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Innate Immunity

A Genetic Variation of Lipopolysaccharide Binding Protein Affects the Inflammatory Response and Is Associated with Improved Outcome during Sepsis

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ABSTRACT

LPS binding protein (LBP) is an important innate sensor of microbial cell wall structures. Frequent functionally relevant mutations exist and have been linked to influence susceptibility to and course of bacterial infections. We examined functional properties of a single nucleotide polymorphism resulting in an exchange of phenylalanine to leucine at position 436 of LBP (rs2232618) and compared the frequent variant of the molecule with the rare one in ligand binding experiments. We then stimulated RAW cells with bacterial ligands in the presence of serum obtained from individuals with different LBP genotypes. We, furthermore, determined the potential effects of structural changes in the molecule by in silico modeling. Finally, we analyzed 363 surgical patients for this genetic variant and examined incidence and course of sepsis following surgery. We found that binding of LBP to bacterial ligands was reduced, and stimulation of RAW cells resulted in an increased release of TNF when adding serum from individuals carrying the F436L variant as compared with normal LBP. In silico analysis revealed structural changes of LBP, potentially explaining some of the effects observed for the LBP variant. Finally, patients carrying the F436L variant were found to be similarly susceptible for sepsis. However, we observed a more favorable course of severe infections in this cohort. Our findings reveal new insights into LPS recognition and the subsequent activation of the innate immune system brought about by LBP. The identification of a genetic variant of LBP influencing the course of sepsis may help to stratify individuals at risk and thus reduce clinical complications of patients. *ImmunoHorizons*, 2021, 5: 972–982.

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[1]O.K. and K.G. contributed equally to this manuscript.

Abbreviations used in this article: CI, confidence interval; ICU, intensive care unit; ID, identifier; LBP, LPS binding protein; OR, odds ratio; PDB, Protein Data Bank; Phe, phenylalanine; PLTP, phospholipid transfer protein; SNP, single nucleotide polymorphism.

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INTRODUCTION

The LPS binding protein (LBP) is an acute-phase protein primarily secreted from the liver with substantial concentrations also released from pulmonary and gut epithelial cells (1–4). It belongs to a larger family of phospholipid transfer proteins (PLTPs), which transfer lipid derivates and lipopeptides throughout the body influencing lipid homeostasis (5, 6). These functions are associated with common diseases like vascular and heart disease but are also known to be involved in innate immunity (7, 8). It has recently been shown that LBP particularly facilitates the transfer of multimers of LPS to its sensing receptor consisting of CD14, TLR4, and MD2, initiating the inflammatory response (9–11). Furthermore, it plays a role in detoxification of LPS by transferring LPS into lipoproteins (4, 12–15). In addition, LBP is also able to bind lipopeptides originating from both Gram-negative and Gram-positive bacteria and to mediate their proinflammatory effects (16–18). Recently, it has been postulated to be associated with bacterial translocation in the gut, potentially adding function to this molecule by sensing/scavenging bacterial material entering the body through a deranged gut wall (19). Finally, it has been shown that LBP, in a similar manner as other members of the genetically related family of PLTPs, such as cholesterol ester transfer protein and PLTP, can bind and transfer phospholipids. This mechanism most likely is important for LPS transfer from micelles to HDL particles and into membranes but potentially may represent a general lipid transport system (20, 21).

In critically ill patients, LBP levels are markedly increased severalfold (22). LBP effects within the host depend strongly on its concentrations in serum, in which lower concentrations are responsible for effective initiation of LPS sensing. However, high acute-phase concentrations of LBP exert a rather inhibiting effect on the immune response (23–25). Underlying mechanisms of this phenomenon are not yet completely understood but may involve facilitation of LPS internalization brought about by LBP (26).

Hereditary factors have been studied in the context of sepsis, and consequently, a growing number of genetic factors was identified that contribute to risk and the course of severe forms of sepsis (27, 28). Single nucleotide polymorphisms (SNPs) of the innate immune system have been described that influence individual responses to invading pathogens (29).

For the LBP gene on chromosome 20, at least two studies were able to show association of mutations with altered inflammatory responses (30, 31), and haplotype studies were able to associate genetic alteration to outcome (32, 33). In a very recent comprehensive review of this year, it was concluded that genetic variations of LBP alter the risk for inflammatory complications (34). For the purposes of this study, nonsynonymous LBP SNPs were deemed as appropriate candidates to assess structural changes in the molecule in combination with functional and clinical data. Other LBP SNPs identified with regard to infections and sepsis were mostly promotor variants or synonymous SNPs (34). In this study, we compared patients carrying different variants of LBP regarding a coding SNP (rs2232618) at position 436, leading to an amino acid exchange from phenylalanine (Phe) to leucine in the LBP molecule (31, 35, 36). We previously studied a different SNP (rs2232613) leading to a change at amino acid position 333 in a comparable experimental setting with patient data from the same cohort also partly included in this study. These results showed an altered molecular response and an influence on clinical outcome (37). Patients carrying this SNP were excluded from the primary analysis. The SNP further evaluated in this study has been shown by others to be associated with altered sepsis prevalence and mortality in Chinese trauma victims (38, 39).

The SNP was initially thought to cause an exchange from leucine to Phe, but recent sequence analysis revealed that the cystine to thymidine exchange is more frequent in Western European inhabitants. Therefore, the c1341t genotype (nucleotide sequence: TTC leading to Phe) was considered to be the common variant. As a continuation of a previous study, we show in this study how functional properties of the LBP molecule are altered by this SNP (37). We assessed binding of several ligands to the variants of LBP. As molecular patterns originating from bacteria, we used LPS and lipopeptides. Phosphatidylethanolamine was used as a nonbacterial ligand to assess transfer ability to LBP as has been done in previous experiments (21). Furthermore, we used the murine RAW 246.7 cell line and stimulated the cells with the ligands in the presence and absence of serum from patients carrying the rare variant of LBP. We used this as a macrophage model for LPS recognition and measured the in vitro release of a proinflammatory cytokine (TNF). In this way, we were able to simulate the structural and biochemical influence of this SNP on the basis of the recently discovered molecular structure of the whole LBP molecule using different bioinformatic tools (40). We also examined this functionally relevant SNP in a cohort of 363 surgical intensive care unit (ICU) patients from European ancestry and associated it with the susceptibility to postoperative infections and outcome. We hypothesized that the variant of LBP studied in this study would affect its ability to confer its main biological function. We sought to determine whether structural changes in the molecule could explain this behavior. Finally, we tried to determine whether these findings of altered structure and function would influence the clinical course of patients with postoperative infections carrying this SNP.

MATERIALS AND METHODS

Binding assays of LBP-containing sera of distinct genotypes with bacterial and synthetic ligands

For binding assays, either LPS (*Escherichia coli* O111:B4 "smooth" LPS, Sigma-Aldrich, Taufkirchen, Germany), Pam_2Cys [Pam_2 Cys-Ser-$(Lys)_4$ × 3 trifluoroacetic acid, EMC Microcollections, Tübingen, Germany], Pam_3Cys (Pam_2Cys-Ser-Lys_4, EMC Microcollections), or 3-sn-PE (3-sn-phosphatidylethanolamine, Sigma-Aldrich) were placed on an ELISA plate in a concentration of 30

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μg/ml each in carbonate buffer (pH 8.2). Plates were incubated with sera containing LBP of individuals carrying either the common or the rare genotype in increasing concentrations (0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25%, respectively). These sera were diluted 1:2 from a maximal concentration of 25%. We used sera from different individuals with a mean LBP concentration of 17 ng/ml for common variant sera and 15.8 ng/ml for sera of heterozygous individuals. Following incubation, the photometric reaction was stopped with 1 M H_2SO_4. OD of the bound ligands was photometrically measured at 450 nm in an ELISA reader (Photometer Spectra Fluor Plus, Tecan, Crailsheim, Germany).

Stimulation of murine macrophage cells

Murine macrophage cells (RAW 264.7, Leibniz-Institut DSMZ, Braunschweig, Germany) were stimulated with 1, 10, and 100 ng/ml of two different LPS types (smooth LPS [*E. coli* 0111:B4] and "rough" LPS [*Salmonella minnesota* Re595, Sigma-Aldrich]) and two different lipopeptides (Pam_2Cys [Pam_2Cys-Ser-$(Lys)_4$ × 3 TFA, EMC Microcollections] and Pam_3Cys [Pam_3Cys-Ser-$(Lys)_4$, EMC Microcollections]) in addition to serum of individuals with different genotypes (concentrations of either 1, 2, and 5%). Concentrations of TNF were determined at 4 h using ELISA (DPC Biermann, Bad Nauheim, Germany). All measurements were performed in duplicate, resulting in four values per experiment.

In silico analysis

The sequence information about human LBP SNP rs2232618 was retrieved from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). MutPred was used to determine the effect of the amino acid substitutions (41). To investigate the secondary structures of common and rare variants, PSIPRED was used (42). I-Mutant 3.0 (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) was applied for the determination of free energy change (ΔΔG) and protein stability changes owing to single-site mutations (43). The crystal structure of the LBP protein was obtained from Protein Data Bank (PDB identifier [ID]: 4M4D) (44). This was used as a template for generating the three-dimensional structure of common and rare variant human LBP. The three-dimensional structures were generated and energy minimized with SWISS-MODEL and Swiss-PdbViewer, respectively (45, 46). For the purpose of validating these structures, PROSA, ProQ, and SAVES (https://saves.mbi.ucla.edu/) were used (47, 48). Finally, the modeled structures of human LBP common and rare variant types were visualized with PyMOL. Docking of LPS with variants of human LBP were carried out using PatchDock and Firedock software (49, 50). The software DUET (http://biosig.unimelb.edu.au/duet/) was applied to study the role of mutation on the protein structure, using a combination of support vector machine and machine learning algorithms. The implication of mutation on the domain core stability was predicted using ELASPIC (51). ProFunc was used to figure out the alterations between common and rare variants of the proteins regarding functional clefts and cavities (52). ConSurf estimated the conservation profile of the key residues related to functional regions in the structures (53). The differences in the conserved residues of the major functional clefts were studied. CASTp was employed to examine the prevalence of functional pockets in the modeled structures (54). The solvent-accessible area and changes because of mutations were determined with GETAREA (55).

We also subjected the sequence information from the L variant of LBP SNP rs2232613 described in (37) to MutPred, PSIPRED, and I-Mutant 3.0, respectively, and analyzed the modeled structures of this variant with DUET, ELASPIC, ProFunc, ConSurf, CASTp, and GETAREA.

Genotyping

The frequency of the LBP SNP F436L (rs2232618) was determined by real-time PCR assays with subsequent melting curve analysis using the LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany). Sequenced controls representing different genotypes were included in each reaction. Oligonucleotides used for genotyping were as follows: primers, forward: 5′-TTTGCTTTTCCCAAGCGTT-3′ and reverse: 5′-GAGCCCTGTTTTCCAAGTCC-3′; and probes, sensor: 5′-CTATTACATCCTTAACACCCTCTAC-FL-3′ and anchor: red 5′-640-CCAAGTTCAATGGTAAGAATCACTGTGG-3′. One reaction volume of 20 μl contained 2 μl 10× PCR Buffer, 2 mM $MgCl_2$, 125 μM NTPs, 5 U Taq polymerase, 3 μg BSA, primers at 0.5 μM (LBP forward/reverse), fluorescence probes at 0.2 μM each, and 5–20 ng DNA. On the LightCycler 1.5 platform, PCR parameters were as follows: initial denaturation at 95°C for 4 min, 40 cycles of denaturation (95°C for 1 s), annealing (56°C for 10 s), and extension (72°C for 8 s) with subsequent melting curve analysis: 1 cycle at 95°C for 10 s, 40°C for 30 s, followed by an increase of temperature to 80°C at a slope of 0.1°C/s. All oligonucleotides were manufactured by TIB MOLBIOL (Berlin, Germany). PCR reagents were obtained from Rapidozym (Berlin, Germany).

Patient selection

The local ethics committee of the Charité – Universitätsmedizin Berlin approved this clinical study (AA3/03/45). DNA testing was permitted by a signed broad written consent including DNA testing before surgery. All steps were performed according to the Helsinki declaration. Statistical analysis was carried out after anonymization of the patients' data. Definition of sepsis (systemic inflammatory response syndrome, sepsis, severe sepsis, and septic shock) was based on published criteria (56). We did not reclassify the patients according to new sepsis definitions because recent publications show that matching is not reliable (57, 58).

The patient cohort from this study was previously described (59). Infections were defined as described by the clinical classification for nosocomial infections of the National Institutes of Health (60). A total number of 363 patients fulfilled the inclusion criteria. Severity of disease was assessed by the Simplified Acute Physiology Score on admission (61). Patients were followed up until discharge from the hospital. Patient DNA was extracted

from blood or tissue specimens collected prior to surgery and were examined for the LBP SNPs.

Then, 675 individuals from a group of 692 volunteers (healthy individuals and blood donors) served as controls for frequencies of these SNPs. Characteristics of these individuals were published recently (62). All individuals consented to genetic testing. Blood donors were anonymized, and the healthy individuals gave written informed consent. Either blood or oral swabs were used for DNA extraction using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany).

Statistical analysis

Contingency tables were statistically tested with the χ^2 test or Fisher exact test where appropriate for differences in frequencies. Odds ratios (OR) were determined using the χ^2 test. Differences in numerical data were compared with unpaired *t* test or Mann–Whitney *U* test. For statistical analysis, the IBM SPSS Statistics 20.0 software package (IBM) and the Prism 8 Software package (GraphPad Software) were used. A two-tailed $p < 0.05$ was considered statistically significant.

RESULTS

Binding of bacterial or synthetic ligands to serum of carriers with the F436L genotype

We hypothesized that the F436L variant of LBP was associated with altered binding of bacterial ligands. To study this, we incubated plates containing different bacterial ligands to the binding of progressively diluted serum of individuals with different genotypes. For all tested ligands, we were able to record reduced binding in carriers of the F436L mutation of LBP as shown in Fig. 1. Very low concentrations showed only little influence that was not statistically significant. When reaching increased concentrations (3.13% up to 25% of serum), carriers of the mutation bound ligands to a lesser degree.

This was detectable for ligands originating from Gram-negative bacteria (LPS 0111:B4) for serum concentrations of 6.25 and 12.5%, in which binding of LPS differed significantly between common and rare variant LBP (6.25: 0.507 versus 0.354, $p = 0.048$ and 12.5: 0.734 versus 0.526, $p = 0.010$) (Fig. 1A). This was as detectable in ligands from Gram-positive bacteria, although to a lesser degree. In this study, binding of the lipopeptide Pam_2Cys was markedly reduced in all experiments (0.78%: 0.169 versus 0.155, $p = 0.026$; 1.56%: 0.231 versus 0.179, $p = 0.002$; 3.125%: 0.248 versus 0.187, $p < 0.001$; 6.25%: 0.254 versus 0.187, $p < 0.001$; 12.5%: 0.250 versus 0.196, $p < 0.001$; and 25%: 0.234 versus 0.194, $p < 0.001$) (Fig. 1B). Similar results were seen in binding of the lipopeptide Pam_3Cys, although only for higher concentrations of serum (6.25%: 0.291 versus 0.226, $p = 0.047$; 12.5%: 0.327 versus 0.245, $p = 0.034$; and 25%: 0.311 versus 0.21, $p = 0.003$). (Fig. 1C). Binding of the artificial ligand 3-*sn*-phosphatidylethalonamine was also markedly reduced in patients carrying the genetic variation (0.78%: 0.175 versus 0.136, $p = 0.004$; 1.56%: 0.274 versus 0.198, $p = 0.006$; 3.125%: 0.361 versus 0.251, $p < 0.001$; 6.25%: 0.378 versus 0.263, $p < 0.001$; 12.5%: 0.426 versus 0.297, $p = 0.002$; and 25%: 0.491 versus 0.354, $p = 0.003$) (Fig. 1D). An additional finding was a saturation effect seen with increasing serum concentrations. This effect was detectable for all ligands, confirming previous observations (12). We saw no influence of the genotype with this regard.

Differences in genotypes affect LPS stimulation of murine macrophage cell lines

After we found an effect on binding of ligands induced by the protein changes brought about by the genetic variations of LBP, we investigated whether this would affect their ability to facilitate the LPS transfer to TLR4 and thus the release of the proinflammatory cytokine TNF. We incubated RAW cells with LPS and other ligands in the absence and presence of sera obtained from individuals differing in their LBP genotype. As expected, we found the LPS-induced release of TNF to depend on the addition of LBP-containing serum. Mean concentrations of TNF induced by the smooth LPS 0111:B4 (containing carbohydrates in addition to the lipid A core) increased markedly stronger as compared with the rough variant of LPS Re595 (see Supplemental Table I). As is shown in Fig. 1E, the addition of LBP-containing serum induced a severalfold stronger release of TNF as compared with LPS 0111:B4 alone. In detail, 100 ng/ml LPS 0111:B4 with 1% serum resulted in a mean ± SEM of 6514 ± 667.0 ng/ml TNF with serum of rare variant LBP compared with 8655 ± 538.6 ng/ml TNF with added common variant serum (*p* value: 0.012; Mann–Whitney *U* test).

In the experiments with lower concentrations of LPS 0111:B4 (1 and 10ng/ml) with 1% serum either from common variant or rare variant, the results in individuals was lower TNF values with a similar trend between the genotypes just failing to reach statistical significance. Mean ± SEM of TNF was 4248 ± 1006 ng/ml for the rare variant and 2327 ± 626.8 ng/ml for common variant serum ($p = 0.16$, unpaired *t* test). Interestingly, adding 2 or 5% serum to the above-mentioned doses resulted in a less pronounced difference (LPS 0111:B4 10 ng/ml + 5% serum, $p = 0.98$; LPS B4 100 ng/ml + 5% serum, $p = 0.61$, unpaired *t* test) (Fig. 1E, Supplemental Table I). Overall, the "response curve" was flatter, with individuals carrying the rare variant.

Adding rough LPS (Re595) to the LBP-containing sera resulted in an overall lower induction of TNF and no statistically significant differences between the genotypes. Other experiments with lipopeptides (Pam_2Cys or Pam_3Cys) and LBP-containing sera with different genotypes showed an increase in TNF concentrations, but there was no statistical difference between the genotype groups (see Supplemental Table I). Of note, the number of repetitions in these experiments was low because of sparse amounts of human sera available for the different genotypes.

Computational analysis of the LBP SNP F436L

We assessed a potential impact of the F436L LBP genotype on protein function on a structural level by computer modeling.

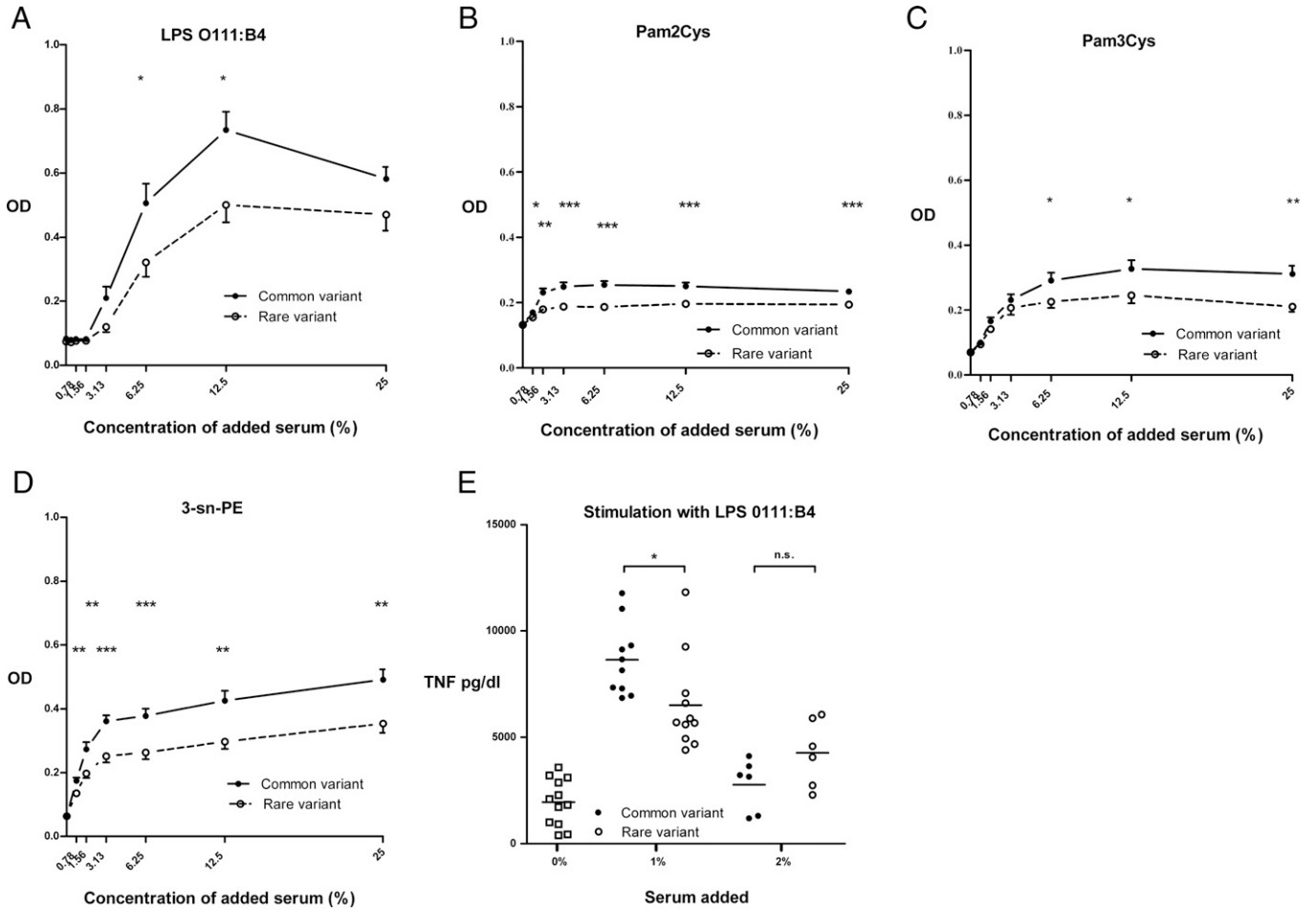

FIGURE 1. (A) Binding of progressively diluted serum of individuals with common and rare LBP variant with the known LBP ligands LPS O111:B4 and (B) Pam_2Cys, (C) Pam_3Cys, and (D) 3-sn-PE.

Binding was assessed by OD. OD values were compared using the Mann–Whitney nonparametric test. All data are expressed as mean ± SEM. Error bars are shown either above or below respective values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ denote statistically significant differences. (**E**) Stimulation of RAW 264.7 cells with 100 ng LPS 0111:B4. Left shows stimulation with no additional LBP. Middle and right show costimulation with LBP-containing serum 1 and 2%, respectively, from carriers with either common or rare variants. *$p < 0.05$. n.s., not statistically significant.

Three-dimensional structural models of the common and rare variant human LBP protein were generated by homology modeling based on the crystal structure of LBP recently published (37, 63). The LBP molecule is boomerang shaped with three functionally distinct parts. The first is the N-terminal part, which is believed to primarily interact with LPS (9). The C-terminal portion is potentially interacting with CD14, and this subsequently leads to TLR4 interaction and thus may initiate signaling. The groove in human LBP is a unique region differing from related proteins such as bactericidal/permeability increasing protein (21). Position 436 is buried and located inside the C-terminal domain of LBP between the A′ loop and the Phe core at the tip of the molecule. In both models, F436/L436 was positioned within an α-helix (helix B) lining the inner surface of the C-terminal phospholipid binding pocket formed by helix A and B and the β2 and β6 sheets (23). Fig. 2A shows the three-dimensional structure of the LBP variant and the location of the F436L exchange in the groove region.

We compared hydrophobicity, surface charge, and the intactness of LBP-specific structural features such as the Phe core and the LPS binding groove formed by the A′ loop (21). The close ups and top view portrayed the nature of the interior surface of common and rare variant protein in Fig. 2B–E.

When analyzing the inner surface of this pocket, we noted that one continuous hydrophobic channel is formed in the common variant model, similar to the murine LBP crystal structure in which both phospholipid acyl chains that were seen in the crystal could be accommodated in the channel (23). In the L436 model, however, the leucine side chain protruded into this channel, thus effectively blocking the channel and reducing the accessible size of the pocket by 50%. This means in the L436 structure, only one acyl chain could be accommodated as

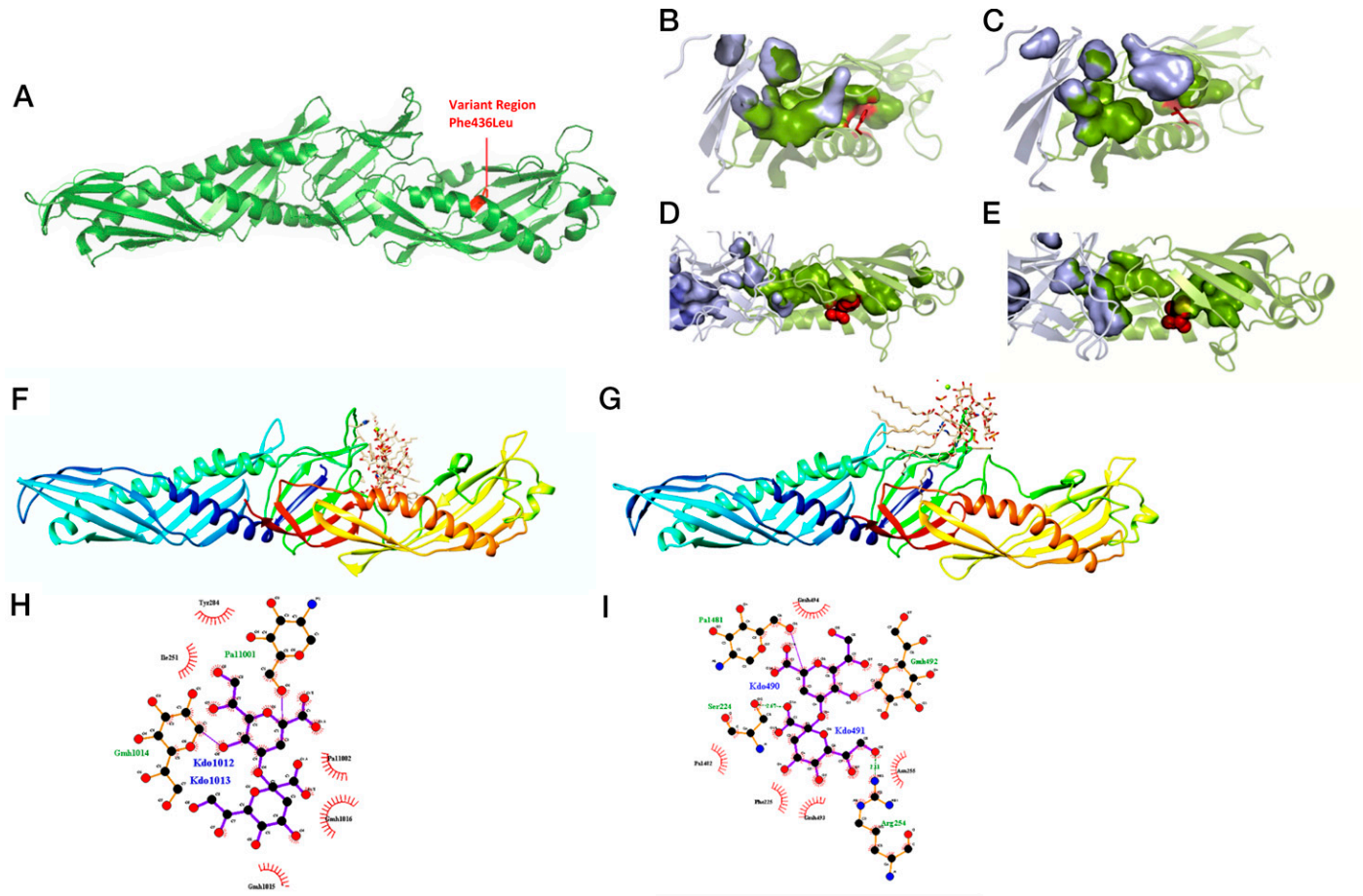

FIGURE 2. Overview of the F436L and the common variant with regard to location, effects on molecular structure, and LPS binding caused by mutational changes in the amino acid chain.

(**A**) Ribbon diagram of the three-dimensional structures of human LBP highlighting the location of the variant region in red. Left, central and right portion of the structures represented N-terminal, central, and C-terminal regions. (**B** and **C**) Close up of the interior surface of the common variant showing either Phe (F436) residue (B) or leucine (L436) residue (C) in red. (**D** and **E**) Top views of the interior surfaces from the common (D) and rare human LBP variant (E) showing conformational differences as a result of the amino acid change. (**F** and **G**) Docking of LPS to the common variant (F) in the groove region shows a marked difference as compared with the rare human LBP (G) variant with LPS. (**H** and **I**) The comparison of ligand plots of the common human LBP variant (H) and the rare variant (I) shows that there are potentially changes in the molecular interaction between the LBP residues and LPS based on our analysis.

evident from superpositions with phospholipid molecules. We also found that the rare variant differed in size of the first major cleft from the common variant (2845.0 versus 2416.08 $Å^3$). These clefts are known to harbor functional residues (64). The highlighted alterations in surface topography may therefore cause changes in molecular function.

Molecular docking simulations show the interaction between human LBP and LPS. Fig. 2 H–I demonstrates the differences in docking residues of human LBP with LPS in both variants in the groove region. In the common variant, Ile251 and Tyr284 were the supporting residues for a potential interaction with LPS. Interestingly, Ser224 and Arg254 residues can interact with LPS in the rare variant in addition to other supporting residues. This difference in docking residues in the common variant and rare variant could cause structural conformational rearrangements influencing molecule function. These results are supported by the observations from MutPred, I-Mutant 3.0, DUET, and ELASPIC, which indicate a loss of stability of the rare variant LBP. This is further supported by solvent accessibility analysis showing a change in polar energy, surface atoms, and buried atoms.

The results are presented in Tables I and II. An additional comparison with the rs2232613 rare variant described in a previous study is presented in Supplemental Table III.

Distribution of genotypes in the study cohorts

To evaluate if the tested genotypes would potentially influence susceptibility to and course of clinical infections, we examined a cohort of patients following surgery. In this cohort, 290 of 363 patients carried the more frequent variant. In 73 patients,

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TABLE I. Comparison of structural stability between human LBP common and rare variant (F436L) using in silico tools

Analysis with MutPred (Result of nsSNP rs2232618 [F436L])			
nsSNP	Amino acid change	Loss of stability	Loss of helix
rs2232618	F436L	$p = 0.07$	$p = 0.1299$
Analysis of Stability Changes in LBP Variants Using I-Mutant3.0, DUET, and ELASPIC			
Amino acid change	I-Mutant3.0	DUET	ELASPIC domain core
F436L	−1.29 (destabilizing)[a]	−0.151 (destabilizing)[a]	−0.794 (destabilizing)[a]

[a]All values shown as ΔΔG/kcal/mol.

the LBP SNP F436L (rs2232618) was present. Of these, 69 patients were heterozygous and 4 were homozygous carriers of the mutated alleles. We found a similar distribution of frequency in the control group consisting of 675 healthy volunteers: 555 were carriers of the common variant alleles only, 112 were heterozygous, and 8 were homozygous. The resulting allele frequencies were 0.091 and 0.095, respectively, as shown in Table III. The SNP was in complete Hardy–Weinberg equilibrium in both groups (patients: $\chi^2 < 0.002$, $p = 0.96$; control group: $\chi^2 = 0.75$, $p = 0.39$). The distribution of the SNP is in accordance with available data (http://www.ensembl.org/Homo_sapiens/Transcript/Haplotypes?db=core;g=ENSG00000129988;r=20:38346482-38377013;t=ENST00000217407).

Influence of LBP SNP F436L on infection susceptibility and clinical course in ICU patients

Comparison of clinical characteristics between the genotype groups revealed no differences with regard to age, gender distribution, or preexisting conditions. Overall, preadmission disease severity scores (American Society of Anesthesiologists classification and Simplified Acute Physiology Score) were equal, as shown in Supplemental Table II. After performing risk calculation, none of these conditions influenced susceptibility to infections or outcome (data not shown). Of the 363 studied patients, 202 (55.7%) developed infections in the ICU. In terms of sepsis susceptibility, no influence of the studied genotype could be found (common variant: 163/290 [56.2%]; F436L: 39/73 [53.4%]), which was also the case for the type of infection. These results are presented in Table IV. The most frequent infection in three groups was pneumonia followed by abdominal and wound infections. The genotypes had no influence on the prevalence of infections nor on the causing pathogens. The distribution of pathogens was equal in all groups. In ∼10% of the patients, there was more than one pathogen identified, and in a similar number of patients, no pathogen could be retrieved by microbiological examination. Both facts were not different between the groups and did not influence further outcome (data not shown).

As compared with the prevalence of infections, we found an association with severity of infection between the genotype groups. The F436L genotype group showed a lower risk for septic shock (OR 0.34; 95% confidence interval [CI] 0.11–1.02; $p < 0.05$) as compared with the common variant group. Furthermore, patients carrying the F436L allele had a lower mortality associated with overall infectious complications (2.6 versus 10.4%) or septic shock (25.0 versus 39.0%) as compared with common variant patients. The latter fact was not statistically significant (OR 0.23; 95% CI 0.03–1.75; $p = 0.21$ for infectious complications;

TABLE II. Structural comparison of human LBP common and rare variant (F436L) using in-silico tools

Difference in Secondary Structure between LBP Variants		
Feature	CV[a]	RV[a]
Coil	32.01	30.56
Helix	26.40	26.40
Strands	41.58	43.03
Difference in Highly Conserved Residues of Cleft 1 and Cleft 2 between LBP Variants		
Clefts	CV	RV
Cleft 1	287, 289	287, 289, 316, 379, 400
Cleft 2	38, 39, 93, 142, 205, 209, 215, 236, 276, 451	38, 39, 50, 93, 142, 205, 209, 215, 236, 276, 451, 478
Analysis of LBP Variants for Functional Pockets Using CASTp		
No. of pockets	CV	RV
	64	65
Comparison of the Solvent Accessibility Analyzed by GETAREA between LBP Variants		
Feature	CV	RV
Polar energy	8726.01	8781.85
No. of surface atoms	2137	2149
No. of buried atoms	1446	1408

[a]Values as percentage of structural components.
CV, common variant; RV, rare variant.

TABLE III. Distribution of genotypes in the studied cohorts

	Number of Individuals (%)		Allele Frequencies	
	Patients (n = 363)	Controls (n = 675)	Patients	Controls
Common variant F436L	290 (77.3%)	555 (82.2%)		
Heterozygous	69 (18.4%)	112 (16.6%)	0.091	0.095
Homozygous	4 (1.1%)	8 (1.2%)		

The genotyping data reported in this study reveal a similar distribution of alleles as compared with HapMap data and The Innate Immunity Program for Genomic Applications data regarding populations of European ancestry (HapMap: 0.084 and 0.103, The Innate Immunity Program for Genomic Applications: 0.043; p = 0.794, using χ^2 test).

OR 0.47; 95% CI 0.05–4.92; p = 0.64 for septic shock, respectively). We found no difference in the clinical course of homozygous compared with heterozygous patients.

Interestingly, we found that the previously studied P333L SNP showed almost opposite effects in most of the studied aspects (37). With the additional genotyping data for the study cohort, we were able to directly compare both rare variants. We found mortality was significantly higher in the P333L group than in the F436L group (21.2%: 7/33 versus 2.4%: 1/39; p = 0.02 tested with Fisher exact test). However, we noted differences in the rate of preexisting conditions between the genotype groups that did not reach statistical significance, except for diabetes. More details for the comparison of these variants are provided in the supplemental material (Supplemental Table IV). Overall, the genotype groups did not differ with regards to risk factors for worse outcome.

DISCUSSION

This work shows a series of phenotypic effects of a genetic variation in the LBP gene on either functional properties of the molecule as well as clinical effects. We found that ligand binding to LPS was reduced when the serum of carriers of the rare variant was added as compared with serum of individuals carrying the common variant. This might be explained by a physically impaired adherence of these molecules to the ligand binding domain of LBP. To effectively exert its function, LBP must bind to its ligands to induce CD14 and thus TLR binding and consecutively induce intracellular signal transduction (65). The experimental concept employed in this study aimed at simulating macrophage function in the presence of serum proteins that are also part of the ligand transfer like MD-2 or CD14. In our cell stimulation experiments, we found lower cytokine concentrations induced in macrophages by bacterial ligands associated with the rare variant of LBP. This was particularly the case with high concentrations of LPS and the serum of individuals carrying the rare variant. Taken together, it appears that reduced binding of LBP ligands associated with the rare variant leads to lesser pronounced induction of proinflammatory cytokines. In the early response to bacterial infection, the release of high concentrations of proinflammatory cytokines might be associated with a more pronounced inflammatory reaction. Recent sepsis definitions articulate a dysregulated or uncontrolled immune reaction as a major factor for morbidity and mortality in this setting (66). However, the host ability to combat bacteria might also be related to adequate amounts of cytokines released (67).

The function of LBP is not only restricted to the transfer from LPS or other ligands to its innate immune receptors but also has immune inhibiting properties, particularly in higher acute-phase concentrations, by scavenging LPS (23). The observed genetic changes might affect this function of the molecule as well. In our experiments, higher concentrations of LBP did not result in increased binding of the molecule. However, we also could confirm previous findings, which show that high concentrations of serum tend to scavenge LPS, but this effect was reduced in the rare variant (Fig. 1A). This effect was also seen with binding to bacterial lipopeptides, although the effect was less pronounced. Interestingly, binding to a nonbacterial ligand did not show this effect.

TABLE IV. Clinical characteristics of patients with infections (n = 202)

Characteristic	Common Variant Group (n = 163)	Rare Variant Group F436L (n = 39)	p Value
Site of infection (No. [%])			
Pneumonia	67 (41.1)	17 (43.6)	0.86
Peritonitis	29 (17.8)	5 (12.8)	0.63
Abscess	45 (27.6)	12 (30.8)	0.70
Urinary tract infections	4 (2.5)	–	1.00
Other	18 (11.0)	5 (12.8)	0.78
Type of microorganism (No. [%])[a]			
Gram negative	95 (58.3)	24 (61.5)	0.86
Gram positive	61 (37.4)	18 (46.2)	0.36
Fungi	12 (7.4)	2 (5.1)	1.00
Outcome (No. [%])			
Sepsis	122 (74.8)	35 (89.7)	–
Septic Shock	41 (25.2)	4 (10.3)	<0.05
Mortality	17 (10.4)	1 (2.6)	0.21

[a]Numbers not adding up to 100% because of missing data or more than one detected microorganism in a patient. Statistical analysis for contingency tables using χ^2 test except for mortality, where Fisher's exact test was applied.

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In an in silico approach, we tried to explain the results through molecular modeling of the rare variant as compared with the common variant. In the F436L variant, the changed amino acid is situated close to the groove of the molecule near the center. This means that in the L436 structure, only one acyl chain could be accommodated, as evident from superpositions with phospholipid molecules. Although this has not been formally proven, from a structural perspective, it appears plausible that the two hydrophobic channels in LBP contribute to LPS or lipopeptide binding. Therefore, the F436L variant might alter the ability to bind these hydrophobic substances. Analysis of MutPred implied change in function of the protein because of destabilization.

Examination of the three-dimensional structures revealed some structural changes because of amino acid change from F436L. This is further supported by the destabilization occurring owing to free energy change based on I-Mutant 3.0, DUET, and ELASPIC analysis. Additionally, docking analysis revealed a shift in the amino acids residues binding to LPS in the rare variant compared with the common variant. Topographical and conformational changes associated with the amino acid change from F436 to L436 is evident from the altered binding of the rare variant to LPS. An increase in size of cleft 1 in the rare variant of LBP demonstrated that it had a higher number of active sites and was more receptive for ligand binding interactions owing to extra structural rearrangements. Finally, functional pockets and the overall number of highly conserved residues from two major functional clefts of the rare variant were higher in number as compared with the common variant. Comparatively higher number of pockets in the rare variant point to the fact that it may be slightly more flexible than the common variant.

From the clinical data of our study, we show a lower risk of septic shock in the presence of the rare LBP variant. It was not associated with sepsis prevalence and susceptibility to infection. Although not statistically significant, we also found an association with reduced mortality. Clinical effects of LBP variants have been observed in previous studies showing an influence on susceptibility to and also severity of infections or other inflammatory diseases (34). As we have shown in a previous study, another functional LBP SNP (P333L and rs2232613) was associated with a more severe course of sepsis (37). By direct comparison of these SNPs in our clinical cohort, we observed a marked difference in the clinical course of infections, suggesting an opposite effect.

In other studies, LBP variants like an LBP haplotype variation were associated with an increased rate of Gram-negative infections in recipients of homolog bone marrow for hematologic malignancies (30). In other patients, LBP haplotype variation was a risk factor for ventilator-associated pneumonia and sepsis in pediatric patients (32, 33). The LBP haplotypes variations did include wildtype and mutated variants. Therefore, no conclusive picture regarding risk alleles could be drawn from these data. Interestingly, the F436L SNP examined in this study was associated with a higher prevalence of sepsis in a cohort of trauma victims of Han Chinese origin with a different demographic composition (38, 39).

In conclusion, this study shows a functional relevant SNP in the LBP gene that is associated with changes in its properties regarding binding capability and function with regard to stimulation of macrophages. In silico analysis revealed molecular alterations that could be associated with these functional changes. We found a favorable outcome following severe infections in patients after surgery. We propose several potential ways of how a loss of function or a gain of function could be explained by this variant and potentially contribute to patient outcome.

There are potential limitations of our study. First, in our experimental approach, we used a xenogenic model, including mouse macrophages and human serum. In our view, this is justified by the fact that human macrophages (i.e., derived from transformed THP-1 cells) are not readily available and might not display full functional capacity. Human serum used in this study, in addition, contains functional molecules that are needed for sufficient signal transduction in contrast to recombinant protein. The retrospective analysis of only two variants of LBP, furthermore, is a limitation of this study. The association found should be studied prospectively in a broader approach, including other nonsynonymous SNPs.

To further delineate functional consequences regarding concentration-dependent effects of this SNP, however, more experiments may be necessary. Elucidating the complex cascade of events leading from recognition of pathogens to systemic inflammation and disease may in the future lead to novel intervention strategies currently needed to improve the outcome of sepsis. Furthermore, a genetic risk stratification may allow for better prevention of clinical complications in patients at risk for infectious diseases.

DISCLOSURES

The authors have no financial conflicts of interest.

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REFERENCES

1. Schumann, R. R., C. J. Kirschning, A. Unbehaun, H. P. Aberle, H. P. Knope, N. Lamping, R. J. Ulevitch, and F. Herrmann. 1996. 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: 3490–3503.

2. Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science* 249: 1429–1431.
3. Knapp, S., S. Florquin, D. T. Golenbock, and T. van der Poll. 2006. Pulmonary lipopolysaccharide (LPS)-binding protein inhibits the LPS-induced lung inflammation in vivo. *J. Immunol.* 176: 3189–3195.
4. Vreugdenhil, A. C., M. A. Dentener, A. M. Snoek, J. W. Greve, and W. A. Buurman. 1999. Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *J. Immunol.* 163: 2792–2798.
5. Albers, J. J., S. Vuletic, and M. C. Cheung. 2012. Role of plasma phospholipid transfer protein in lipid and lipoprotein metabolism. *Biochim. Biophys. Acta* 1821: 345–357.
6. Alva, V., and A. N. Lupas. 2016. The TULIP superfamily of eukaryotic lipid-binding proteins as a mediator of lipid sensing and transport. *Biochim. Biophys. Acta* 1861(8, 8 Pt B)913–923.
7. Gautier, T., and L. Lagrost. 2011. Plasma PLTP (phospholipid-transfer protein): an emerging role in 'reverse lipopolysaccharide transport' and innate immunity. *Biochem. Soc. Trans.* 39: 984–988.
8. Levels, J. H., D. Pajkrt, M. Schultz, F. J. Hoek, A. van Tol, J. C. Meijers, and S. J. van Deventer. 2007. Alterations in lipoprotein homeostasis during human experimental endotoxemia and clinical sepsis. *Biochim. Biophys. Acta* 1771: 1429–1438.
9. Ryu, J. K., S. J. Kim, S. H. Rah, J. I. Kang, H. E. Jung, D. Lee, H. K. Lee, J. O. Lee, B. S. Park, T. Y. Yoon, and H. M. Kim. 2017. Reconstruction of LPS Transfer Cascade Reveals Structural Determinants within LBP, CD14, and TLR4-MD2 for Efficient LPS Recognition and Transfer. *Immunity* 46: 38–50.
10. Tobias, P. S., K. Soldau, N. M. Iovine, P. Elsbach, and J. Weiss. 1997. Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. *J. Biol. Chem.* 272: 18682–18685.
11. Tapping, R. I., and P. S. Tobias. 1997. Cellular binding of soluble CD14 requires lipopolysaccharide (LPS) and LPS-binding protein. *J. Biol. Chem.* 272: 23157–23164.
12. Schumann, R. R. 2011. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. *Biochem. Soc. Trans.* 39: 989–993.
13. Wurfel, M. M., S. T. Kunitake, H. Lichenstein, J. P. Kane, and S. D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J. Exp. Med.* 180: 1025–1035.
14. Vreugdenhil, A. C., A. M. Snoek, C. van 't Veer, J. W. Greve, and W. A. Buurman. 2001. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. *J. Clin. Invest.* 107: 225–234.
15. Mueller, M., K. Brandenburg, R. Dedrick, A. B. Schromm, and U. Seydel. 2005. Phospholipids inhibit lipopolysaccharide (LPS)-induced cell activation: a role for LPS-binding protein. *J. Immunol.* 174: 1091–1096.
16. Schröder, N. W., H. Heine, C. Alexander, M. Manukyan, J. Eckert, L. Hamann, U. B. Göbel, and R. R. Schumann. 2004. Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. *J. Immunol.* 173: 2683–2691.
17. Schröder, N. W., B. Opitz, N. Lamping, K. S. Michelsen, U. Zähringer, U. B. Göbel, and R. R. Schumann. 2000. Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by *Treponema* glycolipids. *J. Immunol.* 165: 2683–2693.
18. Weber, J. R., D. Freyer, C. Alexander, N. W. Schröder, A. Reiss, C. Küster, D. Pfeil, E. I. Tuomanen, and R. R. Schumann. 2003. Recognition of pneumococcal peptidoglycan: an expanded, pivotal role for LPS binding protein. *Immunity* 19: 269–279.
19. Nyström, J., J. Stenkvist, A. Häggblom, O. Weiland, and P. Nowak. 2015. Low levels of microbial translocation marker LBP are associated with sustained viral response after anti-HCV treatment in HIV-1/HCV co-infected patients. *PLoS One* 10: e0118643.
20. Wurfel, M. M., and S. D. Wright. 1997. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. *J. Immunol.* 158: 3925–3934.
21. Yu, B., E. Hailman, and S. D. Wright. 1997. Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids. *J. Clin. Invest.* 99: 315–324.
22. Cunningham, S. C., D. L. Malone, G. V. Bochicchio, T. Genuit, K. Keledjian, J. K. Tracy, and L. M. Napolitano. 2006. Serum lipopolysaccharide-binding protein concentrations in trauma victims. *Surg. Infect. (Larchmt.)* 7: 251–261.
23. Zweigner, J., H. J. Gramm, O. C. Singer, K. Wegscheider, and R. R. Schumann. 2001. 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: 3800–3808.
24. Lamping, N., R. Dettmer, N. W. J. Schröder, D. Pfeil, W. Hallatschek, R. Burger, and R. R. Schumann. 1998. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J. Clin. Invest.* 101: 2065–2071.
25. Hamann, L., C. Alexander, C. Stamme, U. Zähringer, and R. R. Schumann. 2005. Acute-phase concentrations of lipopolysaccharide (LPS)-binding protein inhibit innate immune cell activation by different LPS chemotypes via different mechanisms. *Infect. Immun.* 73: 193–200.
26. Kopp, F., S. Kupsch, and A. B. Schromm. 2016. 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. *Biochim. Biophys. Acta* 1863: 660–672.
27. Arcaroli, J., M. B. Fessler, and E. Abraham. 2005. Genetic polymorphisms and sepsis. *Shock* 24: 300–312.
28. Nakada, T. A., W. Takahashi, E. Nakada, T. Shimada, J. A. Russell, and K. R. Walley. 2019. Genetic polymorphisms in sepsis and cardiovascular disease: do similar risk genes suggest similar drug targets? *Chest* 155: 1260–1271.
29. Kumpf, O., and R. R. Schumann. 2010. Genetic variation in innate immunity pathways and their potential contribution to the SIRS/CARS debate: evidence from human studies and animal models. *J. Innate Immun.* 2: 381–394.
30. Chien, J. W., M. J. Boeckh, J. A. Hansen, and J. G. Clark. 2008. Lipopolysaccharide binding protein promoter variants influence the risk for Gram-negative bacteremia and mortality after allogeneic hematopoietic cell transplantation. *Blood* 111: 2462–2469.
31. Barber, R. C., and G. E. O'Keefe. 2003. Characterization of a single nucleotide polymorphism in the lipopolysaccharide binding protein and its association with sepsis. *Am. J. Respir. Crit. Care Med.* 167: 1316–1320.
32. Flores, C., L. Pérez-Méndez, N. Maca-Meyer, A. Muriel, E. Espinosa, J. Blanco, R. Sangüesa, M. Muros, J. G. Garcia, and J. Villar; GRECIA and Gen-SEP groups. 2009. A common haplotype of the LBP gene predisposes to severe sepsis. *Crit. Care Med.* 37: 2759–2766.
33. Jabandziev, P., M. Smerek, J. Michalek, M. Fedora, L. Kosinova, J. A. Hubacek, and J. Michalek. 2014. Multiple gene-to-gene interactions in children with sepsis: a combination of five gene variants predicts outcome of life-threatening sepsis. *Crit. Care* 18: R1.
34. Meng, L., Z. Song, A. Liu, U. Dahmen, X. Yang, and H. Fang. 2021. Effects of Lipopolysaccharide-Binding Protein (LBP) Single Nucleotide Polymorphism (SNP) in Infections, Inflammatory Diseases, Metabolic Disorders and Cancers. *Front. Immunol.* 12: 681810.
35. Hubacek, J. A., F. Stüber, D. Fröhlich, M. Book, S. Wetegrove, M. Ritter, G. Rothe, and G. Schmitz. 2001. Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide

binding protein in sepsis patients: gender-specific genetic predisposition to sepsis. *Crit. Care Med.* 29: 557–561.
36. Korhonen, T., S. Grauling-Halama, N. Halama, S. Silvennoinen-Kassinen, M. Leinonen, and P. Saikku. 2006. Rapid genotyping of lipopolysaccharide-binding protein (LBP) C(1341)-->T (Leu(436)-->Phe) polymorphism by LightCycler real-time PCR. *J. Immunol. Methods* 317: 171–174.
37. Eckert, J. K., Y. J. Kim, J. I. Kim, K. Gürtler, D. Y. Oh, S. Sur, L. Lundvall, L. Hamann, A. van der Ploeg, P. Pickkers, et al. 2013. The crystal structure of lipopolysaccharide binding protein reveals the location of a frequent mutation that impairs innate immunity. *Immunity* 39: 647–660.
38. Zeng, L., W. Gu, A. Q. Zhang, M. Zhang, L. Y. Zhang, D. Y. Du, S. N. Huang, and J. X. Jiang. 2012. A functional variant of lipopolysaccharide binding protein predisposes to sepsis and organ dysfunction in patients with major trauma. *Ann. Surg.* 255: 147–157.
39. Lu, H. X., J. H. Sun, D. L. Wen, J. Du, L. Zeng, A. Q. Zhang, and J. X. Jiang. 2018. LBP rs2232618 polymorphism contributes to risk of sepsis after trauma. *World J. Emerg. Surg.* 13: 52.
40. Niroula, A., and M. Vihinen. 2016. Variation Interpretation Predictors: Principles, Types, Performance, and Choice. *Hum. Mutat.* 37: 579–597.
41. Li, B., V. G. Krishnan, M. E. Mort, F. Xin, K. K. Kamati, D. N. Cooper, S. D. Mooney, and P. Radivojac. 2009. Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics* 25: 2744–2750.
42. McGuffin, L. J., K. Bryson, and D. T. Jones. 2000. The PSIPRED protein structure prediction server. *Bioinformatics* 16: 404–405.
43. Bava, K. A., M. M. Gromiha, H. Uedaira, K. Kitajima, and A. Sarai. 2004. ProTherm, version 4.0: thermodynamic database for proteins and mutants. *Nucleic Acids Res.* 32: D120–D121.
44. Berman, H. M., J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, and P. E. Bourne. 2000. The Protein Data Bank. *Nucleic Acids Res.* 28: 235–242.
45. Guex, N., M. C. Peitsch, and T. Schwede. 2009. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. *Electrophoresis* 30(S1, Suppl 1)S162–S173.
46. Waterhouse, A., M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T. A. P. de Beer, C. Rempfer, L. Bordoli, et al. 2018. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* 46(W1): W296–W303.
47. Wiederstein, M., and M. J. Sippl. 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res.* 35: W407–W410.
48. Wallner, B., and A. Elofsson. 2003. Can correct protein models be identified? *Protein Sci.* 12: 1073–1086.
49. Zhang, C., G. Vasmatzis, J. L. Cornette, and C. DeLisi. 1997. Determination of atomic desolvation energies from the structures of crystallized proteins. *J. Mol. Biol.* 267: 707–726.
50. Mashiach, E., D. Schneidman-Duhovny, N. Andrusier, R. Nussinov, and H. J. Wolfson. 2008. FireDock: a web server for fast interaction refinement in molecular docking. *Nucleic Acids Res.* 36: W229–W232.
51. Brender, J. R., and Y. Zhang. 2015. Predicting the Effect of Mutations on Protein-Protein Binding Interactions through Structure-Based Interface Profiles. *PLOS Comput. Biol.* 11: e1004494.
52. Laskowski, R. A., J. D. Watson, and J. M. Thornton. 2003. From protein structure to biochemical function? *J. Struct. Funct. Genomics* 4: 167–177.
53. Ashkenazy, H., E. Erez, E. Martz, T. Pupko, and N. Ben-Tal. 2010. ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res.* 38: W529–W533.
54. Liang, J., H. Edelsbrunner, and C. Woodward. 1998. Anatomy of protein pockets and cavities: measurement of binding site geometry and implications for ligand design. *Protein Sci.* 7: 1884–1897.
55. Fraczkiewicz, R., and W. Braun. 1998. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J. Comput. Chem.* 19: 319–333.
56. Levy, M. M., M. P. Fink, J. C. Marshall, E. Abraham, D. Angus, D. Cook, J. Cohen, S. M. Opal, J. L. Vincent, and G. Ramsay; SCCM/ESICM/ACCP/ATS/SIS. 2003. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit. Care Med.* 31: 1250–1256.
57. Shankar-Hari, M., G. S. Phillips, M. L. Levy, C. W. Seymour, V. X. Liu, C. S. Deutschman, D. C. Angus, G. D. Rubenfeld, and M. Singer; Sepsis Definitions Task Force. 2016. Developing a new definition and assessing new clinical criteria for septic shock: for the third international consensus definitions for sepsis and septic shock (sepsis-3). *JAMA* 315: 775–787.
58. Poutsiaka, D. D., M. C. Porto, W. A. Perry, J. Hudcova, D. J. Tybor, S. Hadley, S. Doron, J. A. Reich, D. R. Snydman, and S. A. Nasraway. 2019. Prospective Observational Study Comparing Sepsis-2 and Sepsis-3 Definitions in Predicting Mortality in Critically Ill Patients. *Open Forum Infect. Dis.* 6: ofz271.
59. Kumpf, O., E. J. Giamarellos-Bourboulis, A. Koch, L. Hamann, M. Mouktaroudi, D. Y. Oh, E. Latz, E. Lorenz, D. A. Schwartz, B. Ferwerda, et al. 2010. Influence of genetic variations in TLR4 and TIRAP/Mal on the course of sepsis and pneumonia and cytokine release: an observational study in three cohorts. *Crit. Care* 14: R103.
60. Garner, J. S., W. R. Jarvis, T. G. Emori, T. C. Horan, and J. M. Hughes. 1996. CDC definitions for nosocomial infections. In *PIC Infection Control and Applied Epidemiology: Principles and Practice.* R. N. Olmsted, ed. Mosby, St. Louis, p. A1–A20.
61. Le Gall, J. R., S. Lemeshow, and F. Saulnier. 1993. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *JAMA* 270: 2957–2963.
62. Oh, D. Y., S. Taube, O. Hamouda, C. Kücherer, G. Poggensee, H. Jessen, J. K. Eckert, K. Neumann, A. Storek, M. Pouliot, et al. 2008. A functional toll-like receptor 8 variant is associated with HIV disease restriction. *J. Infect. Dis.* 198: 701–709.
63. Kubarenko, A. V., S. Ranjan, E. Colak, J. George, M. Frank, and A. N. Weber. 2010. Comprehensive modeling and functional analysis of Toll-like receptor ligand-recognition domains. *Protein Sci.* 19: 558–569.
64. Laskowski, R. A., N. M. Luscombe, M. B. Swindells, and J. M. Thornton. 1996. Protein clefts in molecular recognition and function. *Protein Sci.* 5: 2438–2452.
65. Weiss, J., and J. Barker. 2018. Diverse pro-inflammatory endotoxin recognition systems of mammalian innate immunity. *F1000 Res.* 7: F1000.
66. Singer, M., C. S. Deutschman, C. W. Seymour, M. Shankar-Hari, D. Annane, M. Bauer, R. Bellomo, G. R. Bernard, J. D. Chiche, C. M. Coopersmith, et al. 2016. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 315: 801–810.
67. Netea, M. G., J. W. van der Meer, M. van Deuren, and B. J. Kullberg. 2003. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol.* 24: 254–258.