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Influence of serum on the immune recognition of a synthetic lipopeptide mimetic of the 19-kDa lipoprotein from *Mycobacterium tuberculosis*

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The innate immune response provides a critical first-line defense against *Mycobacterium tuberculosis*, an intracellular pathogen that represents a major health threat world-wide. A synthetic lipopeptide (LP) mimicking the lipid moiety of the cell-wall associated 19-kDa lipoprotein from *M. tuberculosis* has recently been assigned an important role in the induction of an antibacterial immune response in host macrophages. Here, we present experimental data on the biological activities and the biophysical mechanisms underlying cell activation by synthetic 19-kDa *M. tuberculosis*-derived lipopeptide (Mtb-LP). Investigation of the geometry of the LP (*i.e.* the molecular conformation and supramolecular aggregate structure) and the preference for membrane intercalation provide an explanation for the biological activities of the mycobacterial LP. Cell activation by low concentrations of Mtb-LP was enhanced by the lipopolysaccharide–binding protein and CD14. However, surprisingly, we found that activation of human macrophages to induce pro- as well as anti-inflammatory mediators (tumor necrosis factor(TNF)-α, Interleukin(IL)-6, IL-8, and IL-10) in response to the Mtb-LP is strongly reduced in the presence of serum. This observation could be confirmed for the immune response of murine macrophages which showed a strongly enhanced TNF-α release in the absence of serum, suggesting that the molecular mechanisms of immune recognition of the Mtb-LP are tailored to the ambient conditions of the lung.

Keywords: Monocytes/macrophages, cell activation, inflammation, lipopeptide, lung

Abbreviations: BMDM, bone marrow derived macrophages; FCS, fetal calf serum; FRET, Förster resonance energy transfer; IL, interleukin; LBP, lipopolysaccharide binding protein; LP, lipopeptide; LPS, lipopolysaccharide; Mtb-LP, synthetic 19-kDa *M. tuberculosis*-derived lipopeptide tripalmitoyl-*S*-glyceryl-L-Cys-SSSNKSTTGSGETTTA; Pam_3CSK_4, tripalmitoyl-*S*-glyceryl-L-Cys-Ser-$(Lys)_4$; PS, phosphatidylserine; TLR, Toll-like receptor; TNF, tumor necrosis factor.

Introduction

Recognition of bacterial virulence factors by receptors of the innate immune system provides a rapid and effective immune response via the induction of pro-inflammatory and anti-microbial mediators. In mammals, the Toll-like receptor (TLR) family of pattern-recognition receptors participates in conferring sensitive recognition of

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bacterial pathogens. Toll-like receptors 2 and 4 have both been assigned important roles in the innate immune defense against bacterial infection, recognising cell-wall associated compounds of bacterial pathogens such as lipopolysaccharides (LPSs) and lipoproteins, respectively.[1,2]

Tuberculosis caused by infection with *Mycobacterium tuberculosis* is a world-wide health threat with over 2 billion infected people, and strains with multiple antibiotic resistances are emerging increasingly. The 19-kDa antigen is a cell-wall associated lipoprotein expressed in *M. tuberculosis*. Besides in some slow-growing mycobacterial strains, homologues of this antigen have not been identified.[3] The biological function of the 19-kDa protein is still obscure. In *M. tuberculosis*, the 19-kDa protein has been identified as a major antibody-inducing antigen,[4,5] and was the first antigen that was demonstrated to induce a specific T-cell response.[6,7] Later, it was shown to induce a variety of biological responses in the course of tuberculosis infection.[8–12] The induction of host defense mechanisms targeted to mycobacterial killing is centered on the activation of host macrophages, representing the major target cell of *M. tuberculosis*.

Macrophages infected with mycobacteria induce a variety of intracellular signalling events, including MyD-88-dependent TLR downstream signaling and the activation of the triad of mitogen-activating kinases, leading to the induction of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α) and phagocyte chemoattractants (IL-8). Cytokine-activated T-cells provide interferon-γ (IFN-γ) as stimulus for macrophages to produce reactive oxygen species which have been implicated as a potent antimicrobial effector mechanism. Activation of macrophages has been assigned to several molecules of the mycobacterial cell wall, including phosphatidylinositolmannosides, lipomannans, lipoarabinomannans, and LP.[14–18] However, most of these molecules represent rather weak stimuli of macrophage activation and their relative contribution to an effective immune response remains to be elucidated.[19–21] In a previous study, we have shown that physicochemical parameters are important determinants for the biological activities of LP.[22] However, lipoproteins or LP from *M. tuberculosis* have not been characterized physicochemically so far. Recently, a synthetic LP mimicking the lipid moiety of the 19-kDa lipoprotein has been assigned an important function in the induction of an antimicrobial immune response to *M. tuberculosis* by stimulation of a TLR2/TLR1-dependent signalling pathway. Gene expression profiling of human monocytes and macrophages in a gene array approach identified that stimulation with the synthetic *M. tuberculosis*-derived LP induces the expression of vitamin D receptor and vitamin D_1-hydroxylase genes and the downstream induction of antimicrobial peptides.[12] This signaling cascade was found to be accompanied by improved mycobacterial killing and could subsequently be assigned to the expression and activity of cathelicidin,[23] suggesting a significant contribution of the mycobacterial LP to the containment of the infection.

In the present study, we have investigated the response of macrophages to the synthetic LP mimicking the lipid moiety of the 19-kDa lipoprotein of *M. tuberculosis* (Mtb-LP) and characterised the physicochemical properties in relation to the bioactivity of this molecule. Our results suggest that the mechanisms of innate immune recognition of the Mtb-LP are adapted to the environment of the lung, thus allowing a sensitive recognition by macrophages in this environment.

Materials and Methods

Synthesis

The Mtb-LP mimicking the lipid moiety of the 19-kDa lipoprotein from *M. tuberculosis* Pam3-CSSSNKSTTGSGETTTA and the LP Pam_3CSK_4 were synthesized and analyzed by EMC microcollections GmbH according to published procedures.[24–26] The structural formula of the synthetic compound tripalmitoyl-*S*-glyceryl-L-Cys-SSSNKSTTGSGETTTA (Mtb-LP) is shown in Figure 1A. A peptide CSSSNKSTTGSGETTTA without the lipid anchor was synthesized with an amidated C-terminus by the solid-phase peptide synthesis technique on an automatic peptide synthesizer (model 433 A; Applied Biosystems) and purified by reversed-phase HPLC. Purity was determined by matrix-assisted laser-desorption-time-of-flight mass spectrometry (MS; Bruker).

Reagents

Deep rough mutant LPS (Re LPS) was extracted from *Salmonella enterica* sv. Minnesota strain R595 according to the phenol/chloroform/light petroleum procedure.[27] The LPS preparation was lyophilized and used in the natural salt form. The chemical purity of the LPS preparation was confirmed by MS. The LPS from *Escherichia coli* strain K235 was purchased from List Biochemicals Inc. (Campbell, CA, USA) and purified by phenol-water extraction according to a published procedure.[28] Smooth LPS from *Salmonella enterica* sv. Friedenau was a kind gift from Helmut Brade (Research Center Borstel, Germany). The LPS was suspended in PBS (Biochrom, Berlin, Germany) by thorough vortexing. The suspensions were temperature-cycled twice between 4°C and 56°C, each cycle being followed by intense vortexing for a few minutes, and then stored at 4°C for at least 12 h prior to measurement.

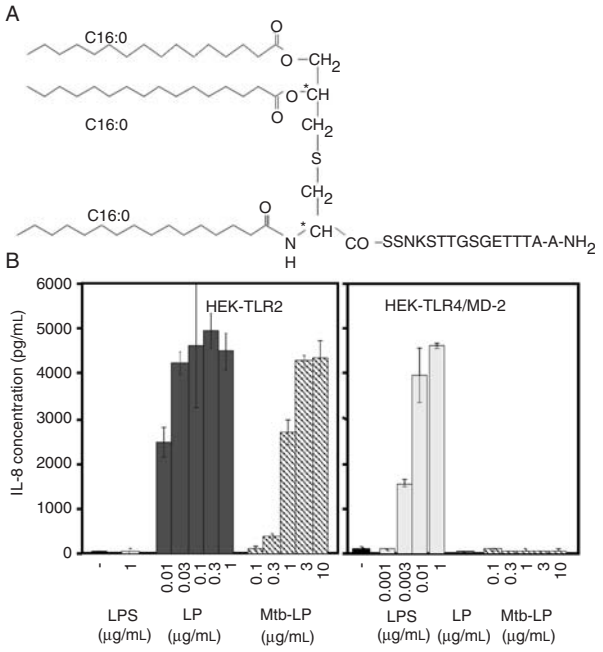

Fig. 1. Chemical structure of a synthetic Mtb-LP and activation of innate immune receptors by the lipopeptide. (A) Chemical structure of synthetic tripalmitoyl-*S*-glyceryl-L-Cys-SSSNKSTTGSGETTTA (Mtb-LP). (B) HEK293 cells expressing either TLR2 or TLR4/MD-2 were stimulated with LPS (smooth LPS from *E. coli* strain K235), Pam_3CSK_4 (LP) or Mtb-LP at the indicated concentrations. After 24 h, supernatants were collected for the determination of the chemokine IL-8. Data shown are mean ± SD of triplicate samples of one experiment representative of at least three independent experiments.

Suspensions were aliquoted and stored at −20°C. Phosphatidylserine (PS) from bovine brain was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. The fluorescent dyes *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and *N*-(rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Molecular Probes (Eugene, OR, USA). Recombinant human LBP (456 amino acid holoprotein $rLBP_{50}$) in 10 mM HEPES, pH 7.5 was kindly provided by XOMA LLC (Berkeley, CA, USA). Anti-CD14 antibody biG 14 was purchased from Biometec (Greifswald, Germany), control mouse IgG_{2a} was obtained from BD Biosciences (Heidelberg, Germany).

Lipid sample preparation

The Mtb-LP was dissolved as stock (10 mg/mL) in DMSO. Pam_3CSK_4 was dissolved as stock (1 mg/mL) in endotoxin-free water (Braun, Melsungen, Germany). For this, the LP was suspended directly in buffer, thoroughly vortexed, temperature-cycled twice between 4°C and 56°C and then stored for at least 12 h before measurement. Aliquots of stock solutions were stored at −20°C. Working solutions were prepared from stocks by dilution in 20 mM HEPES, pH 7.0.

Activation of human macrophages

Human mononuclear cells (MNCs) were isolated from peripheral blood of healthy donors by the Hypaque-Ficoll gradient method and cultivated at 37°C with 6% CO_2 in Teflon bags in RPMI 1640 medium (endotoxin ≤0.01 EU/mL, Biochrom, Berlin, Germany) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 4% heat-inactivated human serum type AB from healthy donors. Cells were cultured in the presence of 2 ng/mL M-CSF for 7 days to differentiate monocytes to macrophages. To determine cytokine induction after cell stimulation, macrophages were seeded in 200 mL aliquots of 1×10^6 cells/mL into 96-well tissue culture dishes (Nunc, Wiesbaden, Germany) in RPMI 1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, with or without 4% human serum and stimuli were added as indicated in the respective experiment. Cell-free supernatants were collected 4 h after stimulation for the determination of TNF-α and 24 h after stimulation for the determination of IL-6, IL-8 and IL-10, respectively, and stored at −20°C until determination of cytokine content. Data shown are mean (±SD) of triplicate samples of one experiment and representative of at least three independent experiments with cells from different donors.

Activation of murine macrophages

Murine bone marrow derived macrophages (BMDMs) from C57BL/6 mice were generated as previously described.[29] For stimulation, macrophages were cultured in DMEM (PAA, Pasching, Austria) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 10 mM glutamine, 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany). Triplicates of BMDMs were incubated for 6 h with increasing concentrations of the Mtb-LP or LPS (*S. enterica* sv. Friedenau, 10 ng/mL) in the absence or presence of 1% and 10% FCS. Data shown are mean (±SD) of triplicate samples of one experiment and representative of three independent experiments.

Stimulation of HEK293 cells

HEK-TLR2 cells were established by transfection of HEK293 cells with expression plasmids coding for human TLR2 (the expression plasmid for TLR2 was a kind gift of Douglas Golenbock, University of

Massachusetts Medical School, Worcester, MA, USA). Transfected cells were selected in the presence of geniticin (G418). Stable clones were generated by limited dilution technique and maintained at 37°C under an atmosphere of 5% CO_2 in DMEM (Biochrom AG, Berlin, Germany) containing 10% fetal calf serum (FCS; Linaris, Bettingen am Main, Germany), 0.5 U/mL penicillin, 0.5 μg/mL streptomycin (Biochrom AG), and 0.4 μg/mL G418. The generation of a HEK-TLR4/MD-2 cell line was described in detail earlier.[30] Cell lines were grown at 37°C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% FCS, 0.5 U/mL penicillin, 0.5 μg/mL streptomycin, and 0.4 μg/mL G418 (HEK293-TLR2) or 500 U/mL hygromycin and 0.4 μg/mL G418 (HEK293-TLR4/MD-2). For cell stimulation experiments, HEK293 cell lines were plated in 200 μL aliquots at a density of 2.5×10^5 cells/mL in 96-well plates in DMEM supplemented with 10% FCS, 0.5 U/mL penicillin, 0.5 μg/mL streptomycin. Cells were stimulated with the TLR4-dependent ligand LPS (smooth LPS from *E. coli* strain K235), the TLR2-dependent LP Pam_3CSK_4 (100 ng/mL) or Mtb-LP at the indicated concentrations for 24 h. Cell-free supernatants were collected and stored at −20°C until determination of the chemokine IL-8 by ELISA.

Cytokine determination

Human TNF-α and human IL-6 were determined in combined cell-free supernatants of stimulated cells by sandwich ELISA using monoclonal mouse antibody against human IL-6 and POD-conjugated rabbit anti-human IL-6 antibody, and monoclonal mouse antibody against human TNF-α and POD-conjugated rabbit anti-human TNF-α antibody, respectively (Intex, Muttant, Switzerland) as stated in detail elsewhere.[31] Human IL-8 was determined by sandwich ELISA using IL-8 cytoset from Biosource (Solingen, Germany) exactly according to the manufacturer's protocol. Human IL-10 and murine TNF-α were determined in cell-free supernatants using a Ready-Set-Go! kits from eBiosciences (San Diego) and DuoSet ELISA kits from R&D Systems (Wiesbaden, Germany), respectively.

Förster resonance energy transfer spectroscopy

The Förster resonance energy transfer (FRET) technique was used as a probe dilution assay[32] to obtain information on the intercalation of the synthetic LP into phospholipid liposomes. For preparation of liposomes, PS was solubilised in chloroform and double-labeled with NBD-PE and Rh-PE in chloroform [PS]:[NBD-PE]:[Rh-PE] at 100:1:1 molar ratios. The solvent was evaporated under a stream of nitrogen, and the lipids were resuspended in the appropriate volume of PBS pH 7.0, mixed thoroughly, sonicated with a Branson sonicator for 1 min (1 mL solution), and temperature-cycled as described above. A preparation of 900 μL of the double-labeled liposomes (10^{-5} M) at 37°C was excited at 470 nm (excitation wavelength of NBD-PE), and the intensities of the emission light of the donor NBD-PE (531 nm) and acceptor Rh-PE (593 nm) were measured simultaneously on the fluorescence spectrometer SPEX F1T11 (SPEX Instr., Edison, NY, USA). Mtb-LP was added to liposomes after 50 s at a 1 μM concentration, PBS as a dilution control or recombinant LBP at 5.5 μg/mL were added after 100 s. Since FRET spectroscopy is used here as a probe dilution assay, intercalation of unlabeled molecules causes an increase of the distance between donor and acceptor and, thus, leads to a reduced energy transfer. This again causes an increase of the donor and decrease of the acceptor intensities. For a qualitative analysis of experiments, the ratio of the intensities of the donor dye and the acceptor dye are plotted against time. The data shown are representative for three independent experiments.

Fourier-transformed infrared spectroscopy

The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). For phase transition measurements, the lipid sample was placed between CaF_2 windows with a 12.5 μM Teflon spacer. Temperature scans were performed automatically between −10°C and 70°C with a heating rate of 0.6°C/min. Every 3°C, 50 interferograms were accumulated, apodized, Fourier-transformed, and converted to absorbance spectra. For the identification of particular functional groups, infrared spectra of the LP at 95% water content were analysed. The vibrational bands typical for the hydrophobic region (symmetrical and antisymmetrical) ν_s and ν_{as} stretching vibration of $-CH_2-$ groups around 2920 cm^{-1} and 2850 cm^{-1}, respectively, in the IR-spectra of LP are sensitive markers of acyl chain order.

X-Ray diffraction

The measurements of X-ray diffraction were performed at the European Molecular Biology Laboratory (EMBL) outstation at the Hamburg synchrotron radiation facility HASYLAB using the SAXS camera X33.[33] Diffraction patterns in the range of the scattering vector $0.1 < s < 1.0\ nm^{-1}$ ($s = 2\sin\theta/\lambda$, 2θ scattering angle and λ the wavelength = 0.15 nm) were recorded at 40°C with exposure times of 1 min using an image plate detector with online readout (MAR345; MarResearch, Norderstedt, Germany). The s-axis was calibrated

with Ag-Behenate which has a periodicity of 5.84 nm. The diffraction patterns were evaluated as described previously assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures. The cubic structures are most relevant here. They are characterized by the following features. The different space groups of these non-lamellar three-dimensional structures differ in the ratio of their spacings. The relation between reciprocal spacing $s_{hkl} = 1/d_{hkl}$ and lattice constant 'a' is:

$$s_{hkl} = [(h^2 + k^2 + l^2)/a]^{1/2} \quad (1)$$

where hkl = Miller indices of the corresponding set of plane.

RESULTS

The synthetic LP mimetic Mtb-LP induces a TLR2-dependent innate immune response

The 19-kDa lipoprotein from *M. tuberculosis* has been shown to induce a TLR2-dependent macrophage response.[8,10,34] In order to analyse the ability of the synthetic LP mimetic of the 19-kDa lipoprotein to activate receptors of the innate immune system, we investigated the activation of HEK293 cell lines heterologously expressing TLR2 or TLR4/MD-2. Clearly, the Mtb-LP showed a TLR2-dependent activation of HEK293 cells to produce the chemokine IL-8 starting at 1 μg/mL and increasing with increasing dose of the LP (Fig. 1B, left panel). In contrast, activation of a TLR4/MD-2-dependent response was not observed up to a concentration of 10 μg/mL of the Mtb-LP, whereas LPS induced a sensitive and dose-dependent activation of these cells (Fig. 1B, right panel). These data confirm a TLR2-dependent biological activity of the synthetic Mtb-LP. Interestingly, the efficacy of cell activation by the Mtb-LP was about 100-fold reduced compared to the efficacy of the synthetic LP Pam_3CSK_4 which induced cell activation already at 0.01 μg/mL suggesting that the peptide part of the LP has a strong impact on the biological activity.

Physicochemical characterization of the Mtb-LP

A variety of investigations into the prerequisites of cell activation by amphiphilic molecules like phospholipids and glycolipids have revealed that physical parameters are strong determinants of biological activity: Above a threshold concentration, defined as the critical micellar concentration (CMC), these molecules form aggregates in an aqueous environment. The type of aggregate structure, uni- and multilamellar, cubic direct or inverted, hexagonal H_I or inverted H_{II} strongly impacts their behavior in biological systems[35] – for review, see Seydel *et al.*[36] For LPS (endotoxin) from the outer membrane of Gram-negative bacteria, we have shown that only compounds with a lipid A moiety adopting cubic inverted aggregate structures (which are formed by conically shaped molecules) exhibit high biological activity, whereas lamellar aggregate structures formed by cylindrical molecules exhibit low or no biological activity. Accordingly, biologically highly active enterobacterial LPS with its lipid anchor lipid A has been shown to predominantly adopt cubic inverted aggregates.[37–39] Therefore, we here investigated the three-dimensional aggregate structure of aqueous aggregate dispersions of the Mtb-LP using synchrotron radiation small-angle scattering (SAXS). In Figure 2A (left panel) diffraction patterns are presented at temperatures between 5–75°C. These patterns are indicative of a complex non-lamellar aggregate structure. In Figure 2B (right panel), the pattern at 40°C is plotted showing five peaks, some of which can be inter-related: For example, the reflexion at 4.91 nm obeys the relation $4.91\,\text{nm} = 7.00\,\text{nm}/\sqrt{2}$. Furthermore, the relation $9.11\,\text{nm} = 5.26\,\text{nm} \times \sqrt{3}$ holds. These peak positions are in good correlation with the pattern found for the aggregate structure of Pam_3CSK_4[22] and are characteristic for cubic phases[40] which are associated with high biological activity.

An important aspect of cell activation by glycolipids and LP is their transport by the serum-protein LPS-binding-protein (LBP) to the host cell membrane and the surface antigen CD14 on myelomonocytic cells. Förster resonance energy spectroscopy was applied to investigate whether the LP is able to incorporate into artificial membranes. Indeed, the LP itself showed only a weak tendency to intercalate into phospholipid liposomes spontaneously (Fig. 2B, black line, addition of the LP at t = 50 s is indicated by arrow 1). However, the addition of LBP at t = 100 s (indicated by arrow 2) induces a much stronger increase of the FRET-signal than in the absence of the LP (dashed line) which is indicative of a transport of the LP into the liposomal membrane. Analogously to what has been reported for the transport of LPS from Gram-negative bacteria,[32] LBP also operates as a transport protein for the Mtb-LP. This transport may facilitate the delivery of the LP to receptor proteins in the cytoplasmic membrane.

Lipids exhibit a characteristic phase behavior. Depending on the fluidity of the acyl chains, lipids can either be in a gel (β)-phase or in a fluid liquid-crystalline (α)-phase. Between both phases, a main phase transition occurs at a characteristic temperature T_c. The fluidity of the acyl chains may modulate the three-dimensional structure of aggregates, their stability, their interaction with phospholipid membranes as well as their interaction with proteins, and thus may modulate the

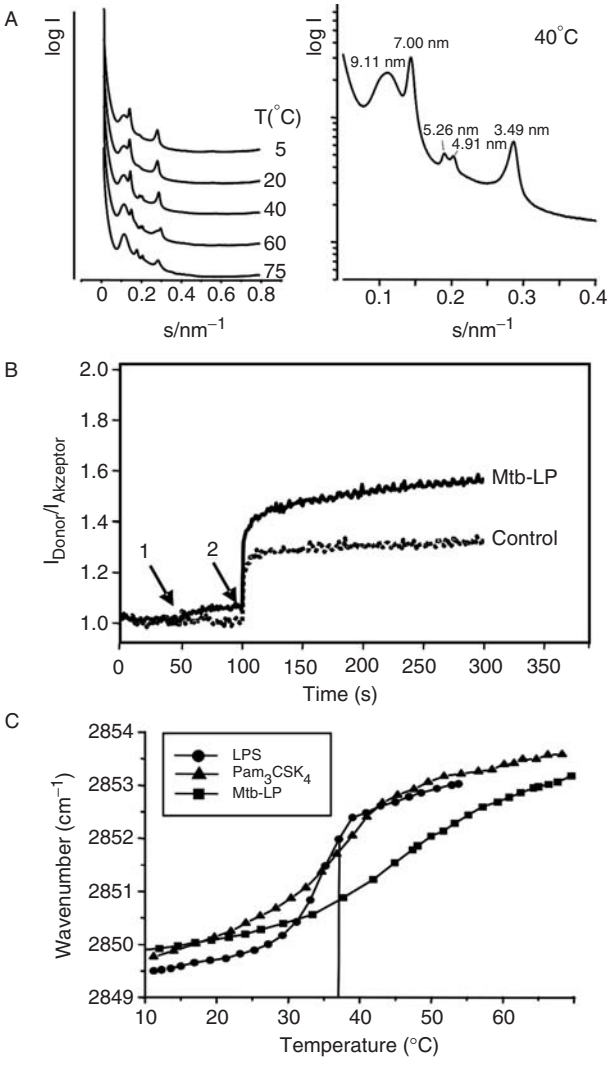

Fig. 2. Physicochemical analysis of the Mtb-LP. (A) Small-angle X-ray diffraction patterns of the Mtb-LP at 95% water content and five temperatures (left) and at 40°C (right). The logarithm of the scattering intensity is plotted versus the scattering vector s (=1/d; d = spacing). (B) Incorporation of the Mtb-LP into PS liposomes by Förster resonance energy transfer spectroscopy (FRET). The FRET signal ID/IA is plotted versus time. The Mtb-LP was added at T = 50 s to PS-liposomes to a final concentration of 1 μM, at time point 100 s human recombinant LBP protein was added to a final concentration of 5.5 μg/mL. Data are representative for three independent experiments. (C) Peak position of the symmetric stretching vibrational band of the methylene groups versus temperature for deep rough mutant LPS (Re LPS) from *S. enterica* sv. Minnesota strain R595, Pam_3CSK_4, and the Mtb-LP determined with Fourier-transform infrared spectroscopy (FTIR). In the gel phase, the peak position lies around 2850 cm^{-1}, in the liquid crystalline phase between 2852.5 cm^{-1} and 2853.0 cm^{-1}.

biological activity. We employed infrared spectroscopy to investigate the phase behavior of the Mtb-LP, and have compared the data with those from well-known rough mutant LPS and lipopeptide Pam_3CSK_4. In Figure 2C, the peak position of the symmetric stretching vibration of the methylene groups of the acyl chains are plotted versus temperature. As can be seen, the curves for LPS R595 and Pam_3CSK_4 are rather similar showing a phase transition temperature at 35°C and a wave-number value of around 2851.7 cm^{-1} at 37°C corresponding to a relatively high fluidity of the acyl chains. In contrast, the phase transition of the Mtb-LP is much broader with an approximate value around 45°C. Parallel to this, the wave-number value at 37°C lies around 2850.8 cm^{-1} corresponding to a relatively low fluidity.

Pro-inflammatory response of human macrophages to the Mtb-LP

Macrophages are the primary target cell of *M. tuberculosis*. We investigated the activation of human monocyte-derived macrophages to characterize the cellular pro-inflammatory response to the synthetic Mtb-LP. Stimulation of macrophages with the Mtb-LP in the presence of 4% human serum led to a dose-dependent production of the pro-inflammatory cytokine TNF-α (Fig. 3A). Under these conditions, 3–10 μg/mL Mtb-LP were required to induce TNF-α production. In contrast, a synthetic peptide corresponding to the Mtb-LP without the lipid anchor did not induce cytokine induction up to a concentration of 100 μg/mL (Fig. 3A). Cell activation by a variety of bacterial virulence factors depends on the presence of the myelomonocytic surface antigen CD14 (mCD14) or the corresponding soluble CD14 (sCD14) in serum. In order to elucidate the role of CD14 in the response of macrophages to the Mtb-LP, we employed a neutralizing antibody to CD14. In the presence of anti-CD14 antibody, the production of TNF-α induced by low doses of the Mtb-LP (100 ng/mL and 300 ng/mL) was reduced, whereas a control IgG antibody had no effect on the cytokine response (Fig. 3B, upper panel). Analysis of the data from cells of three different donors confirmed the inhibitory effect of anti-CD14 antibody at low concentrations of Mtb-LP (100 ng/mL and 300 ng/mL; Fig. 3B, lower panel). However, at microgram concentrations of the LP, the presence of the anti-CD14 antibody did not inhibit the cytokine response. In case of cell stimulation by LPS, the same concentration of anti-CD14 antibody completely inhibited the activation of macrophages by LPS at a concentration (10 ng/mL) that induces maximal cytokine production. These data suggest that the cellular response to low doses of the Mtb-LP is modulated by CD14 and suggests that CD14 does play a role in this activation pathway.

Cell activation by the Mtb-LP is strongly enhanced under serum-free conditions

The main cellular compartment of macrophages encountering contact with *M. tuberculosis* is the lung.

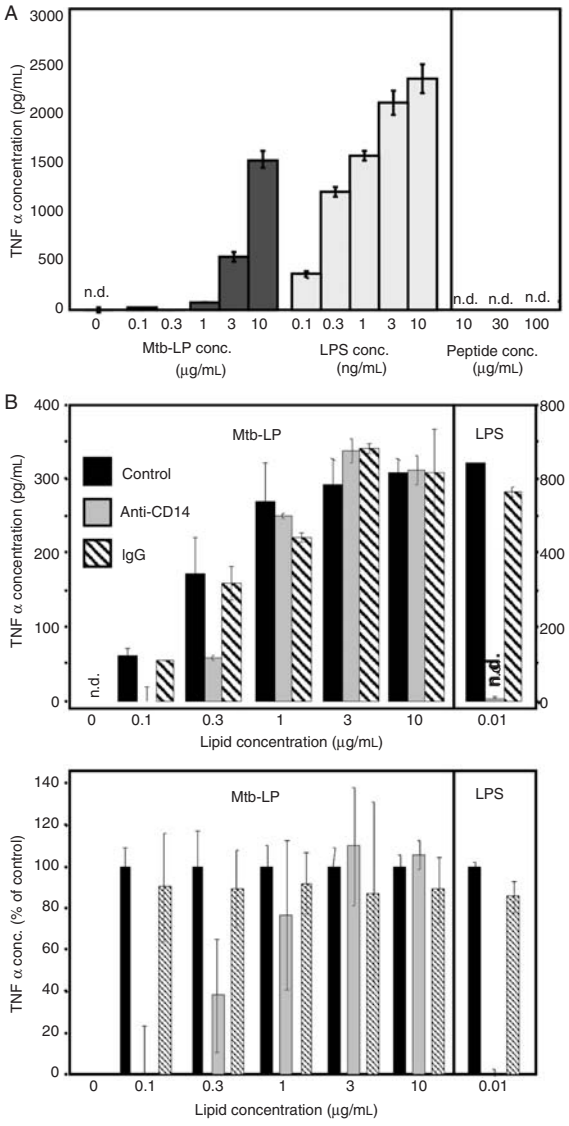

Fig. 3. The Mtb-LP induces a pro-inflammatory response in human macrophages. (A) Human mononuclear cells from healthy donors were *in vitro* differentiated to macrophages in the presence of 4% human AB-type serum and 2 ng/mL M-CSF. At day 7, cells were collected, resuspended at 1×10^6 cells/mL in RPMI medium containing 4% human serum, seeded at 200 μL aliquots in 96-well dishes, and stimulated with the Mtb-LP, LPS, or a peptide corresponding to Mtb-LP without the lipid part (CSSSNKSTTGSGETTTA) at the indicated concentrations. After 4 h, cell free-supernatants were collected and analysed by ELISA for their content of TNF-α. (B) To determine the participation of CD14 in cell activation by the Mtb-LP, human macrophages were incubated with a neutralizing antibody to CD14 (biG14) at 10 μg/mL or control IgG antibody at 10 μg/mL for 10 min and subsequently stimulated with Mtb-LP or LPS (*E. coli* strain K 235) at the indicated concentrations. After 4 h, supernatants were collected for the determination of TNF-α. (Upper panel) The data represent mean ± SD from one representative experiment out of three, and the error bars result from the determination of TNF-α in triplicate. n.d., not detectable. Lower panel: analysis of the data from three independent experiments. To account for donor variability in the biological response, TNF-α production after stimulation with the indicated concentrations of Mtb-LP or LPS were set 100% (control, black bar) and calculated as percentage of control in the presence of anti-CD14 antibody (grey bar) and control IgG (dashed bar).

Macrophages of the pulmonary compartment are specialized cells of the pulmonary innate immune response to infections and are naturally adapted to very low serum or serum-free conditions in the lung under physiological conditions. To characterize the pro-inflammatory immune response of macrophages under these conditions, we investigated the activation of human monocyte-derived macrophages to stimulation with the Mtb-LP under serum-free culture conditions. Surprisingly, stimulation of the cells with the Mtb-LP in serum-free culture medium led to a strong enhancement of cytokine production indicating a potent increase in sensitivity of the cells to low amounts of the LP. In contrast to serum-containing conditions where cell activation started at a concentration of the Mtb-LP of 1 μg/mL, TNF-α production was already induced by 100 ng/mL of Mtb-LP under serum-free conditions and increased dose-dependently with increasing concentrations of the LP (Fig. 4A). In contrast, the pro-inflammatory response to Pam_3CSK_4 was very similar in the presence and in the absence of serum, as shown exemplarily for the production of TNF-α (Fig. 4A). Similar results for the cell activation by the Mtb-LP could be observed for the late pro-inflammatory mediators IL-6 and IL-8 which both showed a dose-dependent enhancement under serum-free conditions after 24 h of stimulation (Fig. 4B). Analogous data were also obtained for the induction of the late anti-inflammatory cytokine IL-10 by Mtb-LP (Fig. 4C). These data indicate that the immune recognition of Mtb-LP is strongly modulated by serum proteins: compounds in serum attenuate cell activation by the Mtb-LP, whereas in a serum-free environment a highly sensitive recognition is provided, suggesting that the capacity of the Mtb-LP to activate immune responses is compartment-defined.

Serum and lipopolysaccharide-binding protein (LBP) differentially affect macrophage activation by the Mtb-LP

The serum protein LBP is important in the innate immune recognition of a variety of pathogen-associated molecules, including LPS and LP from Gram-negative bacteria. It has been shown to interact with LPS and enhance cell activation at low concentrations of LPS. However, at high concentrations of LBP as they appear in acute-phase serum, inhibitory effects of LBP on cellular responses to LPS have been reported.[41–43] Since LBP has been found to interact also with LP,[44–46] we postulated that LBP may be the component in serum modulating the cellular response to the Mtb-LP. To gain more information on the role of LBP in the process of cell activation by the Mtb-LP, we titrated recombinant human LBP to serum-free culture medium and

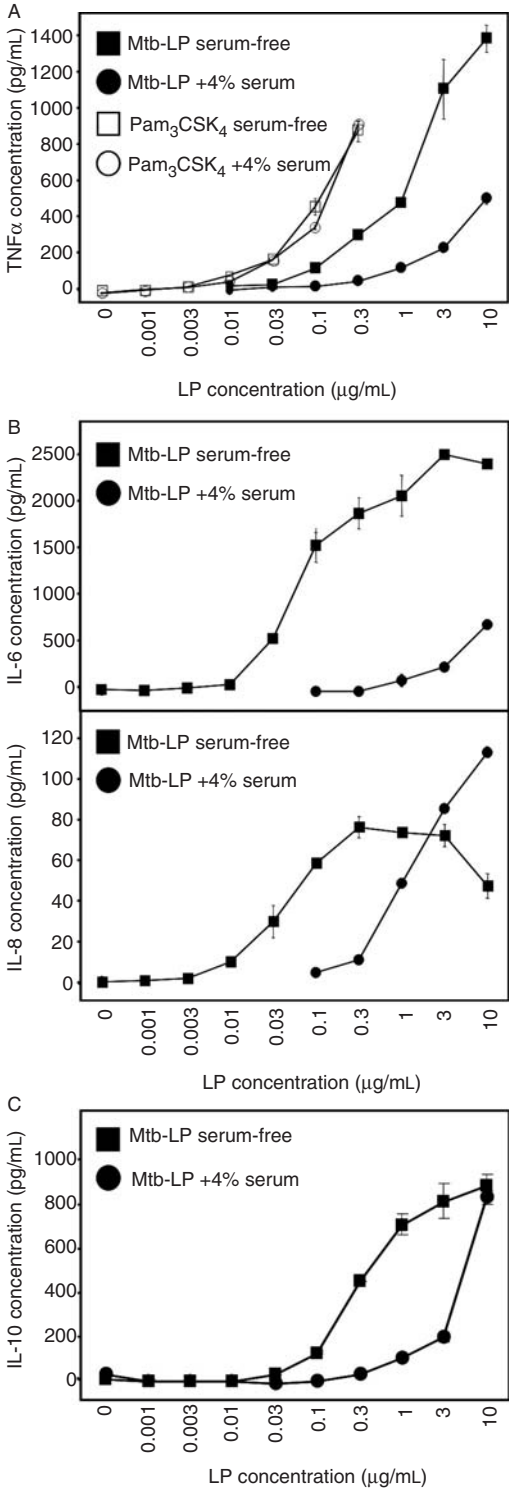

Fig. 4. Activation of human macrophages by the 19-kDa LP is strongly enhanced under serum-free conditions. Human macrophages were stimulated with the Mtb-LP or Pam_3CSK_4 at the indicated concentrations in culture medium containing 4% human AB-type serum or in serum-free medium. After 4 h, cell-free supernatants were collected for the determination of TNF-α (A) and after 24 h for the determination of IL-6, IL-8 (B), and IL-10 (C), respectively. The data represent mean ± SD from one representative experiment out of three, and the error bars result from the determination of the mediators in triplicate.

stimulated human macrophages with 10 ng/mL Mtb-LP. The production of the early pro-inflammatory cytokine TNF-α clearly showed a dose-dependent enhancement by LBP after 4-h stimulation at low concentrations of the Mtb-LP (Fig. 5A). The effects of LBP were observed at 5 μg/mL and 10 μg/mL LBP, a concentration range that corresponds to the physiological concentration of LBP in normal human serum (5–10 μg/mL). However, addition of only 1% of human serum completely abolished cytokine production by 10 ng/mL Mtb-LP under the same experimental conditions. The enhancing role of LBP in cell activation by the Mtb-LP is corroborated by the observed transport of the Mtb-LP by LBP into phospholipids membranes (Fig. 2B). Thus, LBP at physiological concentrations does not appear to be a negative regulator of cell activation by the Mtb-LP and thus is unlikely to be responsible for the observed attenuating effect of serum.

In contrast to the results obtained with LBP, titration of human AB-type serum to serum-free culture medium showed that serum almost completely abolishes the pro-inflammatory response to 10–100 ng of the LP and showed a strong and dose-dependent inhibition at 1–10 mg/mL of the LP already at a serum content of 2% (Fig. 5B). These results could be confirmed in titration experiments using fetal calf serum providing similar results (data not shown). Thus, serum and LBP differentially affect the biological activity of Mtb-LP: LBP dose-dependently enhances cell activation at physiological concentrations, whereas even very low amounts of serum strongly attenuate the cellular response over a wide range of concentrations.

A great wealth of our knowledge on the cellular immune response of macrophages to *M. tuberculosis* has been obtained from experiments using murine cells. We, therefore, sought to investigate whether the observed effects of serum on the response to the Mtb-LP are species-specific. We used murine bone marrow-derived macrophages and stimulated these cells under serum-free and serum-containing conditions with the Mtb-LP. In line with the data obtained from human macrophages, the murine macrophages showed an increased sensitivity to low amounts of the Mtb-LP under serum-free culture conditions and resembled the dose-response of macrophages derived from human blood (Fig. 5C). Compared to serum-free conditions, the production of TNF-α under serum-containing conditions was strongly attenuated already at a serum content of 1% fetal calf serum and even more strongly attenuated at a concentration of 10% fetal calf serum, thus decreasing with increasing amounts of serum. In contrast, the response of murine macrophages to LPS was not inhibited by 1% or 10% serum, suggesting a strong specificity of this inhibitory effect for the LP.

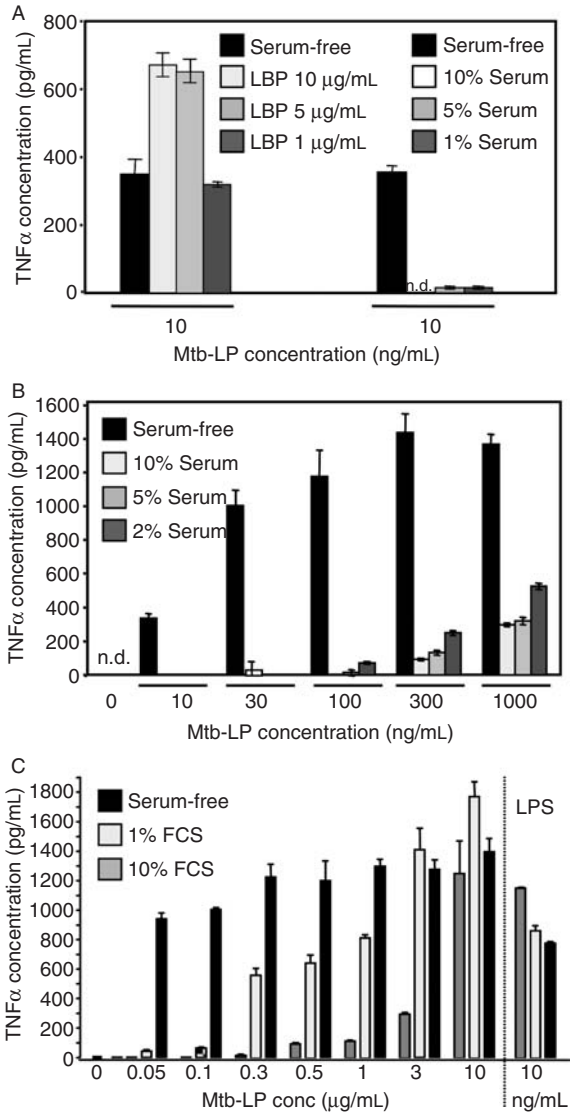

Fig. 5. Lipopolysaccharide-binding-protein (LBP) and serum differentially affect cell activation by the Mtb-LP. (A) Human macrophages resuspended in serum-free medium were stimulated with 10 ng/mL Mtb-LP in the presence of recombinant human LBP (left panel) or human AB-type serum (right panel) at the indicated concentrations. After 4 h, cell-free supernatants were collected for the determination of TNF-α. (B) Human macrophages resuspended in serum-free medium were stimulated with a wide range of concentrations of Mtb-LP in the absence or presence of 1–10% human AB-type serum. (C) Bone marrow-derived macrophages from C57BL/6 mice were incubated with the indicated concentrations of the 19-kDa LP in the absence or presence of 1% and 10% fetal calf serum. Supernatants were harvested 6 h after stimulation and TNF-α concentrations were determined by ELISA. The data represent mean ± SD from one representative experiment out of three, and the error bars result from the determination of the mediators in triplicate. n.d., not detectable.

DISCUSSION

Development of protective immunity against tuberculosis has been a continuing challenge during the last decades. The number of *M. tuberculosis* strains with multiple antibiotic resistances is steadily increasing causing major problems in the therapy of this infection.[47] The mechanisms of the complex host response in tuberculosis are not very well understood. A major problem in investigating molecular mechanisms of the innate immune defense against the intracellular pathogen is the lack of defined and pure compounds from mycobacteria. The complex three-dimensional structure of the multilayered mycobacterial cell wall makes the isolation and purification of biologically active fractions a challenging task. Besides being a prominent target of antibody response, the 19-kDa antigen has been identified as a cell-wall associated pathogenicity factor in tuberculosis and attributed to diverse biological effects such as apoptosis, induction of a TH1-centered immune response and the production of pro-inflammatory mediators.[8,10,19,48] However, so far, there is only limited data available on the innate immune recognition of this molecule. Recently, in *M. avium* subsp. *paratuberculosis*, a lipopentapeptide also has been demonstrated to induce a strong antibody response in the host.[49] Thus, the synthetic Mtb-LP mimicking the biologically active part of the 19-kDa lipoprotein represents a valuable tool to learn more about the molecular basis underlying its immunological activity.

The family of Toll-like receptors constitutes a group of highly conserved receptors conferring sensitive immune recognition of pathogens and mediate effective host-response mechanisms to a variety of infectious pathogens. Thus, TLR4 initiates cell activation by LPS in concert with the extracellular protein MD-2.[50–52] For TLR2, a variety of different ligands has been described including lipoteichoic acid, lipoarabinomannan, bacterial lipoproteins and LP.[2] The immune stimulatory capacity of a variety of lipoproteins has been attributed to the LP anchor of these molecules.[53–55] Toll-like receptor 2 has also been shown to associate with co-receptors.[56] Together with Dectin-1, it recognizes infections by yeasts[57,58] and mycobacteria.[59] Via heteromeric receptors complexes with TLR1 and TLR6, TLR2 also recognizes a variety of bacterial LP.[60,61] Only recently, it was suggested that CD36 may have a role in the recognition of the diacylated mycoplasma-derived LP MALP-2.[62] We have assigned a role of TLR2 and TLR4 in the host response to infection with *M. tuberculosis* bacteria; however, the relative contribution of Toll-like receptors was described controversially under different conditions of infection.[21,63,64] The 19-kDa lipoprotein of *M. tuberculosis* has been assigned to a TLR2/TLR1-dependent recognition. In this study, we confirm TLR2-dependent cell activation for the synthetic Mtb-LP (Fig. 1B). Interestingly, the capacity of the Mtb-LP to activate cells via TLR2 is by an order of 100-fold attenuated when compared to the prototypic LP Pam_3CSK_4, which has an identical lipid anchor but only

a short peptide of one serine and four lysine residues (Figs 1B and 4A). These data demonstrate that the peptide part has considerable influence on the biological activity of the LP. Of note is that a synthetic peptide with the amino acid composition of the Mtb-LP but without the lipid moiety did not induce cell activation (Fig. 3A), confirming that the lipid part is critically important for the biological activity. Furthermore, although the LP anchor represented by Pam_3CSK_4 is sufficient to induce cell activation, a longer peptide chain attached to the lipid anchor affects the biological activity as demonstrated here. This interdependency has not been investigated so far and will need further attention in the future for a better understanding of the principles of immune recognition of bacteria-derived lipoproteins and LP. As outlined below, there is strong evidence that the acyl chain fluidity at 37°C, *i.e*. the mobility within the aggregates, is at least partially responsible for this.

In contrast to the LPS from Gram-negative bacteria, there is virtually no information on the physicochemical basis of biological activity of amphiphilic cell-wall components from mycobacteria. The present paper is the first combined systematic physicochemical and biological analysis of a LP structure of mycobacterial origin. There are considerable differences in the chemical structures between LPS and LP. The investigated synthetic Mtb-LP compound has a zwitterionic, but electrically neutral, peptide backbone instead of the negatively charged sugar moiety of lipid A and three acyl chains instead of six acyl chains in those lipid A expressing high biological activity. Nevertheless, we found some striking similarities to LPS: the Mtb-LP showed induction of the pro-inflammatory cytokine TNF-α (Figs 3A,B and 4A) and has a strong preference to adopt cubic structures (Fig. 2A), which has also been reported for lipid A.[65] A similar correlation of the aggregate structure with biological activity has recently been reported for the synthetic LP Pam_3CSK_4 and Pam_2CSK_4, respectively, indicating that this correlation represents a general principle in innate immune recognition.[22]

Another characteristic attribute to lipids is the fluidity of their acyl chains. We have shown in earlier investigations on LPS, that the phase behavior and the fluidity of the acyl chains may modulate the immune response to these amphiphiles.[66–68] This can be understood considering that the acyl chain fluidity has pleiotropic effects on: (i) the packing density of the acyl chains; (ii) the molecular conformation; and (iii) the binding energy of molecules within the supramolecular aggregates. As can be deduced from Figure 2C, our data are clearly indicative of a decrease of the fluidity in the Mtb-LP as compared to the fluidity of LPS and Pam_3CSK_4 at 37°C, which should result in more stable aggregates and a decrease in biological activity. For a more detailed analysis, however, a more systematic study is necessary to assess the influence of the acyl chain fluidity of LP by comparing compounds with an identical hydrophobic moiety and variable peptide chains. In support of this interpretation is our finding that Mtb-LP exhibited only a very weak tendency to intercalate spontaneously into membranes resembling the composition of a macrophage membrane (Fig. 2B). This observation is in contrast to what we and others have reported for the synthetic Pam_3Cys containing the same tri-acylated lipid anchor than the Mtb-LP: these were shown to intercalate readily into artificial and cellular membranes without the need of a transport.[69–72] However, an LBP-dependent transport of the Mtb-LP was observed, as has been shown for LPS and other synthetic LP from Gram-negative bacteria.[22,32,41] Since the specific physicochemical properties of LP molecules such as the molecular conformation and the fluidity of the acyl-chains are largely different from that of the membrane-constituting phospholipids, the integration of the acyl chains of the LP into the cytoplasmic membrane of host cells is likely to affect the functionality of the cytoplasmic membrane und can thus be assumed to be important for the process of signal transduction.

Serum proteins have been shown to modulate the biological activity of a variety of microbial TLR ligands. Immune recognition of LPS is readily enhanced by physiological doses of LBP in serum[73] and cell activation by di- and tri-acylated LP has been reported to be enhanced in the presence of LBP.[46] In agreement with these data, we found an enhancing effect of LBP on the cell activation by the Mtb-LP at physiological concentrations of the protein (Fig. 5A), suggesting that delivery of the Mtb-LP to the host cell surface by LBP as demonstrated above represents an important pathway to amplify cell activation by low doses of the LP. Although the lung does not contain serum, LBP has been demonstrated to be present in the alveolar fluid. The concentration of LBP under physiological conditions is assumed to be 10–100 ng/mL in the alveolar fluid, corresponding to a serum concentration of 0.1–1%. In patients suffering from acute lung injury, bronchoalveolar concentrations of LBP have been documented to rise by 100-fold.[74,75]

When we investigated the role of serum in cell activation by the Mtb-LP, we made the surprising observation that the pro-inflammatory immune response to the Mtb-LP was strongly attenuated in the presence of extremely low amounts of serum (Figs 4 and 5B,C). This unexpected behaviour suggests that the mechanisms of immune recognition of this LP are different from the so far known microbial ligands, highlighting the complex ligand- and compartment-specific adaptation of mechanisms of innate immune recognition. Differential signalling mechanisms with respect to serum requirement have also been described in the context of the induction of

surface activation markers on neutrophils by LPS and the 19-kDa lipoprotein from *M. tuberculosis*.[76] However, in this study, the lipoprotein was found to induce neutrophil differentiation independent of the presence of serum. Another example of an inhibitory effect of serum on the immune recognition of a microbial ligand has recently been reported for lipoteichoic acid, a cell wall component of Gram-positive bacteria.[77] The authors showed in this study, that the binding of lipoteichoic acid to LBP leads to the formation of inactive complexes subsequently causing the attenuation of the immune response to lipoteichoic acid by serum. However, the mode of inactivation of the Mtb-LP by serum does not appear to be mediated by a similar mechanism. From our data, it is clear that the interaction of the Mtb-LP with LBP is not responsible for the observed attenuation of the cellular response (Fig. 5A). Possible modes of inactivation may include the binding and neutralization of the LP by specific antibodies, binding to proteins, proteolytic cleavage of the peptide, or binding to lipoprotein complexes. The specific mechanisms will have to be elucidated in future investigations.

Recent evidence has indicated a novel contribution of the synthetic Mtb-LP in mounting a vitamin D_3-dependent antibacterial host response via the induction of antibacterial cathelicidin.[12,23] The current data contribute insights into the molecular basis of the biological activity of this LP. The surprising observation that Mtb-LP exhibits a highly sensitive immune recognition accompanied by a broad pro-inflammatory response of host macrophages under serum-free conditions supports the conclusion that the innate immune response has adapted the mechanisms of immune recognition of this pathogen-associated molecule to the specific conditions of the lung.

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