to the soluble stimuli. This denotes that again only few molecules bound to the polypropylene surface. Fluorescence measurements with rhodamine-labelled LTA showed that about 4% of the 2 µg/well LTA employed had bound to the polypropylene surface after 2 h incubation ($n=4$).

As an example of a surface material with low binding capacity, we also performed incubations in silicone borosilicate tubes. Hardly any IL-1β or IL-8 was secreted by blood upon stimulation with soluble LTA in comparison to polypropylene tubes (Fig. 6), while LPS induced similar IL-8 and IL-1β levels in silicone borosilicate and polypropylene tubes. These data indicated that in fact LTA is only recognized by immune cells when it is presented on a surface.

To rule out other possible effects of the silicone-coated glass, we coupled LTA to polymer beads and used these to induce cytokine secretion in the glass vials. The efficiency of coupling LTA to the beads could not be determined by fluorescence measurements as the beads are opaque. Based on the relations of beads and LTA employed in the labelling reaction, the 200 µg/ml beads employed in the experiment could have been loaded with a maximum of 1 µg LTA. However, as the supernatant was still highly immunostimulatory after the reaction, it is likely that the labelling efficiency was much lower. These LTA-linked beads, like live and inactivated *S. aureus* bacteria, were able to stimulate cytokine secretion in silicone borosilicate tubes, showing that the silicone borosilicate surface itself did not interfere with cytokine induction by LTA (Fig. 7). This cytokine induction was comparable to that in polypropylene tubes. These data confirmed that presentation of LTA is crucial for its recognition by immune cells. Uncoupled but quenched beads induced no significant cytokine release (data not shown).

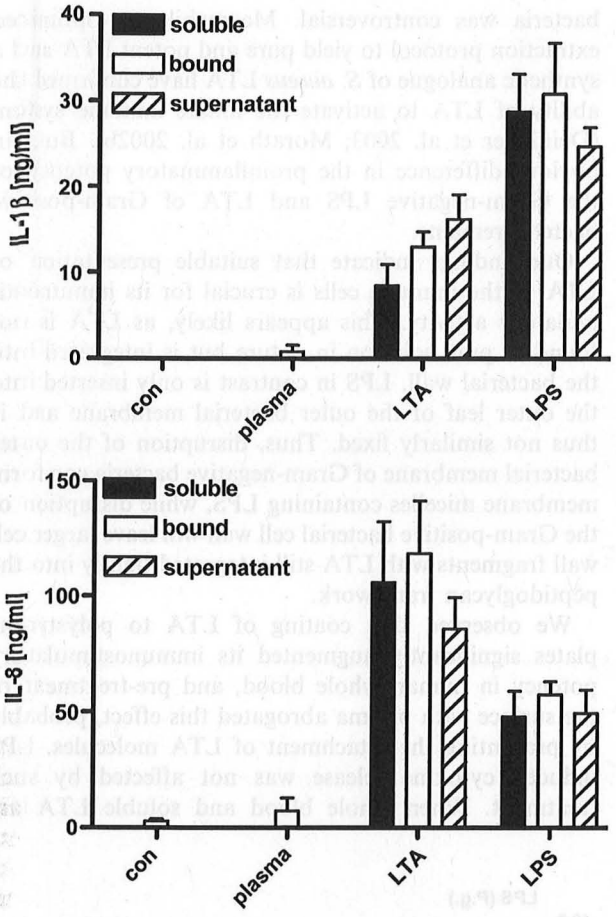

Fig. 5. Binding of LTA to polypropylene has less effect on inducible cytokine release. 2 µg/well LTA and LPS were either coated for 2 h at 37 °C before transfer of the supernatant to a new well (supernatant) and subsequent rinsing to remove unbound stimulus (bound), or employed directly (soluble). IL-1β and IL-8 release by human whole blood diluted 1:5 in RPMI was measured by ELISA after overnight incubation. Significance was determined by one-way ANOVA and Dunn's multiple comparison test, $^{*}p<0.05$ versus soluble, $n = 8$–16, pooled data from four independent experiments.

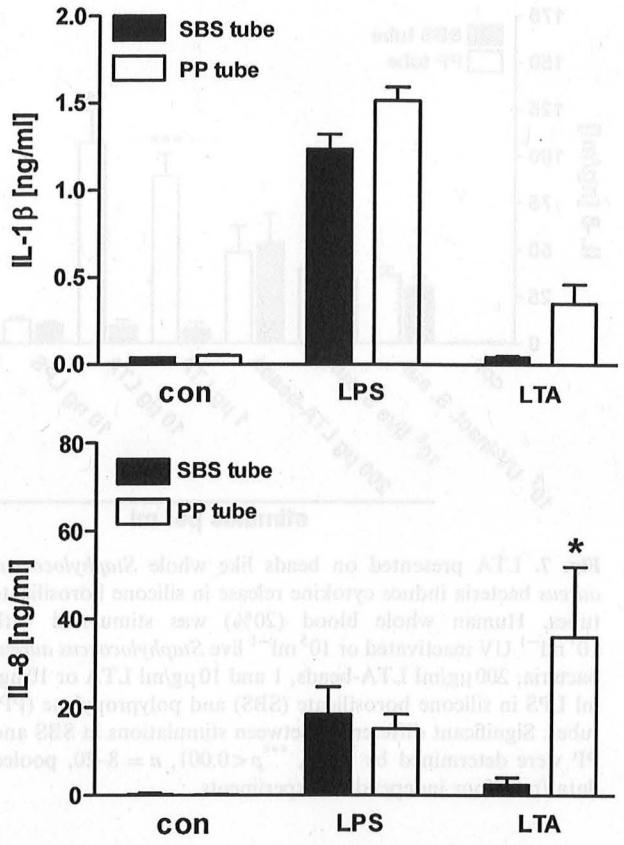

Fig. 6. Whole blood incubation in silicone borosilicate tubes leads to a loss of cytokine induction by LTA. IL-1β and IL-8 release of 20% human whole blood in response to stimulation with 0.2 µg/well LTA or LPS in silicone borosilicate (SBS) or polypropylene (PP) tubes. Significant difference between SBS and PP was determined by t-test, $^{*}p<0.05$.

Role of presentation for the recognition of other TLR2 agonists

Next we asked whether the immunostimulatory potency of other TLR2 agonists also depends on presentation. Peptidoglycan (PGN) of *S. aureus*, the synthetic lipopeptide Pam_3Cys and LPS of *P. gingivalis* are described to be TLR2 agonists like LTA. We preincubated silicone borosilicate, polypropylene and polystyrene with these stimuli. After incubation overnight at 37 °C, wells were washed twice with physiological saline to remove unbound molecules. The potency of the attached stimuli was compared with that of the respective soluble stimulus by measurement of cytokine release in human whole blood (Fig. 8). In contrast to LTA, Pam_3Cys induced less IL-1β after coating on polypropylene and polystyrene in comparison to the respective soluble stimulus. But, interestingly, Pam_3Cys preincubated with silicone borosilicate was a significantly more potent inducer of IL-1β than Pam_3Cys in solution. Coating of LPS (*P.g.*) or PGN to the three different materials resulted in a lower IL-1β induction than stimulation with soluble stimuli, indicating that no presentation effect similar to that of LTA occurred. Thus, only Pam_3Cys showed amplified cytokine induction by binding in one setting. Although it was not possible to follow whether or how much of these stimuli actually bound to the different surfaces, the comparison suggests that surface presentation likely does not play as large a role for these stimuli as it does for LTA.

Discussion

For a long time, the role of lipoteichoic acid (LTA) as the immunostimulatory molecule of Gram-positive

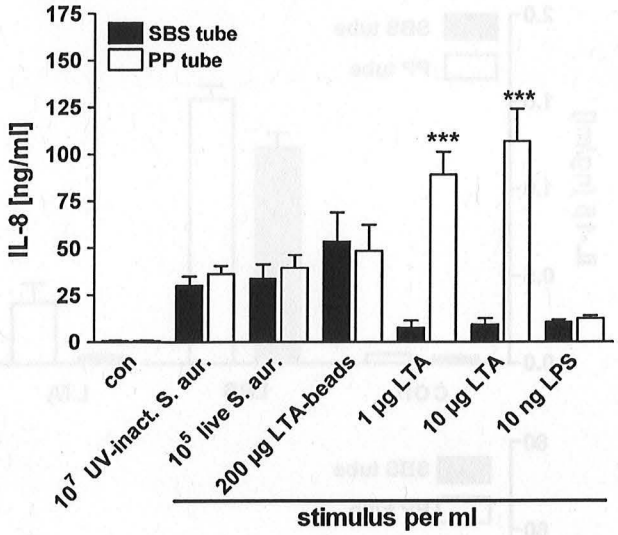

Fig. 7. LTA presented on beads like whole *Staphylococcus aureus* bacteria induce cytokine release in silicone borosilicate tubes. Human whole blood (20%) was stimulated with $10^7\,ml^{-1}$ UV inactivated or $10^5\,ml^{-1}$ live *Staphylococcus aureus* bacteria, 200 µg/ml LTA-beads, 1 and 10 µg/ml LTA or 10 ng/ml LPS in silicone borosilicate (SBS) and polypropylene (PP) tubes. Significant differences between stimulations in SBS and PP were determined by *t*-test, $^{***}p<0.001$, $n = 8$–20, pooled data from four independent experiments.

bacteria was controversial. Meanwhile an optimized extraction protocol to yield pure and potent LTA and a synthetic analogue of *S. aureus* LTA have confirmed the ability of LTA to activate the innate immune system (Deininger et al. 2003; Morath et al. 2002b). But, an obvious difference in the proinflammatory potency of the Gram-negative LPS and LTA of Gram-positive bacteria remains.

Our findings indicate that suitable presentation of LTA to the immune cells is crucial for its immunostimulatory activity. This appears likely, as LTA is not found in pure solution in nature but is integrated into the bacterial wall. LPS in contrast is only inserted into the outer leaf of the outer bacterial membrane and is thus not similarly fixed. Thus, disruption of the outer bacterial membrane of Gram-negative bacteria can form membrane micelles containing LPS, while disruption of the Gram-positive bacterial cell wall will leave larger cell wall fragments with LTA still integrated tightly into the peptidoglycan framework.

We observed that coating of LTA to polystyrene plates significantly augmented its immunostimulatory potency in human whole blood, and pre-treatment of the surface with plasma abrogated this effect, probably by preventing the attachment of LTA molecules. LPS induced cytokine release was not affected by such treatment. When whole blood and soluble LTA are

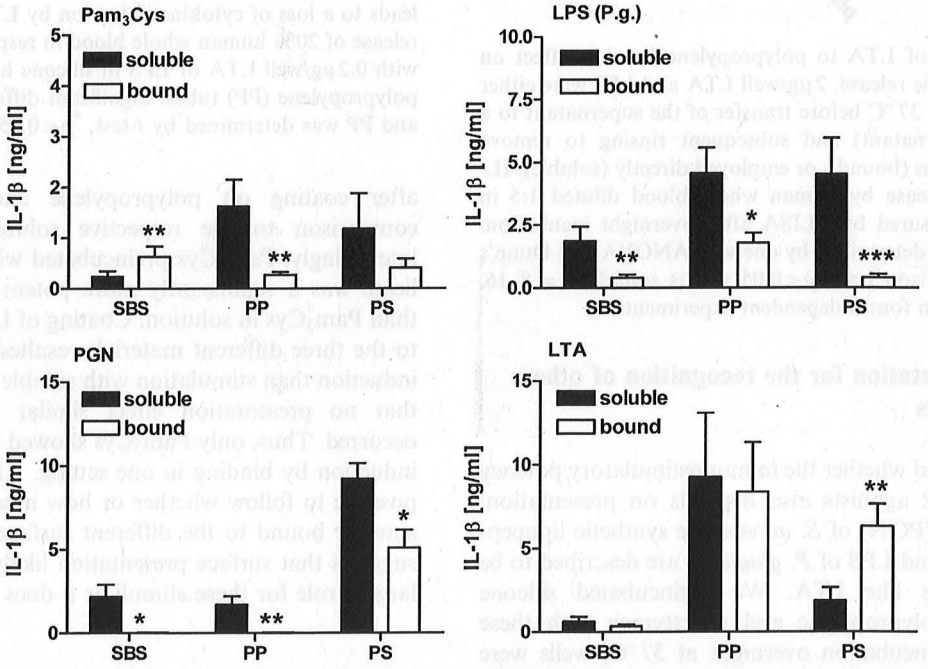

Fig. 8. Comparison of cytokine induction by various soluble and bound TLR2 agonists on different surface materials. Stimulation of human whole blood (20%) in silicone borosilicate (SBS), polypropylene (PP) and polystyrene (PS) vessels with 10 µg/ml Pam_3Cys, LPS (P.g.), PGN or LTA, either coated overnight and washed (bound) or employed in soluble form (soluble). IL-1β secretion was determined by ELISA. Significant differences between bound and soluble form was determined by *t*-test, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus soluble, $n = 12$–16, pooled data from four independent experiments.

pipetted into the vessel at the same time, the LTA molecules probably compete with the plasma components for binding to the surface. Moreover, LTA molecules may bind to plasma components (Levels et al. 2003), possibly masking them from immune cells.

Cytokine induction in human blood demonstrated that the LTA coating process occurs already after a few seconds and that saturation is obtained after about 2 h. Fluorescence measurements with rhodamine-labelled LTA revealed that only about 1% of the LTA had bound to the polystyrene surface. This was consistent with the high residual cytokine inducing potency of the supernatant of this pre-treatment step (Fig. 1). Combined with a highly increased cytokine release, this results in an impressive increase in potency.

The release of all the proinflammatory cytokines IL-1β, TNF and IL-6 and the chemokines IL-8 and G-CSF was greater in case of stimulation with bound LTA compared to soluble LTA. This was not the case for the induction of anti-inflammatory IL-10. These data agree with our result that whole *S. aureus* bacteria also only induce low levels of IL-10 in human whole blood (data not shown). The lack of modulation of IL-10 induction upon presentation may be linked with findings showing that the induction of IL-10 is distinct from that of proinflammatory cytokines and depends on the transcription factors Sp1 and Sp3 without a contribution from Rel, C/EBP (CCAAT/enhancer-binding protein), or AP-1 (Brightbill et al. 2000; Tone et al. 2000). Whether the lack of increased activation of this pathway upon LTA presentation is due to a limited activation capacity of this pathway, e.g. by limited availability of components, or to the involvement of co-receptors that are not affected in their activation capacity by the presentation of LTA remains to be elucidated.

Since the attachment of the LTA molecules to a surface appeared to be important for its potency, we also examined the materials polypropylene and silicone borosilicate, two other materials commonly used for incubation experiments. The binding properties of LTA should be altered depending on the surface properties. Upon incubation in polypropylene vessels, LTA preincubation and prior plasma-preincubation had no significant effect on LTA or LPS induced cytokine release. In contrast, the immunostimulatory potency of LTA was almost completely lost in silicone borosilicate vessels, whereas the potency of LPS was not affected. Information given by the material manufacturers indicates that the polystyrene materials are the most hydrophilic of the three materials tested. Silicone-coated borosilicate is considered to be a surface that is resistant to binding of substances and is therefore specifically used when this should be avoided, e.g. for the determination of the concentration of LPS in a solution by LAL assay. The silicone borosilicate vessels did not inhibit cytokine production *per se* because the cytokine response to LPS stimulation was normal. Therefore, it seems that surface presentation of LTA is essential for recognition by the immune cells. As the cytokine induction levels of LTA bound to polypropylene and to polystyrene were comparable and were as high as the levels induced by soluble LTA in polypropylene vessels (see Fig. 8, panel D), the interaction with the surface may occur in a similar way in both materials. However, we are currently unable to determine how the bound LTA is oriented on these surfaces.

We covalently coupled LTA to the surface of small polystyrene beads and used these as well as UV inactivated and live *S. aureus* bacteria to stimulate blood in silicone borosilicate vessels. In line with our hypothesis, both the bacteria and the LTA-beads induced cytokine release in silicone borosilicate. Thus, both LTA adhering to a surface and LTA covalently coupled to a surface are able to induce cytokine release.

Surface presentation of LTA may modulate its cytokine-inducing activity in the same way as crosslinking of LTA by anti-LTA antibodies does (Mancuso et al. 1994) or modelling crosslinked LTA in the form of a synthetic bisamphiphilic LTA, which displayed a greater cytokine induction potency than the monoamphiphilic analogue (Stadelmaier et al. 2006). It has been suggested that in the latter two cases the crosslinked LTA modulates the clustering of receptors. Whether this is in fact the case for surface presented LTA remains to be investigated.

Lipid raft formation of LTA and its receptor molecules is necessary for activation and signalling, similar to LPS (Pfeiffer et al. 2001; Triantafilou et al. 2004). Indeed, for LPS it was found that soluble LPS is a more potent stimulus for TNF production than particulate, bacterium-bound LPS or LPS coated onto latex-beads (Leeson and Morrison 1994). There must thus be a difference in the ability of LTA and LPS to evoke receptor clustering.

Finally, we asked whether the immunostimulatory potency of other TLR2 agonists is also determined by presentation. The TLR2 agonists Pam_3Cys, LPS from *P. gingivalis* and PGN from *S. aureus* were incubated in silicone borosilicate, polystyrene or polypropylene vessels in bound or soluble form. Coating of these stimuli to polystyrene did not amplify cytokine release as seen for LTA, and also did not occur in the other reaction vessels. This means that the effect of increasing the potency by binding and presentation appears to be limited to LTA. As sole exception, Pam_3Cys induced higher amounts of IL-1β when bound to silicone borosilicate than the soluble stimulus. This difference in the presentation efficacy of the different surfaces for LTA and Pam_3Cys likely depends on differences in their chemical structure. However, since no fluorescence-labelled stimuli were available, actual binding rates could not be determined.

In bacteria, LTA is immobilized in the framework of the murein sacculus and protrudes through the PGN. Generally, PGN is purified by sonification of the whole bacteria, extensive boiling in SDS and enzymatic digestion of proteins and DNA. This procedure does not necessarily eliminate LTA. Travassos et al. (2004) reported that highly purified PGN, freed from lipoproteins and LTA by hydrofluoric acid treatment no longer activates TLR2 in transfected cells. They concluded that the TLR2-dependent immunostimulatory activity of PGN is due to LTA or lipoprotein remnants. Our results support this conclusion by showing that small amounts of LTA presented in the murein framework can potently induce cytokine release.

Taken together, the presentation of LTA determines its immunostimulatory activity and is affected by the incubation vessel used in an experiment. This strong effect has a bearing on the safety testing of medical devices. Until now, mostly only rinsing solutions of medical devices have been tested to exclude pyrogenic contaminations and the focus has been on detecting LPS contaminations using the LAL assay. Because the potency of a contamination may be critically dependent on its form of presentation on the device, new test settings should be established which include testing the overall inflammatory activity of the material or the product directly (Mazzotti et al. 2007).

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