Mechanisms of immune activation during infection
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CHAPTER 6

Lipopolysaccharide binding protein deficient mice have a normal defense against pulmonary mycobacterial infection

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Submitted
Abstract

Lipopolysaccharide (LPS) binding protein (LBP) facilitates the transfer of LPS of Gram-negative bacteria to the pattern recognition receptor CD14, resulting in activation of immunocompetent cells. LBP can also facilitate the binding of lipoarabinomannan, a major cell wall component of mycobacteria, to immune cells. To determine the role of LBP in the immune response to pulmonary *M. tuberculosis* infection, LBP gene deficient (---) and normal wild type (WT) mice were intranasally infected with *M. tuberculosis*. LBP-/- mice displayed a similar survival and mycobacterial outgrowth in lungs and liver, although they demonstrated a reduced lymphocyte recruitment and activation during the early stages of infection. The clearance of pulmonary infection with the non-pathogenic *M. smegmatis* was also unaltered in LBP- - mice. These data suggest that LBP does not contribute to an effective host response in *M. tuberculosis* infection.
Introduction

Lipopolysaccharide binding protein (LBP) is an acute phase reactant predominantly derived from the liver (1). LBP plays an important role in the activation of immunocompetent cells by lipopolysaccharide (LPS) from Gram-negative bacteria. LBP binds and disaggregates LPS, strongly promoting delivery to the pattern recognition receptor CD14 (2). Subsequent LPS-induced intracellular activation proceeds via Toll-like receptor (TLR) 4 in the presence of an additional extracellular protein, MD-2 (3-5). Elimination or inhibition of LBP largely prevents LPS-induced toxicity in experimental animals, indicating that LBP is required for the transfer of LPS to its receptor complex in vivo (6-9). Interestingly, LBP gene deficient (-/-) mice were unable to mount an effective early inflammatory response to the Gram-negative bacteria *Salmonella typhimurium* (10-13), *Klebsiella pneumoniae* (14, 15) and *Escherichia coli* (16), which resulted in an enhanced bacterial outgrowth and an increased lethality when compared to normal wild type (WT) mice. Together, these data suggest that LBP is important in the chain of events that leads to an adequate innate immune response to at least some Gram-negative infections by virtue of its capacity to present LPS to the CD14/TLR4 receptor complex.

*Mycobacterium tuberculosis* is responsible for approximately two million deaths per year worldwide (17, 18). The organism is primarily transmitted via the respiratory route and pulmonary tuberculosis is the most common disease manifestation. The induction of an appropriate innate immune response is essential for the control of the infection (19). Lipoarabinomannan (LAM) is a cell wall component of mycobacteria that shares many physicochemical properties with LPS (20). LAM isolated from *M. tuberculosis* strains is capped with mannose residues at the nonreducing arabinofuranosyl termini (ManLAM) (21), whereas LAM derived from rapidly growing nonpathogenic mycobacteria lacks mannose caps (AraLAM) (22). AraLAM is much more potent in eliciting inflammatory responses by isolated mononuclear cells than ManLAM (23-25), although in whole blood in vitro and in mouse lungs in vivo ManLAM is able to produce inflammatory responses that resemble LPS effects (26-29). In cell lines transfected with CD14 and or TLR’s, the effects of AraLAM are mediated via LBP and CD14, after which signal transduction occurs via TLR2 (30-33). Although ManLAM induces virtually no effect in this in vitro system (31, 34), various effects of ManLAM on monocyctic THP-1 cells and macrophages can be inhibited by anti-CD14
antibodies, suggesting that CD14 may play a role in at least some ManLAM induced intracellular responses (25, 35).

LBP can be produced in the lung by alveolar epithelial cells (36), and patients with various inflammatory lung diseases display elevated LBP concentrations in their bronchoalveolar lavage fluid (37, 38). Recently, locally expressed LBP was found to be important for host defense against Gram-negative pneumonia (15). Although to our knowledge LBP levels have not been reported in the pulmonary compartment of patients with lung tuberculosis, the serum concentrations of LBP are elevated in such patients (39). In the present study we sought to determine the role of endogenous LBP in host defense against lung tuberculosis. For this purpose we intranasally inoculated LBP-/- and LBP+/+ WT mice with virulent M. tuberculosis and monitored survival, mycobacterial outgrowth and host responses. In addition, the outgrowth of avirulent M. smegmatis, expressing AraLAM rather than ManLAM, was compared in LBP-/- and LBP+/+ WT mice.

Materials and Methods

Mice

LBP-/- mice, backcrossed 11 times to a C57Bl/6 background, were generated as described previously (9). Normal LBP+/+ C57Bl/6 WT mice were purchased from Harlan Sprague Dawley Inc. (Horst, the Netherlands). Female mice were used at age 8-10 weeks, and maintained in biosafety level 3 facilities. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Experimental infection

Pulmonary tuberculosis was induced exactly as described previously (40-42). In brief, a virulent laboratory strain of M. tuberculosis H37Rv was grown in liquid Dubos medium containing 0.01% Tween 80 for 4 days. A replicate culture was incubated at 37°C, harvested at mid-logarithmic phase, and stored in aliquots at -80°C. For each experiment, a vial was thawed and washed two times with sterile 0.9% NaCl. Mice were lightly anesthesized by inhalation with isoflurane (Upjohn, Ede, the Netherlands) and intranasally inoculated with 50 μl of mycobacterial suspension. The intranasal route of infection has been described previously by us and others, and results in a reproducible infection of the lung with
subsequent dissemination to liver and spleen (40-44). Exact inoculum strength was determined by plating tenfold dilutions of the suspension on 7H11 Middlebrook agar plates immediately after inoculation. Mice were inoculated with $10^5$ Colony Forming Units (CFU) *M. tuberculosis*. After 2 and 6 weeks, groups of 6-7 mice per time point were anesthetized by FFM (fentanyl citrate 0.079 mg ml, fluanisone 2.5 mg ml, midazolam 1.25 mg ml in H₂O) and sacrificed by bleeding out the vena cava inferior. Lungs and one lobus of the liver were removed aseptically and homogenized with a tissue homogenizer (Biospec Products, Bartlesville, OK) in 5 volumes of sterile 0.9% NaCl and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days of incubation at 37°C. CFU are provided as total per gram lung or liver tissue. In an additional experiment mice were intranasally inoculated with *M. smegmatis* (ATCC 14468, Rockville, MD). *M. smegmatis* was grown in the exact same way as *M. tuberculosis* and plated on Middlebrook 7H11 agar plates to determine inoculum strength. Mice were intranasally inoculated with $10^5$ Colony Forming Units (CFU) *M. smegmatis* and were sacrificed 24 and 72 hours after inoculation. Lungs were homogenized and plated. Lung bacterial colonies were counted after 4 days.

For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer (150 mM NaCl, 15 mM Tris, 1mM MgCl₂·H₂O, 1 mM CaCl₂, 1% Triton X-100, 100 μg/ml Pepstatin A, Leupeptin and Aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g for 15 min after which the supernatants were sterilized using a 0.22 μm filter (Corning, Corning, NY) and stored at -20°C until further use.

**Histologic examination**

Lungs for histologic examination were harvested at the designated time points, fixed in 10% formaline and then embedded in paraffin. Four μm thick sections were stained with haematoxylin and eosin, and analyzed for inflammation and granuloma formation by a pathologist who was blinded for groups.

**Flow cytometry**

Pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System: Dako, Glostrup, Denmark) and processed as described previously (40, 45). Total leukocytes in lung cell suspensions were counted by using a hemacytometer (Beckmann Coulter, Fullerton, CA). The number of macrophages, granulocytes and
lymphocytes were calculated from these totals, using cytopsin preparations stained with modified Giemsa stain (Diff-Quick. Baxter, McGraw Perk. IL). For FACS analysis, cells were brought to a concentration of $4 \times 10^6$ cells/ml FACS buffer (PBS supplement with 0.5% BSA, 0.01% NaN₃, and 100 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using directly labeled Abs against CD3 (anti-CD3 PE). CD4 (anti-CD4 CyChrome), CD8 (anti-CD8 FITC, anti-CD8 PerCP), CD25 (anti-CD25 FITC) and CD69 (anti-CD69 FITC). All Abs were used in concentrations recommended by the manufacturer (PharMingen. San Diego, CA). To correct for non-specific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. T-cells were analyzed by gating the CD3+ population. The number of positive cells was obtained by setting a quadrant marker for nonspecific staining. FACS analysis was performed using Cellquest (Becton Dickinson Immunocytometry Systems. San Jose, CA). The results are expressed as the percentage of CD4+, CD8+, CD25+ and CD69+ T-cells within the CD3+ population in the lungs.

Cytokine measurements

Cytokines were measured in lung homogenates by specific ELISA's according to the manufacturer's instructions: interferon (IFN)-γ, interleukin (IL)-4, tumor necrosis factor-α (TNF) and IL-6 (all R&D Systems. Minneapolis. MN).

Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test. For comparison of survival curves Kaplan-Meier analysis with a log rank test was used. $P < 0.05$ was considered to represent a statistically significant difference.

Results

Survival

To investigate the role of LBP in the long-term response to tuberculosis, mice were intranasally inoculated with *M. tuberculosis* and monitored for 29 weeks. As shown in Figure 1. 5 of 11 (45%) LBP−/− and 4 of 11 (36%) WT mice died (non significant for the difference between groups).
Role of LBP in mycobacterial infection

**Figure 1.** LBP deficiency does not influence the outcome of *M. tuberculosis* infection. Survival in LBP-/- and WT mice after intranasal inoculation with $10^7$ CFU *M. tuberculosis*. N = 11 mice per group.

*Mycobacterial outgrowth*

Next, we determined mycobacterial numbers in the lungs in earlier phases of infection. At 2 and 6 weeks post-infection, the numbers of CFU recovered from lungs of LBP-/- and WT mice were not different. Moreover, lungs of LBP-/- and WT mice that survived 29 weeks of infection did not differ in mycobacterial lung counts (Figure 2A). Since mycobacterial infections tend to disseminate in mice, mycobacterial numbers were also determined in a distant organ - the liver. The mycobacterial load in liver tissue was similar in LBP-/- and WT mice 2, 6 and 29 weeks after *M. tuberculosis* inoculation (Figure 2B).

**Figure 2.** LBP deficiency does not influence mycobacterial outgrowth. Mycobacterial outgrowth in lungs (A) and liver (B) of LBP-/- and WT mice 2, 6 and 29 weeks after intranasal inoculation with $10^7$ CFU *M. tuberculosis*. Data are mean ± SEM of 8 mice per group at 2 and 6 weeks; data at 29 weeks are from mice that survived this period as shown in Figure 1 (6 LBP-/- and 7 WT mice).
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Histology

At 2 weeks after infection, histopathology slides of lungs of both WT and LBP-/- mice displayed granulomas, generally located around small bronchi and vessels. These granulomas were mainly composed of macrophages and lymphocytes (Figure 3A and B). At 6 weeks after infection, the inflammation became more diffuse in WT and LBP-/- mice, covering 50 – 70% of the lung parenchyma area. Whereas the infiltrate in lungs from WT mice was still predominantly composed of lymphocytes and macrophages (Figure 3C), granulocytes were more prominent in lungs of LBP-/- mice (Figure 3D). Moreover, edema was more pronounced in LBP-/- than in WT mice.

Figure 3. Representative lung histology of WT (A and C) and LBP-/- mice (B and D) 2 weeks (A and B) and 6 weeks (C and D) after intranasal inoculation with *M. tuberculosis*. Data are representative of 5 mice per group. H&E staining, magnification ×10.

Cellular composition of lung infiltrates

To obtain more insight into the cellular composition of the pulmonary infiltrates, we determined leukocyte counts and differentials in whole lung cell suspensions (Table I). At 2
weeks post-infection, lungs of LBP-/- and WT mice contained similar numbers of leukocytes. At 6 weeks post-infection however, lungs of LBP-/- mice contained less leukocytes than those of WT mice ($P = 0.05$). In accordance with the histopathology, the decreased leukocyte number in LBP-/- mice was largely due to lower numbers of lymphocytes. Since T cells are important for protective immunity against *M. tuberculosis* infection (46-48), we next studied the phenotype and activation status of T cells in the lungs in the presence and absence of LBP by flow cytometry. The decrease in lymphocyte numbers in lungs of LBP-/- mice 6 weeks post infection was proportionally distributed over the CD3/CD4+ and the CD3/CD8+ populations compared to WT mice (data not shown). Strikingly, 2 weeks after infection, CD4+ and CD8+ lymphocytes of LBP-/- mice were significantly less activated than CD4+ and CD8+ lymphocytes of WT mice as assessed by the activation markers CD25 and CD69 (Figure 4; all $P < 0.05$ vs. WT). These differences had largely disappeared 6 weeks after infection (data not shown).

<table>
<thead>
<tr>
<th>2 weeks post-infection</th>
<th>6 weeks post-infection</th>
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<tbody>
<tr>
<td>LBP-/-</td>
<td>WT</td>
</tr>
<tr>
<td>leukocytes ($x10^4$)</td>
<td>18.0 ± 4.8</td>
</tr>
<tr>
<td>macrophages (%)</td>
<td>29.6 ± 2.6</td>
</tr>
<tr>
<td>granulocytes (%)</td>
<td>38.0 ± 3.8*</td>
</tr>
<tr>
<td>lymphocytes (%)</td>
<td>32.3 ± 3.7</td>
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</table>

Table 1. Cell subsets in the lungs of LBP-/- and WT mice during pulmonary tuberculosis. Cell subsets in the lungs of LBP-/- and WT mice infected with *M. tuberculosis* 2 and 6 weeks post-infection. Total leukocyte counts in lungs and differential counts as percentage of total leukocytes. Data are mean ± SE of 6-7 mice per group at each time point. * $P < 0.05$ vs. WT at the same time point.

**Cytokine response**

A type 1 immune response is pivotal in the early host defense against *M. tuberculosis* infection (19). We therefore investigated whether the absence of LBP was associated with a change in the concentrations of the type 1 cytokine IFN-$\gamma$ within the pulmonary compartment early in infection. No difference in IFN-$\gamma$ levels, measured in lung homogenates 2 and 6 weeks post-infection, between LBP-/- and WT mice could be detected (Figure 5A). The Th2 cytokine IL-4 was not detectable in lung homogenates. To determine the ability of both mouse strains to mount a proinflammatory cytokine response, TNF and IL-6 were measured. At 2 weeks, TNF and IL-6 levels were similar in LBP-/- and WT mice. At 6 weeks
however, TNF and IL-6 lung concentrations were significantly elevated in LBP−/− mice compared with WT mice ($P < 0.05$ vs. WT; Figure 5B and C).

**Figure 4.** Impaired CD4+ and CD8+ lymphocyte activation in LBP−/− mice 2 weeks after *M. tuberculosis* infection. Flow cytometry results are expressed as the percentage of CD4+CD25+, CD4+CD69+, CD8+CD25+ and CD8+CD69+ T cells within the CD3+ population in the lungs. Data are mean ± SE of 6 mice per group. *$P < 0.05$ vs. WT.

**Figure 5.** Lung cytokine concentrations. IFN-γ (A), TNF (B) and IL-6 (C) concentrations in lung homogenates of LBP−/− and WT mice 2 and 6 weeks after *M. tuberculosis* infection. Data are mean ± SEM of 7-8 mice. *$P < 0.05$ vs. WT.
Role of LBP in mycobacterial infection

*M. smegmatis* infection.

*M. tuberculosis* bacilli contain ManLAM in their cell walls. Rapidly growing mycobacteria, however, such as the nonpathogenic *M. smegmatis*, contain AraLAM in their cell walls. To investigate whether LBP is important for host defense responses in infections with AraLAM expressing mycobacteria, LBP-/- and WT mice were intranasally infected with *M. smegmatis*. In preliminary experiments we assessed that infection of mice with this mycobacterial species does not result in growth in the lungs, such as after infection with *M. tuberculosis*, but rather that the mycobacteria are cleared from the lungs within 2 weeks. We compared mycobacterial loads in LBP-/- and WT mice 24 and 72 h after intranasal infection with *M. smegmatis*. At both time points lungs of LBP-/- and WT mice contained similar number of *M. smegmatis* (Table II).

<table>
<thead>
<tr>
<th></th>
<th>LBP-/- (× 10^5 CFU/g lung)</th>
<th>WT (× 10^5 CFU/g lung)</th>
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<tbody>
<tr>
<td>24 h</td>
<td>13.6 ± 4.9</td>
<td>27.5 ± 7.9</td>
</tr>
<tr>
<td>72 h</td>
<td>6.1 ± 2.2</td>
<td>11.0 ± 1.8</td>
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**Table II.** Outgrowth of *M. smegmatis* is not influenced by the absence of LBP. Mycobacterial outgrowth in lungs of LBP-/- and WT mice 24 and 72 hours after intranasal inoculation with 10^6 CFU *M. smegmatis*. Data are mean ± SEM of 6-7 mice.

Discussion

LBP is an acute phase protein mainly produced in the liver. However, LBP is also produced in the pulmonary compartment in response to inflammatory stimuli and is considered to be important for the recognition of LPS and for the transfer of bacterial LPS to CD14 (49) in the lung. Since LAM present in the mycobacterial envelope shares many properties with LPS, including its interaction with LBP, we were interested in the role of endogenous LBP in the immune response to pulmonary tuberculosis. We here demonstrate that LBP is not important for host defense against lung infection with *M. tuberculosis*, as reflected by similar responses in LBP-/- and WT mice with respect to survival and mycobacterial outgrowth. We furthermore showed that the clearance of avirulent *M. smegmatis* was unaffected in LBP-/- mice.
M. tuberculosis strains contain ManLAM in their cell wall (21), whereas LAM derived from rapidly growing nonpathogenic mycobacteria, such as M. smegmatis, express AraLAM (22). The vast majority of in vitro studies examining the signal pathways utilized by LAM have used AraLAM, showing that it utilizes LBP to activate the CD14/TLR2 complex (30-33). Other studies have suggested that ManLAM may also induce cell activation via CD14 (25, 35). In light of the different pathways that AraLAM and ManLAM may use to activate cells, we also investigated the role of LBP in infection with a nonpathogenic, AraLAM expressing, mycobacterium, M. smegmatis. These experiments established that the absence of LBP did not influence the capacity of the host to clear the infection. In vitro experiments with Chinese hamster ovary (CHO) cells transfected with CD14 and/or TLR2 or TLR4 have revealed that, at least in this system, viable M. tuberculosis induces intracellular signaling via both TLR2 and TLR4 by a mechanism that does not require CD14 or LBP (31). In addition, a blocking anti-LAM monoclonal antibody was incapable of preventing M. tuberculosis induced activation of CHO cells via TLR2 or TLR4, suggesting that LAM is not involved in TLR-dependent intracellular signaling by M. tuberculosis (31). Together with our current results, these data indicate that LBP is not part of the in vivo mechanism by which mycobacteria are presented to cells involved in innate immunity, most likely because LAM (either AraLAM or ManLAM) is not important for this process.

We found discrete differences between the immune response of LBP-/- and WT mice early after infection with M. tuberculosis, which included a reduced influx of lymphocytes to the lungs and a diminished activation of CD4+ and CD8+ T cells. Apparently, these alterations were not biologically significant enough to impact on antibacterial defense, although at 6 weeks LBP-/- mice tended to have slightly more M. tuberculosis CFU in their lungs than WT mice. We do not have a definitive explanation for these findings considering that LBP is unlikely to have a direct influence on lymphocyte functions. Our finding of increased neutrophil numbers in infected lungs of LBP-/- mice suggests that the relative lack of lymphocytes at the site of inflammation may lead to an enhanced neutrophil migration in a LBP independent compensatory response. Indeed, we recently observed a similarly increased neutrophil influx in lungs of CD44-/- mice with a deficient lymphocyte recruitment after infection with M. tuberculosis (41). The increased neutrophil content, together with the modestly higher mycobacterial load, may have contributed to the elevated levels of TNF and IL-6 in lungs of LBP-/- mice at 6 weeks post-infection. Of note, neutrophils have been found to produce TNF in response to viable M. tuberculosis bacilli in vitro (50).
Although there is abundant evidence that *M. tuberculosis* and mycobacterial antigens can be recognized by TLRs *in vitro* (for review see (51)), recent studies on the role of TLR4 and TLR2 in the protective immune response to *M. tuberculosis* *in vivo* have yielded variable results. TLR4 mutant mice were reported to have a reduced capacity to eliminate mycobacteria from their lungs in a model of pulmonary tuberculosis induced by aerosol infection, resulting in 100% mortality after 30 weeks (i.e. an observation period similar to that in the present study) (52). However, in other studies TLR4 mutant mice demonstrated an unaltered antibacterial defense and survival after aerosol challenge with *M. tuberculosis* (53, 54). TLR2-/- mice had a reduced survival only after a high-dose aerosol challenge with *M. tuberculosis*, which also caused 100% mortality in normal C57BL/6 WT mice within 30 weeks, whereas TLR2 did not contribute to antibacterial defense after low dose *M. tuberculosis* infection (53). In yet another investigation, TLR2-/- mice were found to have an only slightly and transiently impaired defense against airborne infection with the Kurono strain of *M. tuberculosis* (55). CD14-/- mice displayed an unimpaired resistance against *M. tuberculosis* infection, although the follow up in this study was relatively short (14 weeks) (53). Together with our study these data indicate that if TLR4 or TLR2 contribute to host defense against lung tuberculosis their role likely does not depend on LBP.

LBP is of eminent importance for the recognition of LPS by the host and thereby for the induction of an adequate innate immune response to Gram-negative bacteria. Although LAM shares many properties with LPS, and can interact with LBP *in vitro*, we here show that endogenous LBP is not important for host defense against either virulent *M. tuberculosis* or avirulent *M. smegmatis*.

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References


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