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Influence of Lipopolysaccharide Binding Protein on Pulmonary Inflammation in Gram-negative Pneumonia

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Abstract

Introduction—Lipopolysaccharide binding protein (LBP) is upregulated as part of the acute phase response. LBP has a known multifunctional role in potentiating the recognition, clearance, and killing of Gram-negative bacteria. In a *Klebsiella pneumoniae* model we previously demonstrated that LBP^{-/-} mice experience increased mortality when compared to wild-type (Wt) mice (98 vs. 59%).

Hypothesis—We hypothesize that LBP is essential to bacterial clearance from the lung and its absence leads to alteration of the pulmonary inflammatory response to pneumonia.

Methods—12–16 week old female C57Bl/6 Wt mice and age matched LBP^{-/-} mice were administered 1×10^3 colony forming units (CFU) of *Klebsiella pneumoniae* by intratracheal injection. Animals were euthanized at 6, 12, 24, or 36 hours post inoculation. Lung tissue and bronchoalveolar lavage samples were obtained. Lung homogenate samples were assayed to determine quantitative bacteria load per whole lung, proinflammatory cytokine concentrations, myeloperoxidase activity, and assessment of pulmonary leukocyte populations. *In vitro* production of inflammation mediators were also assayed following lipopolysaccharide stimulation of peritoneal macrophages isolated from Wt, Toll-like receptor 4 (TLR4)^{-/-}, and LBP^{-/-} mice.

Results—LBP^{-/-} mice demonstrated significantly elevated levels of bacteria in the lung at 24 and 36 hours when compared to wild-type controls. The average lung levels of proinflammatory cytokines IL-1 β , IL-6, KC, and MIP-2 were greater in the LBP^{-/-} mice and remained elevated longer when compared to the Wt mice. Myeloperoxidase activity, an indicator of neutrophil content, was significantly increased at time 36 hours in the LBP^{-/-} mice. Following *in vitro* stimulation of peritoneal macrophages with LPS, production of IL-1 β , IL-6, IL-10, KC, and MIP-1 α were suppressed in LBP^{-/-} and TLR4^{-/-} mice compared to Wt.

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Conclusions—Absence of a functional LBP gene results in diminished clearance of Gram-negative bacteria from the pulmonary system. Failure to recognize and clear Gram-negative bacteria via the LBP/TLR4 axis results in an initial delayed inflammatory response. This delay in LBP^{-/-} mice is followed by excessive amplification and prolonged elevation of proinflammatory mediators and neutrophil sequestration within the lungs.

Keywords

Pneumonia; Innate immunity; Cytokine; Lipopolysaccharide binding protein; Alveolar macrophage

Introduction

In the instance of Gram-negative pathogens, bacteria lipopolysaccharide (LPS) plays a principle role in the activation of the inflammatory response. The amphipathic LPS molecule is a major component in the outer membrane of Gram-negative bacteria, and is recognized by immune cells via the CD14-Toll like receptor 4 (TLR4) complex.(24) Transfer of LPS to the CD14-TLR4 receptor complex is greatly facilitated by lipopolysaccharide-binding protein (LBP).(7, 17) *In vivo*, LBP has been shown to potentiate the cellular response to LPS upwards of 1000 fold.(17)

The role of LBP in sensitizing cells to LPS has been demonstrated in studies where mice treated with a monoclonal antibody against LBP were protected from lethal endotoxemic shock following intraperitoneal injection of LPS.(15) Studies using LBP gene-deficient mice (LBP^{-/-}) have shown that LBP is necessary for the induction of a rapid innate immune response to small amounts of Gram-negative bacteria. In peritonitis models using *Escherichia Coli* or *Salmonella typhimurium*, LBP^{-/-} mice demonstrated increased mortality, greater bacteria load, earlier bacteria dissemination into the blood stream, and reduced neutrophil sequestration within the peritoneal cavity compared to wild-type or heterozygous controls.(9, 11) In addition to its role in transferring LPS to CD14, LBP has been shown to potentiate the phagocytosis of Gram-negative pathogens by binding directly to LPS in the outer membrane of bacteria,(10, 25) and facilitating neutralization/clearance of LPS by passing it to high- and low-density lipoproteins.(13, 26) In total, these results indicate a severely impaired ability for LBP-deficient animals to adequately respond to and clear Gram-negative pathogens.

While LBP is predominately produced by hepatocytes, respiratory epithelial cells are also able to produce LBP in response to interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α .(4) Levels of LBP in the lung have been found to be increased in patients with acute respiratory distress syndrome.(17, 18) In mice, its concentration in the lungs was increased nearly 7-fold in response to intrapulmonary administration of LPS.(2) These findings led us to believe that LBP plays an important role in the induction of pulmonary inflammatory responses to Gram-negative bacteria. Our laboratory previously demonstrated increased mortality among LBP^{-/-} mice using a murine model of *Klebsiella pneumoniae*.(5) We have also shown that reinstatement of LBP activity in LBP^{-/-} animals, with systemic adenoviral vector gene transfer, restores survival in these animals to a level similar to wild-

type controls.(8) In addition, overexpression of LBP in wild-type mice with systemic gene transfer improves survival from *Klebsiella* pneumonia when compared to sham-gene transfer in wild-type controls.(8)

Our objective in these experiments was to further investigate the role LBP plays in pulmonary recognition of Gram-negative bacteria and the innate immune response to pneumonia. We hypothesized that the diminished survival in LBP^{-/-} mice is associated with impaired clearance of bacteria from the lungs. Furthermore, we hypothesized that impaired bacterial clearance in LBP^{-/-} mice would lead to measurable derangements in the local pulmonary immune response in a TLR4 dependent manner.

Materials And Methods

Animals

LBP^{-/-} mice were a gift from Douglas Golenbock (Boston University School of Medicine). (21) These mice had been backcrossed into the background C57BL/6 strain at least 12 times before being acquired into our colony. They were housed in a specific pathogen-free environment and allowed to breed. Female LBP^{-/-} mice ranging from 12 to 16 weeks in age and appropriate age-matched female C57BL/6 wild-type (Wt) or TLR4^{-/-} mice (Harlan, Indianapolis, IN) were used in all of the experiments. The Wt or TLR4^{-/-} mice were housed under specific pathogen-free conditions and were allowed to acclimate to their new surroundings for 1 week prior to being used in experiments. All experiments were performed in accordance with National Institutes of Health guideline for care and use of animals. Approval for the experimental protocol was obtained from the University of Michigan Committee on Use and Care of Animals.

Pneumonia model

Klebsiella pneumoniae, strain 43816, serotype 2 (American Type Culture Collection, Manassas, VA) was a gift from Theodore Standiford (Pulmonary Medicine, University of Michigan Medical School). This microbial strain was cultured in trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) with reinoculation the following morning into fresh medium to bring the bacteria into the log growth phase. The bacteria were centrifuged at 2000 rpm at 4°C for 10 min (Sorvall RT 6000D, Kendro, Newtown, CT), washed with sterile 0.9% normal saline, centrifuged again, and then resuspended in sterile saline. Optical density was read on a spectrophotometer (Milton Roy, Rochester, NY) at a wavelength of 600 nm. Appropriate serial dilutions were made to achieve a concentration of 1000 CFU's of bacteria per 30 µL of inoculum, the LD₅₀ for C57BL/6 Wt mice using this strain of *Klebsiella*. Mice were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL) and inoculated with 30 µL of the bacterial suspension via sterile cutdown and intratracheal injection. Pneumonia groups consisted of Wt and LBP^{-/-} mice euthanized at different time points post inoculation.

Lung homogenate and quantification of lung bacteria

At 6, 12, 24 or 36 hours post inoculation, mice were euthanized, the thoracic cavity opened, and lungs flushed of blood with 2 mL of 0.9% sterile saline. Both lungs were removed and

homogenized in 1 mL of 0.9% sterile saline. Serial 10-fold dilutions were made in 0.9% sterile saline and 100 μ L volumes of homogenate were plated on 5% blood agar plates (Thermo Fisher Scientific, Remel Products, Lenexa, KS). Plates were incubated overnight at 37°C and colony forming units (CFU's) counted after 16 hours. Lung homogenate samples were also collected from Wt and LBP-/- mice that underwent the same handling but were not subjected to intratracheal injection of bacteria. These samples were used to obtain normal baseline values.

Bronchoalveolar lavage

At 6, 12, 24 or 36 hours post inoculation, mice were anesthetized and a tracheal cutdown performed. The lungs were lavaged with 2 mL of sterile saline using a syringe, angiocatheter and three way stopcock apparatus. Bronchoalveolar lavage (BAL) samples were centrifuged at 400 g for 10 minutes. Following centrifugation the supernatant was separated from the cells and these samples were stored frozen at -80°C until use. Spun down cells were resuspended and used in the cytospin or flow cytometry assay. BAL samples were also collected from Wt and LBP-/- mice that underwent the same handling but were not subjected to intratracheal injection of bacteria. These samples were used to obtain the normal baseline values.

Cytospins and histology

The BAL cellular pellet was resuspended in RPMI with 1% fetal calf serum. Following pretreatment with Zap-Oglobin (Coulter, Miami, FL), cell counts were performed using a Coulter Counter (Coulter Electronics, Hialeah, FL). Based on cell counts, samples were diluted to a concentration of 5×10^6 cells per mL. Cytospins were performed by loading 100 μ L containing 5×10^5 cells into each cuvette and spinning the slides at 350g for 10 minutes. Slides were fixed with Diff-Quik (Baxter, Detroit, MI) and stained with a Wright-Giemsa stain for histologic examination

***In vivo* cytokine and chemokine analysis**

The remaining lung homogenates were centrifuged at 3000 g for 15 minutes at 4°C. Supernatants were collected and diluted 1:1 in lysis buffer (1x PBS, 1% Triton X-100, 1 tablet Complete X protease inhibitor [Roche, Indianapolis, IN], pH 7.4). Pellets were saved for later use in the myeloperoxidase assay. Samples were stored at -80°C until use. IL-1 β , IL-6, TNF- α , macrophage-inflammatory protein-2 (MIP-2), and keratinocyte-derived chemokine (KC) were measured in BAL supernatant or lung homogenate using prefabricated ELISA kits, according to the manufacturers protocol (R&D Systems, Inc, Minneapolis, MN). These cytokines and chemokines reflect commonly used markers of inflammation found to be altered in previous studies of LBP.(5, 12, 14) Plates were read using a microplate reader (Biotek Instruments, Winooski, VT) at 450 and 540 nm and cytokine concentrations calculated using a 6-point standard curve and are expressed as pg/mL.

Flow cytometry analysis

Pulmonary cell populations from BAL and enzymatically digested (Collagenase A, Roche Diagnostics, Indianapolis, IN) lungs were assessed using fluorescence antibody staining and flow cytometry techniques. A total of 2×10^6 cells were used with viability staining performed with a Live/Dead staining kit (Invitrogen-Molecular Probes, Carlsbad, CA). The cells were washed and re-suspended in flow cytometry buffer consisting of Dulbecco's PBS with 1% fetal bovine serum. Cells were divided into two equal sets containing 1×10^6 cells each. Fc receptors (CD16/CD32) were blocked using mouse Fc block antibodies (BioLegend, San Diego, CA) for 10 min at 4°C. Diluted fluorescent antibodies specific for the different surface markers or control antibodies were added to the cell suspension and incubated for 10 min at 4°C. The cells were washed, formalin-fixed, washed again and re-suspended in flow buffer and assayed the same day. The following monoclonal antibodies were obtained from BD Biosciences: Gr-1-PE, CD11c-PE, CD4-FITC; from BioLegend: Ly6C-FITC, F4/80-Alexa Fluor 647, F4/80-Alexa Fluor 700, A-I/b-Alexa Fluor 647, CD11b-PE-Cy7, Annexin V-PE, and CD11c-APC-Cy7. Data were collected using a LSR II BD Biosciences (San Jose, CA) flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Forward light scatter low Gr-1+ CD11b+ F4/80- cells were identified as neutrophils and forward light scatter high F4/80+ CD11b+ cells were identified as macrophages.

Detection of neutrophil sequestration (Myeloperoxidase assay)

Pellets recovered from centrifugation of lung homogenates were re-suspended in 1 mL C-TAB buffer consisting of dibasic potassium phosphate, cetyltrimethylammonium bromide, and acetic acid (Sigma Aldrich, Milwaukee, WI). The suspensions were sonicated (Branson Sonifier 250; Branson Ultrasonics Corp, Danbury, CT) on ice for 40 seconds for membrane disruption and myeloperoxidase (MPO) release. Samples were centrifuged for 15 minutes at 16,000g and 4°C and the supernatants collected. Supernatants were stored at -80°C until use. Immediately prior to use, samples were activated by incubation in a 60°C water bath for 2 hours.

20 µL standards (Calbiochem, Gibbstown, NJ) or samples were added to a 96-well immunosorbent micro-plates (NUNC, Rochester, NY), followed by the addition of 155 µL of 20mM TMB/DMF consisting of 3,3',5,5'-tetramethylbenzidine/N,N-dimethylformamide in 115 mM potassium phosphate buffer (Fischer Scientific, Pittsburgh, PA) to each well. Samples were mixed well, after which 20 µL of 3 mM H₂O₂ was rapidly added to each well. The reaction was stopped immediately by adding 50 µL/well of 0.061 mg/mL Catalase (Roche, Indianapolis, IN). The plates were read using a microplate reader at 620 nm. Myeloperoxidase (MPO) concentrations were calculated using a linear standard curve and adjusted for previous dilution. The final concentrations were expressed as µg/mL.

In-vitro cytokine and chemokine production

For elicitation of peritoneal macrophages, Wt, TLR4^{-/-}, and LBP^{-/-} mice were injected i.p. with 1.5 mL 2.4% thioglycollate medium (Becton Dickinson, Franklin Lakes, NJ).(1) Peritoneal macrophages were harvested 4 days later by peritoneal lavage. After centrifugation, cells were resuspended in RPMI 1640 (25 mM HEPES, 100 U/ml penicillin-

streptomycin, [both from Life Technologies], 0.1% BSA [Sigma-Aldrich]) and plated at 2×10^6 cells/mL in polystyrene culture plates and incubated at 37°C, 5% CO₂. Peritoneal macrophages were diluted and plated at 0.3×10^6 cells/mL and stimulated with 50ng/mL of LPS for 8 h in serum-free medium. Cell culture supernatants were assayed using BioRad BioPlex technology (Life Science Research, Hercules, CA) according to the manufacturer's recommendations.

Statistical methods

All statistical analysis and graphs were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Results are presented as mean values $\pm$ the standard deviation unless otherwise noted. CFU values were analyzed using a Mann-Whitney test. Continuous variables were analyzed using an unpaired two-tailed Student's *t*-test. Statistical significance was defined as a *p*-value < 0.05 .

Results

LBP^{-/-} mice have impaired pulmonary clearance of Gram-negative bacteria

Quantitative culture techniques were used to assess the CFU's of bacteria present in lung homogenates at different time points post-inoculation. At 6 (8.5×10^2 vs. 1.7×10^3 , $p = 0.06$) and 12 (2.7×10^3 vs. 4.7×10^4 , $p = 0.2$) hours post intratracheal injection of *Klebsiella pneumoniae*, Wt mice and LBP^{-/-} mice displayed no significant difference in the level of lung bacteria present in quantitative culture assay (Figure 1A). By 24 (2.0×10^4 vs. 8.7×10^5 , $p = 0.0002$) and 36 (1.4×10^4 vs. 8.3×10^5 , $p = 0.0001$) hours after inoculation LBP^{-/-} mice demonstrated significantly higher levels of bacteria in the lung when compared to Wt controls.

Representative photomicrographs from samples of BAL cytospin preparations are shown in Figure 1B. In the infected lungs of a Wt mouse, the macrophages are of the usual size and show few cytoplasmic vesicles. Macrophages from an LBP^{-/-} mouse are considerably larger and contain numerous cytoplasmic vacuoles with rounded substances of undetermined composition.

Gram-negative pneumonia in LBP^{-/-} mice results in elevated pro-inflammatory cytokine/chemokine levels within the lung

Levels of the proinflammatory cytokines IL-1 β , IL-6, and TNF- α along with C-X-C chemokines KC and MIP-2, murine analogues of IL-8 instrumental in neutrophil recruitment, were assayed in lung homogenates. Six and 12 hours after inoculation with *Klebsiella*, there were no significant differences in the levels of any of these proinflammatory cytokines between Wt and LBP^{-/-} mice (Figure 2A). At 24 hours the level of these inflammatory mediators was markedly increased in the LBP^{-/-} mice and significant differences were present between the Wt and LBP^{-/-} animals for IL-1 β ($2932 \text{ pg/mL} \pm 409$ vs. $5782 \text{ pg/mL} \pm 634$), IL-6 ($1673 \text{ pg/mL} \pm 334$ vs. $3750 \text{ pg/mL} \pm 501$), KC ($1577 \text{ pg/mL} \pm 343$ vs. $4505 \text{ pg/mL} \pm 562$) and MIP-2 ($715 \text{ pg/mL} \pm 142$ vs. $1626 \text{ pg/mL} \pm 213$). Thus at 24 hours post-inoculation, the LBP^{-/-} mice had evidence of both elevated inflammation and impaired pulmonary bacteria clearance. By 36 hours, the level of pulmonary inflammation

as measured by cytokine and chemokine analysis was trending downward, but the LBP^{-/-} mice had a decreased absolute level of decline when compared to the Wt mice. A significant difference persisted in the level of the chemokine KC between the Wt and LBP^{-/-} mice (164 pg/mL $\pm$ 15 vs. 990 pg/mL $\pm$ 339).

In BAL fluid, a similar pattern of results was found for the levels of the inflammatory mediators IL-1 β (129 pg/mL $\pm$ 43 vs. 475 pg/mL $\pm$ 159), IL-6 (94 pg/mL $\pm$ 21 vs. 293 pg/mL $\pm$ 59), KC (91 pg/mL $\pm$ 17 vs. 971 pg/mL $\pm$ 203) and MIP-2 (144 pg/mL $\pm$ 42 pg/mL vs. 430 $\pm$ 68) with each being markedly increased in the LBP^{-/-} mice at 24 hours post-inoculation when compared to the Wt animals (Figure 2B). No difference was detected in TNF- α between Wt and LBP^{-/-} mice at any of the measured time points (data not shown).

LBP^{-/-} mice have lower initial levels of recruited macrophages within the lung at 24 hours following Gram-negative pneumonia

Flow cytometry was used to evaluate the recruitment of inflammatory cells into alveoli and lung parenchyma of *K. pneumoniae* challenged animals. There was no difference detected in the total number of BAL cells between Wt and LBP^{-/-} mice at 24 hours post-inoculation (data not shown). In addition, the total numbers of neutrophils and macrophages in the BAL fluid of LBP^{-/-} mice were comparable to Wt mice (data not shown). In the lung parenchyma however, analysis of the distribution of macrophages did reveal a decrease in the total recruitment of these cells in LBP^{-/-} animals (Figure 3).

***In vitro*, LBP^{-/-} thioglycollate-elicited peritoneal macrophages produce decreased amounts of inflammatory cytokines, Th-2 cytokines, and chemotactic factors following stimulation with LPS**

Our *in vitro* experiments demonstrated that an absence of LBP or TLR4 significantly decreased LPS-induced production of soluble mediators by peritoneal macrophages. Products released include T-cell stimulatory and pro-inflammatory cytokines. IL-1 β , IL-6, IL-10, KC and MIP-1 α production was uniformly suppressed in both LBP^{-/-} and TLR4^{-/-} mice (Figure 4). MCP-1, MIP-1 β , RANTES and TNF- α production were suppressed to a greater extent in TLR4^{-/-} mice compared to LBP^{-/-} mice (Figure 4).

LBP^{-/-} mice demonstrate a sustained increase in neutrophil sequestration within the lung during Gram-negative pneumonia

Lung myeloperoxidase levels were less at 6 hours in the LBP^{-/-} mice compared to the Wt. MPO levels increased from the 6 hour to the 24 hour time point for the Wt and LBP^{-/-} mice following induction of pneumonia (Figure 5). As pulmonary bacteria were cleared in the Wt mice the MPO level declined from 24 to 36 hours. However, in the LBP^{-/-} mice, in which pulmonary bacterial clearance was impaired, there was increased MPO activity at 36 hours when compared to the Wt mice.

LBP^{-/-} mice display increased apoptosis and necrosis of neutrophils

Flow cytometry analysis of BAL macrophages and neutrophils revealed that almost all of the neutrophils isolated from LBP^{-/-} mice were apoptotic or necrotic 24 hours after bacterial challenge (Figure 6). Conversely, the Wt mice exhibited mostly live neutrophils at the same

time point. LBP^{-/-} mice also show evidence of apoptotic and necrotic macrophages, however the shift in towards these cell populations was not as pronounced as it was for neutrophils. Altogether, LBP^{-/-} mice displayed increased numbers of inflammatory cells in BAL fluid compared to Wt C57B6 mice, with many of these cells being apoptotic or necrotic neutrophils.

A summary of pulmonary inflammatory responses to *Klebsiella sp.* infection in Wt (left) and LBP^{-/-} (right) mice, based on our results is shown in Figure 7.

Discussion

The LPS recognition pathway, and specifically LBP, has been proposed as a potential target in the prevention of sepsis. By catalyzing the transfer of LPS from Gram-negative bacteria to CD14, LBP serves to potentiate CD14/TLR4 mediated inflammatory responses to LPS. (17) Early studies suggested this ability was largely detrimental, sensitizing the host to the toxic effects of LPS.(6, 15) However, models of infection in LBP-deficient mice using live bacteria established an essential and protective role for LBP in the host immune response.(2, 5, 9, 11, 14) Demonstrated functions of LBP include sensitization of cells to low levels of LPS (7, 17), opsonization of Gram-negative bacteria (10, 25), and neutralization of LPS (13, 26). The importance of one or all of these potential functions in the lung is not yet fully understood. In the present study, we investigated the impact of LBP on the proinflammatory response and bacterial clearance in an attempt to further explain its protective role in Gram-negative pneumonia.

Our results indicate that loss of LBP function contributes to a profound dysregulation of the host innate immune system and its ability recognize and clear Gram-negative bacteria from the lung. Previous experiments with Gram-negative models of pneumonia in LBP^{-/-} mice have found augmented levels of proinflammatory cytokines at 18 and 24 hours post-inoculation.(2, 5) Our experiments agreed with and expanded upon these results, demonstrating that LBP^{-/-} macrophages display evidence of a suboptimal early local inflammatory response when exposed to LPS *in vitro*. This reduction in inflammatory mediator production by LBP^{-/-} macrophages following LPS stimulation was identical to the response generated in TLR4^{-/-} macrophages. We also demonstrated that LBP^{-/-} mice have decreased levels of neutrophils in the lung 6 hours after inoculation and less macrophages in the lung 24 hours after inoculation with bacteria compared to Wt controls. An initial depression in chemokine levels and inflammatory cell recruitment has also been demonstrated in models of Gram-negative peritonitis in LBP^{-/-} animals (11), and likely reflects diminished early sensitivity of these animals to a low concentration of LPS.

TLR4 has been shown to mediate virtually all of the observed gene expression in response to Gram-negative bacteria in the lungs of mice, indicating that macrophage activation via this receptor must remain a major factor in the inflammatory response even without the contribution LBP.(16, 19) In addition, mice deficient in LBP remain responsive to pulmonary LPS at high concentrations.(12) In fact, LBP^{-/-} mice not only remain responsive to high intratracheal doses of LPS, but they actually exhibit enhanced responses compared to Wt mice.(12) Our quantitative culture results suggest that impaired initial clearance leads to

eventual higher levels of Gram-negative bacteria, and thus LPS, in the lungs of LBP^{-/-} mice. By 24 hours there is direct stimulation of TLR4 leading to augmentation of the inflammatory response.

When LBP is expressed in high concentrations consistent with the acute phase response it can inhibit LPS-induced host cell activation and protect mice from an otherwise lethal intraperitoneal injection of LPS or Gram-negative bacteria by transferring LPS into high- and low-density lipoprotein, effectively neutralizing the endotoxin.(23, 26) The ability of LBP to both enhance and suppress immune cell activation by LPS gives this protein a buffer-like property. Mice lacking LBP are hyporesponsive to low levels of bacteria; however, the same animals may exhibit exaggerated proinflammatory responses to higher levels of bacteria cell exposure. Our findings indicate that the higher bacteria load and the loss of LBP-mediated LPS neutralization enhances the risk of developing an unrestrained inflammatory response leading to sepsis and ultimately death in these animals.

The response to intratracheal bacteria produced an initial reduction in neutrophil sequestration into the lung as measured by myeloperoxidase assay at 6 hours. Despite equivalent recruitment of neutrophils into the alveolar spaces at 24 hours, this initial hyporesponsiveness caused by absence of LBP places the host at immediate disadvantage with regard to microbial clearance and tips the homeostatic balance in favor of overwhelming bacterial proliferation. Given that an absence of LBP diminishes phagocytosis and bacterial killing of Gram-negative bacteria by alveolar macrophages and neutrophils *in vitro*, this suggests that the neutrophils that were finally recruited into the lung were less successful at ingesting and killing the bacteria.(10, 25) In the presence of exogenous LBP, neutrophils obtained from LBP^{-/-} animals had an unaltered ability to phagocytose bacteria and to generate a respiratory burst *in vitro*.(9) Therefore, it is doubtful that the poor bacterial clearance observed in these experiments is due to any intrinsic loss of function of the LBP^{-/-} animal's phagocytic cells, but rather the loss of a necessary contribution from LBP. In cytospin preparations, we observed notable changes in the morphology of macrophages in the absence of LBP. At 24 hours the macrophages and neutrophils present in BAL fluid of LBP^{-/-} mice displayed high levels of apoptosis and necrosis. These changes suggest a possible disordered bacterial clearance mechanism that is associated with degenerative changes in these cells and persistence of bacteria intracellularly.

Our results have several limitations. First is that the murine model of sepsis has been called into question over its ability to accurately replicate human sepsis.(20). The murine model we utilized may not accurately mimic the preinfected immunologic state of a patient prior to infection with pneumonia causing bacteria. (3) Second, we chose *Klebsiella pneumoniae* as the bacteria with which to induce pneumonia in the mice. *Klebsiella* sp. are known to be quite virulent in mice pneumonia models and only require on the order of 500 to 1000 CFU's per inoculum to create >50% mortality. Mice are known to be more resistant to pneumonia from other Gram-negative bacteria such as *Pseudomonas aeruginosa*, which requires on the order of 1×10^6 CFU's in the inoculum to produce >50% mortality.(22) In the clinical setting *Pseudomonas* is often the more difficult bacterial pneumonia to treat in humans. Lastly, the

knockout model itself is limited in that to survive without a particular gene and functioning protein unmeasured compensatory responses not found naturally may occur.

In summary, these results indicate that LBP plays an important protective role in not only the initiation, but also the control of host immune defense to Gram-negative pneumonia. The loss of LBP-mediated functions results in derangements of the inflammatory response and bacterial clearance mechanisms. In contrast, augmenting LBP function may serve to enhance the efficiency of the immune response. Previous work in our lab demonstrated improved survival from Gram-negative pneumonia in Wt mice altered with gene therapy to over express LBP.(8) Because the role of LBP in the immune response is multifaceted, investigations into potential therapies that are capable of enhancing all of LBP's reported functions might prove most effective.

Conclusion

Absence of a functional LBP gene resulted in marked derangements of the innate immune response to *Klebsiella pneumoniae*. LBP^{-/-} mice exhibited diminished clearance of Gram-negative bacteria from the pulmonary system. This is associated with suboptimal early chemokine production and neutrophil sequestration, followed by augmented and prolonged local proinflammatory response as evidenced by increased levels of IL-1 β , IL-6, KC, MIP-2, and MPO activity at 24 and 36 hours. These results indicate that LBP has an important protective role in the lung, and is necessary for proper function and coordination of the immune response following invasion of Gram-negative *K. pneumoniae*.

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Figure 1.

Quantification of lung bacteria levels. All values are Wt vs. LBP^{-/-} and represent median group values of CFU's in 100μL of the whole lung homogenate. (A) CFU's at 6, 12, 24, and 36 hours post inoculation of *Klebsiella pneumoniae*. At 6 hours N=5, at 12 hours N=5, at 24 hours N=22, and at 36 hours N=10 for both Wt and LBP^{-/-} species. (*p < 0.05). (B) BAL macrophages from *Klebsiella* infected lungs. Upper frame, high power magnification (x100) of BAL macrophages (stained with toluidine blue) obtained from *Klebsiella* sp. infected Wt mouse lungs 12 hr after lung challenge. Lower frame, similarly obtained BAL macrophages from *Klebsiella* sp. infected LBP^{-/-} mouse lungs. Arrows show the inclusions within vesicles of the cytosol.

Figure 2.

In vivo cytokine and chemokine analysis. (A) Wt vs. LBP^{-/-} mice lung homogenates at 6, 12, 24, and 36 hours. (B) Wt vs. LBP^{-/-} BAL fluid at 6, 12, 24, and 36 hours. (**p* < 0.05). Normal group represents baseline values from mice at 0 hours that did not undergo bacterial challenge and were undetectable for all measured cytokines. At 6 hours N=12, at 12 hours N=11, at 24 hours N=24, and at 36 hours N=10 for both Wt and LBP^{-/-} species.

Figure 3.

Flow cytometric analysis of inflammatory cell populations in lung parenchyma at 24 hours post-inoculation. **(A)** Significantly reduced recruitment of macrophages into lung parenchyma was found in LBP^{-/-} animals compared to the Wt. **(B)** F4/80⁺ CD11b⁺ CD11c⁺ macrophages represented a greater percentage of the total population of lung parenchymal cells in Wt mice (19% vs 9%). (* $p < 0.05$). N=4 for both Wt and LBP^{-/-} species.

Figure 4.

In vitro cytokine and chemokine analysis. Thioglycollate-elicited peritoneal macrophages were stimulated with 50ng/mL of LPS for 8 hours in serum-free medium. Supernatants were assayed for inflammatory mediators. N=4 for Wt, LBP^{-/-}, and TLR4^{-/-} species.

Figure 5.

Lung myeloperoxidase assay. Wt vs. LBP^{-/-} mice MPO activity in lung homogenates at 6, 12, 24, and 36 hours. MPO activity was significantly higher in Wt mice vs. LBP^{-/-} mice at 6 hours (0.094 μg/mL ± 0.012 vs. 0.037 μg/mL ± 0.03, $p < 0.05$). At 36 hours, MPO activity was significantly higher in LBP^{-/-} mice compared to Wt (0.352 μg/mL ± 0.03 vs. 0.123 μg/mL ± 0.012, $p < 0.05$). At 6 hours N=5, at 12 hours N=5, at 24 hours N=22, and at 36 hours N=10 for both Wt and LBP^{-/-} species.

Figure 6.

Flow cytometric analysis cells in bronchial lavage fluid (BAL) at 24 hours post bacteria inoculation. BAL macrophages were identified as forward light scatter high, F4/80+, CD11c +, CD11b+ cells. BAL neutrophils were identified as FSC low, Gr-1+, CD11b++, F4/80- cells. Apoptotic cells were identified as Annexin V+ Live/Dead-. Necrotic cells were identified as Annexin V+ Live/Dead+. (* $p < 0.05$). N=4 for both Wt and LBP-/- species.

Figure 7.

Summary of changes in Wt lungs (left) and LBP^{-/-} lungs (right) after challenge with *Klebsiella* sp. Wt lungs showed fewer CFUs (arrows indicating *Klebsiella* organisms). Macrophages were more numerous and PMNs eventually declined to lower levels. In LBP^{-/-} lungs (right), many more *Klebsiella* prevail, the number of PMNs is considerably greater, and intrapulmonary cytokine levels are considerably higher, possibly due to a heightened load of bacteria.