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Lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus for neutrophil recruitment

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Abstract

Lipoteichoic acid (LTA) is a major immunostimulatory principle of Gram-positive bacteria. Intranasal application of LTA from *S. aureus* to mice resulted in greatly increased neutrophil and macrophage counts in the bronchoalveolar lavage as well as increased levels of the chemokine KC. The potential of highly pure, bioactive LTA from *S. aureus* to induce neutrophil recruitment and activation was investigated further in the human system.

Although neutrophils expressed the key known receptors, CD14, TLR2 and TLR6, LTA did not induce or prime neutrophils for oxidative burst, or release of chemokines, bactericidal permeability-increasing protein or myeloperoxidase. However, LTA induced a strong release of the chemoattractants LTB_4, IL-8, C5a, MCP-1 and the colony-stimulating factor G-CSF in whole blood comparable to stimulation with the same concentration of LPS (*S. abortus equi*). Further, the cytokine and chemoattractant pattern induced by LTA correlated well with that induced by live *S. aureus* of the same strain.

LTA does not appear to activate neutrophils directly, but is a strong stimulus for the recruitment of phagocytes to the site of infection.

Abbreviations: BALF = bronchoalveolar lavage fluid; BPI = bacterial permeability-increasing protein; C5a = complement factor 5a; fMLP = N-formyl-L-methionyl-L-leucyl-L-phenylalanine; G-CSF = granulocyte colony-stimulating factor; LTA = lipoteichoic acid; LT = leukotriene; MCP-1 = macrophage chemoattractant protein-1; MPO = myeloperoxidase; PMA = phorbol myristate acid; TLR = toll-like receptor; TNF = tumor necrosis factor.

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Introduction

Lipoteichoic acid (LTA) is anchored in the cytoplasmic membrane of Gram-positive bacteria. There have been reports on the immunostimulatory activity of LTA from many different bacterial species. However, it was found that commercially available LTA was often contaminated with lipopolysaccharide (LPS) or other molecules with immunostimulatory capacity (Gao et al., 2001; Kusunoki et al., 1995; Morath et al., 2002a), thereby raising doubt with regard to previous findings and making repurification steps necessary. Surprisingly, in contrast to LTA from other species, repurified LTA from *Staphylococcus aureus* was biologically inactive in the systems tested (Hermann et al., 2002; Morath et al., 2002a). Recently, we succeeded in preparing highly pure and biologically active LTA from *S. aureus* by a more gentle preparation procedure using butanol instead of phenol extraction (Morath et al., 2001). Structural comparison of this LTA with commercially available *S. aureus* LTA showed that alanine substituents are lost by traditional purification procedures and that this was the reason for the loss of bioactivity (Morath et al., 2002a). This was not so for commercial preparations of LTA from *B. subtilis* or *S. pyogenes*: although these preparations were also contaminated with endotoxin, they retained immunostimulatory activity on repurification (Morath et al., 2002a). LTA from *S. aureus* was synthesized chemically on the basis of the proposed structure and found to have comparable biological activity to purified natural LTA (Morath et al., 2002b). Further studies on the functionality of the structural components in immunostimulation were performed using modifications of the synthetic LTA (Deininger et al., 2003).

S. aureus represents a predominant cause of pus-associated skin infections, e.g. abscesses, impetigo, furuncles, carbuncles, wound infections and Gram-positive sepsis. Consequently, we are now investigating the spectrum of the immunostimulatory potential of LTA from *S. aureus*, especially focusing on whether it plays a role in the formation of pus during infection. Pus is mainly made up of serum, necrotic tissue and dead neutrophilic granulocytes. In this study, we investigated, whether LTA activates or primes neutrophils directly, whether it plays a role in their attraction to the site of infection and whether it affects their turnover.

Materials and Methods

Intranasal application of LTA

Pathogen-free 10-week-old female C57BL/6 mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Mice were inoculated intranasally with 100 μg LTA ($n = 8$) or sterile saline ($n = 6$) in a total volume of 50 μl. After 6 hours, mice were anesthetized with Hypnorm^R (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, The Netherlands) and bronchoalveolar lavage was performed.

The trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalveolar lavage was performed by instilling two 0.5 ml aliquots of sterile saline. Approximately 1 ml of lavage fluid (BALF) was retrieved per mouse. Total cell numbers were counted from each sample using a hemocytometer (Türck chamber), BALF differential cell counts were done on cytospin preparations stained with Giemsa and KC concentration in the lavage fluid was determined by ELISA (R&D, Wiesbaden, Germany).

Stimuli

Lipoteichoic acid (LTA) from *Staphylococcus aureus* was prepared in-house according to the published method (Morath et al., 2001). Endotoxin contamination was excluded by negative Limulus amoebocyte lysate assay (less than 25 pg LPS per mg LTA, QCL-1000, Charles River Endosafe, Charleston, WV, USA). The batch of lipopolysaccharide (LPS) from *Salmonella abortus equi* (Sigma, Deisenhofen, Germany) used had no TLR2 agonistic activity as determined by stimulation of bone marrow cells from TLR2 knock-out mice in comparison to controls. N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), phorbol myristate acid (PMA) and C5a were from Sigma. *S. aureus* (DSM 20233) and *E. coli* K12 (JM 109, a kind gift from Dr. Gerald Gruetz, University Clinic Charité, Berlin, Germany) were grown in LB medium and taken from the log phase.

Differential blood counts

Differential blood counts were routinely performed on the blood of all volunteers with a Pentra 60 (ABX

Diagnostics, Montpellier, France) to rule out acute infections.

Whole blood incubations

Stimuli were added to 800 µl RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 100 IU penicillin, 100 µg/ml streptomycin and 2.5 IU/ml heparin (Liquemin®, Hoffmann LaRoche, Grenzach-Whylen, Germany), and 200 µl heparinized whole blood in 1.5 ml polypropylene tubes (Eppendorf, Hamburg, Germany). After incubation at 37 °C and 5% CO_2 for the times indicated, vials were shaken up, cells were sedimented by centrifugation, and supernatants were frozen in aliquots at − 80 °C until mediator measurement.

Mediator measurements

ELISA were based on antibody pairs against G-CSF, MCP-1 (R&D, Wiesbaden, Germany), TNF-α, IL-8 and IFNγ (Endogen, Biozol, Eching, Germany). C5a was measured with an ELISA kit from DRG (IBL, Hamburg, Germany), the BPI ELISA was from HyCult biotechnology (Trinova Biochem, Giessen, Germany) and the LTB_4 EIA was from Cayman (SPI Europe, Gif sur Yvette Cedex, France). All assays were performed according to the manufacturers' instructions. Myeloperoxidase (MPO) was measured by diluting supernatant samples or standard in 50 nM potassium phosphate (pH 6.0) with 5 mg/ml hexadecyltrimethylammonium bromide in a volume of 50 µl and adding 100 µl/well TMB liquid substrate solution (all Sigma). The reaction was stopped with 1 M sulfuric acid and measured at 450 nm against 620 nm.

Toll-like receptor staining

Whole blood was stained directly or after incubation as described above. Cells were resuspended and stained with anti-CD45-PerCP, anti-CD14-FITC (BD Biosciences, Heidelberg, Germany) and anti-TLR2-PE, anti-TLR4-PE or isotype control (eBioscience, San Diego, CA, USA) at room temperature. Erythrocytes were lysed using FACS lysing solution and fluorescence was measured after washing in a FACS Calibur (BD Biosciences). Cells were differentiated according to their expression of CD45 and CD14: all leukocytes express CD45, monocytes express high levels of CD14, neutrophils express low levels of CD14 and lymphocytes express no detectable levels of CD14.

Toll-like receptor 6 mRNA expression

Peripheral blood mononuclear cells (PBMC) of healthy volunteers were prepared with CPT™Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, USA). Monocytes were isolated from these with CD14 MicroBeads (Miltenyi Biotec) to a purity of >95% according to the manufacturer's instructions. Highly pure neutrophils were prepared as described below. RNA was prepared with the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including DNA digestion with the RNase-free DNase Set (Qiagen). 10.2 µl RNA was reverse transcribed in a sample volume of 20 µl containing 2.5 µM oligo-dT16 (Gibco BRL, custom primer), $MgCl_2$ (5 mM), dNTP (1 mM each), RNAse inhibitor (1 U/µl), murine leukemia virus reverse transcriptase (2.5 U/µl) in PCR buffer (all PE Applied Biosystems, Germany). Samples were incubated at 21 °C for 10 minutes, 42° C for 15 minutes, 94 °C for 5 minutes and 5 °C for 5 minutes in a GeneAmp PCR System 2400 (PE Applied Biosystems). 2 µl cDNA was added to PCR master mix, containing 4 mM $MgCl_2$, 400 nM sense, respectively antisense primers for toll-like receptor-6 adapted from Hornung et al. (2002) and Faststart LightCycler DNA Master SYBR Green1 (Roche Diagnostics, Germany). PCR was performed in a LightCycler™ Instrument using thermal settings as follows: denaturation and activation of the polymerase at 95 °C for 10 minutes and 50 cycles of 95 °C for 10 s, 68 °C for 10 s touching down to 58 °C in 0.5 °C step sizes, 72 °C for 16 s. Melting curve analysis and agarose gels were performed after PCR to verify the specificity of the reaction and the size of the PCR product.

Intracellular cytokine staining

Whole blood incubations were performed as above in the presence of 5 µg/ml Brefeldin A (Sigma). After incubation, cells were resuspended and stained with anti-CD45-PerCP and anti-CD14-FITC at room temperature for 30 minutes. After washing once, cells were incubated with 1 ml Cell Fix/Perm for 20 minutes at 4 °C. After two washes with Cell Perm/Wash, cells were stained with anti-TNF-α-PE or anti-IL-8-PE for 30 minutes and 20 000 cells were measured after two more washes in a FACS Calibur (all BD Biosciences). Cell populations were gated as described above.

Standard neutrophil isolation

Heparinized blood was diluted 1:1 with PBS (pH 7.4) and loaded onto a Ficoll (Pharmacia, Uppsala, Sweden) and Histopaque (Sigma) gradient. After centrifugation for 20 minutes at 320 g at room temperature, the neutrophil fraction was collected and washed with RPMI with 0.05% human serum albumin (CLB, Amsterdam, The Netherlands). The remaining erythrocytes were lysed for 45 s with distilled water, after which concentrated PBS was added to re-establish isotonic conditions. After washing, cells were counted and resuspended in RPMI.

Chemiluminescence experiments

1×10^5 neutrophils in 100 µl HBSS with 1% pooled human serum were primed for 30 minutes at 37 °C with LPS from *Salmonella minnesota* strain R595 (ReLPS, Sigma) or LTA, then placed in a luminometer (Autolumat LB 953, Berthold, Wildbad, Germany). Cells were stimulated by automatic injection of 100 µl fMLP (final concentration 1 µM) in the presence of 600 µl 150 µM luminol-balanced salt solution (LBSS, ExOxEmis, San Antonio, TX, USA), resulting in a total volume of 800 µl for each sample. The chemiluminescence response was measured over 10 minutes and data were analyzed with the AXIS software package (ExOxEmis).

Preparation of highly pure neutrophils

Heparinized blood was spun at 270 g for 20 minutes at room temperature. The buffy coat was mixed with 0.72% dextran T500 (Sigma) in 50 ml PBS and left to stand for 30 minutes for the erythrocytes to sediment. The leukocytes in the supernatant were spun for 7 minutes at 850 g, washed once with HEPES buffer and the cells were transferred to a discontinuous Percoll (BD Pharmacia)/HEPES buffer gradient (density 1.093, 1.088, 1.072, 1.059) and spun for 15 minutes at 450 *g*. The neutrophil band was transferred to a new tube. Contaminating monocytes were removed by positive selection with CD14 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol under sterile conditions. CD14 expression of neutrophils is too low to select them positively under these conditions. Purity was determined flow cytometrically after staining with anti-CD45-PerCP and anti-CD14-PE. 20 000 cells were counted, the remaining contamination with CD14 positive monocytes was less than 0.2% for all blood donors. 1×10^6 neutrophils in 200 µl medium with 10% autologous serum were stimulated in polypropylene cups. Whole blood incubations with blood from the same donors were run in parallel.

Statistics

T-test or repeated measure analysis of variance followed by Dunnett's multiple comparison test were performed using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant. Unless otherwise indicated, data are means ± SEM of the number of blood donors indicated, calculated per ml blood, i.e. corrected for the dilution factor of 5 in the whole blood incubation, or per cell count.

Results

LTA induces strong leukocyte infiltration in vivo

10-week-old female C57BL/6 mice were given 100 µg LTA or saline intranasally. The bronchoalveolar lavage after 6 hours showed a strong infiltration of cells in response to LTA ($73 \pm 15 \times 10^3$ cells compared to $6 \pm 2 \times 10^3$ in controls, $p < 0.01$). The infiltrating cells were mainly neutrophils but some alveolar macrophages were also recorded (Fig. 1). The levels of the chemokine KC in the lavage samples were 793 ± 89 pg/ml in lavage from LTA-treated vs. 69 ± 12 pg/ml in lavage from saline-treated animals, $p < 0.001$. This experiment indicated that LTA is a potent stimulus for neutrophil recruitment and accumulation *in vivo* and stimulated the investigation of how LTA exerts this action.

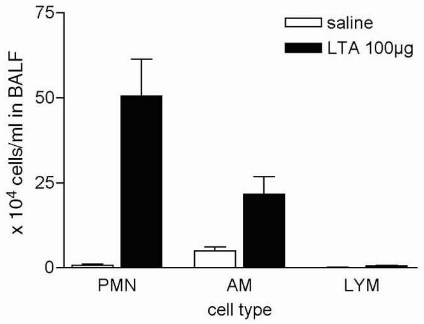

Fig. 1. Intranasal application of LTA into the mouse lung causes infiltration of neutrophils and macrophages. C57BL/6 mice were inoculated intranasally with 100 µg LTA or saline. Differential blood cell counts were done on cytospins of bronchoalveolar lavage fluid 6 hours after application. Data are means ± SEM of 8 mice treated with LTA and 6 controls.

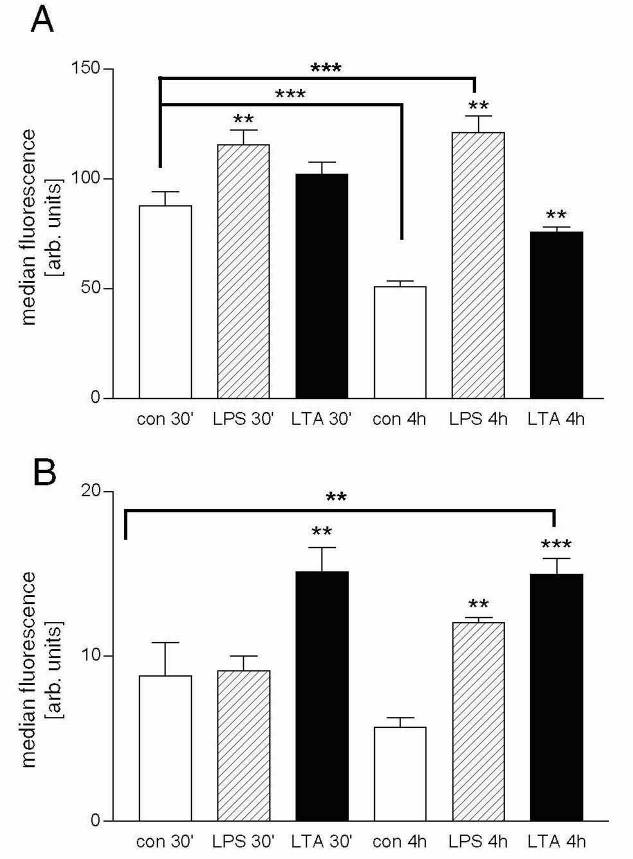

Fig. 2. Modulation of monocyte TLR2 (A) and TLR4 (B) expression by LTA and LPS. Whole blood was incubated with 10 µg/ml LTA (*S. aureus*) or LPS (*S. abortus equi*) for 30 minutes or 4 hours. Then, resuspended cells were stained with anti-CD45 and anti-CD14 antibodies to gate monocytes and with anti-TLR2 (A), anti-TLR4 (B) or isotype antibodies. Data show mean fluorescence minus isotype control of monocytes from 4 donors ± SEM from one representative experiment of three. **, $p < 0.01$; ***, $p < 0.001$ against respective control or as shown.

Potential of LTA to activate neutrophils

We have shown previously that, apart from CD14, highly purified LTA requires the toll-like receptor 2 (TLR2) (Lehner et al., 2001) for signaling, in contrast to LPS which requires TLR4. Flow cytometric analysis of whole blood stained for these TLRs demonstrated that both monocytes and neutrophils express TLR2 and TLR4 receptors, and lymphocytes express neither (data not shown). Both receptors are expressed at higher levels on monocytes than on neutrophils. As no suitable antibodies are commercially available to determine TLR6 protein expression in leukocytes, expression of TLR6, which appears to cooperate functionally with TLR2 (Bulut et al., 2001), was examined at the level of RNA. Both monocytes and neutrophils from 4 donors each expressed TLR6 as was verified by the size of the PCR product on an agarose gel (data not shown).

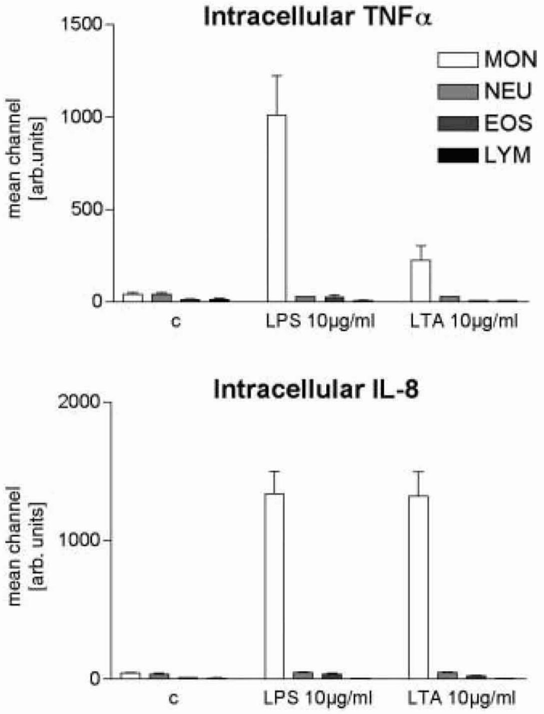

Fig. 3. Monocytes but not neutrophils produce TNF-α and IL-8 in response to LTA or LPS stimulation. Whole blood was incubated with 10 µg/ml LTA (*S. aureus*) or LPS (*S. abortus equi*) in the presence of 5 µg/ml Brefeldin A for 5 hours. Then, resuspended cells were stained with anti-CD45 and anti-CD14 antibodies, permeabilized and stained for intracellular TNF-α or IL-8. Data show average median fluorescence of blood from 4 donors ± SEM from one representative experiment of three.

Incubation of blood with 10 µg/ml LPS or LTA caused modulation of TLR expression on monocytes (Fig. 2). Stimulation with the TLR4 agonist LPS induced up-regulation of monocytic TLR2 over the control value at 30 minutes, while stimulation with the TLR2 agonist LTA induced up-regulation of TLR4 over the 30 minute control value. In the unstimulated samples, TLR expression decreased between 30 minutes and 4 hours, however, LPS-induced TLR2 expression and LTA-induced TLR4 expression remained significantly increased over the respective control values at both time points. Four hours after stimulation with LTA, TLR2 expression was not significantly different from control expression after 30 minutes, although it was significantly elevated over control expression after 4 hours. Likewise, 4 hours after LPS stimulation, TLR4 expression was the same as control expression after 30 minutes, but significantly elevated over control expression after 4 hours. These results indicate that engagement either of TLR2 or TLR4 results in the maintenance of the expression of the respective receptor and in the up-regulation of the other

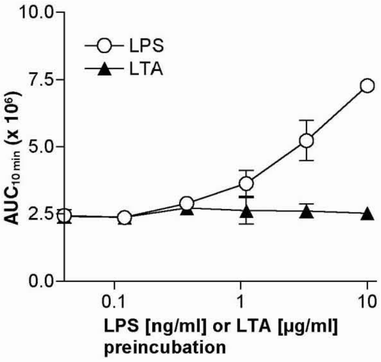

Fig. 4. LTA does not prime for fMLP-induced chemiluminescence. Isolated neutrophils (standard isolation procedure) were preincubated with LPS (100 pg/ml to 10 ng/ml) or LTA (100 ng/ml to 10 µg/ml) for 30 minutes before luminol-enhanced chemiluminescence was induced with 1 µM fMLP. Data are given as the mean area under the curve of duplicate values, showing minimum and maximum from one representative experiment of three.

receptor on monocytes in comparison to the expression on unstimulated cells under the same conditions. Expression of TLRs on neutrophils was not modulated significantly by either stimulus.

Lack of direct activation of neutrophils by LTA

Five hours (Fig. 3) as well as 24 hours (data not shown) after stimulation of whole blood with LPS or LTA in the presence of Brefeldin A, tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) could both be detected intracellularly in monocytes, but not in lymphocytes. LPS but not LTA induced a minor but significant ($p < 0.01$) induction of IL-8 in neutrophils after 24 hours, although intracellular TNF-α could not be detected in neutrophils in response to either stimulus, not even after 24 hours (data not shown). These data indicate that monocytes are the major producers of the cytokines TNF-α and IL-8 in whole blood in response to LPS or LTA, and that neutrophils are not activated directly by LTA to release these cytokines despite expression of TLR2, TLR6 and CD14, while they may contribute to IL-8 production in response to LPS.

To verify these results, highly purified neutrophils (monocyte contamination less than 0.2%) were stimulated with LPS, LTA or PMA parallel to a whole blood incubation with blood from the same donors. Neither stimulus induced the release of detectable amounts of TNF-α from neutrophils (< 5 pg/10^{6} neutrophils), though all three stimuli induced TNF-α release in whole blood (LPS: 7.8 ± 0.8 ng/ml blood; LTA: 0.86 ± 0.2 ng/ml blood and PMA: 2.0 ± 1.2 ng/ml blood after 24 hours). LTA again did not induce IL-8 release from the purified neutrophils although

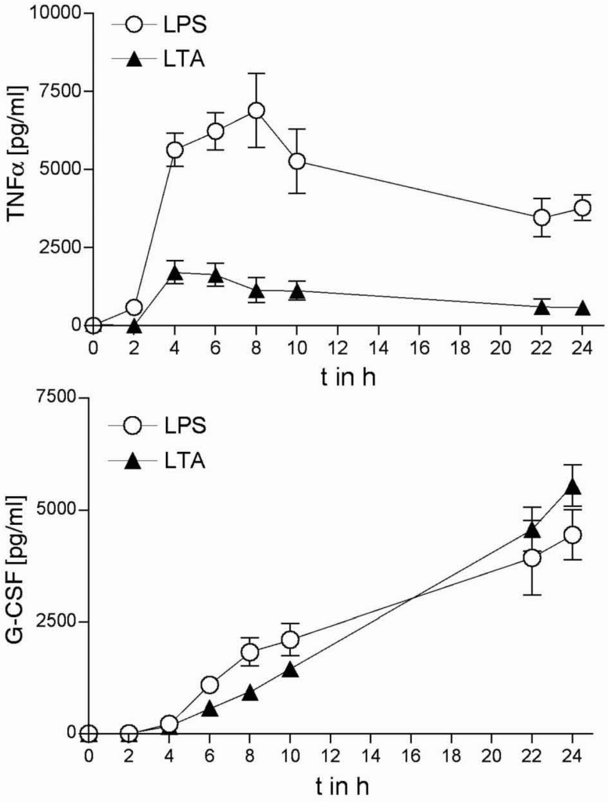

Fig. 5. LTA induces less TNF-α but the same G-CSF as LPS at equal concentrations. Time course study of 20% whole blood from 4 healthy donors stimulated with 10 µg/ml LTA *(S. aureus)* or LPS *(S. abortus equi)* over 24 hours. Cytokines were measured in the supernatants by ELISA. Data are means ± SEM from one representative experiment of three.

both LPS and PMA did so, verifying the flow cytometric data that neutrophils contribute to IL-8 production in response to LPS (control, < 0.34 ng; LPS, 12.8 ± 3.4 ng; LTA, 0.55 ± 32 ng; PMA, 45.3 ± 3.7 ng per 10^{6} neutrophils, $p < 0.001$ for LPS or PMA vs. control) and that they were still responsive.

PMA induced the release of myeloperoxidase (MPO) from the neutrophils (1.3 ± 0.2 U/ml vs. control 0.6 ± 0.2 U/ml, $p < 0.001$), however, LPS and LTA did not induce MPO release. Bactericidal permeability-increasing protein (BPI) release into the supernatant of a whole blood incubation was measured over a time period of 24 hours. LPS-induced degranulation (120 ± 19 ng/ml after 24 hours, $p < 0.05$) was significantly greater than spontaneous degranulation over this time period (70 ± 13 ng/ml after 24 hours), however, LTA did not induce significant degranulation of BPI.

Another key indicator of neutrophil activation is oxidative burst, i.e. formation of reactive oxygen species. LTA at concentrations of 100 ng/ml to 10 µg/ml was not able to directly induce luminol-enhanced chemiluminescence in whole blood in

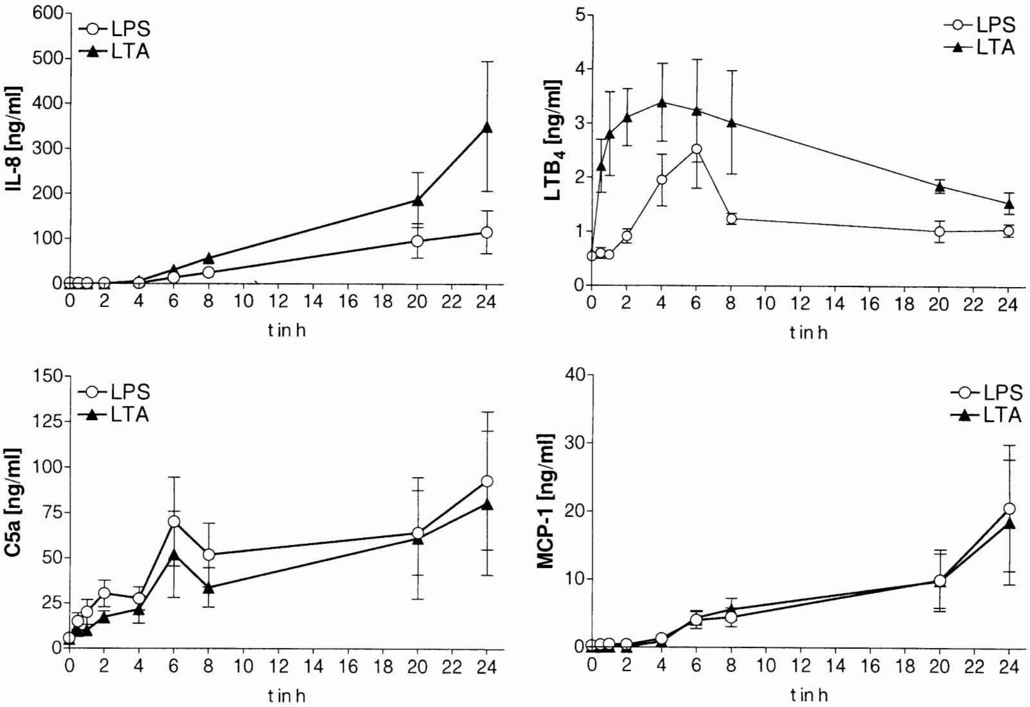

Fig. 6. LTA is a potent inducer of chemoattractant release. Time course study of 20% whole blood from 4 healthy donors stimulated with 10 μg/ml LTA or LPS over 24 hours. Mediators were measured in the supernatants by ELISA or EIA. Data are means ± SEM from one representative experiment of three.

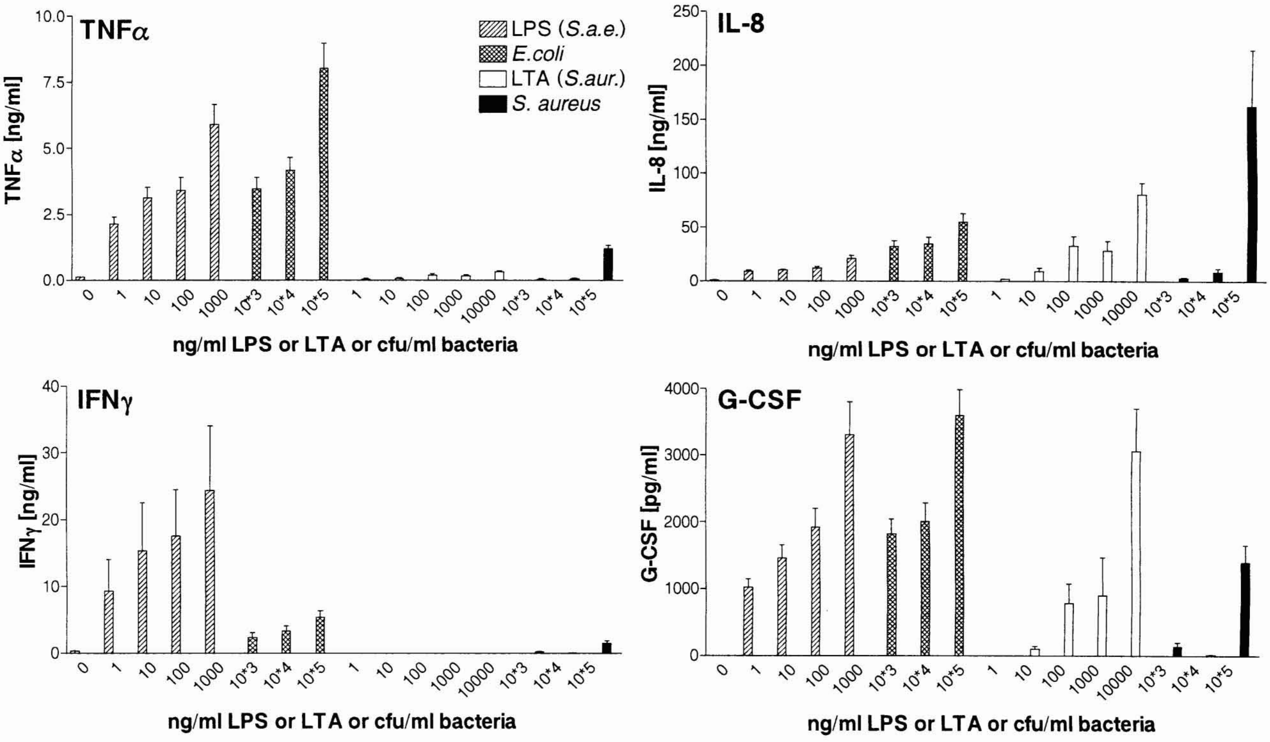

Fig. 7. Comparison of cytokine and chemokine induction by LTA and live *S. aureus*. 20% whole blood from 12 healthy donors was stimulated with the given concentrations of LPS or LTA or with the given cfu of UV-inactivated *E. coli* or live *S. aureus*. Mediators were measured in the supernatants by ELISA. Data are means ± SEM.

comparison to the positive control, 5 nM phorbol myristate acid (PMA), as shown by the area under the curve over 10 minutes (2.5×10^8 vs. control 1.8×10^7, $p < 0.001$). Pre-incubation of isolated neutrophils with LPS (0.1 to 10 ng/ml) for 30 minutes enhanced chemiluminescence induced with 1 µM fMLP, as shown in Fig. 4 as an increase in the area under the curve, however, pre-incubation with LTA (100 ng – 10 µg/ml) did not affect fMLP-induced chemiluminescence.

In summary, no direct effects of LTA on neutrophils could be recorded.

LTA is a potent inductor of chemoattractants and G-CSF

In a time course experiment, the release of TNF-α and G-CSF by human whole blood in response to equal concentrations (both 10 µg/ml) of LPS (*S. abortus equi*) and LTA was compared over 24 hours. Although the kinetics were similar for both cytokines, LTA induced much less TNF-α release than LPS but equal amounts of G-CSF release (Fig. 5).

As G-CSF has been attributed with chemoattractive properties apart from its central role in the production and release of neutrophilic granulocytes from the bone marrow, we also compared the induction of other chemoattractants IL-8, C5a, leukotriene B_4 (LTB_4) and macrophage chemotactic protein (MCP-1) over 24 hours after stimulation with LPS and LTA at the same concentrations (Fig. 6). LTB_4 was released fast in response to LTA stimulation and in amounts at least comparable with this strong stimulation with LPS. Similarly, C5a, IL-8 and MCP-1 were released with comparable kinetics and in comparable amounts in response to LTA and LPS.

We compared the pattern of cytokine induction of LTA and live *S. aureus* as well as of LPS and UV-inactivated *E. coli* (Fig. 7). The patterns corresponded very well between the Gram-negative and the Gram-positive stimuli for all cytokines measured.

Discussion

An explanation for the discrepancies in the existing literature on the immunostimulatory properties of LTA from *S. aureus* has only recently been put forward. Often, commercial preparations of LTA were used, which were contaminated with various other substances, especially lipoproteins and LPS (Gao et al., 2001; Kusunoki et al., 1995; Morath et al., 2002a). Although repurification steps were performed once these problems became known, these led to negative results for LTA from *S. aureus*, as the material had already been inactivated by dealanylation during the isolation procedure employing phenol (Morath et al., 2002a). Now that highly pure, biologically active LTA from *S. aureus* can be prepared (Morath et al., 2001), the immunostimulatory activity of this LTA must be re-evaluated. For many of the comparative experiments, equal amounts of LTA and LPS were chosen. This maximal stimulation with LPS shows the extent of the response capacity of the leukocytes.

The animal experiment indicated that LTA from *S. aureus* is able to recruit both neutrophils and monocytes/macrophages to the lung *in vivo*. We also found increased levels of the chemokine KC in the BALF, indicating how the inflammatory cells were recruited. We investigated further in human cells which leukocytes express receptors for LTA, whether LTA can activate neutrophils directly and which chemoattractants LTA can induce.

In addition to expression of CD14, neutrophils also express the TLR2 protein, though in lesser amounts than monocytes. This observation is in line with a recent publication (Sabroe et al., 2002). Expression of TLRs on monocytes was up-regulated over that of unstimulated cells 30 minutes after stimulation with an agonist of the opposite receptor, i.e. the TLR2 agonist LTA induced relative up-regulation of TLR4 and *vice versa*. However, the expression of TLR2 was not modulated by either stimulus on neutrophils, even after 4 hours, as was reported for incubation with G-CSF or GM-CSF (Kurt-Jones et al., 2002). Furthermore, neutrophils and monocytes were both shown to express TLR6 mRNA. These data demonstrate that neutrophils display the receptors found to be necessary to respond to LTA, i.e. TLR2 and CD14 (Lehner et al., 2001; Opitz et al., 2001), although they appear to display them in lesser numbers than monocytes, as well as the putative co-receptor TLR6. Also, the low TLR4 expression on neutrophils indicates that other receptors are involved in the response of these cells to LPS as already implied by other studies (Haziot et al., 2001).

Intracellular staining of stimulated whole blood indicated that only the monocytes produce TNF-α and IL-8 in response to LTA, although neutrophils were shown to contribute to LPS-induced IL-8 production. These data were verified using highly purified neutrophils and measuring cytokine release into the supernatant. There is an ongoing discussion whether neutrophils are able to release cytokines or not. Most published observations on this subject were made in isolated neutrophils purified by Percoll or Ficoll separation. Although a purity of 98% can be achieved by these methods, the few percent of monocytes left are sufficiently potent to release the low amounts of cytokines measured in such experiments (Kurt-Jones et al., 2002; Lichtenberger et al., 1999; Reglier et al., 1998). Measurement of cytokines locked inside the cells inhibited in their Golgi function (by Brefeldin A) is a useful method of pinpointing the cells which produce the cytokine in response to a stimulus, as these can be clearly identified by counterstaining. Another method which can support those findings is the repurification of isolated cell populations by magnetic separation with the MACS system. Our data show with both these methods that neutrophils do not release significant amounts of TNF in response to any of the stimuli employed, however, they were able to release IL-8 in response to both LPS and PMA measured after 24 hours. The data on LPS stimulation concur with results reported for TNF and IL-8 release of neutrophils (Reglier et al., 1998).

Further neutrophil functional assays were performed to determine whether LTA can prime or activate neutrophils directly, including measurement of BPI and MPO degranulation and oxidative burst. Each of our approaches provided the same results: neutrophils were neither primed nor directly activated by LTA. Two different neutrophil isolation methods were employed in these studies, as the experiments were performed in different laboratories with the in-house established procedures and with different aims for the different experiments.

It is unclear whether LTA from *S. aureus* interacts with neutrophils but does not stimulate them or whether there is no interaction because the number of relevant receptors expressed is too low. To investigate this point further we plan to label LTA fluorescently. It has been reported that LTA from group A streptococci was only able to stimulate the release of reactive oxygen metabolites on co-incubation and possible cross-linking with anti-LTA-antibodies (Ginsburg et al., 1988).

In the process of pus formation, the priming and activation of neutrophils is preceded by their attraction to the inflammatory focus by means of chemoattractants. The chemoattractants are a vast family of mediators with different structures and different receptor requirements. IL-8, C5a and LTB_4 are strong recruitment signals for neutrophils, while MCP-1 is chemoattractive mainly for monocytes. Measurement of the release of chemoattractants showed LTA to be a strong inducer of chemoattractant release in whole blood, at least comparable to LPS from *S. abortus equi* on an equal weight basis (10 μg/ml). Also, LTA was able to induce the same strong induction of G-CSF release despite being a weaker inducer of TNF-α than LPS.

Although there is as yet no method to measure serum levels of LTA from *S. aureus*, there are reports of an immunoassay detecting teichoic and lipoteichoic acids of *Streptococcus pneumoniae* (Schneider et al., 1999; Stuertz et al., 1998). Concentrations of LTA/teichoic acid of up to 26 μ/ml were measured in the cerebrospinal fluid of patients with diagnosed *S. pneumoniae* infection with a median of 285 ng/ml in 30 patients (Schneider et al., 1999). These data imply that local concentrations of LTA may reach even higher levels, indicating that the concentrations employed in these experiments are in the relevant range.

The cytokine pattern of LTA correlated very well with that of live *S. aureus*. A rough estimate based on our average preparation yields is that 10^5 *S. aureus* carry about 1 μg LTA. Indeed, the cytokine induction by 10^5 *S. aureus* corresponded to that induced by μg quantities of LTA.

Taken together, these data show that LTA is not simply like a weak LPS. Instead, it has qualities different to LPS. It is a strong inducer of chemoattractant release, as well as G-CSF production, despite apparently not activating neutrophils directly. The animal experiment showed that application of LTA alone is sufficient to induce infiltration of neutrophils and macrophages into the lung. The data from this study strongly support our hypothesis that LTA plays a major role in the recruitment of neutrophils to the site of infection leading to the formation of pus.

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