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The Essential Role of Lipopolysaccharide-Binding Protein in Protection of Mice Against a Peritoneal Salmonella Infection Involves the Rapid Induction of an Inflammatory Response

Jan-Michael Heinrich,* Martin Bernheiden,* Gabriela Minigo,* Kang Kang Yang,* Christine Schütt,* Daniela N. Männel,† and Robert S. Jack 2*

Acute and chronic hyperinflammation are of major clinical concern, and many treatment strategies are therefore directed to inactivating parts of the inflammatory system. However, survival depends on responding quickly to pathogen attack, and since the adaptive immune system requires several days to adequately react, we rely initially on a range of innate defenses, many of which operate by activating parts of the inflammatory network. For example, LPS-binding protein (LBP) can transfer the LPS of Gram-negative bacteria to CD14 on the surface of macrophages, and this initiates an inflammatory reaction. However, the importance of this chain of events in infection is unclear. First, the innate system is redundant, and bacteria have many components that may serve as targets for it. Second, LBP can transfer LPS to other acceptors that do not induce inflammation. In this study, we show that innate defense against a lethal peritoneal infection with Salmonella requires a direct proinflammatory involvement of LBP, and that this is a major nonredundant function of LBP in this infection model. This emphasizes that blocking the LBP-initiated inflammatory cascade disables an essential defense pathway. Any anti-inflammatory protection that may be achieved must be balanced against the risks inherent in blinding the innate system to the presence of Gram-negative pathogens. The Journal of Immunology, 2001, 167: 1624–1628.

A bacterial infection is held in check over the first few days by a complex array of innate defense mechanisms. These are essential to provide a breathing space within which the time-consuming processes of clonal selection and expansion can generate a specific immune response. The innate system relies on receptors directed against structures commonly found on microorganisms, but absent from our own cells (1). The interactions of these receptors with their ligands provides a means of both detecting an infection and targeting the response to the pathogen. Receptors that recognize the outer membrane glycolipid LPS are believed to play a major role in innate defense against Gram-negative bacteria (2). The major high-affinity cellular receptor for LPS is the CD14 molecule (3), which is expressed principally on monocytes, macrophages, and, to a lesser extent, granulocytes. The CD14 molecule is bound to the surface of the cell via a GPI anchor, and since it has no cytoplasmic domain, it is incapable of transmitting a signal into the cell (4). The signal transduction across the membrane involves the Toll-like receptor 4 membrane protein (5, 6), although the precise details of the interactions among LPS, CD14, and Toll-like receptor 4 remain to be elucidated.

LPS forms micelles in aqueous solution, and the concentration of the biologically active monomer form is extremely low (7). Because of this, the interaction between LPS and CD14 is slow unless catalyzed by the serum protein LPS-binding protein (LBP) (8–10). In vitro experiments have established a number of additional possible roles for LBP. Under appropriate conditions it can transfer LPS not only to CD14, but also to high density lipoprotein (11, 12) and can take part in transfer reactions involving a number of phospholipids and membrane structures (13, 14). These interactions between LBP, LPS, lipoproteins, and cells in vitro are complex (15), but their relevance to LBP function in vivo is uncertain. In vivo, two functions for LBP have been established in mice. First, it is required for the release of proinflammatory mediators such as TNF (16) in response to a challenge with purified bacterial LPS. These mediators initiate a pleiotropic cascade of events, including activation of the coagulation system and recruitment of phagocytes (17). Second, LBP is an acute phase protein, and in high concentrations it has an anti-inflammatory effect that is thought to be due to LPS sequestration (18).

Given this plethora of suggested functions we sought to narrow the in vivo role of LBP during the course of an infection by tracing the initial steps that require LBP for the control of a Gram-negative pathogen in mice. For this purpose we have generated an LBP-deficient mouse line (LBP<sup>−/−</sup>) and shown that these animals are defective in their ability to mount an inflammatory response, as judged by the serum titer of TNF when challenged with purified LPS (16). To investigate the response of these animals to a pathogen we have used a peritoneal infection with Salmonella. In this model system a lethal infection can be initiated with a very low number of bacteria.

Salmonella, like all pathogens, must solve problems at three quite different levels. First, it must cross the physical barriers that

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constitute our first line of defense. Having done this it must survive whatever the innate defense system undertakes against it, and finally, any survivors must face up to the specific immune system (19). There are complex interactions taking place at each of these three levels. However, since our interest centers on the role of the innate defense system, we avoid the complexities associated with the breaching of the physical barriers by injecting the Salmonella directly into the peritoneum and ignore the contribution from the specific immune system by concentrating on early events in the infection.

To determine the role of LBP in this infection, we have made use of mice in which LBP function has been specifically ablated by gene targeting. We showed that the LBP-deficient animals are incapable of controlling the Salmonella infection and succumb to a dose of the pathogen that their heterozygous littermate survives. Here we show that the inability of these mice to control an i.p. Salmonella infection is a direct result of the loss of LBP function by demonstrating rescue of the animals by supplementing them with recombinant mouse LBP. We then show that in wild-type mice the infection results in the rapid elaboration of an inflammatory response, as evidenced by the release of detectable levels of IL-6 within the first hours of infection, and that this inflammatory response is deficient in LBP−/− animals. Finally, we show that the requirement for LBP in these animals can be replaced by injection of the principle proinflammatory mediator TNF. The ability to substitute TNF for LBP indicates that a major nonredundant role for LBP in this infection model involves the generation of an inflammatory response.

Materials and Methods

Reagents

Recombinant murine TNF was purchased from Life Technologies (Karlsruhe, Germany), and recombinant human TNF was obtained from BASF Bioresearch (Ludwigshaven, Germany). Histidine-tagged recombinant murine LBP was prepared as previously described (20) from supernatants of transfected Chinese hamster ovary cells by metal chelate chromatography and stored frozen in PBS at −20°C until required. Samples for injection were diluted in pyrogen-free PBS (Sigma, Düsseldorf, Germany).

Mice

BALB/c and CBA/J mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). The generation of LBP-deficient mice on a strain 129 background has been previously described (16). The animals used in the experiments described here are progeny of LBP−/− heterozygotes obtained from a second backcross generation of the LBP−/− animals to strain CBA. All of the animals carried the resistance allele of the NRAMP1 gene (21).

Genotypic characterization

Genotypes were determined by PCR analysis of genomic DNA prepared from the tail tip. Tail tips were digested overnight with 100 μg/ml proteinase K (Roche, Mannheim, Germany) in 100 mM Tris (pH 8.5), 200 mM EDTA, 5 mM DTT, and 0.02% SDS. The digest was extracted three times with phenol, precipitated with an equal volume of isopropanol, and redissolved in 100 μl of TE buffer (10 mM Tris (pH 8.0) and 1 mM EDTA). LBP−/− and LBP+/+ animals were distinguished by PCR. The LBP−/− NRAMP−/− progeny were identified by PCR using DNA prepared from the tail tip. The LBP genotype was determined using the LBP-specific primers 5′-GGGCCCAAGGAGGGCCTGG-3′ and 5′-CCAGTACCTTGAGGAGGCTG-3′ and the neoR primers 5′-TGCACCAGAAAGGCCTCT-3′ and 5′-GCCACGAGCACTCTTTCA-3′. This PCR yields a fragment of 266 bp, which is readily distinguished from the 285-bp fragment from the null allele.

ELISA determinations

ELISAs for murine IL-6 and murine TNF-α were purchased from BD PharMingen (San Diego, CA). The ELISA for mouse LBP was obtained from Biometec (Greifswald, Germany).

Salmonella infection

Salmonella enterica serotype typhimurium (ATCC 14028s, American Type Culture Collection, Manassas, VA; Salmonella typhimurium) transfected with the plasmid pFGV251.1 coding for green fluorescent protein was a gift from Dr. M. Hensel (Max von Pettenkofer Institut, Munich, Germany). A single colony was picked and grown overnight in Trypто-C Yeast Extract broth containing 100 μg/ml ampicillin. The overnight culture was diluted 1/100 into fresh Trypто-C Yeast Extract without antibiotics and grown at 37°C with vigorous shaking for 2.5 h. Cells were collected by centrifugation and were washed in PBS. The number of bacteria was estimated from the OD at 550 nm by reference to a standard curve. The actual number of CFU used in each experiment was determined by plating samples of the injected suspension. For determination of TNF and IL-6, animals were bled 2 h after infection, and serum was stored at −80°C before analysis by ELISA.

Determination of bacterial load in peritoneum and lung early in infection

Mice were infected with Salmonella and 24 h later were sacrificed by cervical dislocation, and the peritoneal cavity was washed with 10 ml of cold sterile PBS. Appropriate dilutions of the peritoneal wash were plated in duplicate. Plates were incubated for 16 h at 37°C, and colonies were counted. For determination of the bacterial load in the lungs, animals were infected for 48 h, then sacrificed, and the lungs were homogenized in 2 ml of sterile PBS containing 0.2% saponin. Lung homogenates were incubated on ice for 30 min, and suitable dilutions of this extract were plated and counted as described for the peritoneal washes.

Reconstitution of LBP−/− mice with recombinant LBP

LBP−/− mice were injected i.p. with 20 μg of recombinant murine LBP in a final volume of 200 μl sterile pyrogen-free PBS. After 1, 2, 4, and 6 h, groups of three mice were anesthetized with diethyl ether and bled from the orbital complex. The animals were then sacrificed by cervical dislocation, and the peritoneal cavity was washed with 10 ml of cold sterile PBS. The PBS was centrifuged at 1500 × g to remove cells. Serum and cleared peritoneal wash were assayed by ELISA for LBP content. Peritoneal wash data are expressed as total LBP recovered from the peritoneum. LBP levels in serum are expressed as nanograms of LBP per milliliter of serum.

Substitution with recombinant mouse or human TNF

Animals were injected with recombinant mouse TNF (Life Technologies) or with clinical trial grade recombinant human TNF (gift from BASF Bioresearch). TNF (800 ng) was injected i.p. along with the pathogen. In this case the defect in the LBP−/− animals can be rapidly reversed by supplementing them with recombinant murine LBP during the early part of the infection. Alternatively, LBP may play a direct and essential role in the interactions between the host and the pathogen. In this case the defect in the LBP−/− animals will be rapidly reversed by supplementing them with recombinant murine LBP during the early part of the infection.

Results and Discussion

As previously reported, LBP−/− animals are susceptible to an infection with a dose of S. typhimurium that produces no evidence of disease in their heterozygous littermates (16). The inability of LBP−/− animals to adequately cope with an i.p. Salmonella infection may be due to either of two mechanisms. First, LBP may play a direct and essential role in the interactions between the host and the pathogen. In this case the defect in the LBP−/− animals will be rapidly reversed by supplementing them with recombinant murine LBP during the early part of the infection. Alternatively, LBP may play no direct role in this infection model, but, rather, may be essential for the proper development of the innate defense system. If the architecture of the innate defense system developing in animals entirely devoid of LBP were to be seriously deformed, then the system might no longer be able to adequately respond to the pathogen. In this case the defect would not be able to be rapidly complemented by supplementing adult LBP−/− mice with recombinant murine LBP.
Fate of recombinant murine LBP in vivo

We first determined the fate of recombinant LBP injected i.p. into LBP<sup>−/−</sup> animals. Groups of LBP<sup>−/−</sup> mice were injected i.p. with 20 µg of recombinant murine LBP. One, 2, 4, and 6 h later animals were bled, then sacrificed by cervical dislocation, and the peritoneum was washed with PBS. The amount of LBP present in serum and peritoneal wash was determined by ELISA. Despite considerable individual variation in the titers it is clear that the injected recombinant LBP is quite rapidly lost from the peritoneum, with a half-life of ~1 h (Fig. 1a). Concomitantly LBP starts to appear in the circulation and reaches a maximal concentration of about 5 µg/ml at 2 h, after which it decays with a half-life of ~1 h, reaching a value of about 0.3 µg/ml at 6 h (Fig. 1b). For comparison, LBP is normally present in serum at a concentration of 1 µg/ml and rises to 5 µg/ml during an acute phase response (22). These data indicate that the reconstitution that we achieve is modest, and it is unlikely that a significant concentration of the injected LBP is available for more than a brief part of the 7–12 days covered by the lethal infection.

Rescue of the LBP<sup>−/−</sup> phenotype by supplementation with recombinant mouse LBP

To determine whether the injected LBP is able to rescue LBP-deficient animals from a lethal Salmonella infection, we infected the mice i.p. with 50 CFU of <i>S. typhimurium</i> and at the same time injected 2 or 20 µg of recombinant murine LBP. Control animals given Salmonella alone died, while those supplemented with LBP showed a dose-dependent rescue (Fig. 2). Given the brief half-life of the recombinant protein in vivo (Fig. 1), the protection given by the recombinant LBP indicates that one crucial LBP-dependent step in the host response to the pathogen takes place within the first few hours of the infection. The outcome of this LBP-dependent process determines whether the animal will succumb to the infection several days later.

<i>LBP is required to induce an inflammatory response in response to Salmonella infection</i>

Our LBP-deficient animals fail to respond to a bolus injection of LPS by releasing TNF. In contrast, a second LBP knockout that was constructed using an alternative strategy does respond to LPS with the production of TNF (23). The reason for this discrepancy between the two knockout strains is unclear. One possibility is differences in the LPS preparation used. A second possible explanation lies in differences between the two knockout strains. The LBP-deficient mouse of Wurfel et al. (11) does produce an LBP message, and although LBP is not detectable in the blood, the authors speculate that low levels of a functional truncated form of the protein may be sequestered elsewhere, perhaps in the liver. In the case of our mice, in which removal of an internal exon causes a frame shift, the capacity to respond to LPS with the production of TNF is completely abolished (16). To determine whether our LBP<sup>−/−</sup> mice failed to initiate an inflammatory response on infection with <i>S. typhimurium</i> we measured the serum concentrations of the proinflammatory cytokines TNF and IL-6 following infection. TNF, which is released from activated macrophages, is clearly involved in innate resistance to <i>S. typhimurium</i>, since the TNFR1-deficient mice are highly susceptible to this infection (24). However, this cytokine, which has a very short half-life in serum, is not present at detectable levels in the serum of Salmonella-infected mice. In contrast, the more stable proinflammatory cytokine IL-6, which, like TNF, is produced by activated macrophages, is readily detected in serum 2 h after initiating the infection. Individual mice show a broad scatter in the titer, but all animals carrying a wild-type allele of <i>LBP</i> have readily detectable IL-6 in their serum 2 h after infection. The scatter in the IL-6 values is unlikely to be due to variability in the genetic background of the individual animals, since pure-bred BALB/c mice show a similar individual diversity of response. Despite the diversity of the response it is clear that all animals tested to date that carry at least one wild-type <i>LBP</i> allele produce readily detectable levels of IL-6 early in a Salmonella infection. In contrast, the <i>LBP<sup>−/−</sup></i> animals produce no detectable IL-6 (Table I). By this criterion they are deficient in their ability to mount a rapid inflammatory response with the concomitant release of proinflammatory mediators. This inability to mount an effective inflammatory response is the earliest defect we have observed in this infection model. It is important to note that the injection of

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Table I. IL-6 concentration (pg/ml) in sera of individual NRAMP<sup>R/R</sup> LBP<sup>−/−</sup>, NRAMP<sup>R/R</sup>LBP<sup>−/−</sup>, and NRAMP<sup>S/S</sup>LBP<sup>−/−</sup> BALB/c mice 2 h after i.p. infection with 10<sup>4</sup> CFU of <i>S. typhimurium</i>
LBP (20 µg/mouse i.p.) does not in itself cause an inflammatory response (Table II). This shows that the LBP-dependent rescue from the lethality caused by the Salmonella infection documented in Fig. 2 is indeed dependent on a proinflammatory LBP function that is triggered by the bacterial infection.

**Bacterial load in the peritoneum and spreading to the lung early in the infection**

In the early stages of this Salmonella infection, the bacteria first multiply in the peritoneum and then spread out to peripheral organs, including liver, spleen, and lungs. LBP−/− mice on a strain 129 background show an increased rate of growth of Salmonella in the peritoneum compared with their heterozygous littermates (16). This reduced capacity to control the rate of bacterial growth is also evident in LBP−/− animals on a CBA background. Animals infected with 50 CFU Salmonella i.p. have a greater bacterial burden in the peritoneum (Fig. 3a). In addition, we have compared the rate at which the bacteria spread from the peritoneum to the lungs in LBP−/− animals and their LBP+/− littermates. As shown in Fig. 3b, infection of LBP−/− animals i.p. with 50 CFU results in substantially greater bacterial load in the lungs than is evident in their LBP+/− litter mates. Thus, the phenotype of Salmonella-infected LBP-deficient animals includes a decreased inflammatory response 2 h after infection, an increased bacterial load in the peritoneum and lungs at 48 h, and lethality evident 5–7 days after infection.

**Rescue of LBP−/− animals with TNF**

To determine whether the sensitivity of LBP-deficient mice to Salmonella is causally related to their inability to mount an early inflammatory response, we attempted to bypass the requirement for LBP by supplementing the LBP-deficient animals early in the infection with the proinflammatory mediator TNF. LBP-deficient animals infected with 50 CFU of S. typhimurium died within the first week. However, animals that were supplemented with exogenous recombinant murine TNF along with the Salmonella survived (Fig. 4). There are two murine receptors for TNF, both of which bind murine TNF with high affinity. However, only the 55-kDa TNFR1 binds human TNF (25). We therefore repeated the TNF supplementation experiment using recombinant human TNF. As shown in Fig. 5b, recombinant human TNF was also able to rescue these mice, and hence an initial interaction requiring only TNFR1 is sufficient to reverse the lethality associated with lack of LBP. These results show that the requirement for LBP can be entirely replaced by exogenously added TNF.

The fact that the LBP-deficient animals can be rescued by TNF suggests that the induction of proinflammatory mediator production is a major nonredundant function of LBP in this innate defense system. However, this conclusion is only valid if the dose of TNF injected at the start of the infection does not override the normal mechanisms of innate immunity, perhaps through a direct antibacterial action. To rule this out, we made use of strain BALB/c mice, which carry a wild-type allele of the LBP gene and are well able to mount an inflammatory response on infection with S. typhimurium (Table I). They are nevertheless sensitive to the infection and die within 5–7 days because of a mutation in the NRAMP-1 gene. NRAMP-1 encodes a protein that is expressed in macrophages in the endosomal compartment (21, 26). This protein is required to pump divalent metal ions out of the endosome, thus creating an environment hostile to the pathogen’s survival (27). NRAMP 1 is an essential component of the innate defense system and is required so that mice can control the growth and survival of S. typhimurium, which enter host macrophages early in the infection and hide out in the endosome compartment.

![FIGURE 3.](image3.png) **FIGURE 3.** Bacterial growth and spreading in LBP−/− and LBP+/− animals. a, Bacterial growth in the peritoneum of LBP−/− and LBP+/− mice. Groups of five animals were infected with 50 CFU, and 72 h later the peritoneum was washed and bacteria were counted by plating. Values shown are for individual mice. b, Bacterial load in lungs of LBP−/− and LBP+/− mice 48 h after infection with 50 CFU.

![FIGURE 4.](image4.png) **FIGURE 4.** Survival of mice infected with S. typhimurium. Two groups of six LBP−/− animals were infected with 70 CFU of Salmonella. One group (●) was supplemented with 1.6 µg of murine recombinant TNF, whereas the other (■) was not.

![FIGURE 5.](image5.png) **FIGURE 5.** Groups of BALB/c (a) or LBP−/− (b) were infected with 175 CFU of Salmonella. In each case one group (●) was supplemented with 1.6 µg of murine recombinant TNF, whereas the other (■) was not.

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<tr>
<td>200 µl PBS + 20 µg LBP</td>
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<tr>
<td>200 µl PBS + 20 µg LBP + 700 CFU S. typhimurium</td>
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Table II. IL-6 concentration in sera of individual BALB/c mice 2 h after i.p. injection with PBS alone, PBS containing 20 µg of LBP or PBS/LBP plus 700 CFU of S. typhimurium.
ROLE OF LBP IN AN i.p. INFECTION

Two groups of BALB/c mice were injected with 50 CFU of S. typhimurium, and one group was treated with recombinant murine TNF as described for the LBP−/− mice. As shown in Fig. 5a, the susceptibility of these animals to Salmonella is not alleviated by this treatment. Exogenous TNF thus protects the LBP−/− animals, which have a demonstrated defect in their ability to produce an inflammatory response (Fig. 1), but fails to protect animals that are sensitive to the infection due to a defect in a different part of the innate defense system. This rules out the possibility that the injected TNF rescues the LBP-deficient animals by a nonspecific direct bactericidal mechanism unrelated to normal LBP function.

Although numerous studies have shown that an interaction of LPS with LBP leads to macrophage activation and the induction of an inflammatory reaction, it has been unclear how important this pathway may be in combating an infection in vivo. In innate defense is both multifaceted and redundant, and Gram-negative bacteria such as Salmonella contain many components other than LPS, some of which contribute to cell activation (28). Our data make it clear that the LBP-initiated pathway is essential for defense, that it is required for the induction of an inflammatory reaction, and that this is a major nonredundant function of LBP in this Gram-negative peritoneal infection.

The control of acute and chronic inflammatory disorders is currently a major problem in clinical medicine (29, 30), and much effort has been invested in developing therapeutic strategies that block appropriate parts of the inflammatory network. Our results emphasize that any procedure that blocks the LBP-initiated inflammatory cascade will disable an essential defense pathway. Any anti-inflammatory protection that may be achieved must be balanced against the risks inherent in blinding the innate system to the presence of Gram-negative pathogens.

References


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