Biochimica et Biophysica Acta 1863 (2016) 660–672

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Lipopolysaccharide-binding protein is bound and internalized by host cells and colocalizes with LPS in the cytoplasm: Implications for a role of LBP in intracellular LPS-signaling☆

Franziska Kopp, Sarah Kupsch, Andra B. Schromm *

Division of Immunobiophysics, Priority Area Infections, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, 23845 Borstel, Germany

ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form 21 December 2015
Accepted 20 January 2016
Available online 22 January 2016

Keywords:
Lipopolysaccharide
Lipopolysaccharide-binding protein
Macrophage
TLR4

ABSTRACT

The lipopolysaccharide-binding protein (LBP) is critically involved in innate immune responses to Gram-negative infections. We show here that human peripheral blood-derived monocytes, but not lymphocytes, stain positive for endogenous LBP on the cell surface. Studies on human macrophages demonstrate LBP binding at normal serum concentrations of 1–10 μg/ml. Binding was increased in a concentration-dependent manner by lipopolysaccharide (LPS). Fluorescence quenching experiments and confocal microscopy revealed constitutive and LPS-induced internalization of LBP by macrophages. Experiments with macrophages and HEK293 cell lines showed that binding and uptake of LBP do not depend on the LPS receptors CD14 and TLR4/MD-2. Fractionation of Triton X-100 solubilized cytoplasmic membranes revealed that LBP was primarily localized in non-raft domains under resting conditions. Cellular LPS stimulation elevated LBP levels and induced enrichment in fractions marking the transition between non-raft and raft domains. LBP was found to colocalize with LPS at the cytoplasmic membrane and in intracellular compartments of macrophages. In macrophages stimulated with LPS and ATP for inflammasome activation, LBP was observed in close vicinity to activated caspases. Furthermore, LBP conferred IL-1β production by LPS in the absence of ATP. These data establish that LBP serves not only as an extracellular LPS shuttle but in addition facilitates intracellular transport of LPS. This observation adds a new function to this central immune regulator of LPS biology and raises the possibility for a role of LBP in the delivery of LPS to TLR4-independent intracellular receptors.

1. Introduction

The lipopolysaccharide-binding protein (LBP)[4] is a versatile molecule serving different functions in immune regulation and lipid transfer. Like other members of the BPI/LBP/Plunc protein superfamily [1], LBP is a key player in innate immunity defenses against bacterial infections [2,3]. As a class I acute-phase protein LBP is induced by pro-inflammatory cytokines such as interleukins 1 and 6, tumor necrosis factor-alpha (TNF-α), and glucocorticoid hormones in the liver [4,5], and in non-hepatic tissues such as the gut and the lung [6,7]. Serum concentrations of LBP range between 5 and 10 μg/ml during homeostasis, increasing up to 200 μg/ml during an acute-phase response in the course of an infection [8]. LBP expression and function are strongly associated with the recognition and control of bacterial infections. The most prominent task of LBP is its role as part of the sensing apparatus for Gram-negative lipopolysaccharides (LPS, endotoxin) [9]. Upon binding to LBP in serum, LPS is transported via soluble and membrane-anchored cluster of differentiation 14 (CD14) to the Toll-like receptor 4 (TLR4)/MD-2 signaling complex [5,10–13]. LBP can thereby sensitize host cells to minute amounts of LPS [3]. Activation of the TLR4/MD-2 receptor complex triggers a myriad of pro- and anti-inflammatory responses [14]. If dysregulated, these host reactions can have inadvertent outcomes such as severe sepsis or septic shock [15]. In vivo studies showed that LBP-deficient mice generated by targeted deletion of the LBP gene have a reduced inflammatory response and a greater survival rate after LPS challenge compared to their wildtype littermates [16,17]. At the same time LBP-deficient mice have an impaired ability to clear systemic bacterial infections [16,18–20]. Interestingly, LBP-deficient mice show an impaired immune response to pulmonary infections with Gram-negative bacteria with greater bacterial loads and increased mortality [21,22], demonstrating a role of LBP in the immune defense of the

Abbreviations: CD14, cluster of differentiation 14; CHOL, cholesterol; CRP, C-reactive protein; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; FI, fluorescence intensity; GM1, gangliosid M1; LBP, lipopolysaccharide-binding protein; LBP_{A488}, Alexa Fluor 488-conjugated LBP; LBP_{A647}, Alexa Fluor 647-conjugated LBP; LPS, lipopolysaccharide; MNC, mononuclear cells; MD-2, myeloid-differentiation factor-2; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE; Rh-PE, N-(rhodamine B sulfonyl)-PE; SM, sphingomyelin; TLR4, Toll-like receptor 4.

☆ This work was supported by the Leibniz Graduate School "Model systems for infectious diseases" and the Cluster of Excellence "Inflammation at Interfaces" Project 306 O TP4 to A.B.S.

* Corresponding author at: Division of Immunobiophysics, Priority Area Infections, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Parkallee 10, 23845 Borstel, Germany.
E-mail address: aschromm@fz-borstel.de (A.B. Schromm).

http://dx.doi.org/10.1016/j.bbamcr.2016.01.015
0167-4889/

lung. LBP has also been associated with other inflammatory disorders such as metabolic syndrome and artherosclerosis [23,24].

In addition to binding LPS, LBP has been shown to interact with a multitude of bacterial lipids, e.g. lipopeptides, lipomannan, and lipoteichoic acid [2,25]. As a lipid carrier, LBP binds a variety of phospholipids, including phosphatidylcholine and phosphatidylinositol, transporting them to high density lipoproteins [26]. Additionally, we and others have shown previously that LBP intercalates into reconstituted phospholipid bilayers in the absence of LPS [27,28].

The interaction of LBP with host cells has so far mostly been addressed in the context of LPS binding and transport in serum. Based on its membrane activity, we hypothesized that LBP also interacts with the cytoplasmic membrane of host cells. This membrane-associated form of LBP may be involved in LPS-induced immune activation, which would extend the function of LBP beyond that of a mere LPS shuttle protein. In the present study, we investigated the membrane interaction and the internalization of LBP using primary human monocytes and macrophages, as well as a HEK293-derived cell line, expressing recombinant TLR4/MD-2. We analyzed the role of LPS in the interaction of LBP with host cells as well as the localization of LBP in unstimulated and LPS-stimulated cells. Our data demonstrate an inherent ability of LBP to bind to human primary mononuclear cells as well as stable cell lines and support a role for LBP in the intracellular transport of LPS.

2. Experimental procedures

2.1. Chemicals and reagents

Deep rough mutant LPS was extracted from *Escherichia coli* strain WBB01 using the phenol/chloroform/petroleum ether method and lyophilized [29]. Smooth LPS was prepared from wild-type *Salmonella abortus equi* by phenol water extraction [30]. LPS preparations were analyzed by mass spectrometry and used in the natural salt form. Smooth LPS from *E. coli* strain K235 (List Biological Laboratories, Campbell, CA, USA) was purified by phenol-reextraction according to Hirschfeld and colleagues [31]. All LPS preparations were analyzed with respect to TLR-specificity and were found to be devoid of TLR2-activity in HEK293 cell stimulation assays. LPS suspensions were prepared by reconstituting LPS in *A. dest* (B. Braun, Melsungen, Germany) and subjecting it to sonication for 30 min in a water bath. Subsequently, suspensions were temperature cycled three times between 4 °C and 60 °C, 30 min each and stored over night at 4 °C before the first use. For fluorophore-labeling, LPS was mixed with rhodamine-coupled phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL, USA) in chloroform in a molar ratio of 10:1. The chloroform was evaporated under a stream of nitrogen, lipids were reconstituted in *A. dest* and subjected to the same sonication and temperature treatment as described above.

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), sphingomyelin (SM) and cholesterol (Chol) were from Avanti Polar Lipids (Alabaster, AL, USA). The fluorophore-conjugated phospholipids N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and N-(rhodamine B sulfonyl)-PE (Rh-PE) were obtained from Molecular Probes (Life Technologies, Darmstadt, Germany).

Human recombinant LBP was a kind gift of XOMA Corporation (Berkeley, CA, USA). Fluorophore-labeled LBP was prepared using Alexa Fluor® microscale protein labeling kit (Life Technologies, Darmstadt, Germany) according to the manufacturers' protocol. Human serum albumin was obtained from Sigma-Aldrich (München, Germany), human C-reactive protein from R&D Systems (Wiesbaden, Germany) and human IgG control from Life Technologies (Darmstadt, Germany).

Primary anti-LBP antibodies biG33, biG42, and anti-CD14 antibody biG14 were purchased from Biometec (Greifswald, Germany), and mouse anti-TLR4 clone HTA125 was purchased from Imgenex Corp. (San Diego, CA, USA). Goat anti-mouse IgG-A546 and IgG-A647 were from Life Technologies (Darmstadt, Germany), goat anti-mouse IgG-HRP from Jackson ImmunoResearch (West Grove, PA, USA), anti-CD14-PE from BD Biosciences (Heidelberg, Germany), IgG1 and IgG2a isotype control were from Santa Cruz (Heidelberg, Germany). DAPI and Hoechst 34580 were purchased from Sigma-Aldrich (München, Germany). Human TNFα ELISA was from BD Biosciences (Heidelberg, Germany), human IL-1β duoset ELISA was from R&D Systems (Wiesbaden, Germany), and LDH cytotoxicity assay was from Pierce (Thermo Fisher, Braunschweig, Germany). Fugene was purchased from Promega (Mannheim, Germany).

2.2. Cells

Human mononuclear cells (MNCs) were isolated from heparinized peripheral blood of healthy donors. The procedures for blood donation were approved by the ethical committee of the University of Lübeck. MNCs were harvested by density gradient centrifugation and washed three times in serum-free RPMI medium (endotoxin ≤ 0.01 EU/ml, Biochrom, Berlin, Germany) before being used in experiments. For the differentiation into macrophages, MNCs were cultivated in teflon bags in RPMI medium as described previously [32]. Macrophages were harvested on day 7, washed twice in serum-free RPMI, and seeded in serum-free RPMI, containing 200 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for experiments. HEK293 and HEK293-hTLR4/MD-2 cell lines were maintained in DMEM medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (Linaris, Dossenheim, Germany) and 2% L-glutamine/streptomycin/penicillin, at 37 °C in a 5% CO_2 atmosphere. Additionally, HEK293-hTLR4/MD-2 cells were cultivated in the presence of 400 U/ml hygromycin, and 0.5 mg/ml G418 [33].

2.3. Flow cytometry

For the staining of endogenous LBP, freshly isolated human MNCs were aliquoted at 5×10^5 cells/sample, blocked for 30 min in PBS (Biochrom, Berlin, Germany) containing 10% FCS, and either stained for LBP with the primary antibodies biG33 and biG42, or incubated with isotype matched control IgG, followed by goat anti-mouse-biotin, and streptavidin-APC conjugate. Monocytes were counterstained with anti-CD14-PE.

To investigate the binding of recombinant fluorophore-labeled LBP, macrophages were harvested, washed with ice cold PBS, and centrifuged (400 ×*g*, 5 min; 4 °C) in FACS tubes. For each sample 5×10^5 cells were incubated with LBP and LPS as indicated, subsequently washed with PBS or azide-PBS containing 2% FCS, and fixed with 2% paraformaldehyde for 15 min at room temperature. If trypan blue quenching was performed, samples were split into two aliquots. One aliquot was then centrifuged and the supernatant discarded. Directly prior to the measurement the cell pellet was resuspended in 0.2% trypan blue solution. HEK293-hTLR4/MD-2 cells were stained for TLR4 with 20 μg/ml mouse monoclonal anti-TLR4 antibody clone HTA125 for 30 min on ice. An anti-mouse IgG-A647 conjugate served as a secondary antibody. Cells were washed with azide-PBS containing 2% FCS, centrifuged (400 ×*g*, 5 min; 4 °C) and resuspended in azide-PBS with 2% FCS. Flow cytometry was performed using the FACSCalibur™ system from BD Biosciences operated with BD CellQuest software version 6.0 (BD Biosciences, Heidelberg, Germany). For each sample 10,000 events (cells) were measured and data analysis was performed using WinMDI software from Scripps Research Institute (La Jolla, CA, USA).

2.4. Confocal microscopy

Human macrophages, 2×10^5 cells/well, and HEK293-hTLR4/MD-2 cells, 5×10^5 cells/well, were seeded into μ slides VI from Ibidi (Martinsried, Germany), and allowed to settle over night in serum-containing culture medium at 37 °C in a 5% CO_2 atmosphere. Prior to

staining the medium was removed and the cells were washed with serum-free medium. Cells were then incubated with LBP and LPS at 37 °C as indicated, washed twice with ice cold PBS, and fixed with 4% paraformaldehyde for 10 min at room temperature. HEK293-hTLR4/MD-2 cells were stained for TLR4 with 20 μg/ml mouse monoclonal anti-TLR4 antibody clone HTA125 for 60 min, followed by an anti-mouse IgG-A546 conjugate. Cell nuclei were counterstained with Dapi or Hoechst 34580.

For inflammasome staining, human macrophages were seeded at 2×10^5 cells/well in OPTI-MEM medium (Life Technologies, Darmstadt, Germany) into μ-slides VI and incubated for 1 h at 37 °C in a 5% CO_2 atmosphere for adherence. Macrophages were then stimulated with 1 μg/ml ultrapure LPS from *Salmonella abortus equi*, 5 μg/ml LBP_{A647}, and 10 μM of the caspase-1 pseudosubstrate CaspACE ™ FITC-VAD-FMK (Promega, Mannheim, Germany) for 23 h, followed by a 1 h incubation in the presence of 10 mM ATP (Sigma-Aldrich, Hamburg, Germany) for inflammasome activation. Cells were subsequently washed with ice-cold PBS, fixed, and stained for DNA as described above.

Confocal laser scanning microscopy was performed using a TCS SP5 (Leica Microsystems GmbH, Wetzlar, Germany) with the HCX PL APO CS 63×/1.4 oil immersion lens.

2.5. Förster resonance energy transfer (FRET) assay

The interaction of LBP with host cell membranes was investigated using FRET spectroscopy as a probe dilution assay on a Fluorolog 2 spectrometer (Horiba Scientific, Unterhaching, Germany). The incorporation of protein into the liposomes increases the liposomal surface and thus the average distance between the labeled phospholipids, thereby increasing the ratio of donor/acceptor fluorescence intensity (FRET-signal, I_D/I_A). DOPC/SM/CHOL 9:9:2 (M) liposomes were labeled with the fluorescent dyes NBD-PE and Rh-PE at a final molar ratio of [phospholipid]:[NBD-PE]:[Rh-PE] = 100:1:1 as described previously [34]. Liposomes were diluted to a 10 μM concentration in 20 mM HEPES, pH 7.4, adjusted to a temperature of 37 °C, and placed in a glass cuvette with a magnetic stirrer. The emission intensities of the two dyes were equalized at the beginning of each experiment by adjusting the slit width. Fluorescence emission intensities were recorded for 50 s for base line documentation and subsequently proteins were added to a final concentration of 5 μg/ml.

2.6. Fractionation of cytoplasmic membranes

Monocytes from peripheral blood of healthy donors were incubated with 100 ng/ml LPS K235 or serum-free control medium for 30 min at 37 °C. Subsequently, cells were washed with ice-cold PBS and incubated in lysis buffer (10 mM HEPES, 42 mM KCL, 5 mM $MgCl_2$, 1 mM Na-Vanadat, pH 7.4) containing complete mini protease inhibitor (Roche, Mannheim, Germany). The suspension was subjected to ultrasound and subsequently centrifuged with 116.000 ×*g* at 4 °C for 30 min. The supernatant was collected as the cytosolic fraction. The pellet fraction was resuspended in 0.1% Triton X-100 for 1 h and mixed with 85% sucrose solution. The suspension was then overlayed with 30% sucrose and 5% sucrose. The preparation was centrifuged for 19 h at 4 °C with 150.000 ×*g*. The supernatant was collected in fractions of 150 μl each and the pellet as one fraction. Of each fraction 5 μL was pipetted onto nitrocellulose membranes and immunostained for GM1 using HRP-conjugated cholera toxin B and visualized by chemiluminescence. If staining was positive the respective fractions were defined as lipid raft fractions. Membrane fractions were assayed for protein content using Bradford reagent (Biorad, Munich, Germany). Of each fraction 15–20 ng protein was separated via SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were probed for LBP with the biG42 antibody and horseradish peroxidase-conjugated goat anti-mouse IgG-HRP, and visualized by chemiluminescence. To determine differences in the LBP amounts in the cytoplasmic membrane fractions densiometric analysis of immunostained Western blots were performed using ImageJ (National Institute of Health, Bethesda, MD, USA).

2.7. Statistical analysis

All calculations were done using GraphPad Prism (version 5.0; GraphPad Software, San Diego, CA, USA). Details of analysis are noted in respective figure legends. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. LBP binds to mononuclear cells

During homeostasis, LBP is present in serum at constitutive concentrations between 5 and 10 μg/ml [8]. Hence, all cells in the blood stream are constantly exposed to LBP. We sought to determine if LBP could be detected on human monocytes under resting conditions. For this purpose, we incubated human MNCs, freshly isolated from peripheral blood of healthy donors, with two different monoclonal anti-LBP antibodies and analyzed them by flow cytometry. To determine the monocyte population among the MNCs, CD14 staining was performed (Fig.1A). We observed a strong signal from both anti-LBP antibodies in the CD14-positive population (monocytes), whereas no LBP staining was detectable in the CD14-negative population (lymphocytes) (Fig. 1B, C). Statistical analysis of eight independent experiments conducted with macrophages from different donors showed that the signals from both anti-LBP antibodies were significantly higher than the isotype control signal, with $p < 0.01$ for biG42 and $p \leq 0.05$ for biG33 (Fig. 1D). LBP levels in the donor sera were analyzed by ELISA and were found to be within the expected range for normal human serum samples (data not shown). This is the first evidence that LBP is bound to host cells under homeostatic conditions. We continued our investigation by further characterizing the interaction of LBP with primary immune cells.

3.2. LBP interacts with macrophages independently of LPS

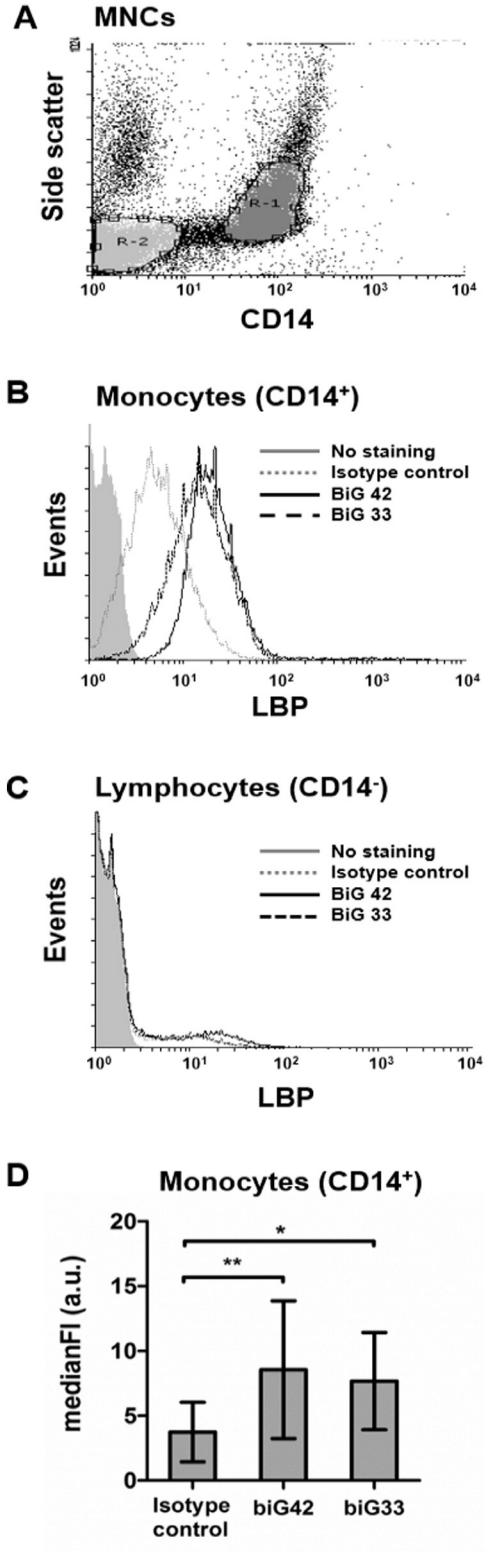
A bacterial infection triggers monocyte differentiation into macrophages. Their responsiveness to LPS is strongly regulated by LBP. Hence, we investigated the binding of LBP to human macrophages with respect to dose-dependence, temperature-dependence, and time. For this purpose, we conjugated recombinant human LBP with the fluorophore Alexa 488, hereafter termed LBP_{A488}, and performed flow cytometry-based binding assays. Biological activity of the LBP_{A488} was retained, as tested in LPS-stimulation experiments (Fig. 2A).

In an initial analysis, we investigated if the association of LBP with host cells is dependent upon the presence of serum components or LPS. Human macrophages were incubated in serum-free medium, in the absence of LPS, with different concentrations of LBP_{A488}, all of which were within the range of homeostatic conditions. Flow cytometry analysis showed a distinct concentration-dependent association of LBP_{A488} with human monocyte-derived macrophages (Fig. 2B). These findings and the described detection of LBP on freshly isolated monocytes demonstrate an inherent cell binding property of LBP in the presence and absence of serum.

Thereafter we conducted assays analyzing cellular LBP binding with regard to time and temperature. Since macrophages are professional phagocytes with a high mobility of their cytoplasmic membrane, we simultaneously addressed the question, if membrane-associated LBP is internalized. For this purpose, we used a quenching protocol based on the diazo dye trypan blue. Addition of the dye to the macrophage suspension eliminated all extracellular fluorescence. Intracellularly originating fluorescence was unaffected, since trypan blue does not pass through cytoplasmic membranes of viable cells. Human macrophages were incubated with LBP_{A488} at 37 °C and on ice for the indicated time

periods. At 37 °C a notable association of LBP_{A488} with macrophages was observed after 5 min of incubation, which increased to significant binding after 15 min ($p < 0.01$) and 30 min ($p < 0.001$). Within the tested incubation time frame of 30 min LBP binding did not go into saturation. Quenching of cells incubated at 37 °C resulted in a decrease of about 50% of the LBP_{A488} signal (Fig. 2C), demonstrating internalization of a considerable portion of LBP_{A488}. Incubation of macrophages with LBP on ice also showed a distinct binding of LBP_{A488} to the cells. However, unlike incubation at 37 °C, binding to macrophages on ice was saturated after 5 min. Quenching of the samples incubated on ice completely abrogated the fluorescence signal (Fig. 2D) indicating that, at low temperatures, LBP remains exclusively surface-bound.

These results oppose the current scientific opinion, which states that LBP exerts a mere extracellular carrier function transporting LPS to the TLR4/MD-2 receptor complex, without any direct interaction with host cells itself. In contrast to this, our findings demonstrate that LBP at normal serum concentrations does bind to human immune cells in the absence of LPS. LBP binding to host cells is time-dependent and temperature-sensitive. Quenching assays showed that, concurrent with binding, LBP is internalized by macrophages.



3.3. LPS enhances the interaction of LBP with macrophages

LBP has been demonstrated to enhance the binding of LPS to host cells, sensitizing host cells to minute amounts of LPS. To investigate if and how LPS in turn affects the interaction between LBP and host cells, we coincubated human macrophages under serum-free conditions with LBP_{A488} and LPS. Flow cytometry analysis revealed a concentration-dependent increasing effect of LPS on the binding of LBP_{488} to macrophages. We also checked for LBP influence on LPS binding, which was enhanced by increasing concentrations of LBP (Fig. 3A, B). In contrast, blocking of CD14 by an inhibitory CD14 antibody did not affect the binding of LBP to human macrophages (Fig. 3C, D). LBP binding in the absence of antibody was not significantly different to binding in the presence of anti-CD14 or isotype control antibody. However, all three groups showed highly significant (**, $p < 0.01$) binding of LBP to macrophages compared to control cells in the absence of LBP.

To analyze the localization of cell-bound LBP in the absence or presence of LPS we performed confocal laser scanning microscopy. For these experiments, we used recombinant LBP labeled with the fluorophore Alexa 647, hereafter termed LBP_{A647}. Human macrophages were incubated in serum-free medium with LBP_{A647} with or without the addition of LPS. In good accordance with our flow cytometry data we found membrane-bound LBP and a punctuate intracellular localization of LBP (Fig. 3E, left column). The addition of LPS enhanced the LBP signal, without notably changing the overall distribution of LBP. Neither did we observe a change in the ratio of membrane-associated LBP to intracellular LBP (Fig. 3E, right column). These findings demonstrate that while the cellular binding property is inherent to LBP itself, LPS does affect the interaction of LBP with host cells. This suggests a possible role of cellular LBP, additionally to serum LBP, in LPS-mediated cell activation.

3.4. TLR4 is not the primary cellular interaction partner of LBP

In the previous experiments we demonstrate that LBP binds to human macrophages independently of LPS and of CD14. To shed further light on the interaction of LBP with host cells we sought to identify the site of LBP membrane interaction. Considering that the TLR4/MD-2 receptor complex is the dominant cellular LPS receptor, we first analyzed its involvement in cellular LBP binding. For this purpose, we performed colocalization experiments using fluorophore-labeled LBP and HEK293

Fig. 1. Freshly isolated human monocytes are LBP positive — human mononuclear cells were isolated from blood of healthy donors and stained for LBP with 10 μg/ml of anti-LBP antibodies biG33 or biG42, or isotype matched control IgG. Secondary staining was performed with goat anti-mouse-biotin and streptavidin-APC conjugate. CD14 staining using anti-human CD14-PE was done to identify the monocyte population. (A) Distribution of CD14-positive (R1) and CD14-negative (R2) cell populations in MNCs. (B, C) LBP staining with antibodies biG42 (solid black line) and biG33 (dashed black line), compared to isotype control IgG (dotted gray line) in CD14-positive monocyte population (B) and in the CD14-negative lymphocyte population (C). The data shown is from one experiment representative of eight independent experiments. (D) Comparison of median fluorescence intensities (FI) of antibody and isotype control staining of monocyte population. The data are depicted as mean ± s.d. of 8 individual donors. Statistical analysis was performed using one-way ANOVA with Dunnett's post-test. Significant differences are marked with * = $p \leq 0.05$ or ** = $p < 0.01$.

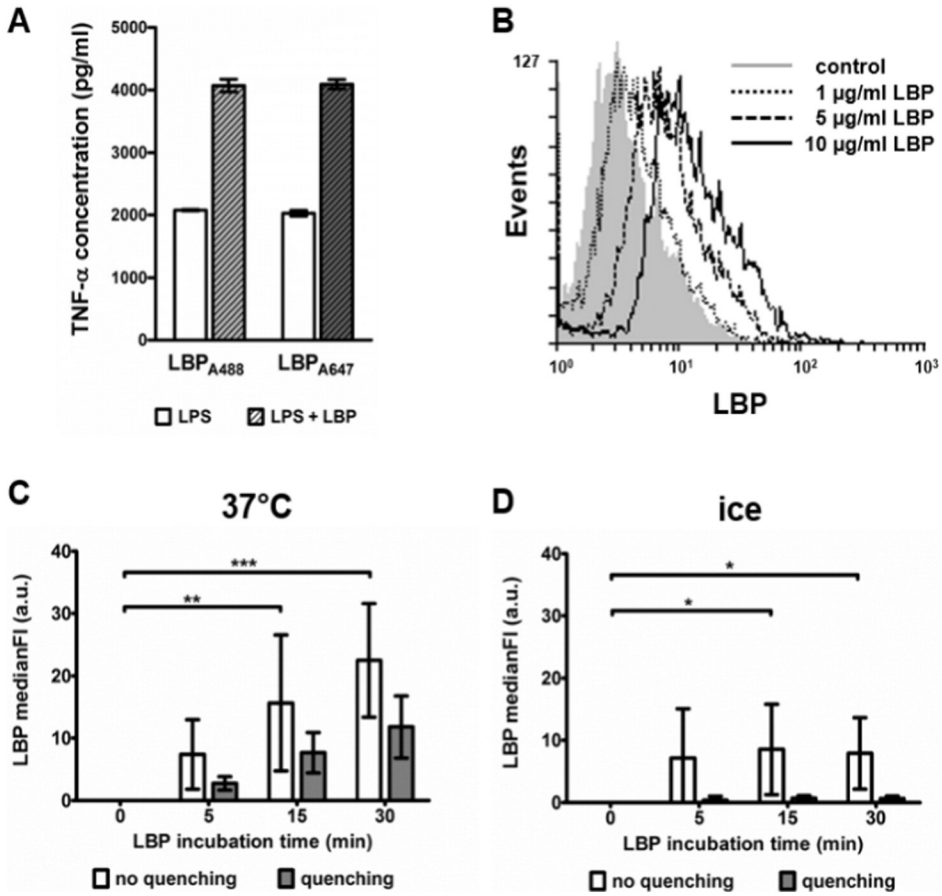


Fig. 2. Human macrophages bind and internalize LBP — (A) TNF-α concentrations measured in cell-free supernatants of human macrophages incubated in serum-free medium in the presence of 1 µg/ml LBP$_{A488}$ (light gray bar), LBP$_{A647}$ (dark gray bar), or control medium (white bars) and subsequently stimulated with 0.1 ng/ml LPS K235 for 4 h at 37 °C. (B) Human monocyte-derived macrophages were incubated with 0, 1, 5, and 10 µg/ml LBP$_{A488}$ in RPMI medium without serum for 15 min at 37 °C, or (C) 20 µg/ml LBP$_{A488}$ for 5, 15, and 30 min at 37 °C, or (D) on ice. Graphs depict median fluorescence intensities, normalized to untreated control cells (0 min) as mean ± s.d. of four independent experiments. White bars represent overall fluorescence (intra- & extracellular). Gray bars represent intracellular fluorescence after trypan blue quenching. Statistical analysis of unquenched samples was performed using one-way ANOVA with Dunnett's post-test. Significant differences in median FI compared to untreated control cells are marked with * = $p \le 0.05$, ** = $p < 0.01$, or *** = $p < 0.001$.

cells recombinantly expressing human TLR4 and MD-2. The cells were incubated with LBP$_{A647}$, subsequently immunostained for TLR4, and analyzed via confocal microscopy. While there was some intracellularly localized LBP$_{A647}$, most of the LBP$_{A647}$ was detected along the cytoplasmic membrane. Colocalization of LBP and TLR4 was not evident (Fig. 4A, upper panel). The fluorescence intensities of LBP$_{A647}$ and antibody-stained TLR4 along the cytoplasmic membrane were inspected in more detail using fluorescence line profiles. Intensity peaks of both proteins alternate with only very little overlay (Fig. 4A, lower panel). Thus, no systematic colocalization between LBP and TLR4 was evident. To exclude the possibility that the absence of colocalization of the two proteins may stem from an interference of the anti-TLR4 primary antibody with LBP binding to the receptor, comparative flow cytometry experiments were conducted. HEK293-hTLR4/MD-2 cells were immunostained for TLR4 with or without prior incubation with LBP$_{A488}$. No difference in the TLR4 staining was observed with regard to LBP$_{A488}$ incubation, proving that the lack of colocalization of LBP and TLR4 is not caused by competing binding sites of LBP and the anti-TLR4 antibody on the receptor complex (data not shown).

To further elucidate whether TLR4 is at all necessary for LBP binding to HEK293 cells, analysis was performed using HEK293 wildtype cells and HEK293-hTLR4/MD-2 cells. FACS analysis showed that LBP binds to both cell lines (Fig. 4B, C), and statistical analysis of four independent experiments revealed no significant difference ($p = 0.392$) in the binding efficiency of LBP to cells with and without the TLR4/MD-2 receptor. Since HEK293 cells do not have endogenous CD14 expression, we conclude that neither CD14 nor TLR4 is a prerequisite for the binding and uptake of LBP by human cells.

3.5. LBP partitioning into eukaryotic membranes

To test the hypothesis of a receptor independent mechanism of host cell interaction, we employed phospholipid liposomes resembling the lipid composition of eukaryotic cell membranes. In a FRET-based membrane interaction assay, addition of LBP to DOPC/SM/CHOL liposomes led to a rapid increase of the FRET-signal, indicating the incorporation of LBP protein into the liposomal membrane (Fig. 5A). In contrast, human serum albumin, the acute phase-protein C-reactive protein, or human IgG molecules did not show intercalation into the liposomes, demonstrating that the observed effect is specific for LBP. These data support the hypothesis that a molecular mechanism based on membrane partitioning contributes to the observed association of LBP with the cytoplasmic membrane of host cells.

3.6. LBP enriches in the cytoplasmic membrane upon LPS stimulation

The proteins of the LPS-sensing apparatus, specifically CD14 and TLR4/MD-2, show a highly regulated spatio-temporal organization in signaling domains of the cytoplasmic membrane that is referred to as lipid rafts. Whereas the GPI-anchored LPS-binding receptor CD14 is constitutively localized in these cholesterol and sphingolipid rich membrane domains, TLR4 has been demonstrated to be localized outside of lipid raft domains

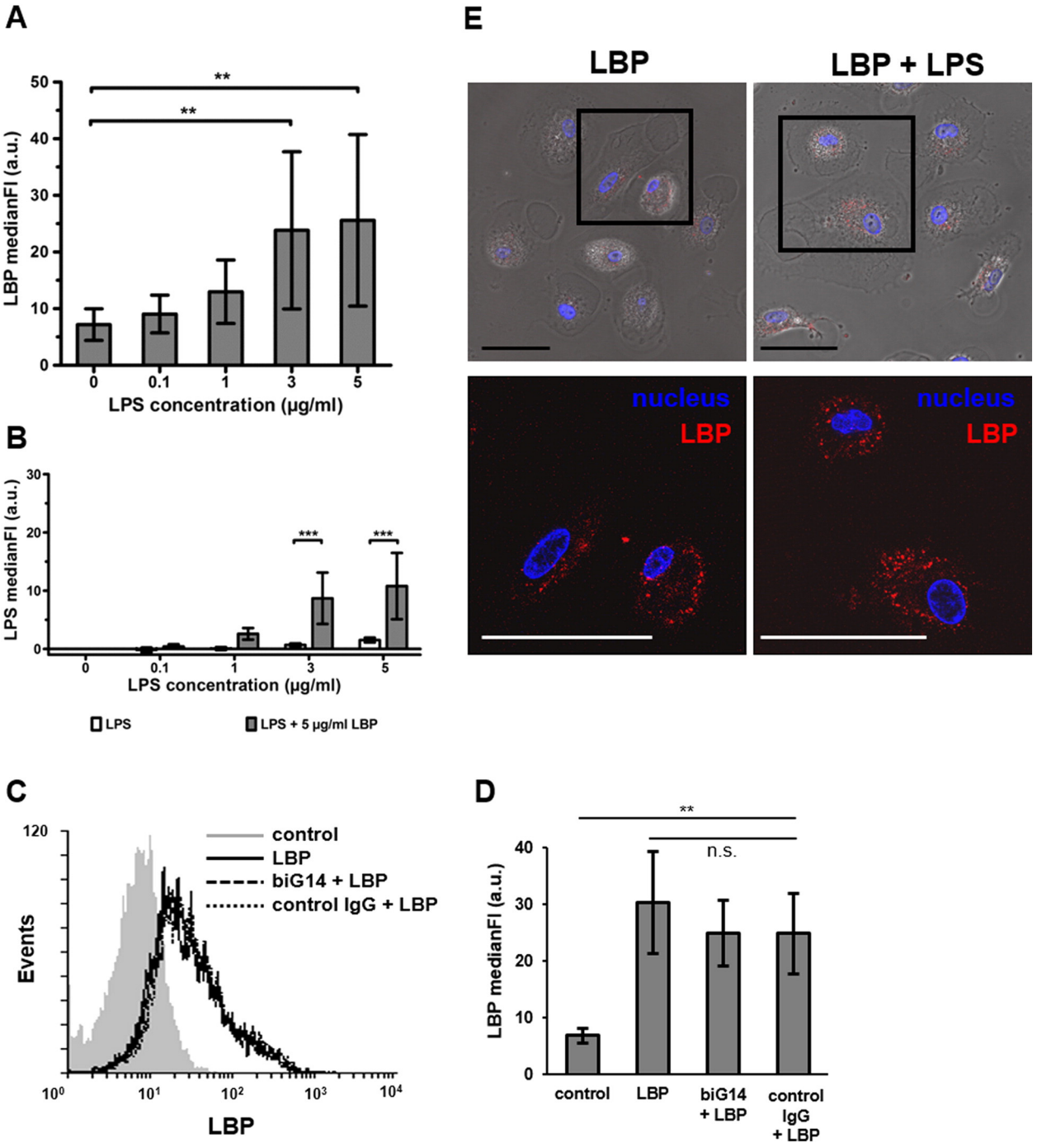


Fig. 3. LPS enhances LBP binding to human macrophages — human macrophages were incubated with 5 μg/ml LBP_{A488} and *E. coli* WBB01 LPS at the indicated concentrations for 15 min at 37 °C. (A) Graph depicts median FI of LBP_{A488} as mean ± s.d. of four independent experiments. Data was normalized to unstained control (no LBP, no LPS). Significance of differences in median FI compared to untreated control groups (0 μg/ml LPS) was determined by one-way ANOVA with Dunnett's post-test (** = $p < 0.01$, *** = $p < 0.001$. (B) Graph depicts median FI of LPS as mean ± s.d. of four independent experiments. Data was normalized to control samples (0 μg/ml LPS). Statistical analysis was performed using one-way ANOVA with Bonferroni post-test. Significant differences in median FI between LPS only and combined LPS + LBP treatment of macrophages are marked as *** = $p < 0.001$. (C) Human macrophages were incubated with anti-CD14 antibody biG14 or isotype matched control antibody at 20 μg/ml for 15 min and then incubated with 10 μg/ml LBP_{A647} for 15 min at 37 °C. Unstained cells are depicted as control. Binding histograms of LBP_{A647} from cells of one donor. (D) Mean ± s.d. of the median FI of LBP_{A647} of three individual donors, cells treated as in C. Statistical analysis by one-way ANOVA with Bonferroni post test (** = $p < 0.01$). Differences between LBP and antibody treatment groups are not significant (n.s.) (E) Human macrophages were incubated with 10 μg/ml LBP_{A647} (red) in the absence (left column) or presence (right column) of 5 μg/ml *E. coli* WBB01 LPS for 10 min at 37 °C. Cell nuclei were stained with DAPI (blue) after cell fixation. Top panel shows overlay images of LBP_{A647} fluorescence with respective phase contrast images. Bottom panel shows enlargements of black rectangles depicted in top panel. White and black bars on bottom left represent 50 μm. Images are taken from a single experiment representative of three independent experiments.

under resting conditions [35]. Upon LPS stimulation, the TLR4/MD-2 complex is recruited to raft domains forming the active signaling complex by heterodimerization upon LPS binding to MD-2 [11,35–37]. We therefore sought to determine the distribution of LBP in lipid raft domains of the cytoplasmic membrane. We stimulated human monocytes with LPS, isolated the plasma membranes and solubilized them in Triton-X 100. Lipid raft domains have been found to be resistant to solubilization with Triton X-100 making them accessible to isolation by this detergent [38]. The

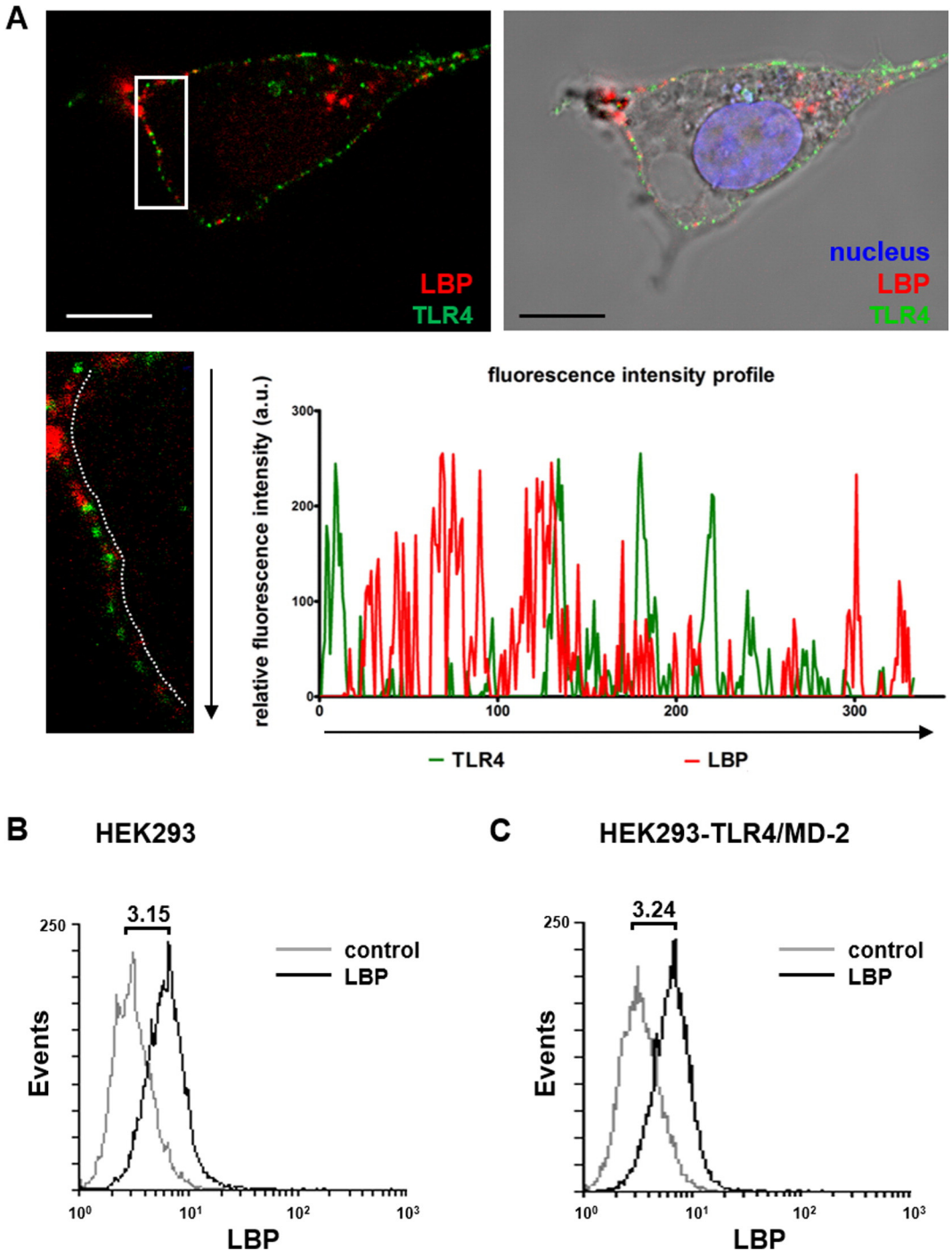


Fig. 4. LBP and TLR4 localize in different domains in the cytoplasmic membrane — (A) HEK293-hTLR4/MD-2 cells were incubated with 20 μg/ml LBP_{A647} (red) for 5 min at 37 °C and immunostained for TLR4 with 20 μg/ml of the anti-TLR4 antibody HTA125 and 2 μg/ml of anti-mouse IgG-A546 antibody (green). Cell nuclei were counterstained with DAPI (blue). Top panel shows the fluorescence image (left) and the overlay with the respective transmission light image (right). Bottom panel shows enlargement of the area marked by a white rectangle (top left) and the fluorescence intensity profile of LBP_{A647} and TLR4/A546 along the dotted white line; black arrows indicate direction. White and black bars on bottom left represent 10 μm. The image was taken from one experiment representative of three independent experiments. (B) HEK293 (left) and HEK293-hTLR4/MD-2 (right) cells were incubated with 10 μg/ml LBP_{A488} for 30 min at 37 °C. Horizontal bars depict the difference in median FI between unstained control sample (gray line) and LBP_{A488} stained sample (black line). Histograms shown are taken from one experiment representative of four independent experiments.

membrane solubilizate was fractionated over a density gradient and fractions were collected and analyzed for gangliosid-M1 (GM1), a typical raft-associated marker glycolipid, to identify raft (GM1-positive) and non-raft (GM1-negative) fractions (Fig. 5B). Probing all fractions with an anti-LBP antibody revealed that LBP was present to some degree in raft-fractions, but was predominantly localized in non-raft fractions of the cytoplasmic membrane (Fig. 5B, left). Upon LPS stimulation, LBP staining strongly increased in the fractions 3 to 5, which mark the transition between raft and non-raft domains (Fig. 5B, right). Densiometric analysis demonstrates an overall increase in LBP signal intensity upon LPS stimulation (Fig. 5C), with statistically significant changes in the non-raft fractions ($p < 0.01$), but not in the raft-fractions ($p = 0.24$). These data are well in line with our previous observation showing increased binding of LBP to macrophages in the presence of LPS (Fig. 3A, E).

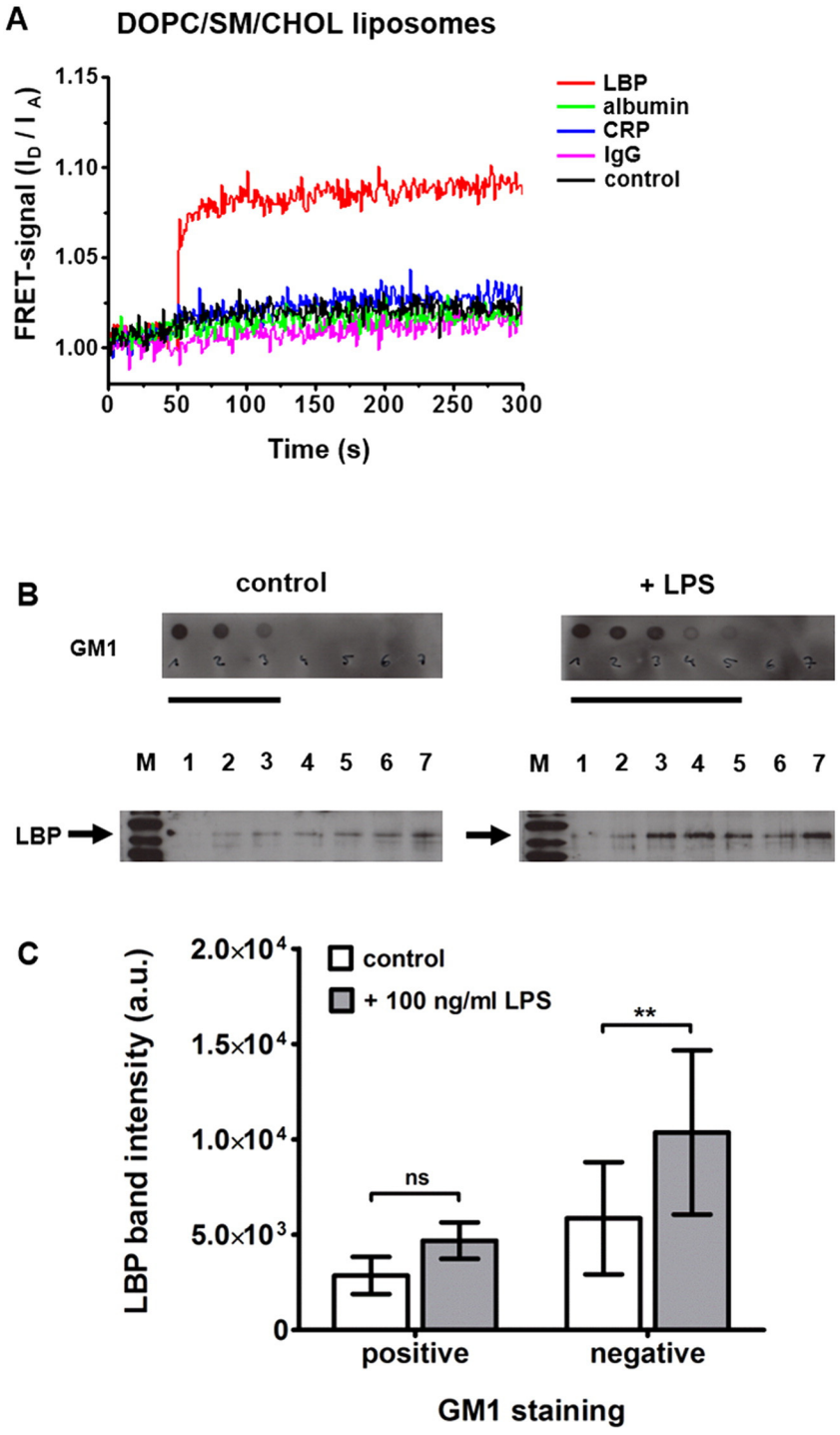


Fig. 5. LBP intercalates into phospholipid bilayers and is enriched in distinct membrane domains upon LPS stimulation — (A) FRET-spectroscopy was performed as a probe dilution assay with eukaryotic model membranes (DOPC/SM/CHOL** 9:9:2 (M); 10 μM liposomes in 20 mM HEPES, pH 7.4) Measurements were conducted at 37 °C and the emission intensities of the two dyes were equalized at the beginning of each experiment. Fluorescence emission intensities were recorded for 50 s for base line depiction and subsequently human recombinant LBP, human serum albumin, human C-reactive protein, human IgG1 or buffer (control) were added at 50 s to a final concentration of 5 μg/ml and signals were recorded for 250 s. The depicted graph is the representative of three independent measurements. (B) Human MNCs were stimulated with 100 ng/ml LPS K235 for 30 min at 37 °C. Cell membranes were solubilized in 0.1% Triton-X100 and subjected to sucrose gradient centrifugation for fractionation. Fractions were collected and immunostained for GM1 using cholera toxin B and analyzed for LBP content. Top panel shows GM1 analysis of fractions and bottom panel shows LBP staining, both of one experiment representative of 5 independent experiments. Horizontal red bars mark GM1-positive raft fractions. M is molecular weight marker. (C) Densiometric analysis of LBP band intensity on Western blot in relation to GM1 staining. Data is shown as mean ± s.d. of 5 independent experiments. Statistical analysis was performed using two-tailed students' *t*-test. Significant differences in LBP staining of control cells compared to LPS-stimulated cells is marked with ** = $p < 0.01$. P values > 0.05 are marked with 'ns'.

3.7. LBP and LPS colocalize in intracellular compartments

As LBP is the primary serum transporter of LPS to the TLR4/MD-2 receptor expressed on the cell surface, we sought to determine if LBP can execute LPS transport also to intracellular compartments. Confocal microscopy analysis of LBP and LPS localization in human macrophages revealed a distinct colocalization of LBP and LPS after 5 to 10 min of incubation at the cytoplasmic membrane (Fig. 6A indicated by arrows)

as well as in intracellular compartments (Fig. 6B). These data demonstrate that LBP mediates a transport of LPS to intracellular locations revealing a hitherto unrecognized function of this lipid-transfer protein.

Potential targets of intracellular LPS transport are cytoplasmic caspases involved in the induction of inflammatory cell activation termed pyroptosis. Mouse caspase-11 and human caspases-4 and -5 have recently been identified to be activated by intracellular LPS [39–42]. The activation of pro-inflammatory caspases by LPS can occur via the canonical pathway, which is dependent on ATP and leads to activation of caspase-1, or via the non-canonical pathway, which does not require ATP, but depends on intracellular delivery of LPS and results in the activation of caspase-4 and -5. To elucidate the potential of LBP for intracellular delivery of LPS to pro-inflammatory caspases, we stimulated human macrophages with LPS and ATP to induce canonical inflammasome activation. The fluorescent pseudosubstrate CaspACE™ which specifically binds to activated caspase-1, -4, and -5, but not to other caspases, was used to localize activated caspases. Confocal microscopy analysis revealed that LBP was in close proximity to activated caspases indicated by accumulation of the fluorescent substrate (Fig. 7A). Interestingly, no direct fluorescence overlap was observed in these structures, but rather activated caspase and LBP were localized adjacent to each other, suggesting that LBP is not part of the inflammasome complex. In macrophages showing full inflammasome activation (Fig. 7B), LBP and activated caspase colocalized in large structures with strong fluorescence overlap of LBP and caspase signals (Fig. 7C). To elucidate a potential role of LBP in the non-canonical inflammasome activation, we stimulated primed human macrophages with LPS delivered into the cells by LBP. The presence of LBP conferred IL-1β release (Fig. 7D) and also a low level of cytotoxicity (Fig. 7E) in a dose dependent manner at normal serum concentrations of 5 and 10 μg/ml.

4. Discussion

LPS is the dominant inducer of Gram-negative sepsis, a pathophysiological complication of bacterial infections with a high risk of multi-organ failure and death [15]. The treatment of patients is still a clinical challenge due to the lack of drugs that enable an effective anti-inflammatory intervention [43]. The activation of an innate immune response to LPS is highly regulated by LBP. However, the actions of LBP are versatile. Depending on its concentration in serum it can amplify, as it does at normal levels of about 5–10 μg/ml [3,8], as well as dampen the inflammatory response to LPS when LBP levels in the blood reach several hundreds μg/ml during an acute-phase reaction [44–46]. In knockout mice, the absence of LBP confers an attenuated immune response and an increased survival rate upon LPS challenge [16,17], while the ability

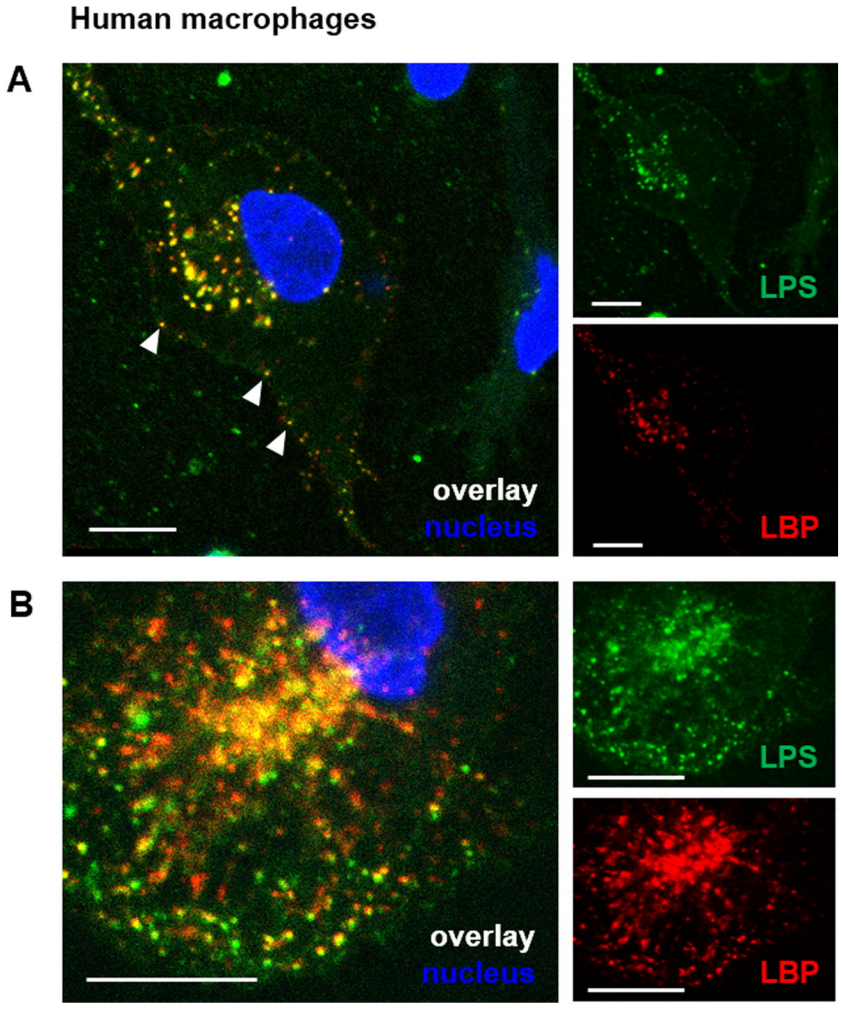


Fig. 6. LPS and LBP colocalize in cellular compartments — human macrophages were incubated with 20 μg/ml LPS (green) and 10 μg/ml LBP_{A647} (red) for 10 min at 37 °C, washed three times with ice-cold PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cell nuclei were counterstained with Hoechst 34580 (blue). (A, B) Overlay of fluorescence images of LPS + LBP treated macrophages (left side). Yellow color indicates colocalization of fluorescence signals. The individual fluorescence images are depicted on the right side. White bar represents 10 μm. Arrow heads point at colocalization of both signals at the cytoplasmic membrane. (B) Close up showing intracellular uptake of LBP and LBP by a macrophage. The images are from one experiment representative of three independent experiments.

to clear bacterial infections is strongly impaired [19,22]. In humans, a recently identified, frequently occurring single nucleotide polymorphism has been associated with an attenuated inflammatory reaction to LPS, increased risk for infectious complications in intensive care patients, and higher mortality in ventilation-associated Gram-negative pneumonia [47].

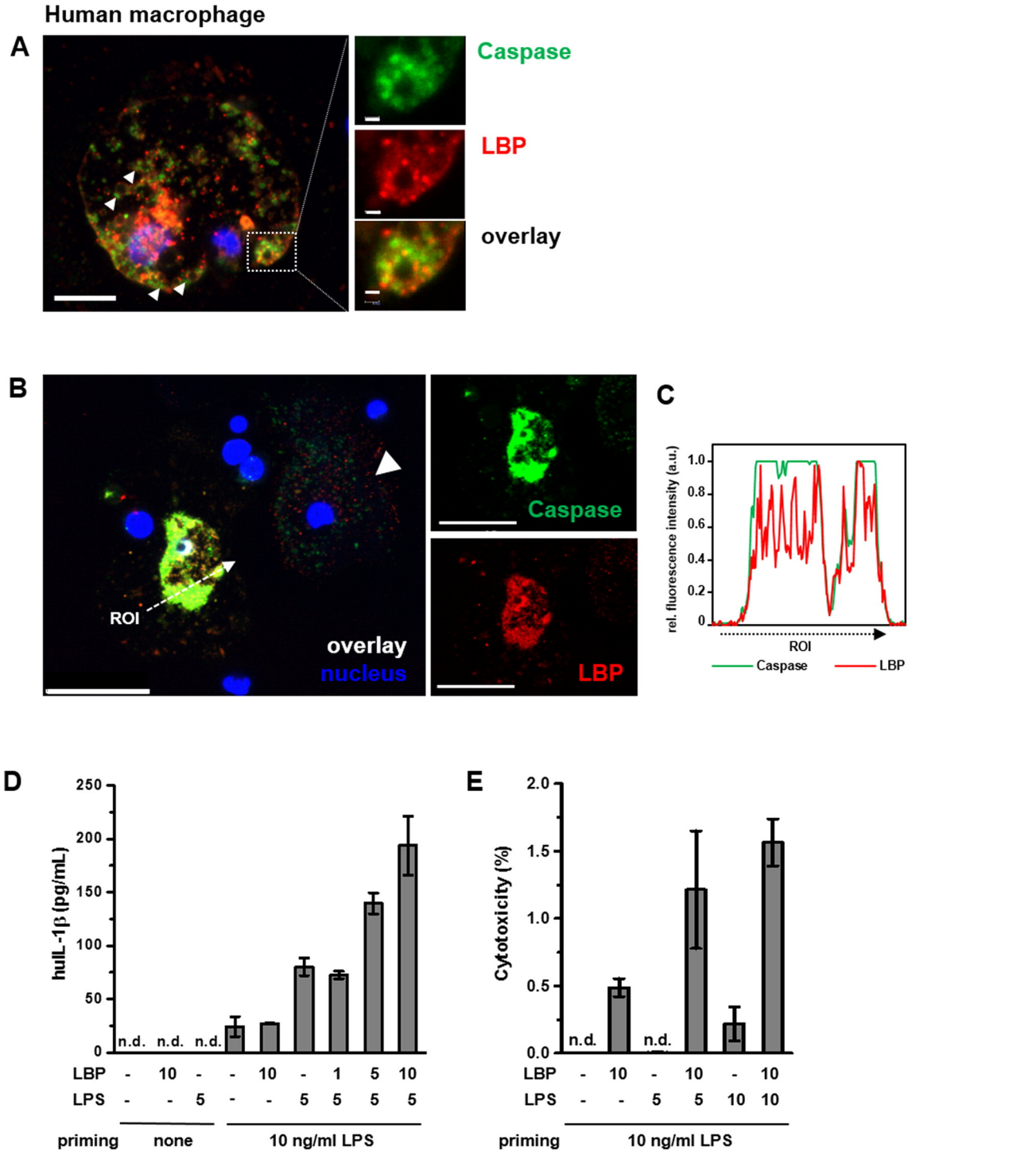


Fig. 7. LBP is localized in close the vicinity of activated caspases — human macrophages were incubated with 1 μg/ml LPS, 5 μg/ml LBP_{A647} (red), and 10 μM of caspase pseudosubstrate FITC-VAD-FMK for 23 h and subsequently stimulated 1 h in the presence of 10 mM ATP for inflammasome activation. Subsequently, cells were washed three times with ice-cold PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cell nuclei were counterstained with Hoechst 34580 (blue). (A) Overlay of fluorescence images of LPS and ATP stimulated macrophage show LBP (red) and accumulation of caspase pseudosubstrate (green). Right panels depict the individual fluorescence images. White bars represent 10 μm (left panel) and 1 μm (right panel). (B) Left cell, strong accumulation of caspase pseudosubstrate during full inflammasome activation; right cell (marked by white arrow), does not show inflammasome activation. Scale bar represents 25 μm. (C) Relative fluorescence intensities of LBP and caspase pseudosubstrate along the dotted line ROI. The images are the representative of three independent experiments. (D, E) Human macrophages were seeded at $1 * 10^5$ cells/well in 96-well dish in OPTI-MEM and allowed to attach for 3 h at 37 °C. Non-adherent cells were washed off, fresh OPTI-MEM medium was added and cells were not primed or primed with 10 ng/ml LPS for 16 h. For complex formation, LPS (5 or 10 μg/ml) was preincubated with LBP (1, 5 or 10 μg/ml) as indicated. LBP, LPS or LPS + LBP were spun onto the cells by centrifugation at 394 ×*g* for 5 min. Cell free supernatants were harvested after 20 h of incubation and assayed for IL-1β by ELISA and for lactate dehydrogenase activity by LDH assay to determine cytotoxicity. Shown is the mean ± s.d. of technical duplicates. Data are the representative of three independent experiments with cells from different donors.

Motivated by previous observations showing that LBP strongly binds a variety of eukaryotic lipids and data demonstrating interaction of the protein with reconstituted model membranes, we investigated the occurrence of LBP on mononuclear cells from peripheral blood. LBP was detected with two LBP-specific monoclonal antibodies on human monocytes but not on lymphocytes. These data demonstrate that serum-derived LBP is bound to the cytoplasmic membrane of cells derived from peripheral blood of healthy donors under resting conditions. Some other studies have shown surface detection of LBP and internalization of the protein on different cell types such as primary macrophages and CHO cells expressing recombinant CD14. Without exception, in these studies cellular LBP binding was demonstrated to be strictly dependent upon the presence of LPS. These observations were therefore primarily interpreted by the interaction of LBP with receptor molecules on the cell surface due to its LPS shuttle function [48–50].

Our binding and internalization studies on human macrophages using recombinant LBP clearly showed that LBP binding is observed in a dose-dependent manner at normal serum concentrations and does occur independently of LPS, demonstrating an inherent ability of LBP to interact with this cell type (Figs. 2, 3). This observation is of importance as it contrasts the established understanding of this pattern recognition protein as a mere carrier, delivering LPS to soluble and membrane-anchored CD14, and thereby to the TLR4/MD-2 receptor complex [12,51–53]. LPS binding was enhanced by LBP (Fig. 3B), which is in line with a number of previously published studies [3,8,54]. Interestingly, also LBP binding was enhanced in the presence of LPS in a concentration-dependent manner, demonstrating an interconnection of both ligands (Fig. 3A).

Our observation, that detection of LBP on primary human MNCs is restricted to the CD14-positive monocyte population (Fig. 1B) as compared to the CD14-negative lymphocyte population (Fig. 1C) clearly demonstrates that the interaction of LBP with host cells underlies a specificity. It appeared obvious that CD14 is a prerequisite for the binding of LBP to the cells, considering that CD14 is the primary interaction partner for LBP during LPS transport [12]. However, LPS-binding to human macrophages was not inhibited by biG14 (Fig. 3C, D), an antibody effectively blocking LPS-induced TNFα production. Experiments using human wildtype HEK293 cells which do not express CD14 proved that LBP does bind to cells in the absence of CD14 (Fig. 4). The TLR4/MD-2 receptor complex, being the dominant cellular LPS receptor, was another potential LBP interaction partner we investigated. Interestingly, our data do not support a primary role for TLR4 in cellular LBP binding under resting conditions, as it was shown to occur at equal intensities to TLR4/MD-2 negative and TLR4/MD-2 positive HEK293 cells (Fig. 4B, C).

In line with the finding that LBP interaction with host cells does not depend on TLR4 is the observation that the distribution of LBP and TLR4 occurred in different domains of the cytoplasmic membrane of TLR4/MD-2 expressing HEK293 cells (Fig. 4A). The TLR4/MD-2 complex has been shown to localize in non-raft domains migrating to lipid raft domains following LPS stimulation [37]. Analysis of the localization and dynamics of LBP in this study demonstrate a distinct compartmentalization of LBP in the cytoplasmic membrane of monocytes. It is primarily present in non-raft domains under resting conditions (Fig. 5B, left panel). This behavior of LBP appeared somewhat similar to TLR4. However, upon LPS exposure, we observed a change in the distribution and an increase in the amount of LBP in the cytoplasmic membrane. LBP enriched in fractions at the border between raft and non-raft domains with the larger portion still present in the non-raft domains (Fig. 5B, right panel), demonstrating a different behavior than TLR4. Notably, the differentiation in raft- and non-raft domains reflects only certain aspects of biological membrane compartmentalization. According to these data, the primary LBP interaction with human cell lines cannot be attributed to the known LPS-related receptor proteins. The FRET-studies on liposomes resembling the eukaryotic membrane demonstrated membrane partitioning of LBP into the liposomal membrane (Fig. 5A), supporting a mechanism that depends on the interaction of LBP with membrane lipids. The recently resolved crystal structure of murine LBP revealed two phospholipid binding pockets in LBP, which may contribute to the observed membrane partitioning [47]. Differences in membrane lipid composition and domain structure of immune cell populations could be possible factors influencing this process. We have demonstrated earlier that LBP does catalyze the intercalation of LPS into reconstituted phospholipid bilayers [28], providing a mechanism of LBP mediated transport of LPS into host cell membranes. Subsequent studies hinted towards an involvement of cell-bound LBP in LPS-mediated macrophage activation [55]. In accordance with this is the observed intracellular colocalization of LBP and LPS (Fig. 6). This raises the question of the biological function of LBP-mediated LPS transport to intracellular compartments. Since host cell interaction of LBP was observed independently of TLR4 and of CD14, it can be concluded that LBP provides a TLR4-independent pathway for intracellular LPS delivery. This is of considerable interest with regard to a previously published study suggesting a role for LBP in TLR4 activation in the endosomal compartment leading to IFN-β induction [56]. This pathway may also be important for TLR4-dependent signaling in cell types lacking surface expression of TLR4 such as epithelial cells of intestines [57] and coronary artery lining [58]. Of note, the recently identified TLR4-independent pathway of LPS induced pyroptosis that depends on intracellular activation of caspases [39–42] represents the first example of a completely TLR4-independent pathway for LPS recognition. The biological mechanism for intracellular delivery of LPS driving caspase activation is not resolved yet. Our data demonstrate a close spatial proximity of LBP to caspases in macrophages activated by LPS and ATP via the canonical pathway (Fig. 7A, B, C). In addition, our data show an involvement of LBP in LPS-induced IL-1β release and pyroptosis via non-canonical inflammasome activation, i.e. in the absence of ATP (Fig. 7D, E). These results demonstrate a role of LBP in inflammasome activation pathways and will be further addressed in future studies.

We show here for the first time that LBP has an intrinsic capacity to interact with human monocytes and macrophages via a mechanism that does not depend on the LPS receptors CD14 and TLR4, and may be potentially based on membrane intercalating properties of LBP. Our results expand the current view of LBP as a mere extracellular transport protein in serum delivering LPS to cell surface receptors (Fig. 8). Our data provide evidence for a function of LBP in the intracellular management of LPS by human immune cells pointing towards a new role of this LPS transporter in regulating the immune response to bacterial infections.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

A.B.S. conceived the study, designed, and interpreted the experiments, and performed the confocal microscopy experiments. F.K. conceived, performed, analyzed, and interpreted the experiments. S.K. performed and analyzed the inflammasome activation experiments. A.B.S. and F.K wrote the manuscript and all authors approved the final version of the manuscript.

Transparency document

The transparency document associated with this article can be found, in online version.

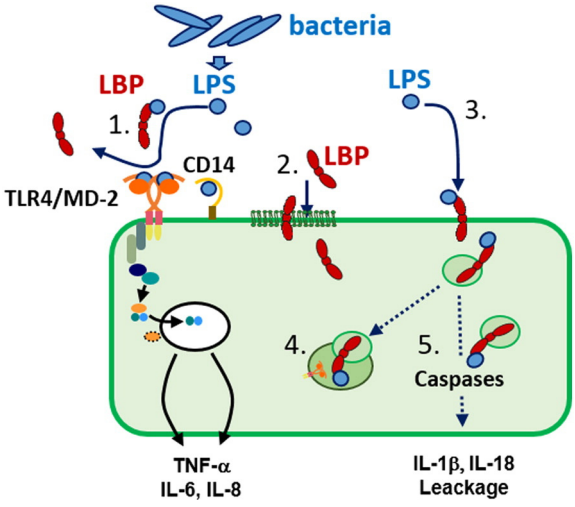


Fig. 8. Model of biological functions of LBP targeting the host cell. (1.) LBP in serum binds LPS from bacteria or LPS-aggregates in the blood stream and mediates the transport of LPS via soluble or GPI-anchored CD14 to the TLR4/MD-2 receptor complex on the cytoplasmic membrane of moncytes and macrophages. Upon delivery of LPS to the cellular receptor proteins, free LBP is released to the serum. This shuttle function of LBP sensitizes the TLR4/MD-2 receptor system to low amounts of LPS and enhances cell activation. (2.) LBP in serum has an intrinsic capacity to integrate into eucaryotic model membranes. In accordance with the observation of LBP membrane partitioning is the finding that monocytes from healthy donors carry LBP protein on the cell surface. (3.) Cell associated LBP can bind LPS and the LBP/LPS complex is internalized to intracellular compartments. The intracelluar transport of LPS by LBP raises the possibiliy of a role for LBP in the delivery of LPS to intracelluar LPS receptors such as (4.) endosomal TLR4 or (5.) cytoplasmic caspases involved in the induction of pyroptosis upon activation by intracellular LPS.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Irina von Cube, Sabrina Groth, and Christine Hamann. We thank Helga Lüthje for the purification of LPS and Buko Lindner for mass spectrometry analysis. Flow cytometry and confocal microscopy experiments were performed at the Flow Cytometry Core Facility. We thank Dieter Adam for critically reading the manuscript

References

[1] C.D. Bingle, C.J. Craven, Meet the relatives: a family of BPI- and LBP-related proteins, Trends Immunol. 25 (2004) 53–55.
[2] N.W.J. Schroeder, R.R. Schumann, Non-LPS targets and actions of LPS binding protein (LBP), J. Endotoxin Res. 11 (2005) 237–242.
[3] R.R. Schumann, S.R. Leong, G.W. Flaggs, P.W. Gray, S.D. Wright, J.C. Mathison, P.S. Tobias, R.J. Ulevitch, Structure and function of lipopolysaccharide binding protein, Science 249 (1990) 1429–1431.
[4] R.R. Schumann, C.J. Kirschning, A. Unbehaun, H.P. Aberle, H.P. Knope, N. Lamping, R.J. Ulevitch, F. Herrmann, The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins, Mol. Cell. Biol. 16 (1996) 3490–3503.
[5] P.S. Tobias, K. Soldau, R.J. Ulevitch, Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum, J. Exp. Med. 164 (1986) 777–793.
[6] M.A. Dentener, A.C.E. Vreugdenhil, P.H.M. Hoet, J.H.J. Vernooy, F.H.M. Nieman, D. Heumann, Y.M.W. Janssen, W.A. Buurman, E.F.M. Wouters, Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells, Am. J. Respir. Cell Mol. Biol. 23 (2000) 146–153.
[7] A.C.E. Vreugdenhil, M.A. Dentener, A.M.P. Snoek, J.-W.M. Greve, W.A. Buurman, Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response, J. Immunol. 163 (1999) 2792–2798.
[8] P.S. Tobias, J. Mathison, D. Mintz, J.-D. Lee, V. Kravchenko, K. Kato, J. Pugin, R.J. Ulevitch, Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation, Am. J. Respir. Cell Mol. Biol. 7 (1992) 239–245.
[9] B. Beutler, E.T. Rietschel, Innate immune sensing and its roots: the story of endotoxin, Nat. Rev. Immunol. 3 (2003) 169–176.
[10] A. Poltorak, X. He, I. Smirnova, M.-Y. Liu, C.V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (1998) 2085–2088.
[11] R. Shimazu, S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, M. Kimoto, MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4, J. Exp. Med. 189 (1999) 1777–1782.
[12] S.D. Wright, R.A. Ramos, P.S. Tobias, R.J. Ulevitch, J.C. Mathison, CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein, Science 249 (1990) 1431–1433.
[13] A.B. Schromm, E. Lien, P. Henneke, J.C. Chow, A. Yoshimura, H. Heine, E. Latz, B.G. Monks, D.A. Schwartz, K. Miyake, D.T. Golenbock, Molecular genetic analysis of an endotoxin nonresponder mutant cell line A point mutation in a conserved region of Md-2 abolishes endotoxin-induced signaling, J. Exp. Med. 194 (2001) 79–88.
[14] M. Rossol, H. Heine, U. Meusch, D. Quandt, C. Klein, M.J. Sweet, S. Hauschildt, LPS-induced cytokine production in human monocytes and macrophages, Crit. Rev. Immunol. 31 (2011) 379–446.
[15] J. Cohen, The immunopathogenesis of sepsis, *Nature* 420 (2002) 885–891.
[16] R.S. Jack, X. Fan, M. Bernheiden, G. Rune, M. Ehlers, A. Weber, G. Kirsch, R. Mentel, B. Fürll, M. Freudenberg, G. Schmitz, F. Stelter, C. Schütt, Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection, Nature 389 (1997) 742–745.
[17] M.M. Wurfel, B.G. Monks, R.R. Ingalls, R.L. Dedrick, R. Delude, D. Zhou, N. Lamping, R.R. Schumann, R. Thieringer, M.J. Fenton, S.D. Wright, D. Golenbock, Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact, J. Exp. Med. 186 (1997) 2051–2056.
[18] J. Fierer, M.A. Swancutt, D. Heumann, D. Golenbock, The role of lipopolysaccharide binding protein in resistance to salmonella infections in mice, J. Immunol. 168 (2002) 6396–6403.
[19] S. Knapp, A.F. de Vos, S. Florquin, D.T. Golenbock, T. van der Poll, Lipopolysaccharide binding protein is an essential component of the innate immune response to *Escherichia coli* peritonitis in mice, Infect. Immun. 71 (2003) 6747–6753.
[20] K.K. Yang, B.G. Dorner, U. Merkel, B. Ryffel, C. Schütt, D. Golenbock, M.W. Freeman, R.S. Jack, Neutrophil influx in response to a peritoneal infection with salmonella Is delayed in lipopolysaccharide-binding protein or CD14-deficient mice, J. Immunol. 169 (2002) 4475–4480.
[21] J. Branger, S. Florquin, S. Knapp, J.C. Leemans, J.M. Pater, P. Speelman, D.T. Golenbock, T.v.d. Poll, LPS-binding protein-deficient mice have an impaired defense against gram-negative but not gram-positive pneumonia, Int. Immunol. 16 (2004) 1605–1611.
[22] M.-H. Fan, R.D. Klein, L. Steinstraesser, A.C. Merry, J.A. Nemzek, D.G. Remick, S.C. Wang, G.L. Su, An essential role for lipopolysaccharide-binding protein in pulmonary innate immune responses, Shock 18 (2002) 248–254.
[23] A. Gonzalez-Quintela, M. Alonso, J. Campos, L. Vizcaino, L. Loidi, F. Gude, Determinants of serum concentrations of lipopolysaccharide-binding protein (LBP) in the adult population: the role of obesity, PLoS ONE 8 (2013), e54600.
[24] J.A. Hubacek, J. Pitha, Z. Skodová, V. AdámkovÁ, I. Podrapska, G. Schmitz, R. Poledne, Polymorphisms in the lipopolysaccharide-binding protein and bactericidal/permeability-increasing protein in patients with myocardial infarction, Clin. Chem. Lab. Med. 40 (2002) 1097–1100.
[25] M. Mueller, C. Stamme, C. Draing, T. Hartung, U. Seydel, A.B. Schromm, Cell activation of human macrophages by lipoteichoic acid is strongly attenuated by lipopolysaccharide-binding protein, J. Biol. Chem. 281 (2006) 31448–31456.
[26] B. Yu, E. Hailman, S.D. Wright, Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids, J. Clin. Invest. 99 (1997) 315–324.
[27] T. Gutsmann, N. Haberer, S.F. Carroll, U. Seydel, A. Wiese, Interaction between lipopolysaccharide (LPS), LPS-binding protein (LBP), and planar membranes, Biol. Chem. 382 (2001) 425–434.
[28] A.B. Schromm, K. Brandenburg, E.T. Rietschel, H.-D. Flad, S.F. Carroll, U. Seydel, Lipopolysaccharide-binding protein mediates CD14-independent intercalation of lipopolysaccharide into phospholipid membranes, FEBS Lett. 399 (1996) 267–271.
[29] C. Galanos, O. Lüderitz, O. Westphal, A new method for the extraction of R lipopolysaccharides, Eur. J. Biochem. 9 (1969) 245–249.
[30] C. Galanos, O. Lüderitz, O. Westphal, Preparation and properties of a standardized lipopolysaccharide from salmonella abortus equi (novo-pyrexal), Zentralbl. Bakteriol. Parasitenkd. Infekt. Hyg. Erste Abt. Orig. Reihe Med. Mikrobiol. Parasitol. 243 (1979) 226–244.
[31] M. Hirschfeld, Y. Ma, J.H. Weis, S.N. Vogel, J.J. Weis, Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2, J. Immunol. 165 (2000) 618–622.
[32] A.B. Schromm, J. Howe, A.J. Ulmer, K.-H. Wiesmüller, T. Seyberth, G. Jung, M. Rössle, M.H.J. Koch, T. Gutsmann, K. Brandenburg, Physicochemical and biological analysis of synthetic bacterial lipopeptides: validity of the concept of endotoxic conformation, J. Biol. Chem. 282 (2007) 11030–11037.
[33] S.P. Keese, K. Brandenburg, M. Roessle, A.B. Schromm, Pulmonary surfactant protein A-induced changes in the molecular conformation of bacterial deep-rough LPS lead to reduced activity on human macrophages, Innate Immun. 20 (2014) 787–798.
[34] J. Jäger, S. Keese, M. Roessle, M. Steinert, A.B. Schromm, Fusion of legionella pneumophila outer membrane vesicles with eukaryotic membrane systems is a mechanism to deliver pathogen factors to host cell membranes, Cell. Microbiol. 17 (2015) 607–620.
[35] M. Triantafilou, K. Miyake, D.T. Golenbock, K. Triantafilou, Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation, J. Cell Sci. 115 (2002) 2603–2611.
[36] B.S. Park, D.H. Song, H.M. Kim, B.-S. Choi, H. Lee, J.-O. Lee, The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex, Nature 458 (2009) 1191–1195.

[37] M. Triantafilou, S. Morath, A. Mackie, T. Hartung, K. Triantafilou, Lateral diffusion of toll-like receptors reveals that they are transiently confined within lipid rafts on the plasma membrane, J. Cell Sci. 117 (2004) 4007–4014.
[38] D.A. Brown, E. London, Structure and origin of ordered lipid domains in biological membranes, J. Membr. Biol. 164 (1998) 103–114.
[39] J.A. Hagar, D.A. Powell, Y. Aachoui, R.K. Ernst, E.A. Miao, Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock, Science 341 (2013) 1250–1253.
[40] N. Kayagaki, M.T. Wong, I.B. Stowe, S.R. Ramani, L.C. Gonzalez, S. Akashi-Takamura, K. Miyake, J. Zhang, W.P. Lee, A. Muszyński, L.S. Forsberg, R.W. Carlson, V.M. Dixit, Noncanonical inflammasome activation by intracellular LPS independent of TLR4, Science 341 (2013) 1246–1249.
[41] J. Shi, Y. Zhao, Y. Wang, W. Gao, J. Ding, P. Li, L. Hu, F. Shao, Inflammatory caspases are innate immune receptors for intracellular LPS, Nature 514 (2014) 187–192.
[42] C.N. Casson, J. Yu, V.M. Reyes, F.O. Taschuk, A. Yadav, A.M. Copenhaver, H.T. Nguyen, R.G. Collman, S. Shin, Human caspase-4 mediates noncanonical inflammasome activation against gram-negative bacterial pathogens, Proc. Natl. Acad. Sci. 112 (2015) 6688–6693.
[43] D.C. Angus, The search for effective therapy for sepsis: back to the drawing board? J. Am. Med. Assoc. 306 (2011) 2614–2615.
[44] N. Lamping, R. Dettmer, N.W. Schröder, D. Pfeil, W. Hallatschek, R. Burger, R.R. Schumann, LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria, J. Clin. Invest. 101 (1998) 2065–2071.
[45] J. Zweigner, H.-J. Gramm, O.C. Singer, K. Wegscheider, R.R. Schumann, High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes, Blood 98 (2001) 3800–3808.
[46] L. Hamann, C. Alexander, C. Stamme, U. Zähringer, R.R. Schumann, Acute-phase concentrations of lipopolysaccharide (LPS)-binding protein inhibit innate immune cell activation by different LPS Chemotypes via different mechanisms, Infect. Immun. 73 (2005) 193–200.
[47] J.K. Eckert, Y.J. Kim, J.I. Kim, K. Gürtler, D.-Y. Oh, S. Sur, L. Lundvall, L. Hamann, A. van der Ploeg, P. Pickkers, E. Giamarellos-Bourboulis, A.V. Kubarenko, A.N. Weber, M. Kabesch, O. Kumpf, H.-J. An, J.-O. Lee, R.R. Schumann, The crystal structure of lipopolysaccharide binding protein reveals the location of a frequent mutation that impairs innate immunity, Immunity 39 (2013) 647–660.
[48] J.A. Gegner, R.J. Ulevitch, P.S. Tobias, Lipopolysaccharide (LPS) signal transduction and clearance — dual roles for LPS binding protein and membrane CD14, J. Biol. Chem. 270 (1995) 5320–5325.
[49] R.I. Tapping, P.S. Tobias, Cell surface binding of LBP—LPS complexes to a protein component distinct from CD14, J. Endotoxin Res. 5 (1999) 52–55.
[50] S.D. Wright, P.S. Tobias, R.J. Ulevitch, R.A. Ramos, Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages, J. Exp. Med. 170 (1989) 1231–1241.
[51] T.L. Gioannini, D. Zhang, A. Teghanemt, J.P. Weiss, An essential role for albumin in the interaction of endotoxin with lipopolysaccharide-binding protein and sCD14 and resultant cell activation, J. Biol. Chem. 277 (2002) 47818–47825.
[52] T.L. Gioannini, A. Teghanemt, D. Zhang, N.P. Coussens, W. Dockstader, S. Ramaswamy, J.P. Weiss, Isolation of an endotoxin–MD-2 complex that produces toll-like receptor 4-dependent cell activation at picomolar concentrations, Proc. Natl. Acad. Sci. 101 (2004) 4186–4191.
[53] E. Hailman, H.S. Lichenstein, M.M. Wurfel, D.S. Miller, D.A. Johnson, M. Kelley, L.A. Busse, M.M. Zukowski, S.D. Wright, Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14, J. Exp. Med. 179 (1994) 269–277.
[54] T.R. Martin, J.C. Mathison, P.S. Tobias, D.J. Letúrcq, A.M. Moriarty, R.J. Maunder, R.J. Ulevitch, Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs, J. Clin. Invest. 90 (1992) 2209–2219.
[55] T. Gutsmann, M. Müller, S.F. Carroll, R.C. MacKenzie, A. Wiese, U. Seydel, Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells, Infect. Immun. 69 (2001) 6942–6950.
[56] A. Kato, T. Ogasawara, T. Homma, H. Saito, K. Matsumoto, Lipopolysaccharide-binding protein critically regulates lipopolysaccharide-induced IFN-β signaling pathway in human monocytes, J. Immunol. 172 (2004) 6185–6194.
[57] M.W. Hornef, B.H. Normark, A. Vandewalle, S. Normark, Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells, J. Exp. Med. 198 (2003) 1225–1235.
[58] S. Dunzendorfer, H.-K. Lee, K. Soldau, P.S. Tobias, Toll-like receptor 4 functions intracellularly in human coronary artery endothelial cells: roles of LBP and sCD14 in mediating LPS responses, FASEB J. 18 (2004) 1117–1119.