Pulmonary Lipopolysaccharide (LPS)-Binding Protein Inhibits the LPS-Induced Lung Inflammation In Vivo

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LPS-binding protein (LBP) facilitates the interaction of the Gram-negative cell wall component LPS with CD14, thereby enhancing the immune response to LPS. Although lung epithelial cells have been reported to produce LBP in vitro, knowledge of the in vivo role of pulmonary LBP is limited. Therefore, in the present study we sought to determine the function of pulmonary LBP in lung inflammation induced by intranasal administration of LPS in vivo. Using LBP-deficient (LBP−/−) and normal wild-type mice, we show that the contribution of LBP to pulmonary LPS responsiveness depended entirely on the LPS dose. Although the inflammatory response to low dose (1 ng) LPS was attenuated in LBP−/− mice, neutrophil influx and cytokine/chemokine concentrations in the bronchoalveolar compartment were enhanced in LBP−/− mice treated with higher (>10 ng) LPS doses. This finding was specific for LBP, because the exogenous administration of LBP to LBP−/− mice reversed this phenotype and reduced the local inflammatory response to higher LPS doses. Our results indicate that pulmonary LBP acts as an important modulator of the LPS response in the respiratory tract in vivo. This newly identified function of pulmonary LBP might prove beneficial by enabling a protective immune response to low LPS doses while preventing an overwhelming, potentially harmful immune response to higher doses of LPS. The Journal of Immunology, 2006, 176: 3189–3195.
Table I. \textit{LBP} concentrations in BALF and plasma\textsuperscript{a}

<table>
<thead>
<tr>
<th>LBP concentrations</th>
<th>BALF (ng/ml)</th>
<th>Plasma (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.7 ± 3.9</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>6 h post-NaCl i.n.</td>
<td>16.8 ± 5.8\textsuperscript{b}</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>6 h post-10 μg of LPS i.n.</td>
<td>47.5 ± 4.7\textsuperscript{bc}</td>
<td>3.5 ± 0.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Wild-type mice (n = 6–8) were inoculated with saline or 10 μg of LPS i.n. and BAL was performed after 6 h. LBP concentrations were measured by ELISA in BALF and plasma. Samples of untreated mice served as controls (n = 6). Data are mean ± SEM.

\textsuperscript{b} p < 0.05 vs untreated controls.

\textsuperscript{c} p < 0.05 vs NaCl-treated mice.

\textbf{Materials and Methods}

\textbf{Mice}

Pathogen-free 10- to 12-wk-old female C57BL/6 wild-type mice were purchased from Harlan Sprague Dawley. LBP\textsuperscript{−/−} mice were generated as described previously (10), backcrossed to C57BL/6 background 11 times and bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands). Age- and sex-matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam, The Netherlands approved all experiments.

\textbf{Induction of lung inflammation}

Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories), and LPS (from \textit{Escherichia coli} O55:B5; Sigma-Aldrich) diluted in 50 μl of sterile saline was instilled intranasally (i.n.). Control mice received sterile saline. In some experiments, mice received purified human LBP (80 ng; HyCult Biotechnology) i.n. simultaneously with LPS. After 6 or 22 h, mice were anesthetized with Hypnorm (Janssen Pharmaceutical) and midazolam (Roche) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes and plasma was stored at −20°C until further usage.

\textbf{Bronchoalveolar lavage}

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott Laboratories). Bilateral BAL was performed by instilling two 0.5-ml aliquots of sterile saline. Approximately 0.9–1 ml of BALF was retrieved per mouse. Total cell numbers were counted from each sample using a hemocytometer (Türck chamber); BALF differential cell counts were done on cytopsin preparations stained with Giemsa. BALF supernatant was stored at −20°C for cytokine and LBP measurements.

\textbf{Histology}

Lungs for histology were harvested at 6 or 22 h after infection, fixed in 10% formalin, and embedded in paraffin. Four-micrometer sections were stained with H&E, and analyzed by a pathologist who was blinded for groups. To quantify lung inflammation and damage, the entire lung surface was semi-quantitatively scored as described previously (24). In brief, the following parameters were analyzed: interstitial inflammation, intra-alveolar inflammation, edema, endotheliolitis, bronchitis, pleuritis, and thrombi formation. Each parameter was graded on a scale of 0–3, with 0: absent, 1: mild, 2: moderate, and 3: severe. The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 21.

\textbf{Assays}

Murine LBP was measured using a commercially available ELISA (HyCult Biotechnology) according to the manufacturer’s instructions; the detection limit was 0.4 ng/ml. TNF-α and KC were measured using specific ELISAs (R&D Systems) according to the manufacturer’s instructions. The detection limits were 31 pg/ml for TNF-α and 12 pg/ml for KC. Protein levels in BALF were measured using the BCA protein kit according to the manufacturer’s instructions (Pierce).

\textbf{LPS responsiveness of alveolar macrophages}

The murine alveolar macrophage cell line MH-S was purchased from American Type Culture Collection and grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 2 mM l-glutamine, 5 mM HEPES buffer, 10% FCS (unless otherwise indicated), penicillin, streptomycin, and 2-ME. In vitro stimulation was conducted in 96-well plates (Greiner) at a density of 1 × 10\textsuperscript{6} cells/ml. Following overnight culture in serum-free medium at 37°C in 5% CO\textsubscript{2}, adherent cells were washed twice in serum-free medium then stimulated overnight with 0.1 ng/ml to 1 μg/ml LPS (E. coli O55:B5; Sigma-Aldrich) in the presence or absence of 0.1 ng/ml to 10 μg/ml human LBP (HyCult Biotechnology) in serum-free medium. Some experiments were performed in the presence of scavenger receptor A (SR-A) blocking Abs (clone: 2F8; Serotec) at a concentration of 10 μg/ml.

\textbf{Statistical analysis}

Serial data were analyzed by one-way ANOVA; differences between two groups were calculated by Mann-Whitney U test. Correlations were calculated by Pearson test. Values are expressed as mean ± SEM. A p value ≤0.05 was considered statistically significant.

\textbf{Results}

\textit{LBP}\textsuperscript{−/−} is detectable in BALF of healthy mice and rapidly increases in acute lung inflammation

To investigate whether pulmonary LBP is detectable in normal BALF and whether acute lung inflammation induces local release of this protein, we measured LBP in BALF of wild-type mice before and 6 h after i.n. administration of 10 μg of LPS or NaCl. Low levels of alveolar LBP were readily detectable in normal mice and LPS induced a significant rise in LBP concentrations (Table I). Plasma LBP levels, which are known to be 100-fold higher, were unaffected by pulmonary inflammation (Table I) and no LBP was detectable in LBP\textsuperscript{−/−} mice. Hence, i.n. administration of LPS induced a rapid increase in alveolar LBP levels, supporting the notion of locally produced LBP in vivo.

\textit{Lung inflammation induced by high dose LPS is enhanced in the absence of LBP}

To determine the specific contribution of pulmonary LBP to acute lung inflammation, we i.n. inoculated wild-type and LBP\textsuperscript{−/−} mice with 10 μg of LPS or saline. LPS induced a strong cell influx that predominantly was caused by polymorphonuclear cells (PMNs) (p < 0.05 LPS-treated wild-type vs saline-treated wild-type mice). Surprisingly, the comparison of wild-type and LBP\textsuperscript{−/−} mice revealed significantly more PMNs as well as higher TNF-α and KC concentrations in BALF of LBP\textsuperscript{−/−} mice (Fig. 1). Hence, these data suggested that pulmonary LBP inhibits lung inflammation induced by 10 μg of LPS in vivo.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{LBP\textsuperscript{−/−} mice demonstrate enhanced lung inflammation upon i.n. administration of 10 μg of LPS. PMN counts, TNF-α, and KC concentrations in BALF of wild-type (■) and LBP\textsuperscript{−/−} mice (□) 6 h after i.n. administration of 10 μg of LPS (n = 6 per strain). Data are mean ± SEM; *, p < 0.05 vs wild-type mice.}
\end{figure}
The influence of LBP on LPS-induced pulmonary inflammation depends on the dose of LPS

This surprising finding of a more pronounced pulmonary inflammation in LBP−/− mice following LPS challenge led us to investigate whether this observation was attributable to the LPS dose. We therefore repeated these in vivo studies with increasing, though lower, LPS doses. Wild-type and LBP−/− mice were inoculated with 1, 10, or 100 ng of LPS i.n. 6 h before BAL was performed, and cell influx and TNF-α and KC responses were evaluated. In mice that received the lowest LPS dose (1 ng), an LBP-dependent increase of pulmonary inflammation was found: 1 ng of LPS induced a significantly higher PMN influx and slightly higher BALF TNF-α levels in wild-type than LBP−/− mice (Figs. 2 and 3). In contrast, PMN influx as well as TNF-α and KC release in LBP−/− mice exceeded values observed in wild-type mice when treated with 100 ng of LPS (Figs. 2 and 3). Results of mice that received 10 ng of LPS were indistinguishable between wild-type and LBP−/− animals (Figs. 2 and 3). Together, the acute pulmonary inflammation induced by LPS doses as low as 1 ng was augmented in the presence of LBP whereas cell influx and cytokine/chemokine production following higher LPS doses (≥10 ng LPS) were independent from or even inhibited by the presence of LBP.

To evaluate these findings over a longer period of time, we repeated the in vivo experiments and assessed pulmonary inflammation in response to different LPS doses (1, 10, and 100 ng) after 22 h. At this late time point, cytokines or chemokines could not be detected anymore in most of the BALF samples. A small amount of TNF-α (65 ± 12 pg/ml) was detected in BALF of LBP−/− mice challenged with 100 ng of LPS. As shown in Fig. 3, all wild-type mice showed a trend toward resolution of the inflammation in terms of a reduction in alveolar PMN counts (as compared with t = 6 h) at this later time point. In contrast, alveolar PMN counts continued to be elevated (10 ng of LPS) or increased even further (100 ng of LPS) in LBP−/− mice. To obtain further proof for enhanced lung inflammation in LBP−/− mice after i.n. administration of 100 ng of LPS, histological analysis of lung slides obtained 6 and 22 h postchallenge were performed. Using the semiquantitative scoring system described in Materials and Methods, we could confirm our findings of enhanced and prolonged pulmonary inflammation in LBP−/− mice that received 100 ng of LPS i.n. As shown on representative slides in Fig. 4, the extent of lung inflammation was more severe in LBP−/− mice when compared with their wild-type counterparts (lung inflammation score at t = 6 h: wild-type: 7.4 ± 1.2, LBP−/−: 11.7 ± 0.8; p < 0.05 and at 22 h: wild-type: 3.0 ± 0.6 and LBP−/−: 7.5 ± 0.8; p < 0.05).

This experiment confirms our previous finding of an exaggerated pulmonary inflammation in the absence of LBP and illustrates furthermore that LBP is involved in the timely resolution of pulmonary inflammation.

Pulmonary LBP concentrations rise in an LPS dose-dependent manner

In Table I, we show elevated LBP protein concentrations within the alveolar compartment following LPS challenge. To study whether this increase in LBP concentrations correlates to the dose of LPS administered, we measured LBP levels in BALF of mice treated with increasing LPS doses (1, 10, or 100 ng). Although in wild-type mice, 1 ng of LPS did not induce any change in LBP levels, a dose-dependent rise in alveolar LBP could be revealed in response to higher LPS doses (≥10 ng LPS) (Fig. 5A). To exclude the possibility that this increase in alveolar LBP levels is a result of extravasated serum proteins, we compared LBP levels with total BALF protein concentrations in BALF of wild-type mice (Fig. 5B): while LBP concentrations increased in a LPS-dose-dependent manner (Pearson correlation LBP dose vs LBP concentration: r = 0.79, p = 0.003), protein levels remained essentially unaltered and no correlation was found between LBP and total protein concentrations in BALF of wild-type mice (Pearson correlation LBP concentration vs total protein concentrations: r = 0.39, p = 0.14). As expected, LBP could not be detected in BALF or plasma of LBP−/− mice whereas in these animals total alveolar protein concentrations increased in response to LPS and correlated with PMN influx (Pearson correlation total protein concentrations vs PMN count: r = 0.79, p = 0.0003) and TNF-α levels (Pearson correlation total protein concentrations vs TNF-α levels: r = 0.82, p = 0.0001). Together, alveolar LBP (but not total protein) concentrations increased in a LPS-dose-dependent manner in wild-type mice. The increase in alveolar protein concentrations most likely reflects the extent of capillary leakage found during acute lung injury and exclusively correlated with alveolar inflammation parameters as reflected by the high levels measured in LBP−/− mice challenged with 100 ng of LPS.

The increased lung inflammation in LBP−/− mice induced by high dose LPS is reversed by exogenous LBP

To investigate whether the augmented lung inflammation in LBP−/− mice following administration of ≥10 ng of LPS is specifically attributable to the lack of LBP, we administered human LBP in a dose of 80 ng together with 100 ng of LPS to...
both wild-type and LBP \( \sim / \) mice and assessed the inflammatory response. Exogenous LBP did not influence LPS responsiveness in the lungs of wild-type mice (Fig. 6). In LBP \( \sim / \) mice, however, the administration of LBP in addition to LPS completely reversed the amplified pulmonary inflammation observed in LBP \( \sim / \) mice treated with LPS only. Thus, the coadministration of LBP virtually transformed LBP \( \sim / \) mice to a wild-type phenotype with regard to both PMN influx and alveolar TNF-\( \alpha \) concentrations, confirming the specificity of the findings.

The LPS responsiveness of alveolar macrophages in vitro depends on LBP

Because we consider the enhanced PMN recruitment a consequence of exaggerated proinflammatory stimuli, we attempted to elucidate the mechanisms involved. In a next step, we therefore examined whether our findings could be reproduced in vitro and whether alveolar macrophages are responsible for our in vivo observations. For this purpose, we stimulated murine alveolar macrophages (MH-S cells) with increasing doses of LPS and LBP in the absence of serum. By doing so, we found a clear LBP and LPS dose-dependent response with respect to TNF-\( \alpha \) production (Fig. 7A). Because it has been reported that “supranormal” LBP concentrations can exert inhibitory actions, we also performed stimulations using LBP concentrations up to 10 \( \mu \)g/ml (Fig. 7B). Even 10 \( \mu \)g/ml LBP further increased TNF-\( \alpha \) levels released by alveolar macrophages in vitro. Another major receptor interacting with LPS responsiveness and expressed on alveolar macrophages is SR-A. In an attempt to investigate whether LBP also transfers LPS to SR-A and thereby causes a damped inflammatory response, we stimulated MH-S cells with LPS and increasing doses of LBP in the presence or absence of a blocking Ab against SR-A. As depicted in Fig. 8, blockage of SR-A increases the inflammatory response irrespective of LBP. Together, no inhibitory properties of LBP were observed in vitro, indicating either that this observation is not reproducible in vitro or that alveolar macrophages are not the primary target cells for the inhibitory properties of LBP seen in vivo.

Discussion

LBP is an important acute phase protein that has attracted much attention as an enhancer of cellular LPS responsiveness via its unique property to transfer LPS to the CD14/TLR4 receptor complex on immune cells (8) thereby facilitating the inflammatory response to LPS and Gram-negative bacteria in vivo (23, 25, 26). Additionally, there is evidence suggesting that very high LBP concentrations, such as found in plasma samples of septic patients, inhibit the proinflammatory effects of LPS (13). Conceivably, the function of LBP depends on diverse aspects, such as concentration, cell types investigated, and–possibly–localization of the stimulus in vivo. The finding of LBP production by respiratory epithelial cells in vitro (19) led us to evaluate the specific in vivo contribution of pulmonary LBP to the inflammatory response induced by LPS. First, we here establish that LPS elicits an increase in alveolar LBP in vivo in a dose-dependent manner, supporting the hypothesis of local, pulmonary LBP production. Second, using LBP \( \sim / \) mice, we were able to demonstrate for the first time that pulmonary LBP–at a naturally occurring low concentration–specifically inhibits and abridges the LPS-induced acute lung inflammation in vivo. These inhibitory properties of pulmonary LBP depended entirely on the LPS-dose administered—with a threshold set at 10 ng of LPS.

While our studies were in progress, Brass et al. (27) reported on the role of endogenous LBP in lung inflammation induced by repeated LPS inhalation during periods up to 4 wk. The authors found reduced cell influx and lower cytokine levels in BALF of LBP \( \sim / \) mice when compared with wild-type mice, indicating that LBP was required for full LPS responsiveness in their model. Of note, the LPS dose administered, albeit repeatedly, was relatively low. Although the precise LPS dose inhaled by an individual mouse is difficult to determine, the approximate LPS concentration in the exposure chambers was determined to be 5 mg of LPS/m\(^3\) air (27). We also found LBP to promote lung inflammation when
FIGURE 6. Exogenous LBP inhibits pulmonary inflammation in LBP−/− mice. Wild-type and LBP−/− mice (n = 5–6/group) received 100 ng of LPS with or without 80 ng of LBP i.n. and PMN counts (A) and TNF-α concentrations (B) were assessed in BALF after 6 h. *, p < 0.05 vs wild-type mice; +, p < 0.05 vs LBP−/− mice treated with LPS only. Data are mean ± SEM.

a low LPS dose (<10 ng) was administered. Of crucial importance, higher LPS doses showed quite the opposite, i.e., a diminished inflammatory response in the presence of LBP.

How can this observation be explained? LBP is a transfer protein that binds anionic structures like LPS and shuttles them to respective receptors or other proteins. Because LBP itself is not directly involved in cellular signaling processes, pulmonary LBP likely transfers LPS to other LPS-binding structures (e.g., receptors, lipid-binding molecules, lipoproteins) that neutralize LPS activity. Alternatively, LBP might actively prevent the transfer of LPS to the CD14/TLR4/MD2 receptor that mediates proinflammatory pathways.

Regarding the latter possibility, the CD14/TLR4/MD2 receptor complex prominently features as "the" LPS-signaling structure. As suggested by a recent report, moderate to high LBP levels inhibit the LPS transfer from CD14 to the TLR4/MD-2 signaling receptor in vitro (28). Thompson et al. (28) showed that LBP removed cell-bound LPS from membrane (m) CD14 and MD-2 resulting in an attenuated inflammatory response. However, the LBP dose required for this to take place was 3 μg/ml, which is comparable to plasma levels, but much higher than the concentrations we found in BALF (although LBP levels in the alveolar lining fluid likely are higher considering the dilution caused by the BAL procedure). We studied the interaction of LPS with alveolar macrophages in vitro and found—in accordance with a previous report—a dose-dependent enhancement of the inflammatory response in the presence of LBP (9). Like Thompson et al. (28), we added LPS a few minutes before LBP but we were unable to find any LBP-related reduction of the inflammatory response even in the presence of LBP doses as high as 10 μg/ml. This in vitro observation is in contrast to the results we found in mice. By stimulating isolated alveolar macrophages of LBP−/− and wild-type mice with LPS in vitro, we observed no difference in their capacity to produce TNF-α, ruling out the possibility of an impaired or exaggerated cytokine secretion of LBP−/− macrophages (data not shown). Already, in 1995, Gnegger et al. (29) described the potential dual role of LBP and mCD14 in LPS signal enhancement and LPS neutralization. They concluded that LBP is also part of the LPS clearance mechanism and that this pathway bifurcates after binding to mCD14. A more recent publication delineated that TLR4 is responsible for LPS signaling while LPS neutralization is mediated by other receptors in vitro (30). Using murine blood monocytes and endothelial cells, Dünzendorfer et al. (30) demonstrated that LBP contributes substantially to the neutralization of LPS via transfer to CD14 as well as to other anionic structure binding receptors, presumably SRs. SRs are expressed on macrophages and able to bind anionic ligands including LPS. It is known that SR-A−/− mice are hypersensitive to LPS, which might be explained by the fact that SR-A can degrade LPS without concomitant release of proinflammatory cytokines in vitro (31). However, when blocking SR-A with an Ab, we could not delineate a role for LBP in the enhanced inflammatory response seen with anti-SR-A-treated cells in vitro (Fig. 8).

Lung epithelial cells have been disclosed to importantly contribute to LPS-induced pulmonary inflammation in vivo via NF-kB-activating pathways (32, 33). Of great interest, lung epithelial cells have also been demonstrated to have the ability to internalize LPS without activation of NF-kB (34). Hamann et al. (34) revealed that the internalization and neutralization of LPS by respiratory epithelial cells depended on the presence of LBP (and partly mCD14) in vitro. In an attempt to investigate the potential role for LBP, we measured the internalization of FITC-labeled LPS by respiratory epithelial cells in the presence or absence of LBP. Although we found LPS rapidly internalized by respiratory epithelial cells, we could not identify a role of LBP herein (data not shown). Furthermore, Hamann et al. (34) described that moderate LBP concentrations enhanced the uptake and signaling of LPS by alveolar macrophages, whereas high LBP concentrations (≥1 μg/ml) diminished the inflammatory response in vitro. Stimulation of alveolar macrophages in the presence of up to 10 μg/ml LBP did not enable us to reproduce this observation in vitro. However, the major difference between our studies and those by Hamann et al. (34) is the LPS chemotype with which cells were stimulated. While Hamann et al. (34) focused on rough LPS mutants, we could not identify a role of LBP herein (data not shown). Furthermore, Hamann et al. (34) focused on rough LPS mutants; we made use of smooth LPS. The vast importance of distinct LPS chemotypes has just been highlighted by a recent article disclosing that the TLR4-MD-2 complex is able to differentiate between these LPS chemotypes (35). Although CD14 is required for MyD88-independent signaling by either LPS chemotype, rough LPS (but not smooth...
LPS can bind and signal via the MyD88-dependent pathway in the absence of CD14 (35). Together, Hamann et al. (34) used rough LPS that also elicits CD14-independent pathways, while our data were generated with CD14-dependent smooth LPS. Our data clearly could not confirm the hypothesis of LBP-mediated neutralization of smooth LPS by respiratory epithelial cells or alveolar macrophages in vitro.

The presence of surfactant proteins (SP) is another unique and specific feature of the pulmonary compartment. SP-A, like LBP, is able to bind anionic structures including rough LPS and to inhibit the inflammatory response of alveolar macrophages to rough or smooth LPS in vitro (36, 37). Furthermore, SP-A−/− mice challenged with intratracheal LPS display increased TNF-α and MIP-2 BALF-concentrations when compared with wild-type controls (38). The potential role of LBP in the SP-A related anti-inflammatory mechanisms is controversial. Although Stamme et al. (37) explained the anti-inflammatory properties of SP-A via its capacity to inhibit the binding of rough LPS to LBP, these data could not be confirmed by Alcorn et al. (36). However, in vivo data thus far only demonstrated the SP-A related decrease in cytokine/chemokine levels whereas PMN influx was not affected (38). Nevertheless, the possibility exists that the anti-inflammatory properties of SP-A are masked in mice lacking LBP, which results in higher pulmonary cytokine/chemokine concentrations that indirectly attract more PMNs.

The airways are a site of constant exposure to inhaled pathogens or LPS and it certainly is of crucial importance to fight these pathogens to prevent pneumonia. Conversely, the continuous daily exposure to minute amounts of inhaled LPS might warrant mechanisms that attenuate an excessive inflammatory response that would be detrimental to the host. In our in vivo findings indicate that LBP could be considered an important regulator of the pulmonary immune response: LBP enables the host to respond to small amounts of LPS with a self-limited inflammatory response while contributing to the neutralization of higher LPS doses and thus preventing the harmful consequences of exaggerated lung inflammation. Although premature at this point, therapeutic implications of these findings may prove beneficial in the future.

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Disclosures

The authors have no financial conflict of interest.

References


