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Critical Role of Lipopolysaccharide-Binding Protein and CD14 in Immune Responses against Gram-Negative Bacteria

Didier Le Roy,* Franco Di Padova,† Yoshiyuki Adachi,‡ Michel Pierre Glauser,* Thierry Calandra,* and Didier Heumann‡‡

LPS-binding protein (LBP) and CD14 potentiate cell activation by LPS, contributing to lethal endotoxemia. We analyzed the contribution of LBP/CD14 in models of bacterial infection. Mice pretreated with mAbs neutralizing CD14 or LBP showed a delay in TNF-α production and died of overwhelming infection within 24 h, after a challenge with 250 CFU of virulent Klebsiella pneumoniae. Blockade of TNF-α also increased lethality, whereas pretreatment with TNF-α protected mice, even in the presence of LBP and CD14 blockade. Anti-LBP or anti-CD14 mAbs did not improve or decrease lethality with a higher inoculum (10⁶ K. pneumoniae) and did not affect outcome following injections of low or high inocula of Escherichia coli O111. These results point to the essential role of LBP/CD14 in innate immunity against virulent bacteria. The Journal of Immunology, 2001, 167: 2759 – 2765.

Plasma LPS-binding protein (LBP)³ and monocyte CD14 are central molecules of the innate immune system, in response to LPS. Due to its essential role in the mediation of the presentation of LPS to CD14 (1, 2), LBP contributes to enhance LPS-induced lethality, as shown in animals with a LBP-disrupted gene (3) or in wild-type mice treated with anti-LBP Abs (4–6). Similarly, mice with a CD14-disrupted gene were resistant to LPS-induced toxicity (7). Abs to CD14 provided therapeutic benefit after in vivo exposure to endotoxin in rabbits and primates (8, 9).

The role of LBP and CD14 in bacterial infections is not clearly defined. LBP and CD14 have been postulated to be a prerequisite in the mechanisms involved in initiation of host defense against Gram-negative bacteria, alarming the host to the presence of minute amounts of LPS (1). In favor of this hypothesis, LBP⁻/⁻ mice, although resistant to LPS, were susceptible to Salmonella typhimurium (3), and the intestinal mucosa of rabbits treated with a neutralizing anti-CD14 mAb exhibited a 50-fold increase in Shigella invasion and more severe injury compared with controls (10).

The role of excess of proinflammatory cytokines in pathogenic events triggered by systemic injections of LPS or high numbers of bacteria has been well documented (11, 12). Yet, in the presence of a low inoculum of bacteria, endogenous production of cytokines and of TNF-α appears pivotal for normal innate immune responses against an invading organism. Ab-mediated blockade of TNF-α or disruption of the TNF-α gene or of the TNFp55 receptor gene was detrimental in models of intracellular facultative bacteria (13–16).

This was also shown for extracellular bacteria, in models of cecal ligation puncture (17), of pneumonia (18–20), or of peritonitis (21). Conversely, treatment with TNF-α improved survival of mice injected with S. typhimurium or undergoing cecal ligation puncture (22, 23). Importantly, LPS-hyporesponsive C3H/HeJ mice were found to be more susceptible to lethal infection with Escherichia coli than normal LPS-responsive mice, a defect that was corrected by administration of TNF-α (24).

Thus a body of data emphasizes the need for an intact mechanism of recognition of LPS leading to cytokine production in initiating host defense mechanisms against Gram-negative infections. In the present study, we investigated the contribution of LBP, CD14, and TNF-α in bacterial sepsis. We hypothesized that blocking of the innate immune responses with Abs to LBP, CD14, or TNF-α may impair the development of normal innate responses to low doses of bacteria, and thus augment lethality.

Materials and Methods

Bacteria

An encapsulated strain of Klebsiella pneumoniae isolated from a bacteremic patient was used in the present experiments (25). This strain was selected among other Klebsiella strains for its high virulence in mice. Bacteria were grown for 2 h in tryptic soy broth (Difco, Detroit, MI) and collected in the exponential growth phase at an OD of 0.18, corresponding to 5.5 × 10⁷ ± 0.15 CFU/ml. Bacteria were then diluted to the desired concentration in saline before injection into mice. E. coli O111:B4 was cultivated as described (26).

Reagents and Abs

Two rat mAbs to mouse LBP described in (6) were studied: 1) the neutralizing mAb (clone M330-9, referred thereafter as to anti-LBP mAb), preventing the binding of LPS to LBP, suppressing LPS-induced TNF-α production and blocking LBP activity in vivo up to 7 h after injection in mice; and 2) the control anti-LBP mAb (clone M306-5, referred thereafter as to control mAb), which does not neutralize LBP activity. 4C1 is a rat mAb that neutralizes mouse CD14 (27). MAb were purified by protein G chromatography, dialyzed into PBS, and stored at ~80°C. Anti-TNF Abs were raised in rabbits and polyclonal IgG from immunized rabbits or control rabbits isolated by protein G chromatography. Recombinant murine TNF-α was a gift from G. Grau (University of Marseille, Marseille, France). Presence of LPS in reagents was determined with the Limulus assay (Chromogenix, Embrach, Switzerland). The LPS content of the mAbs and of the IgG was 1 pg/µg of protein.

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This work was supported by Swiss National Science Foundation Grants 3200-055529.98/1 (to D.H.) and 32-49129.96 (to T.C.). T.C. is a recipient of a career award from the Swiss National Foundation (32-48916-96). MAbs were purified by protein G chromatography. Recombinant murine TNF-α was a gift from G. Grau (University of Marseille, Marseille, France). Presence of LPS in reagents was determined with the Limulus assay (Chromogenix, Embrach, Switzerland). The LPS content of the mAbs and of the IgG was 1 pg/µg of protein.
Galactosamine model

Because 4C1 Ab has never been evaluated in vivo, we assessed whether 4C1 prevented endotoxemia in mice sensitized with α-galactosamine. Mice were injected i.v. with 200 μg of the neutralizing anti-CD14 mAb 4C1 2 h before an i.p. challenge of 50 ng of E. coli O111 LPS (Sigma, St. Louis, MO) given in combination with 20 mg α-galactosamine (Sigma). Mice were bled 1.5 h after LPS challenge to measure plasma TNF-α concentrations. The injection of 4C1 reduced TNF-α production, and prevented death (0 deaths/5 mice) compared with injection of saline (5/5) (data not shown). Similarly to neutralizing anti-LBP mAb, anti-CD14 mAb was effective at blocking LPS-induced cell activation and death for 5–7 h (data not shown).

Bacterial challenge

OF1 female mice, 5– to 6-wk old, were purchased from IFFA Credo (Lyon, France). OF1 mice were injected i.v. with saline or with 100 μg/mouse of rat mAbs in a volume of 250 μl of saline, and with K. pneumoniae or E. coli suspended in 250 μl saline. Plasma was obtained via the tail vein to determine plasma TNF-α concentrations, bacterial counts, and neutrophil counts. To avoid excess bleeding, most of the mice were bled only once (50–μl aliquots), occasionally twice.

TNF bioassay

TNF-α was measured in plasma by bioassay using WEHI clone 13 as targets, as previously described (28).

Neutrophil count determination

Türck Blue (Sigma) was added to whole blood samples, and the total number of white blood cell counts was determined by microscopy. For neutrophil count determination, plasma samples were lysed on ice with 0.1 M NH₄Cl/0.1 M KHCO₃ and cytocentrifuged. The percentage of polymorphonuclear neutrophils (PMN) was determined with May-Grünwald-Giemsa staining. The number of PMN per milliliter of blood was determined taking into account the total number of white blood cells and the percentage of neutrophils.

Statistics

The χ² test was used to assess the significance of differences between treatment groups. The ANOVA test on ranks was used to assess the significance of intergroup differences for the various markers (bacteria, PMN cell, TNF-α).

Results

LBP and CD14 blockade is associated with delayed production of TNF-α in mice injected i.v. with a low inoculum of K. pneumoniae

In preliminary experiments, the LD₅₀ for K. pneumoniae was found to be between 50 and 250 CFU/mouse. We thus used this inoculum to investigate the role of LBP and CD14 in the early steps of infection leading to death.

To investigate the effect of LBP activity blockade on TNF-α production induced by bacterial sepsis, mice were injected with anti-LBP mAbs before a challenge with <250 CFU of K. pneumoniae (Fig. 1). Bioactive TNF-α was not detectable in the blood within the first 5 h, irrespective of treatment (control or anti-LBP mAb). Five hours after infection, TNF-α levels were 0.8 ± 0.4 ng/ml in mice treated with control mAb, and undetectable in mice treated with anti-LBP mAb. There was a 1- to 2-h delay in TNF-α production in mice treated with anti-LBP mAb compared with mice treated with control mAb. Six hours after infection, TNF-α levels were 1 ± 0.5 ng/ml in mice treated with control mAb, and 0.2 ± 0.1 ng/ml in mice treated with anti-LBP mAb. Yet, 7 h after infection, TNF-α levels were higher thereafter in mice treated with anti-LBP mAb.

A similar delay in TNF-α production was obtained in mice treated with anti-CD14 mAb (Fig. 1). Yet, TNF-α levels were still higher in mice treated with anti-CD14 mAb than in mice treated with anti-LBP mAb, 7 h after infection.

LBP and CD14 blockade is associated with impairment of neutrophil recruitment in mice injected i.v. with a low inoculum of K. pneumoniae

In the early hours after infection with K. pneumoniae, neutrophil counts doubled in the first 30 min, irrespective of treatment (Fig. 2). In mice treated with control mAb, neutrophil counts continued to increase to a 4-fold level over basal levels, peaking at 1.5 h, then progressively decreasing to basal levels at 6 h. In contrast, animals treated with anti-LBP mAb did not show any increase of neutrophils from 30 min to 6 h. The same observation was made during the first 3 h after infection, in mice treated with anti-CD14 mAb. Yet, in those mice, numbers of bacteria were similar to controls from 3 to 6 h.

LBP and of CD14 blockade is associated with uncontrolled bacterial proliferation in mice injected i.v. with a low inoculum of K. pneumoniae

Death was associated with elevated bacteremia, and moribund mice had bacterial counts higher than 10⁷ CFU/ml of blood (Fig. 3). Kinetics of bacterial counts were similar in mice receiving saline or control mAb for the first 24 h after bacterial challenge. Fig. 3A illustrates the kinetics of bacterial counts in mice treated with control mAb. Blood bacterial counts increased in survivors and nonsurvivors from 100 to 250 CFU/ml to ~10⁴ CFU/ml during the first 12 h, then plateaued until 48 h, with slightly higher numbers...
containing/H11021 with control mAb had detectable levels of blood TNF-
/H9251 numbers of bacteria (Fig. 4). TNF-
/H9251 with a low inoculum of
/K. pneumoniae mice treated with anti-LBP or anti-CD14 mAb and injected i.v.

We next analyzed whether blood TNF-
/H9251 levels were related to numbers of bacteria (103 vs 10 5 CFU/ml,

Effect of anti-LBP and anti-CD14 mAbs on the blood bacterial counts of mice injected i.v. with a low inoculum (from 60 to 260 CFU/mouse) of
/K. pneumoniae. Mice were injected with 100 µg/mouse of control mAb (A, Survivors, ○; nonsurvivors, ●), anti-LBP mAb (B), or anti-CD14 mAb (C) 5 min before i.v. infection. Data are the mean of five different experiments (42 mice/group). *, p < 0.05, by ANOVA compared to CFU of survivors receiving the control mAb.

in nonsurvivors than in survivors. From day 3, bacteria were cleared from the circulation in survivors, whereas blood bacterial counts progressively increased until death in nonsurvivors. Fig. 3B shows the kinetics of blood bacterial counts in mice injected with anti-LBP mAb (nonsurvivors). Blood bacterial counts were similar (no statistically significant difference) to those measured in survivors or nonsurvivors receiving control mAb for the first 6 h. However, between 7 and 12 h, mice that were moribund all had increased numbers of circulating bacteria than survivors found either in this group of mice or in the group of mice receiving saline or control mAb. Under this condition, bacterial counts remained elevated until death. Kinetics of blood bacterial counts was even more accelerated when mice were treated with anti-CD14 mAb (Fig. 3C, nonsurvivors). All mice had similar numbers of bacteria until 4 h (survivors and nonsurvivors). However, 7 h after bacterial challenge, bacterial numbers were at least 2 logs higher than those of controls (10 3 vs 10 5 CFU/ml, p < 0.0001). Bacterial numbers corresponding to those observed in moribund mice were found in all mice, 14 h after challenge.

Correlation between blood bacterial counts and TNF-α levels in mice treated with anti-LBP or anti-CD14 mAb and injected i.v. with a low inoculum of
/K. pneumoniae

We next analyzed whether blood TNF-α levels were related to numbers of bacteria (Fig. 4). TNF-α was not detected in samples containing <10 4 CFU/ml, irrespective of treatment. Mice treated with control mAb had detectable levels of blood TNF-α, from 60 to 10,000 pg/ml, irrespective of the number of circulating bacteria detected in these samples (from 10 2 to 10 6 CFU/ml). Yet, in mice treated with anti-LBP mAb (or anti-CD14 mAb, data not shown), TNF-α was not detectable or barely detectable in samples in which bacterial counts were <10 5 CFU/ml. However, when blood bacterial counts exceeded 5 × 10 5 CFU/ml, blood TNF-α levels were higher than those found in animals receiving control mAb (p < 0.001). Thus, it appeared that LBP or CD14 blockade induced a delay in early TNF-α production that was associated both with time and number of bacteria (<10 5 CFU/ml). However, as soon as bacteria reached 5 × 10 5 CFU/ml in the blood, TNF-α was produced in higher amounts in mice treated with anti-LBP or anti-CD14 mAb, suggesting LBP- and CD14-independent mechanisms of TNF-α production at high bacterial loads.

Effect of LBP and of CD14 blockade on survival of mice injected i.v. with a low inoculum of
/K. pneumoniae

The Klebsiella strain was very virulent, because 50% in 5 days in control mice receiving saline (Fig. 5). Injection with control mAb had no effect on survival rates. In contrast, injection of anti-LBP or anti-CD14 mAb increased lethality, with a striking increase during the first 24 h.

Ab-mediated blockade of LBP and CD14 could be delayed up to 6 h after the onset of infection, leading to accelerated death rates over controls, indicating that LBP and CD14 are necessary over a sustained period of time to trigger host defense mechanisms (Table I). In most mice, TNF-α measured at 6 h after infection was not detected; TNF-α was only detectable in mice presenting bacterial counts 10 5 CFU/ml at 6 h (data not shown).

Effect of anti-LBP and anti-CD14 mAbs on the survival of mice injected i.v. with a low inoculum (from 60 to 260 CFU/mouse) of
/K. pneumoniae. Mice were injected with saline (○), with 100 µg/mouse of control mAb (●), anti-LBP mAb (■), or anti-CD14 mAb (□) 5 min before i.v. infection. Data are the mean of five different experiments (42 mice/group). No deaths were recorded after 6 days. Survival was assessed by the χ<sup>2</sup> test.

Correlation between blood bacterial counts and TNF-α levels in mice treated with anti-LBP or anti-CD14 mAb and injected i.v. with a low inoculum of
/K. pneumoniae.
Role of TNF-α in mice challenged i.v. with a low inoculum of K. pneumoniae

Experiments with a low inoculum of bacteria indicated a delay in TNF-α production in animals receiving anti-LBP or anti-CD14 mAb, compared with that of controls. This was observed during the initial steps of infection, when bacterial numbers were low. Thus we hypothesized that the absence of TNF-α production may be associated with a defective innate immune response. We investigated the role of TNF-α in that model. As shown in Fig. 6, pretreatment of mice with neutralizing anti-TNF-α Abs augmented early deaths.

TNF-α treatment reverses the effect of LBP or CD14 blockade in mice challenged with a low inoculum of K. pneumoniae

We next investigated whether pretreatment with rTNF-α would improve survival. As shown in Fig. 7, administration of rTNF-α, given at the time of bacterial challenge, induced a good degree of protection in mice challenged with a low inoculum of K. pneumoniae. Yet, TNF has to be given in the early hours after infection, with a failure to protect mice when administered 6 or 10 h after infection (Table II).

Taken together, these experiments suggested that LBP or CD14 blockade, by suppressing TNF-α, induced a defective immune response leading to uncontrolled bacterial multiplication. Thus, we investigated whether pretreatment with rTNF-α would also improve outcome in mice treated with anti-LBP or anti-CD14 mAb. Fig. 8 indicates that administration of rTNF-α almost fully protected mice treated with anti-LBP mAb, that otherwise died rapidly (80% survival compared with 0% survival). Addition of TNF-α also partially restored outcome in mice receiving anti-CD14 mAb (Fig. 9), but protection was less than in mice treated with anti-LBP mAb.

Role of LBP and CD14 in bacterial sepsis induced by a high inoculum of K. pneumoniae

We next investigated how LBP and CD14 blockade affected outcome in mice challenged with high inocula of bacteria. Data obtained ex vivo indicated LBP- and CD14-independent mechanisms of TNF-α production with 10^5 heat-killed K. pneumoniae (data not shown). Mice were pretreated with the three mAbs and injected with 10^5 live K. pneumoniae. Lethality was extremely rapid with this high inoculum. Yet, death rates and bacterial numbers were similar in all groups of mice (Table III), indicating that LBP and CD14 did not contribute to the worsening of infection under these conditions. Plasma TNF-α levels were also not different in the three groups of mice.

Role of LBP and CD14 in bacterial sepsis induced by a low and a high inoculum of E. coli

Finally, we investigated whether LBP and CD14 played a similar role in a systemic challenge of mice with the less virulent Gram-negative bacterium E. coli O111. The LD50 for this particular strain is ~10^8 CFU/mouse. Mice were injected with a nonlethal inoculum (10^5/mouse) to assess whether blockade of the innate system would impair the natural defense mechanisms of mice. As

Table I. Effect of delayed treatment with neutralizing anti-LBP and anti-CD14 mAbs on the lethality of mice injected with 100 CFU of K. pneumoniae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of Administration of mAb (h)</th>
<th>Lethality (%) 24 h Postchallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>12 (1/8)</td>
</tr>
<tr>
<td>Anti-LBP mAb</td>
<td>0</td>
<td>75 (6/8)*</td>
</tr>
<tr>
<td></td>
<td>+2</td>
<td>62 (5/8)*</td>
</tr>
<tr>
<td></td>
<td>+4</td>
<td>75 (6/8)*</td>
</tr>
<tr>
<td></td>
<td>+6</td>
<td>62 (5/8)*</td>
</tr>
<tr>
<td>Anti-CD14 mAb</td>
<td>0</td>
<td>100 (8/8)*</td>
</tr>
<tr>
<td></td>
<td>+4</td>
<td>100 (8/8)*</td>
</tr>
<tr>
<td></td>
<td>+6</td>
<td>50 (4/8)*</td>
</tr>
</tbody>
</table>

* Mice were injected i.v. with bacteria and 100 μg/mouse of Abs given at the same time (t0) or at 2, 4, or 6 h after bacterial challenge. Early deaths observed at 24 h (% in parenthesis, dead/total mice) were recorded and compared to those of mice injected with bacteria and saline given at t0. p < 0.05 by the Fisher exact test, comparing lethality of anti-LBP and anti-CD14 groups vs saline group.

Table II. Effect of delayed treatment with rTNFα on the lethality of mice injected with 80 CFU of K. pneumoniae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of Administration of TNF-α (h)</th>
<th>Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4</td>
<td>8/8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0</td>
<td>1/8*</td>
</tr>
<tr>
<td></td>
<td>+6</td>
<td>3/8</td>
</tr>
<tr>
<td></td>
<td>+10</td>
<td>5/8</td>
</tr>
</tbody>
</table>

* Mice were injected i.v. with bacteria and 50 ng of TNF-α given at the same time (t0) or at 6 or 10 h after bacterial challenge. Deaths were recorded until 6 days. *p < 0.05 by the Fisher exact test.

FIGURE 6. Effect of anti-TNF Abs on survival of mice injected i.v. with a low inoculum (from 60 to 260 CFU/mouse) of K. pneumoniae. Mice were injected with 500 μg/mouse of control rabbit IgG (●) or anti-TNF IgG (○) 5 min before i.v. infection. Data are the mean of two pooled experiments (16 mice/group). No deaths were recorded after 6 days. Survival was assessed by the χ² test.

FIGURE 7. Effect of rTNF-α on survival of mice injected i.v. with a low inoculum (from 60 to 260 CFU/mouse) of K. pneumoniae. Mice were injected with 500 ng TNF-α (○), 100 ng TNF-α (●), or saline (■) 5 min before i.v. infection. Data are the mean of two pooled experiments (16 mice/group). No deaths were recorded after 6 days. Survival was assessed by the χ² test.

FIGURE 8. Administration of rTNF-α almost fully protected mice treated with anti-LBP mAb, that otherwise died rapidly (80% survival compared with 0% survival). Addition of TNF-α also partially restored outcome in mice receiving anti-CD14 mAb (Fig. 9), but protection was less than in mice treated with anti-LBP mAb.

FIGURE 9. Taken together, these experiments suggested that LBP or CD14 blockade, by suppressing TNF-α, induced a defective immune response leading to uncontrolled bacterial multiplication. Thus, we investigated whether pretreatment with rTNF-α would also improve outcome in mice treated with anti-LBP or anti-CD14 mAb. Fig. 8 indicates that administration of rTNF-α almost fully protected mice treated with anti-LBP mAb, that otherwise died rapidly (80% survival compared with 0% survival). Addition of TNF-α also partially restored outcome in mice receiving anti-CD14 mAb (Fig. 9), but protection was less than in mice treated with anti-LBP mAb.

FIGURE 10. Effect of delayed treatment with rTNFα on survival of mice injected i.v. with a low inoculum (from 60 to 260 CFU/mouse) of K. pneumoniae. Mice were injected with 500 μg/mouse of control rabbit IgG (●) or anti-TNF IgG (○) 5 min before i.v. infection. Data are the mean of two pooled experiments (16 mice/group). No deaths were recorded after 6 days. Survival was assessed by the χ² test.
The results demonstrate the significance of coordinated blockade of proximal molecules CD14 and LBP in innate immunity against Gram-negative bacteria. This is the first report of treatment of mice with a neutralizing mAb to mouse CD14. A virulent strain of K. pneumoniae and a less virulent strain of E. coli were selected to assess whether similar innate responses were triggered for these two strains. The results indicated that LBP/CD14 contributed to control infection induced by virulent \( K. \) pneumoniae, not by less virulent \( E. \) coli, suggesting different mechanisms in the elimination of virulent and avirulent bacteria. The two strains display very different characteristics of virulence. \( E. \) coli O111 is the prototype of a Gram-negative organism lacking an invasive phenotype (11). The LD\(_{50}\) for this strain is \( 10^8 \) CFU. Yet, \( E. \) coli O111, when injected in low numbers, are likely to be killed by complement-mediated bacteriolysis or opsonophagocytosis and killing by neutrophils, not requiring the necessary contribution of cytokine production mediated by LPS. In fact, upon exposure to lower numbers of \( E. \) coli O111, LBP- or CD14 blockade did not affect normal bacterial clearance, so that mice survived as control mice. This was not the case for virulent \( K. \) pneumoniae, for which the LD\(_{50}\) is \( K. \) pneumoniae in mice treated with control mAb, anti-LBP-, or anti-CD14 mAbs, up to 4–6 h following injection. This indicated that bacteria were not eliminated but multiplied from the original inoculum of 200 CFU/ml to \( 10^7 \) CFU/ml of blood after 4–6 h. This also indicated that CD14 and LBP blockade did not affect clearance of bacteria in the initial steps of infection. After 6 h, bacteria multiplied dramatically in mice treated with anti-LBP or anti-CD14 mAb, and mice died of overwhelming infection. This implies a critical role for LBP/CD14 in the triggering of host responses that requires time, because the first measurable effects of blockade of the innate system were not observed before 6–7 h of infection.

Mechanisms responsible for aggravation of the disease imply that a vigorous inflammatory response likely triggered by LPS through LBP and CD14 was necessary to eliminate low numbers of virulent bacteria. For this response, TNF-\( \alpha \) stands as a key cytokine, and neutrophil activation and recruitment is of paramount importance. In the \( Klebsiella \) model with a low inoculum, TNF-\( \alpha \) was determinant, as 1) treatment with anti-LBP or anti-CD14 mAb delayed TNF-\( \alpha \) appearance in the blood; 2) treatment of mice with anti-TNF-\( \alpha \) Abs was found to aggravate infection; 3) treatment of mice with rTNF-\( \alpha \) was protective; 4) treatment of mice with rTNF-\( \alpha \) almost totally protected mice treated with anti-LBP mAb, and partially restored survival in mice treated with anti-CD14 mAb, indicating that blockade of CD14 induced more profound alterations of host defense than the sole prevention of TNF-\( \alpha \) production; and 5) TNF-\( \alpha \) had to be present early to prevent death, because administration of rTNF-\( \alpha \) was not effective when given 5–10 h after bacterial challenge.

Bioactive TNF-\( \alpha \) was not detectable when bacterial numbers were \(<10^4\) CFU/ml in the blood, and not before 4 h following infection. Determinant mechanisms for elimination of bacteria (that remain to be defined) happen between 5 and 6 h after infection, and are dependent on host activation by TNF-\( \alpha \). During this 2-h period, anti-CD14- and anti-LBP-treated mice had no detectable bioactive TNF-\( \alpha \). Yet, 1 h later, at 7 h, TNF-\( \alpha \) levels were higher in anti-LBP- and anti-CD14-treated mice than in controls, in relation with the fact that bacterial counts higher than in controls were present in these mice. This suggests that TNF-\( \alpha \) production was not detectable when bacterial numbers were \(<10^4\) CFU/ml in the blood, and not before 4 h following infection.
is dependent on LBP/CD14 in the initial steps, when LPS-triggered monocyte activation occurs. Prophylactic blockade of LBP and CD14 led to the most severe effects. Yet, experiments with a delayed blockade of LBP or CD14 up to 6 h after the start of infection also indicate that these molecules were required during a sustained period of time to protect the mice. From 6 h on, two pathways may be responsible for cell activation, either 1) LPS-dependent (but LBP- and CD14-independent, because concentrations of LPS are too high) mechanisms; or 2) mechanisms dependent on other bacterial products.

Mechanisms responsible for aggravation of the disease following LBP/CD14 blockade are likely not due to prevention of phagocytosis by the Abs, because bacterial numbers were similar in control mice or in mice treated with anti-LBP or anti-CD14 mAbs in the initial steps of infection, whatever the strain of bacteria used. Earlier reports have suggested that phagocytosis of Gram-negative bacteria may occur via a CD14-dependent pathway (29, 30). Yet, these experiments have been performed with heat-killed bacteria, a treatment that breaks capsules and likely allows binding of LBP or CD14 to Gram-negative bacteria. We previously reported that LBP bound efficiently to heat-killed Gram-negative bacteria, but did not bind to most live bacteria, including *Klebsiella* species, whereas it binds to rough live bacteria (31). Binding to CD14 was not observed for the *Klebsiella* strain used in the present study (data not shown). Thus indirect mechanisms, other than prevention of phagocytosis, are responsible for the failure of anti-LBP- and anti-CD14-treated mice to control infection.

Blockade of innate immune responses by anti-LBP or anti-CD14 mAb was associated with an impairment of optimal neutrophil recruitment during the initial steps of infection, which is necessary for host defenses. Concentrations of chemokines have not been measured, but one may speculate that they would also be diminished. Cytokines and chemokines are known to potentiate the function of neutrophils and macrophages, resulting in activation of microbial mechanisms, including generation of superoxide production, reactive oxygen and nitrogen intermediates, or enhancement of phagocytosis, with an increased ability to kill microorganisms (32–35).

It has been largely documented that activation of myelomonocytic cells by LPS via LBP/CD14 occurs only at low doses of LPS, and that LBP or CD14 blockade does not prevent cell activation by high doses of LPS. We approached this question by comparing the effect of CD14 or LBP blockade in mice challenged with a low vs a high bacterial inoculum. The present study indicated a role for LBP and CD14 almost exclusively for low inocula of virulent bacteria. With a high inoculum of bacteria, virulent or not, cytokine synthesis occurred that was LBP- and CD14-independent. Yet, despite triggering host response, bacteria were not eliminated and death occurred rapidly. The present study indicates that under these conditions, administration of anti-LBP or anti-CD14 mAb did not decrease the proinflammatory response.

The detrimental contribution of LBP in models of endotoxemia has been clearly defined (3–6). Its role in infection has been examined in only one study so far. It was reported that LBP was necessary to combat a low-dose infection induced by an i.p. challenge of *S. typhimurium*, presumably because LBP-deficient animals were unable to trigger an adequate response mediating phagocytosis and killing of the microorganism (3). *S. typhimurium* is an intracellular organism, and the present study now extends this observation to extracellular Gram-negative bacteria. With regard to CD14, the present study confirms and extends the conclusion of a recent report showing that blockade of CD14 aggravates experimental shigellosis in rabbits (10). These data as well as the present data may appear at variance with an earlier study that showed that CD14 knockout mice had a better survival than wild-type mice following challenge with *E. coli* (7). There is no explanation for these discrepant results. In the aforementioned study (7), CD14-deficient mice survived to an i.p. inoculum of 5 × 10^9* E. coli* O111, which was lethal to control mice. In the present study, administration of anti-LBP or anti-CD14 mAbs did not alter outcome with a similar inoculum of the same *E. coli* strain given systemically. Studies using gene-deficient animals or animals treated with Abs are not similar.

To conclude, the present study is in agreement with the concept proposed many years ago by Ulevitch and colleagues (1, 2, 36), that LPS shed from Gram-negative bacteria binds to LBP and that the LPS/LBP complexes are presented to CD14 to trigger monocyte activation, leading among other mechanisms to cytokine synthesis, necessary for the host response. This study also stresses the need for critical evaluation of novel therapeutic approaches for the management of patients with severe sepsis or shock.

References


