First publ. in: The Journal of Immunology 185 (2010), 6, pp. 3708-3717

Internalization and Coreceptor Expression Are Critical for TLR2-Mediated Recognition of Lipoteichoic Acid in Human Peripheral Blood

Sebastian Bunk,*,[1] Stefanie Sigel,*,†,‡,[1] Daniela Metzdorf,* Omar Sharif,†,‡ Kathy Triantafilou,§ Martha Triantafilou,§ Thomas Hartung,*,¶ Sylvia Knapp,†,‡ and Sonja von Aulock*,‖

Lipoteichoic acid (LTA), a ubiquitous cell wall component of Gram-positive bacteria, represents a potent immunostimulatory molecule. Because LTA of a mutant *Staphylococcus aureus* strain lacking lipoproteins (Δlgt-LTA) has been described to be immunobiologically inactive despite a lack of ascertained structural differences to wild-type LTA (wt-LTA), we investigated the functional requirements for the recognition of Δlgt-LTA by human peripheral blood cells. In this study, we demonstrate that Δlgt-LTA–induced immune activation critically depends on the immobilization of LTA and the presence of human serum components, which, to a lesser degree, was also observed for wt-LTA. Under experimental conditions allowing LTA-mediated stimulation, we found no differences between the immunostimulatory capacity of Δlgt-LTA and wt-LTA in human blood cells, arguing for a limited contribution of possible lipoprotein contaminants to wt-LTA–mediated immune activation. In contrast to human blood cells, TLR2-transfected human embryonic kidney 293 cells could be activated only by wt-LTA, whereas activation of these cells by Δlgt-LTA required the additional expression of TLR6 and CD14, suggesting that activation of human embryonic kidney 293 cells expressing solely TLR2 is probably mediated by residual lipoproteins in wt-LTA. Notably, in human peripheral blood, LTA-specific IgG Abs are essential for Δlgt-LTA–mediated immune activation and appear to induce the phagocytic uptake of Δlgt-LTA via engagement of FcγRII. In this study, we have elucidated a novel mechanism of LTA-induced cytokine induction in human peripheral blood cells that involves uptake of LTA and subsequent intracellular recognition driven by TLR2, TLR6, and CD14.

Staphylococcus aureus, a common cause of severe infections acquired in hospitals, often colonizes human skin asymptomatically and can be isolated from the nasal mucosa of up to 40% of healthy individuals (1). Having overcome skin or mucosal barriers, *S. aureus* establishes an infection that, although mostly restricted to the skin, may involve tissue or systemic blood bacteremia associated with sepsis and sometimes death (2). Upon contact with immune cells, *S. aureus* initially provokes an innate immune response resulting in secretion of cytokines and chemokines as well as in upregulation of phagocytosis and costimulatory molecules able to trigger adaptive immune responses to control bacteremia (3, 4). This response is set in motion by the recognition of highly conserved bacterial molecules, called pathogen-associated molecular patterns, via dedicated receptors, such as TLRs or cytosolic receptors containing a nucleotide-binding oligomerization domain (5–7).

As for other Gram-positive bacteria, the innate immune recognition of *S. aureus* strongly depends on TLR2 (8, 9). Human TLR2 is expressed on the cell surface and within endosomes of APCs like monocytes, macrophages, dendritic cells, and neutrophils (10). It recognizes various staphylococcal cell wall components including lipoteichoic acid (LTA) (11), lipoproteins (12), and peptidoglycan (13), albeit recognition of the latter is controversially discussed (14, 15). This broad range of different bacterial structures described as TLR2 ligands can be explained by heterodimer formation between TLR2 and other TLRs, such as TLR1 or TLR6 (16, 17). Beside TLR2, the mannose-binding lectin (MBL) and CD36, both able to directly interact with LTA, were found to have an important role during the host immune response against *S. aureus* (8, 18, 19). Whereas membrane-bound CD36 was found to augment TLR2/6-dependent recognition of *S. aureus* and its LTA via improving their intracellular uptake (20), soluble CD36 and MBL were described to enhance LTA-mediated activation by improving LTA delivery to the TLR2/6 heterodimer (18, 19).

Despite strong evidence for a major role of LTA in the immune activation by Gram-positive bacteria (21–23), recent reports suggested that not LTA but lipoproteins are dominant immunobiologically active structures of *S. aureus* (24, 25). The latter authors demonstrated a 100-fold decreased immunostimulatory capacity

*Department of Biochemical Pharmacology and ‖Zukunftskolleg, University of Konstanz, Konstanz, Germany; †Research Center for Molecular Medicine, Austrian Academy of Science; ‡Division of Infectious Diseases and Tropical Medicine, Department of Medicine I, Medical University Vienna, Vienna, Austria; §Infection and Immunity Group, School of Life Sciences, University of Sussex, Brighton, United Kingdom; and ¶Center for Alternatives to Animal Testing, Johns Hopkins University, Baltimore, MD 21205

[1]S.B. and S.S. contributed equally to this work.

This work was supported by the German Research Council (International Research Training Group 1331) and the Zukunftskolleg, University of Konstanz, Konstanz, Germany.

Address correspondence and reprint requests to Dr. Sebastian Bunk, Department of Biochemical Pharmacology, University of Konstanz, 78457 Konstanz, Germany. E-mail address: sebastian.bunk@gmx.net

Abbreviations used in this paper: con, control; E, energy; FRET, fluorescence resonance energy transfer; HEK, human embryonic kidney; IL-1β, β form of pro–IL-1; Δlgt-LTA, lipoteichoic acid of a mutant *Staphylococcus aureus* strain lacking lipoproteins; LTA, lipoteichoic acid; M, marker; MBL, mannose-binding lectin; MDP, muramyl dipeptide; poly I:C, polyinosinic-polycytidylic acid; wt, wild-type.

of LTA preparations derived from a Δlgt deletion mutant *S. aureus* lacking palmitate-labeled lipoproteins compared with LTA from the respective wild-type (wt-LTA) strain. From this finding and from other observations, they concluded contaminating lipoproteins in the LTA preparations to be responsible for LTA-mediated immune activation (24–26). Contrary to the latter investigators (27), we found that LTA of a mutant *S. aureus* strain lacking lipoproteins (Δlgt-LTA) and wt-LTA were equipotent in inducing cytokine release from human whole blood. The major discrepancy in LTA activation capacity observed in the two groups both using human whole blood were puzzling and led us to comparatively investigate the specific requirements for Δlgt- and wt-LTA–mediated cytokine induction in human blood cells.

In this report, we describe that cytokine induction by Δlgt-LTA in human peripheral blood is critically dependent on surface immobilization of LTA and requires the presence of LTA-specific Abs as well as the phagocytic activity of blood cells. We further provide evidence that recognition of Δlgt-LTA requires the coexpression of TLR2, TLR6, and CD14. The results presented in this study not only confirm the equipotent immune activation by Δlgt- and wt-LTA in human whole blood observed previously (28), but also provide a conclusive explanation for contradictory findings obtained in previous experiments analyzing the immunostimulatory capacity of Δlgt-LTA.

Materials and Methods

Stimuli

LTA from *S. aureus* wt (SA 113 wt, wt-LTA) and mutant strain SA 113 *lgt::ermB* (SA 113 Δlgt lacking the lipoprotein diacylglycerol transferase, Δlgt-LTA; both strains were kind gifts from A. Peschel, University of Tübingen, Tübingen, Germany) was isolated by butanol extraction and hydrophobic interaction chromatography as described previously (21). For UV inactivation and subsequent whole blood stimulation, *S. aureus* (SA 113 wt) was cultivated in tryptic soy broth (BD Biosciences, Heidelberg, Germany) for 16 h at 37°C. Harvested bacteria were adjusted to 10^8 bacteria/ml, and 1 ml/well was irradiated on ice (UV-Stratalinker, Stratagene, La Jolla, CA) with an energy density of 1 kN/cm^2 (3 mW/cm^2 × 300 s) for 5 min in a six-well cell culture plate (Greiner Bio-One, Frickenhausen, Germany). The inactivation was controlled by growth on blood agar plates (Columbia-blood agar, Heipa Diagnostika, Eppelheim, Germany) after 24 h at 37°C and 5% CO_2.

Other substances were LPS from *Salmonella enterica* serovar *abortus equi* and cytochalsin D from *Zygosporium masoni* (Sigma-Aldrich, Deisenhofen, Germany), polyinosinic-polycytidylic acid (poly I:C; Invivogen, San Diego, CA), $Pam_2Cys\text{-}SK_4$ and $Pam_3Cys\text{-}SK_4$ (EMC Microcollections, Tübingen, Tübingen, Germany), CpG 2216 (MWG Biotech AG, Ebersberg, Germany), muramyl dipeptides (MDPs; Bachem, Heidelberg, Germany), human IgG Abs (Endobulin, Baxter, Wien, Austria), control IgG1 (Avastin, Roche, Grenzach-Wyhlen, Germany), and anti-LTA IgG1 (Pagibaximab, Biosynexus, Gaithersburg, MD).

Human whole-blood incubation

Heparinized venous blood was obtained from healthy volunteers after informed consent. Differential blood cell counts were routinely determined with a Pentra 60 apparatus (ABX Diagnostics, Montpellier, France) to exclude acute infections. Blood was diluted 5-fold with RPMI 1640 (Lonza, Verviers, Belgium), and 500 μl was incubated in the presence of the different stimuli in polypropylene tubes (Eppendorf, Hamburg, Germany) overnight for 22 h at 37°C and 5% CO_2. For LTA immobilization, 50 μl wt-LTA, Δlgt-LTA in RPMI 1640 at different concentrations was preincubated in tubes for up to 1 h at room temperature before the addition of whole blood and RPMI 1640. In case of LTA immobilization, all other control stimuli, such as LPS or UV-inactivated *S. aureus*, were also preincubated before use for stimulation experiments. To assess the role of phagocytosis in LTA-mediated cytokine induction, diluted human whole blood was first pretreated with 3 μM cytochalasin D for 30 min in siliconized glass tubes (Vacutainer, BD Biosciences) and then applied to the preincubated LTA. The ability of cytochalasin D to inhibit phagocytosis was controlled by stimulation with MDP, CpG, and poly I:C as well as with LPS and $Pam_2Cys\text{-}SK_4$, respectively. After 22 h, blood cells were suspended by gentle shaking and centrifuged at 400 × *g* for 2 min. The cell-free supernatants were stored at −80°C for cytokine determination.

Preparation and stimulation of human PBMCs and monocytes

PBMCs of healthy volunteers were prepared with CPT Cell Preparation Tubes (BD Biosciences). Postcentrifugation at 1600 × *g* for 20 min, PBMCs were collected and washed four times at 300 × *g* for 10 min with RPMI 1640 containing 2.5 IU/ml Liquemin (Hoffmann-La Roche, Basel, Switzerland) to remove serum residues. For experiments employing isolated monocytes or monocyte-depleted PBMCs, PBMCs were isolated from heparinized human whole blood using Lymphoprep as described by the manufacturer (Axis-Shield, Oslo, Norway) and were washed free of serum as described above. Monocytes were isolated from PBMCs by positive or negative magnetic isolation (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, PBMCs were incubated with biotinylated anti-CD14 Ab (positive selection) or with a mixture of biotinylated Abs against CD3, CD7, CD16, CD19, CD56, and CD123 (negative selection) for 10 min on ice. Then cells were incubated with magnetic bead-conjugated anti-biotin Abs for 15 min and applied onto MACS LS columns. In case of positive selection, PBMCs passing the column were collected as monocyte-depleted PBMCs, and the $CD14^+$ monocytes were collected by eluting the bound cell fraction. In case of negatively selected monocytes, the cells passing the column without binding were collected. Prior to stimulation, PBMCs and monocytes were supplemented with 4% autologous serum unless stated otherwise. Serum-free PBMCs were used for stimulation either with or without addition of different concentrations of autologous serum, different human Abs, or IgG-depleted autologous serum. Stimulation of PBMCs or monocytes and the storage of supernatants were performed according to the conditions described for whole blood in a total volume of 220 μl. PBMCs were added at a density of 5 × 10^5 cells/tube, and monocytes were used at a density corresponding to the monocyte count in PBMC samples. For FcγR blockade experiments, PBMCs were preincubated with neutralizing Abs against CD16 (clone 3G8, BD Biosciences), CD32 (clone AT10, Abcam, Cambridge, U.K.), or CD64 (clone 10.1, eBioscience, Hatfield, U.K.) or with the respective isotype control mouse IgG1 Ab (eBioscience) for 30 min at 37°C prestimulation.

Stimulation of transfected human embryonic kidney 293 cells

Human embryonic kidney (HEK) 293 cells stably transfected either with human TLR2 alone or a combination of human TLR2, TLR6, and CD14 as well as wt HEK293 cells were seeded at 2 × 10^5 cells/well in 24-well cell culture plates (BD Biosciences) in DMEM (Life Technologies Invitrogen, Darmstadt, Germany) supplemented with 10% FCS (Life Technologies Invitrogen) and 1% penicillin/streptomycin (Life Technologies Invitrogen). After 48 h, the medium was replaced by FCS-free medium containing different concentrations of wt-LTA, Δlgt-LTA, or $Pam_3Cys\text{-}SK_4$. Cell-free supernatants were collected 22 h poststimulation and stored at −80°C for ELISA experiments.

Fluorescence resonance energy transfer analysis

Fluorescence resonance energy transfer (FRET) is a noninvasive imaging technique used to determine molecular proximity based on nonradiative transfer of energy from the excited state of donor molecules to an appropriate acceptor (29). This energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor (30, 31). In this study, FRET was measured using a method as previously described (30, 31). Briefly, human monocytes on microchamber culture slides (Lab-tek, Life Technologies Invitrogen) were stimulated with 10 μg/ml wt-LTA or Δlgt-LTA or left untreated and then were labeled with 100 μl mixture of donor (Cy3)- and acceptor (Cy5)-conjugated Ab. The cells were rinsed twice in PBS/0.02% BSA and fixed with 4% formaldehyde for 15 min to prevent potential protein reorganization. Cells were imaged on a Carl Zeiss LSM510 confocal microscope (with an Axiovert 200 fluorescent microscope; Zeiss, Oberkochen, Germany) using a 1.4 numerical aperture 63× Zeiss objective. The images were analyzed using LSM 2.5 image analysis software (Zeiss). Cy3 and Cy5 were detected using the appropriate filter sets. Using typical exposure times for image acquisition (<5 s), no fluorescence was observed from a Cy3-labeled specimen using the Cy5 filters, nor was Cy5 fluorescence detected using the Cy3 filter sets.

For calculation, the energy (E) transfer between donor- and acceptor-conjugated Abs was detected as an increase in donor fluorescence (dequenching) after complete photobleaching of the acceptor molecule and calculated according to the formula: $E(\%) \times 100 = 10{,}000 \times [(\text{Cy3 postbleach} \times \text{Cy3 prebleach})/\text{Cy3 postbleach}]$. The scaling factor of 10,000 was used to expand E to the scale of the 12-bit images.

Identification of LTA-binding proteins

For the identification of LTA-interaction components from human serum, the wells of a six-well cell culture plate (Greiner Bio-One) were incubated with either 10 μg Δlgt-LTA/well or with only PBS overnight at 4°C and afterward washed twice with PBS. Then, 1 ml/well 5% human serum in PBS was added for 1 h at room temperature, again followed by two washing steps with PBS. Surface-bound proteins from six wells were pooled in a total volume of 100 μl PBS containing 1% SDS and analyzed by SDS-PAGE and silver staining. The two protein bands at 25 kDa and 50 kDa, found only in the wells preincubated with Δlgt-LTA and not in the PBS controls, were excised from the gel and, after in-gel tryptic digestion, were identified using MALDI-TOF mass spectrometry at the core facility of the Biomedical Centre at the Ludwig-Maximillians-University Munich, Munich, Germany.

Depletion of IgG from human serum and Western blot

IgG was removed from human serum using HiTrap protein A columns (1 ml; GE Healthcare, Munich, Germany). According to the manufacturer's protocol, 1 ml 50% serum in PBS was applied to the column, and the flow-through was collected. To decrease remaining IgG residues, three columns were connected in series. The removal of IgG was confirmed by Western blot analysis. IgG-depleted serum samples were applied to 12% SDS-PAGE and blotted to nitrocellulose membranes (Pall Corporation, Dreieich, Germany). IgG was detected by immunoblotting with HRP-conjugated polyclonal rabbit anti–human-IgG Abs (DakoCytomation, Glostrup, Denmark) and ECL detection using an LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Cytokine measurement

Cytokines released by human whole blood were measured by in-house sandwich ELISA based on commercially available pairs of Abs and standards. Ab pairs against human β form of pro–IL-1 (IL-1β) and IL-6 were purchased from R&D Systems (Minneapolis, MN) and against human TNF, IL-8, and IFN-γ from Endogen (Perbio Science, Bonn, Germany). Recombinant standards for IL-1β, IL-6, TNF, and IFN-γ were obtained from the National Institute for Biological Standards and Control, Hertfordshire, U.K., and rIL-8 was obtained from PeproTech (Tebu, Frankfurt, Germany). Assays were carried out in flat-bottom, ultrasorbent 96-well plates (MaxiSorp, Nunc, Wiesbaden, Germany). Binding of biotinylated secondary Ab was quantified using streptavidin-conjugated HRP (BioSource, Life Technologies Invitrogen), and tetramethylbenzidine substrate (Sigma-Aldrich) was used as substrate. The reaction was stopped with 1 M sulphuric acid, and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm. Cytokine levels are given per milliliter of blood.

Determination of anti-LTA Ab serum titer

The amount of LTA-binding IgG was determined in human serum obtained from 57 healthy volunteers. Flat-bottom 96-well plates (MaxiSorp, Nunc) were coated overnight with 10 μg/ml Δlgt-LTA in PBS at 4°C. Then, wells were blocked with 200 μl 3% BSA in PBS for 2 h at room temperature and washed with PBS preaddition of 4% serum in PBS and further incubation for 1 h at room temperature. After washing, HRP-conjugated polyclonal rabbit anti-human-IgG Ab was added to each well for another 30 min. Enzymatic activity was detected with 3,3′,5,5′,-tetramethybenzidine substrate (Sigma-Aldrich) and stopped with 1 M sulphuric acid. The absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

Statistics

Statistical analysis was performed using the GraphPad Prism 3 Program (GraphPad, San Diego, CA). Three or more groups of data were compared by repeated-measure ANOVA followed by Dunn's posttest. For statistical analysis of two groups of data, the Mann-Whitney *U* test was used. In the figures, *, **, and *** represent p values <0.05, <0.01 and <0.001, respectively.

Results

Immobilized but not soluble LTA of mutant Δlgt S. aureus *induces cytokine release in human whole blood*

There are conflicting reports concerning the immunostimulatory capacity of LTA isolated from an Δlgt mutant *S. aureus* strain that lacks lipoproteins (24, 27, 28). Because we previously reported an increased immune activation by immobilized wt-LTA (32, 33), we analyzed whether immobilization also augments Δlgt-LTA–mediated immune activation. We determined the cytokine induction in human whole blood upon stimulation with Δlgt-LTA and wt-LTA, either used in an immobilized or nonimmobilized form. Immobilization was achieved by preincubating LTA in polypropylene tubes prior to the addition of diluted blood, whereas for nonimmobilizing conditions, LTA was directly added to the blood.

As shown in Fig. 1*A*, whole-blood stimulation with immobilized Δlgt-LTA led to a potent induction of IL-1β and TNF, which was comparable to wt-LTA and LPS (Fig. 1*A*). In contrast, without immobilization, the Δlgt-LTA–induced cytokine release from whole blood was fully abrogated, whereas LPS-mediated cytokine induction was unaffected. Notably, wt-LTA induced also cytokine release when used without prior immobilization, albeit at a significant lower level.

To confirm that LTA does adhere to the polypropylene tubes, we analyzed the cytokine-inducing capacity of only the LTA bound during the immobilization procedure. For this, LTA was incubated in polypropylene tubes for 30 s, and the residual, unbound LTA in the supernatant was transferred to another tube and then incubated for 30 min, followed by another transfer and 30 min incubation, but without subsequent removal of the supernatant. Even postincubation in the vial for only 30 s, the bound Δlgt-LTA and wt-LTA were able to induce IL-1β and TNF, albeit in low amounts, which was strongly enhanced after 30 min preincubation. Interestingly, the cytokine-inducing capacity of bound LTA was not diminished in tubes incubated with supernatants from previous incubations (Fig. 1*B*). No decrease in cytokine induction by the LTA-containing supernatant was observed even when it was transferred up to six times (S. Siegel, D. Metzendorf, and S. Bunk, unpublished observations), indicating that only a small proportion of LTA binds to the tube, thereby enabled to induce cytokine release.

Δlgt-LTA–mediated cytokine release depends on the presence of monocytes and requires the expression of TLR6 and CD14 in addition to TLR2

To determine which type of blood cells respond to stimulation with Δlgt-LTA, we compared cytokine release from PBMCs, isolated monocytes, and monocyte-depleted PBMCs. Stimulation with Δlgt-LTA, wt-LTA, or LPS led to a comparable release of IL-1β and TNF from PBMCs and from monocytes obtained by negative isolation (Fig. 2*A*). Furthermore, PBMCs depleted of CD14-expressing monocytes showed strongly reduced cytokine induction, suggesting that monocytes represent the main Δlgt-LTA–responsive cell type in human peripheral blood. Interestingly, monocytes enriched with anti-CD14 Abs (positive isolation) showed markedly diminished cytokine production poststimulation with Δlgt-LTA but not wt-LTA or LPS, indicating a role for membrane-bound CD14 in the recognition of Δlgt-LTA.

LTA is believed to induce cytokines via TLR2 engagement, but recent studies demonstrated the inability of Δlgt-LTA to activate TLR2-transfected cells (24, 25, 27). To analyze if Δlgt-LTA engages TLR2 independent of its ability to activate cells, we employed FRET analysis to determine Δlgt-LTA–mediated recruitment of TLR2 to lipid rafts of human monocytes (34). For this purpose, we measured the increase in fluorescence intensity (dequenching) of Cy3-labeled donors, such as TLR2, TLR4, or MHC class I, after complete photobleaching of Cy5-labeled acceptors, such as the lipid raft markers CD14 or GM1 ganglioside. Following Δlgt-LTA incubation, increased donor fluorescence of TLR2 was observed after bleaching the acceptors CD14 or GM1, which was not observed for unstimulated monocytes (Table I). In contrast, TLR4 and MHC class I showed unchanged donor fluo-

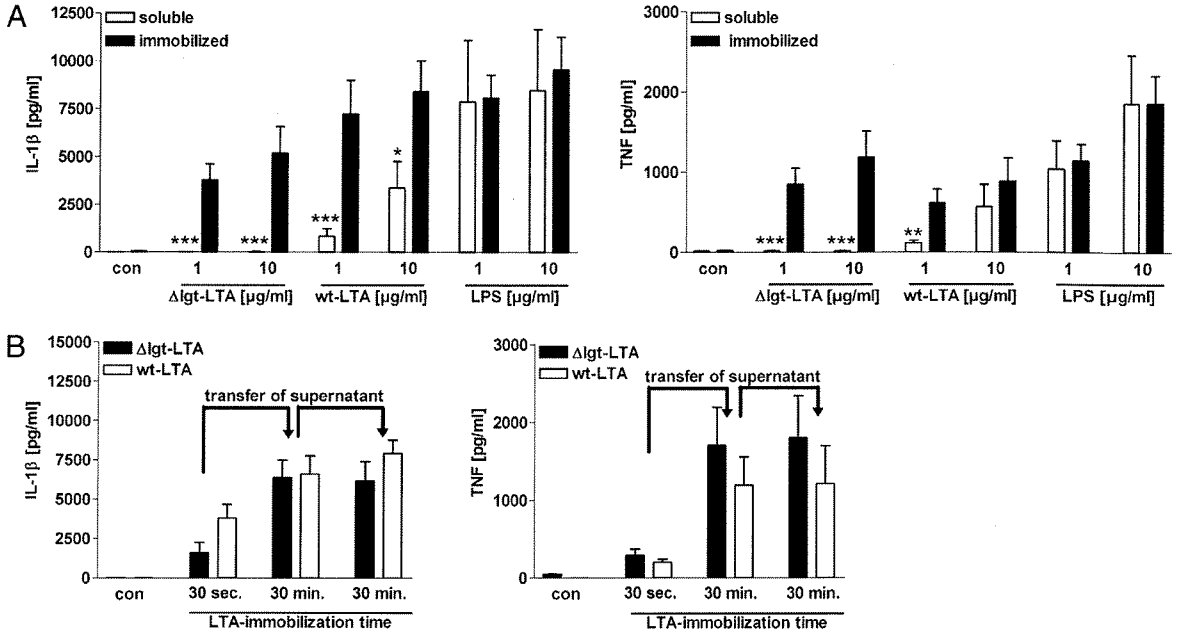

FIGURE 1. Stimulation with immobilized but not soluble Δlgt-LTA induces cytokine release in human whole blood. Whole blood of healthy human donors was stimulated with *S. aureus* wt-LTA or Δlgt-LTA under immobilizing and nonimmobilizing conditions. As controls, stimulations were carried out with LPS or blood was left without stimulus (con). After 22 h, TNF and IL-1β release was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM. *A*, Whole blood of different donors ($n = 11$) was stimulated in polypropylene tubes with 1 μg/ml and 10 μg/ml Δlgt-LTA, wt-LTA, or LPS, respectively. The stimuli were directly applied to the blood (soluble) or were coated for 1 h before blood was added allowing immobilization of stimuli (immobilized). For each stimulus, significant differences between the soluble and the immobilized form were analyzed by Mann-Whitney *U* test. *B*, A total of 10 μg/ml wt-LTA or Δlgt-LTA was immobilized by preincubation in polypropylene tubes for 30 s. Supernatants with unbound LTA were completely removed and transferred to another tube for 30 min, removed again, and incubated in the last tubes for 30 min without subsequent removal of the supernatant. Human whole blood of different donors ($n = 8$) was added directly postimmobilization and samples were incubated for 22 h before cytokine determination by ELISA. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

rescence in the presence of Δlgt-LTA, thus demonstrating that Δlgt-LTA specifically recruits TLR2 to the lipid raft. Comparable results were observed for wt-LTA, suggesting that both LTAs engage the TLR2 receptor. The important role of TLR2 in Δlgt-LTA and wt-LTA recognition was further confirmed in TLR2 knockout mice, which, upon stimulation with both LTAs, showed

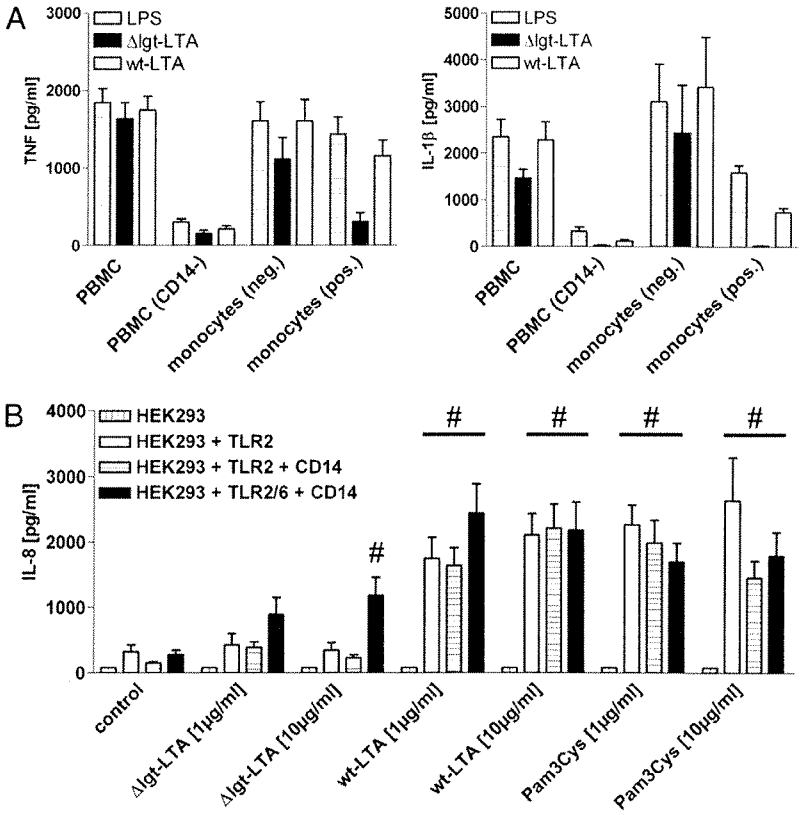

FIGURE 2. Δlgt-LTA mediates cytokine release by human monocytes and requires expression of TLR2, TLR6, and CD14. *A*, Human PBMC (CD14-depleted or nondepleted) or isolated monocytes (obtained by positive isolation employing anti-CD14 Abs or by negative isolation), for which the number was adjusted to the monocyte content of nondepleted PBMCs, were stimulated with 10 μg/ml immobilized Δlgt-LTA, wt-LTA, or LPS in the presence of 4% human autologous serum. After 22 h, the amount of released IL-1β and TNF was measured in the cell-free supernatants by ELISA. Data were obtained from six different healthy donors and are given as means ± SEM. *B*, wt HEK293 cells or HEK293 either stably transfected with TLR2, TLR2/CD14, or TLR2/TLR6/CD14 were stimulated with 1 μg/ml and 10 μg/ml of Δlgt-LTA, wt-LTA, or Pam_3Cys, respectively, or left without stimulation. After 22 h, released IL-8 was determined by ELISA. Data representing five independent experiments (performed in duplicates or triplicates) are given as means ± SEM. For each HEK293 cell type, significant differences in the release of IL-8 between stimulated and nonstimulated conditions (control) were assessed by Kruskal-Wallis test followed by Dunn's posttesting. $\#p < 0.05$.

Table I. *Energy transfer efficiency between donors–acceptor pairs on human monocytes*

	Donor (Cy3)	Acceptor (Cy5)	E ± ΔE (%)[a]
Unstimulated monocytes			
	CD14	CD14	38 ± 1.0
	CD14	GM1	36 ± 1.5
	TLR2	GM1	7 ± 1.5
	TLR2	CD14	6 ± 1.0
	TLR4	GM1	7 ± 0.5
	MHC class I	GM1	5 ± 1.5
Monocytes stimulated with 10 μg/ml wt-LTA			
	CD14	CD14	38 ± 0.2
	CD14	GM1	34 ± 1.5
	TLR2	GM1	38 ± 1.5
	TLR2	CD14	36 ± 0.8
	TLR4	GM1	7 ± 1.0
	MHC class I	GM1	6 ± 1.0
Monocytes stimulated with 10 μg/ml Δlgt-LTA			
	CD14	CD14	37 ± 2.0
	CD14	GM1	32 ± 0.5
	TLR2	GM1	36 ± 1.5
	TLR2	CD14	35 ± 0.5
	TLR4	GM1	7 ± 0.8
	MHC class I	GM1	6 ± 1.0

Data represent means ± SD of a number of independent experiments.
[a]Energy transfer between different pairs was detected from the increase in donor fluorescence after acceptor photobleaching.

significantly reduced cytokine induction compared with wt-mice (Supplemental Fig. 1).

To determine the innate immune receptors required for recognition of Δlgt-LTA and wt-LTA, we analyzed the activation of HEK293 cells stably transfected with human TLR2, TLR2/CD14, or TLR2/TLR6/CD14. In line with previous reports (27), Δlgt-LTA did not activate TLR2-HEK293, whereas these cells showed pronounced IL-8 release poststimulation with wt-LTA or Pam_3Cys (Fig. 2*B*). The same discrepancy between Δlgt-LTA and wt-LTA was observed for HEK293 cells coexpressing TLR2 and CD14. Interestingly, the simultaneous expression of TLR2, CD14, and TLR6 enabled HEK293 cells to effectively recognize Δlgt-LTA. We observed a significant release of IL-8 by TLR2/6/CD14-HEK293 cells upon stimulation with 10 μg/ml Δlgt-LTA. Whereas TLR6 expression was pivotal for Δlgt-LTA recognition, almost no effect was observed for Pam_3Cys and wt-LTA, respectively. Notably, for HEK293 experiments, Δlgt-LTA could not be immobilized prestimulation, a circumstance that might have contributed to the lower potency of Δlgt-LTA compared with wt-LTA. As indicated for isolated monocytes, CD14 was also required for Δlgt-LTA recognition by HEK293 cells, because HEK293 cells transiently transfected with TLR2 and TLR6 did not respond to Δlgt-LTA in the absence of CD14 (data not shown). Taken together, these data demonstrate the important role of TLR2 and the coreceptors TLR6 as well as CD14 in the recognition of Δlgt-LTA by human immune cells.

Δlgt-LTA–induced cytokine release by human PBMCs is abrogated after cytochalasin D treatment and in the absence of human serum

Recognition of LTA was described to be amplified by CD36-mediated phagocytosis and by the presence of serum MBL via a mechanism that requires intracellular uptake of LTA (18, 20). These findings prompted us to investigate the influence of phagocytosis and human serum components on LTA-induced cytokine release in human blood cells. Using cytochalasin D to inhibit phagocytic activity, we analyzed the cytokine-inducing capacity of Δlgt-LTA and wt-LTA in human whole blood. For control purposes, stimulations were carried out with different ligands known to activate immune receptors located either intracellular or extracellular. Surprisingly, in the presence of cytochalasin D, the induction of IL-1β and TNF by Δlgt-LTA was completely abrogated and in the case of wt-LTA strongly attenuated, which was also observed for the intracellular receptor ligands CpG and MDP as well as for poly I:C (Fig. 3*A*). In contrast, recognition of the extracellular TLR ligands LPS and Pam_2Cys was not affected by cytochalasin D treatment, suggesting a pivotal role of phagocytosis for LTA-mediated cytokine induction. To investigate the role of serum components, we determined Δlgt-LTA– and wt-LTA–mediated cytokine release from serum-free PBMCs supplemented with increasing concentrations of autologous human serum. For immobilized but also nonimmobilized (not shown) Δlgt-LTA, we detected no cytokine release from PBMCs in the absence of human serum, which in case of immobilized Δlgt-LTA could be regained by the addition of autologous human serum (Fig. 3*B*). Already at concentrations of 1% serum, significant cytokine induction by Δlgt-LTA was observed. For wt-LTA, a comparable enhancement of cytokine release in the presence of human serum was found, but in contrast to Δlgt-LTA, wt-LTA was already able to induce cytokines under serum-free conditions, albeit at low amounts (Fig. 3*B*). The cytokine induction in PBMCs by Δlgt-LTA and wt-LTA peaked at a concentration of 4% serum, and the amounts of released IL-1β and TNF were comparable between both LTAs, underlining their equal immunostimulatory capacity.

Immobilized Δlgt-LTA interacts with apolipoprotein A1 and cationic Igs from human serum

To identify serum components that possibly interact with immobilized LTA resulting in immune activation, cell-culture plate wells pretreated with or without Δlgt-LTA were incubated with 4% serum from different human donors, and bound components were analyzed by SDS-PAGE. As shown for two exemplary donors in Fig. 4, the SDS-PAGE profiles of serum components derived from wells with immobilized Δlgt-LTA showed two abundant protein bands (25 kDa and 50 kDa) that were absent in serum samples obtained from control wells without LTA. The two protein bands were observed in the SDS-PAGE profiles of six tested donors, albeit the band intensity among these donors was slightly different (data not shown). Using tandem mass spectrometry analysis of peptides derived from the 25-kDa protein band, we identified two different human proteins (i.e., apolipoprotein A1 and Igκ L chain), whereas

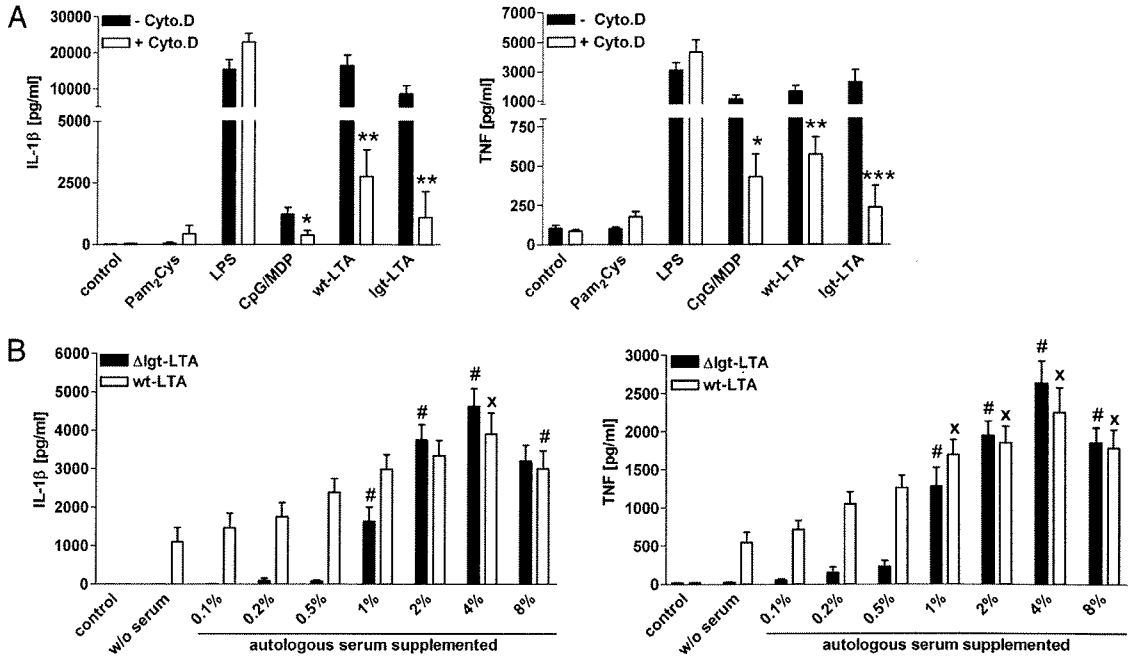

FIGURE 3. Δlgt-LTA–mediated cytokine release is abrogated by cytochalasin D treatment and in the absence of human serum. Whole blood or PBMCs of healthy human donors were stimulated with Δlgt- or wt-LTA immobilized for 1 h to polypropylene tubes. Stimulations were carried out in the presence or absence of the phagocytosis inhibitor cytochalasin D or in the presence of increasing concentrations of autologous serum. After 22 h, TNF and IL-1β release was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM. *A*, Stimulation of cytochalasin D-treated blood samples of different donors (n = 8) with 10 μg/ml Δlgt-LTA or wt-LTA. For control stimulations of extracellular and intracellular TLR receptors, $Pam_2Cys\text{-}SK_4$ (10 μg/ml) or LPS (10 ng/ml) and CpG/MDP (2 μM/500 ng/ml) were used, respectively. For each stimulus, significant differences between samples incubated with or without cytochalasin D were analyzed by Mann-Whitney U test. $*p < 0.05$, $**p < 0.01$; $***p < 0.001$. *B*, Stimulation of human PBMCs of different donors (n = 10) with 10 μg/ml Δlgt-LTA or wt-LTA in the presence of increasing concentrations of autologous sera. For each stimulus, significant differences between the serum-free samples and samples containing serum were assessed by Kruskal-Wallis test followed by Dunn's posttest. # and x represent $p < 0.05$.

the analysis of the 50-kDa protein band revealed only the H chain of Ig (Table II). In addition to the complete Ig L chain sequences with a predicted molecular mass of 23 kDa, a Mascot database search also revealed highest identification scores for the variable domains of L chains derived from anti-DNA and anti-cardiolipin Abs. Interestingly, DNA and cardiolipin share putative epitopes consisting of phosphodiester groups separated by three adjacent carbon atoms together with the polyglycerolphoshate backbone of LTA (35), arguing for a specific interaction between the detected Abs and LTA.

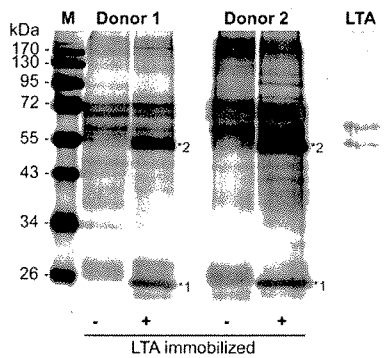

FIGURE 4. Identification of serum proteins interacting with immobilized Δlgt-LTA. Cell-culture plate wells (six-well) were preincubated with or without 10 μg/ml Δlgt-LTA and, after two washing steps, incubated with 4% human serum of different donors. Postremoval of residual soluble serum components, the bound proteins were detached by addition of 1% SDS and analyzed by SDS-PAGE and silver staining. As control, SDS-PAGE analysis was performed with 10 μg Δlgt-LTA. A standard protein marker (M) was used. The two protein bands (25 kDa and 50 kDa) marked with *1 and *2 were excised from the gel, subjected to tryptic digestion, and then analyzed by peptide mass fingerprinting (for results, see Table II).

LTA-specific IgG Abs augment Δlgt-LTA–mediated cytokine induction in PBMCs in a CD32-dependent manner

To analyze the influence of apolipoprotein A1 and specific Igs on the recognition of Δlgt-LTA, we performed supplementation experiments using serum-free PBMCs. The addition of up to 10 μg/ml apolipoprotein A1 was unable to restore cytokine release in serum-free PBMCs stimulated with immobilized Δlgt-LTA (data not shown). In contrast, stimulation of PBMCs supplemented with LTA-specific IgG1 Abs resulted in a marked release of TNF (Fig. 5*A*) and IL-1β (data not shown), which was not observed in the case of control IgG1 Abs. Despite this pronounced effect of LTA-specific Abs, their addition could not fully restore the Δlgt-LTA–induced cytokine secretion observed in PBMCs containing 4% serum. Furthermore, supplementation of PBMCs with human IgG Abs extracted from a large plasma pool of >1000 donors that should contain a certain amount of LTA-specific IgG Abs had no positive effect on Δlgt-LTA–mediated cytokine release in serum-free PBMCs, suggesting that additional serum components contribute to the recognition process of LTA. To further address the role of IgG Abs in Δlgt-LTA recognition, Δlgt-LTA–mediated cytokine release was analyzed in PBMCs supplemented with either normal or IgG-depleted serum. As shown in Fig. 5*B*, the cytokine induction by Δlgt-LTA in PBMCs containing IgG-depleted serum was significantly reduced compared with normal serum supplementation. This reduction was found to be specific for LTA,

Table II. *Identification of serum proteins interacting with immobilized Δlgt-LTA*

Sample	Accession Number	Protein	Tandem Mass Spectrometry Score	Peptides Matched	Molecular Mass (kDa)
1 (25 kDa)	gi90108664	Apolipoprotein A1	702	49	28.0
	gi1407576	Ig L chain, V region (anti-DNA Ab)	369	13	11.9
	gi157838230	Ig κ L chain, C and V region	313	10	23.0
	gi18092618	Ig L chain, V region (anticardiolipin Ab)	236	7	11.9
2 (50 kDa)	gi34365168	Ig H chain	419	41	51.1

because LPS-mediated cytokine release was not affected by IgG depletion.

We next determined the role of FcγRs in cytokine induction by ΔIgt-LTA. PBMCs containing 4% serum were stimulated with ΔIgt-LTA in the presence or absence of anti-human CD16, CD32, or CD64 blocking Abs or an isotype control Ab. As shown in Fig. 6, ΔIgt-LTA–mediated release of IL-1β was fully abrogated in the presence of anti-CD32 Abs, but only marginally or not affected by anti-CD16 or anti-CD64 Abs. Similar results were also observed for TNF release (data not shown). In contrast to ΔIgt-LTA, none of the blocking Abs had an effect on LPS-mediated cytokine release

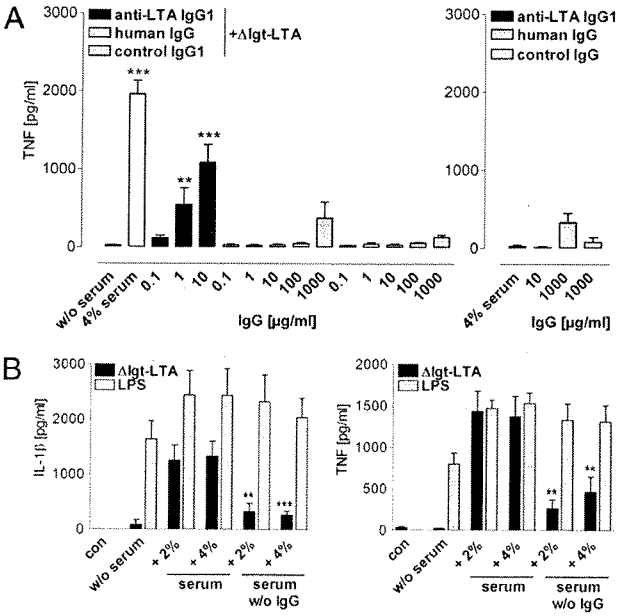

FIGURE 5. ΔIgt-LTA–mediated cytokine release from human PBMCs depends on the presence of specific IgG Abs. Human serum-free PBMCs were stimulated with immobilized ΔIgt-LTA (10 μg/ml) or LPS (10 μg/ml) in the presence of LTA-specific IgG1, human IgG Abs, or control IgG1 Abs or in the presence of autologous or IgG-depleted autologous serum. After 22 h, TNF and IL-1β release was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM. *A*, ΔIgt-LTA–mediated stimulation of serum-free PBMCs from different donors (n = 8) supplemented with or without 4% autologous serum or increasing concentrations of human chimeric anti-LTA IgG1 Abs (Pagibaximab), total human IgG Abs (Endobulin), or human control IgG1 Abs (Avastin) (*left panel*). Significant differences between PBMC samples containing ΔIgt-LTA and the corresponding unstimulated PBMC samples were analyzed by Kruskal-Wallis test followed by Dunn's posttesting. As control, PBMCs (n = 8) containing 4% autologous serum were supplemented with the given concentrations of anti-LTA IgG1, total human IgG, or control IgG1 Abs and left without stimulation (*right panel*). *B*, ΔIgt-LTA– and LPS-mediated stimulation of PBMCs of different donors (n = 10) in presence of 2 or 4% autologous serum, IgG-depleted autologous serum, or in absence of serum. For each stimulus, significant differences between LTA-stimulated samples supplemented with autologous before and after IgG depletion were analyzed by Mann-Whitney U test. $^{**}p < 0.01$; $^{***}p < 0.001$.

(Fig. 6*B*). These data demonstrate the specific requirement of FcγRII (CD32) for ΔIgt-LTA–mediated immune activation in human peripheral blood and thus support the pivotal role of LTA-specific Abs in this process.

The amount of LTA-specific serum IgG correlates with Δlgt-LTA– but not S. aureus*-mediated immune activation in human whole blood*

Immune recognition of whole *S. aureus* strongly depends on internalization of bacteria, because the decreased phagocytosis found in CD36-deficient mice or cytochalasin D-treated murine immune cells associates with attenuated or abrogated immune activation by *S. aureus* (18, 20). In the current study, we found that LTA-mediated cytokine induction is sensitive toward cytochalasin D treatment and requires LTA-specific Abs, suggesting an opsonization-dependent LTA uptake and recognition process that could also contribute to the recognition of whole *S. aureus* presenting LTA at their surface. To investigate this, we correlated LTA-specific IgG titers in the sera of different donors with the respective cytokine

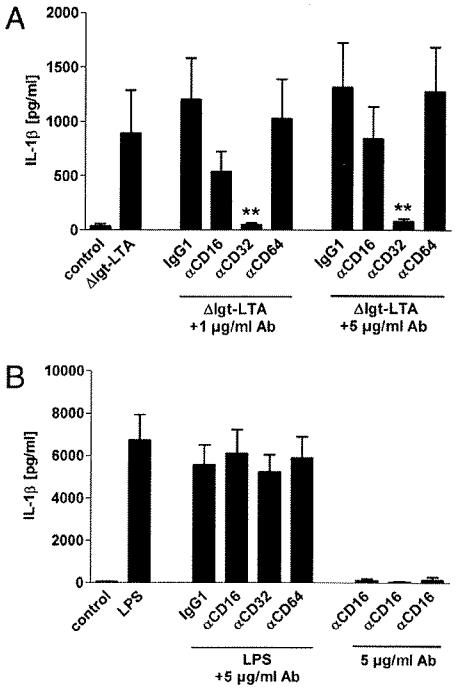

FIGURE 6. ΔIgt-LTA–mediated cytokine release from human PBMCs depends on the expression of CD32. Human PBMCs from different donors (n = 8) containing 4% autologous serum were preincubated for 30 min with 1 μg/ml or 5 μg/ml neutralizing IgG Ab against human CD16, CD32, or CD64 or with isotype control IgG1 Ab. Subsequently, they were stimulated with 10 μg/ml immoblized ΔIgt-LTA (*A*) or LPS or left without stimulation (*B*). After 22 h, released TNF was determined by ELISA. Data are given as means ± SEM. For each stimulus, significant differences of samples containing neutralizing Abs compared with samples containing the corresponding amount of isotype control Ab were assessed by Kruskal-Wallis test followed by Dunn's posttesting. $^{**}p < 0.01$.

response of their whole blood poststimulation with Δlgt-LTA, whole *S. aureus*, or LPS. Among 57 human donors, we observed pronounced differences in LTA-specific IgG titers, as revealed by ELISA experiments using immobilized Δlgt-LTA (Fig. 7). Consistent with the results above, compared with blood from donors with high titers of specific IgG, blood from donors with low titers showed a significantly decreased induction of IL-1β and TNF poststimulation with Δlgt-LTA (Fig. 7*B*). For IL-6, which was strongly induced by Δlgt-LTA in comparison with whole *S. aureus* stimulation, we observed no significant difference between low- and high-titer donors. Furthermore, as previously described for wt-LTA (36), Δlgt-LTA was unable to induce IFN-γ in human whole blood. When whole blood stimulation was carried out with UV-inactivated *S. aureus* or LPS, no differences in the cytokine induction between donors with low and high titers of LTA-specific Abs were observed. The latter result suggests that, despite the necessity of LTA-specific IgG Abs to recognize LTA, these Abs are dispensable for the immune recognition of whole *S. aureus* by human peripheral blood, possibly due to uptake of the bacteria via alternative mechanisms.

Discussion

The immunostimulatory capacity of Δlgt-LTA is controversially discussed and is of seminal interest for the field of innate immunity, as it questions the ability of LTA per se to induce cytokine release. Although fully synthetic LTA was found to mirror the immunostimulatory activity of butanol-extracted LTA from wt *S. aureus* (22, 23), Hashimoto et al. (24, 27) propose that the activity of the latter entirely stems from purported, highly immunostimulatory lipoprotein contaminations not present in Δlgt-LTA, which was inactive in their cellular systems and human whole blood. However, using the same bacterial strains to extract LTA, we have previously reported equipotent immunostimulatory activity of wt-LTA and Δlgt-LTA in human bloods cells (28). Notably, the current study, initiated to address these discrepancies, uncovered a novel mechanism of Δlgt-LTA recognition in human peripheral blood and suggests that LTA-mediated immune activation in general is more complex than previously thought. In our experiments, the recognition of Δlgt-LTA was critically dependent on: 1) surface immobilization and opsonization by IgG Abs; 2) internalization through FcγRII-mediated uptake; and 3) recognition via TLR2, TLR6, and CD14. The latter observation argues for an essential role of the TLR2/TLR6 heterodimer in the recognition of Δlgt-LTA, which has also been described for macrophage-activating lipopeptide 2 (37), a highly active natural TLR2 ligand (38). Recently, the importance of TLR2 and TLR6 coexpression for the recognition of lipoprotein-deprived LTA was also shown in mice using *S. aureus* LTA further purified from contaminants by phenol (19). In addition to TLR2 and TLR6, the authors demonstrated the essential role of CD14 during activation of mouse macrophages by this LTA preparation. Comparable to the experiments in mice, we found that CD14 is indispensable for Δlgt-LTA–mediated activation of HEK293 cells and human monocytes, in which the positive selection process employing anti-CD14 Abs abrogated the response to Δlgt-LTA.

Our study revealed an important role of internalization during LTA-induced cytokine induction in human peripheral blood. The activation of blood cells by Δlgt-LTA was completely abrogated after the inhibition of phagocytosis using cytochalasin D, suggesting that Δlgt-LTA recognition via TLR2/6 and CD14 is mediated within the phagosome. Interestingly, the importance of phago somal uptake of LTA has been described in two recent studies

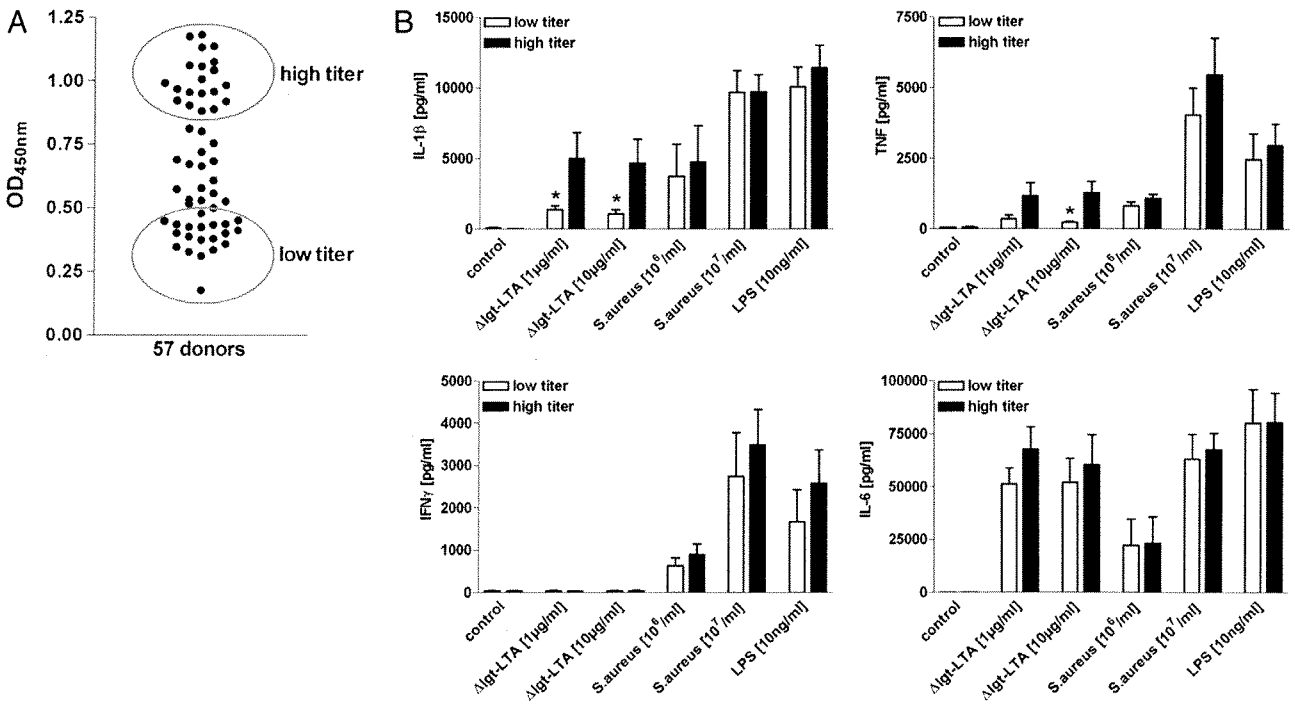

FIGURE 7. LTA-specific serum IgG titers determine immune activation in human whole blood poststimulation with Δlgt-LTA but not *S. aureus* or LPS. *A*, Δlgt-LTA (10 μg/ml) immobilized in 96-well plates was used to determine the titer of LTA-specific IgG Abs in serum samples obtained from 57 healthy blood donors. Blood from the donors showing the highest (n = 20) and the lowest LTA-specific IgG titers (n = 20) was used for further experiments. *B*, Whole blood from donors with either high or low titers of LTA-specific Abs was stimulated with immobilized Δlgt-LTA (1 μg/ml and 10 μg/ml), UV-inactivated *S. aureus* (10^6 and 10^7/ml), or LPS (10 ng/ml). After 22 h, the amount of released IL-1β, TNF, IFN-γ, and IL-6 was determined by ELISA. Data representing six different donors of each group are given as means ± SEM. For each stimulus, significant differences in cytokine release between donors with low and high titers of LTA-specific Abs were assessed by Mann-Whitney U test. $*p < 0.05$.

showing that CD36 (20) and MBL (18) strongly enhance TLR2/6-mediated recognition of LTA when delivered to the phagosome. Furthermore, CD36 expression itself was found to trigger the internalization of whole *S. aureus* and its LTA in mouse macrophages and human TLR2/6-transfected HEK293 cells, thereby allowing intracellular recognition (20). Despite this role of CD36 in LTA uptake, in our experiments with human peripheral blood, the internalization of Δlgt-LTA was mediated by FcγRII after its opsonization by IgG Abs. Abs recognizing the polyglycerol phosphate backbone of LTA have been detected in human serum, and their titers can become elevated during streptococcal infection (39, 40). Our data revealed pronounced differences in the level of LTA-specific IgG Abs among 57 healthy human donors, which were associated with the amount of released IL-1β and TNF in Δlgt-LTA–stimulated peripheral blood. The mechanism of Δlgt-LTA–induced immune activation proposed in this study involving Abs and FcγRII engagement would explain the inability of LTA to induce the release of IL-12 and subsequent IFN-γ, because ligation of phagocytic receptors on immune cells during stimulation with TLR ligands has been found to selectively downregulate IL-12 transcription (41–43). Recently, an indispensable role of *S. aureus*-specific IgG Abs has been demonstrated for the activation of human immune cells by whole *S. aureus* (44). The authors described an FcγRIIA-dependent uptake process of Ab-opsonized bacteria to be responsible for the activation of plasmacytoid dendritic cells by *S. aureus*. However, poststimulation with *S. aureus*, we observed no differences in cytokine release from blood donors with high or low LTA-specific Ab titers, suggesting that whole bacteria in peripheral blood were taken up by alternative mechanisms.

In our experiments, immobilization of Δlgt-LTA was pivotal for cytokine induction. The lack of activity observed for soluble Δlgt-LTA possibly relates to its decreased accessibility for innate immune receptors (e.g., due to formation of micelles or binding to inhibitory serum components). Indeed, serum lipoproteins have been described to bind soluble LTA with very fast kinetics (45) and were found to inhibit LTA-mediated immune activation under nonimmobilizing conditions (46, 47). In line with this, we identified human serum apolipoproteins that abrogate cytokine induction by soluble Δlgt-LTA (S. Sigel, S. Deininger, J. Hoffmann, S. v.Aulock, T. Meergans, and S. Bunk, manuscript in preparation). The interactions with inhibitory serum components might also contribute to the lower pyrogenic potential of soluble LTA preparations in vivo compared with LPS (48). In contrast to LTA preparations, naturally occurring LTA anchors in the bacterial cell membrane of *S. aureus* via its two fatty acids and projects through the peptidoglycan, an arrangement that would shield the fatty acids from interactions with serum apolipoproteins. We speculate that the immobilization of Δlgt-LTA on the hydrophobic incubation tubes observed in this study is mediated by the fatty acids, which as a result are protected from binding to inhibitory serum components, thereby preserving the activity of LTA.

The presented data clearly confirm an equipotent peripheral blood cytokine induction by Δlgt-LTA and LTA from wt *S. aureus*, but also indicate different requirements for the recognition of Δlgt-LTA and wt-LTA. As described previously (32, 33), wt-LTA–induced cytokine release was also potentiated postimmobilization, but in contrast to Δlgt-LTA, was also detected under nonimmobilizing conditions. In addition, cytokine induction by wt-LTA was less dependent on serum supplementation than Δlgt-LTA, and its recognition by TLR2-transfected HEK293 cells was independent from TLR6 and CD14 coexpression. As Δlgt-LTA does not contain any lipoprotein contaminants, and we and others found no structural differences between Δlgt-LTA and wt-LTA (C. Rockel, S. Sigel, M. Borisova, S. Deininger, C. Draing, O. Dehus, A. Ulmer, M. Pfitzenmeier, A. Geyer, F. Götz, T. Hartung, S. Bunk, C. Hermann, and S. v.Aulock, manuscript in preparation and Ref. 24), it appears that this residual activity of wt-LTA results from remaining lipoproteins, which were shown by Hashimoto and coworkers (24, 25) to elute in the same hydrophobic interaction chromatography fractions than wt-LTA. This assumption would also correlate with the ability of wt-LTA to stimulate TLR2-transfected HEK293 cells in the absence of TLR6 and CD14, a common ability of lipopeptides or lipoproteins (38). Recent reports analyzing the structure of the TLR2/TLR1 (16) and the TLR2/TLR6 (17) heterodimer in complex with TLR2 ligands emphasized the role of the ligands' two fatty acids as being the main driving force for TLR2 binding. This role of fatty acids likely explains why both Δlgt-LTA and wt-LTA initiate the recruitment of TLR2 to the membrane lipid raft of stimulated human monocytes. The selective requirement of TLR2/TLR6 heterodimerization for the recognition of Δlgt-LTA support the specificity of LTA-induced immune activation, because the fatty acid-driven binding to TLR2 alone has only a limited capability in discriminating bacterial ligands from the abundant diacetylated molecules found in humans, like phospholipids (16, 17). However, the ability of lipoproteins to activate TLR2-transfected cells in absence of TLR6 indicates that specific immune activation by other TLR2 ligands can also occur without TLR2/TLR6 heterodimerization. Lipoproteins were found to play an important role in the host defense against *S. aureus* in mice (49), and, as described in this paper, lipoprotein contaminants of wt-LTA do induce cytokines from human whole blood. In our experiments using conditions conducive to Δlgt-LTA–mediated immune activation, we found no differences in the immunostimulatory capacity of Δlgt-LTA and wt-LTA, arguing for a limited contribution of lipoprotein contaminants to the activity of wt- LTA. Our results also suggest that the stimulatory capacity of LTA in general is strongly underestimated in our experimental setup, because only a low proportion of LTA becomes immobilized (1–4%) (32) and, thus, is able to activate immune cells.

Based on our data, we suggest a mechanism for LTA-mediated immune activation in human blood cells that involves opsonization of immobilized LTA by specific Abs, thereby enabling phagocytic uptake of LTA and subsequent intracellular recognition driven by TLR2, TLR6, and CD14. Although the Ab-mediated uptake of LTA might play only a minor role in the recognition of LTA anchoring in the cell membrane of *S. aureus*, the current study uncovers important requirements for LTA-induced immune activation in general that should be taken into consideration when working with purified LTA. Furthermore, it offers a satisfying explanation for the discrepant immunobiologically activity of Δlgt-LTA observed in different laboratories.

Acknowledgments

We thank Corinna Hermann, Christof Hauck, and Christian Hesslinger for helpful discussions and Tobias Speicher for conceptual support. We also thank Leonardo Cobianchi and Margarete Kreuer-Ullmann for technical assistance and Sonja Erath for experimental help.

Disclosures

The authors have no financial conflicts of interest.

References

1. von Eiff, C., K. Becker, K. Machka, H. Stammer, G. Peters; Study Group. 2001. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N. Engl. J. Med.* 344: 11–16.
2. Todd, J. K. 2005. Staphylococcal infections. *Pediatr. Rev.* 26: 444–450.
3. Jonsson, I. M., S. K. Mazmanian, O. Schneewind, T. Bremell, and A. Tarkowski. 2003. The role of *Staphylococcus aureus* sortase A and sortase B in murine arthritis. *Microbes Infect.* 5: 775–780.
4. Thomas, C. A., Y. Li, T. Kodama, H. Suzuki, S. C. Silverstein, and J. El Khoury. 2000. Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. *J. Exp. Med.* 191: 147–156.

5. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20: 197–216.
6. Inohara, Chamaillard, C. McDonald, and G. Nuñez. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* 74: 355–383.
7. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–801.
8. Hoebe, K., P. Georgel, S. Rutschmann, X. Du, S. Mudd, K. Crozat, S. Sovath, L. Shamel, T. Hartung, U. Zähringer, and B. Beutler. 2005. CD36 is a sensor of diacylglycerides. *Nature* 433: 523–527.
9. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol.* 165: 5392–5396.
10. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168: 4531–4537.
11. Seo, H. S., S. M. Michalek, and M. H. Nahm. 2008. Lipoteichoic acid is important in innate immune responses to gram-positive bacteria. *Infect. Immun.* 76: 206–213.
12. Buwitt-Beckmann, U., H. Heine, K. H. Wiesmüller, G. Jung, R. Brock, and A. J. Ulmer. 2005. Lipopeptide structure determines TLR2 dependent cell activation level. *FEBS J.* 272: 6354–6364.
13. Dziarski, R., and D. Gupta. 2006. The peptidoglycan recognition proteins (PGRPs). *Genome Biol.* 7: 232.
14. Travassos, L. H., S. E. Girardin, D. J. Philpott, D. Blanot, M. A. Nahori, C. Werts, and I. G. Boneca. 2004. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep.* 5: 1000–1006.
15. Dziarski, R., and D. Gupta. 2005. *Staphylococcus aureus* peptidoglycan is a toll-like receptor 2 activator: a reevaluation. *Infect. Immun.* 73: 5212–5216.
16. Jin, M. S., S. E. Kim, J. Y. Heo, M. E. Lee, H. M. Kim, S. G. Paik, H. Lee, and J. O. Lee. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 130: 1071–1082.
17. Kang, J. Y., X. Nan, M. S. Jin, S. J. Youn, Y. H. Ryu, S. Mah, S. H. Han, H. Lee, S. G. Paik, and J. O. Lee. 2009. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* 31: 873–884.
18. Ip, W. K., K. Takahashi, K. J. Moore, L. M. Stuart, and R. A. Ezekowitz. 2008. Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome. *J. Exp. Med.* 205: 169–181.
19. Jimenez-Dalmaroni, M. J., N. Xiao, A. L. Corper, P. Verdino, G. D. Ainge, D. S. Larsen, G. F. Painter, P. M. Rudd, R. A. Dwek, K. Hoebe, et al. 2009. Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2. *PLoS ONE* 4: e7411.
20. Stuart, L. M., J. Deng, J. M. Silver, K. Takahashi, A. A. Tseng, E. J. Hennessy, R. A. Ezekowitz, and K. J. Moore. 2005. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. *J. Cell Biol.* 170: 477–485.
21. Morath, S., A. Geyer, and T. Hartung. 2001. Structure-function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*. *J. Exp. Med.* 193: 393–397.
22. Morath, S., A. Stadelmaier, A. Geyer, R. R. Schmidt, and T. Hartung. 2002. Synthetic lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus of cytokine release. *J. Exp. Med.* 195: 1635–1640.
23. Deininger, S., A. Stadelmaier, S. von Aulock, S. Morath, R. R. Schmidt, and T. Hartung. 2003. Definition of structural prerequisites for lipoteichoic acid-inducible cytokine induction by synthetic derivatives. *J. Immunol.* 170: 4134–4138.
24. Hashimoto, M., K. Tawaratsumida, H. Kariya, A. Kiyohara, Y. Suda, F. Krikae, T. Kirikae, and F. Götz. 2006. Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in *Staphylococcus aureus*. *J. Immunol.* 177: 3162–3169.
25. Hashimoto, M., M. Furuyashiki, R. Kaseya, Y. Fukada, M. Akimaru, K. Aoyama, T. Okuno, T. Tamura, T. Kirikae, F. Kirikae, et al. 2007. Evidence of immunostimulating lipoprotein existing in the natural lipoteichoic acid fraction. *Infect. Immun.* 75: 1926–1932.
26. Hashimoto, M., K. Tawaratsumida, H. Kariya, K. Aoyama, T. Tamura, and Y. Suda. 2006. Lipoprotein is a predominant Toll-like receptor 2 ligand in *Staphylococcus aureus* cell wall components. *Int. Immunol.* 18: 355–362.
27. Hashimoto, M., M. Furuyashiki, and Y. Suda. 2007. Response to Comment on "Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus" *J. Immunol.* 178: 2610; author reply 2610–2611.
28. von Aulock, S., T. Hartung, and C. Hermann. 2007. Comment on "Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus". *J. Immunol.* 178: 2610, author reply 2610–2611.
29. Wu, P., and L. Brand. 1994. Resonance energy transfer: methods and applications. *Anal. Biochem.* 218: 1–13.
30. Kenworthy, A. K., and M. Edidin. 1998. Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of <100 A using imaging fluorescence resonance energy transfer. *J. Cell Biol.* 142: 69–84.
31. Kenworthy, A. K., and M. Edidin. 1999. Imaging fluorescence resonance energy transfer as probe of membrane organization and molecular associations of GPI-anchored proteins. *Methods Mol. Biol.* 116: 37–49.
32. Deininger, S., S. Traub, D. Aichele, T. Rupp, T. Baris, H. M. Möller, T. Hartung, and S. von Aulock. 2008. Presentation of lipoteichoic acid potentiates its inflammatory activity. *Immunobiology* 213: 519–529.
33. Nilsen, N. J., S. Deininger, U. Nonstad, F. Skjeldal, H. Husebye, D. Rodionov, S. von Aulock, T. Hartung, E. Lien, O. Bakke, and T. Espevik. 2008. Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: role of CD14 and CD36. *J. Leukoc. Biol.* 84: 280–291.
34. Triantafilou, M., M. Manukyan, A. Mackie, S. Morath, T. Hartung, H. Heine, and K. Triantafilou. 2004. Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. *J. Biol. Chem.* 279: 40882–40889.
35. Kalsi, J., C. T. Ravirajan, A. Rahman, and D. A. Isenberg. 1999. Structure-function analysis and the molecular origins of anti-DNA antibodies in systemic lupus erythematosus. *Expert Rev. Mol. Med.* 1999: 1–28.
36. Hermann, C., I. Spreitzer, N. W. Schröder, S. Morath, M. D. Lehner, W. Fischer, C. Schütt, R. R. Schumann, and T. Hartung. 2002. Cytokine induction by purified lipoteichoic acids from various bacterial species—role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN-gamma release. *Eur. J. Immunol.* 32: 541–551.
37. Takeuchi, O., T. Kawai, P. F. Mühlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13: 933–940.
38. Zähringer, U., B. Lindner, S. Inamura, H. Heine, and C. Alexander. 2008. TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. *Immunobiology* 213: 205–224.
39. Markham, J. L., K. W. Knox, R. G. Schamschula, and A. J. Wicken. 1973. Antibodies to teichoic acids in man. *Arch. Oral Biol.* 18: 313–319.
40. Gargir, A., I. Ofek, D. Hasty, S. Meron-Sudai, H. Tsubery, Y. Keisari, and A. Nissim. 2001. Inhibition of antibody-dependent stimulation of lipoteichoic acid-treated human monocytes and macrophages by polyglycerolphosphate-reactive peptides. *J. Leukoc. Biol.* 70: 537–542.
41. Sutterwala, F. S., G. J. Noel, R. Clynes, and D. M. Mosser. 1997. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J. Exp. Med.* 185: 1977–1985.
42. Marth, T., and B. L. Kelsall. 1997. Regulation of interleukin-12 by complement receptor 3 signaling. *J. Exp. Med.* 185: 1987–1995.
43. Grazia Cappiello, M., F. S. Sutterwala, G. Trinchieri, D. M. Mosser, and X. Ma. 2001. Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. *J. Immunol.* 166: 4498–4506.
44. Parcina, M., C. Wendt, F. Goetz, R. Zawatzky, U. Zähringer, K. Heeg, and I. Bekeredjian-Ding. 2008. *Staphylococcus aureus*-induced plasmacytoid dendritic cell activation is based on an IgG-mediated memory response. *J. Immunol.* 181: 3823–3833.
45. Levels, J. H., P. R. Abraham, E. P. van Barreveld, J. C. Meijers, and S. J. van Deventer. 2003. Distribution and kinetics of lipoprotein-bound lipoteichoic acid. *Infect. Immun.* 71: 3280–3284.
46. Grunfeld, C., M. Marshall, J. K. Shigenaga, A. H. Moser, P. Tobias, and K. R. Feingold. 1999. Lipoproteins inhibit macrophage activation by lipoteichoic acid. *J. Lipid Res.* 40: 245–252.
47. Mueller, M., C. Stamme, C. Draing, T. Hartung, U. Seydel, and A. B. Schromm. 2006. Cell activation of human macrophages by lipoteichoic acid is strongly attenuated by lipopolysaccharide-binding protein. *J. Biol. Chem.* 281: 31448–31456.
48. De Kimpe, S. J., M. Kengatharan, C. Thiemermann, and J. R. Vane. 1995. The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc. Natl. Acad. Sci. USA* 92: 10359–10363.
49. Bubeck Wardenburg, J., W. A. Williams, and D. Missiakas. 2006. Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proc. Natl. Acad. Sci. USA* 103: 13831–13836.