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J Immunol 2009; 183:7411-7419; Prepublished online 4 November 2009;
doi: 10.4049/jimmunol.0804343
http://www.jimmunol.org/content/183/11/7411

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Increased Inflammation, Impaired Bacterial Clearance, and Metabolic Disruption after Gram-Negative Sepsis in Mkp-1-Deficient Mice

W. Joshua Frazier,† Xianxi Wang,* Lyn M. Wancket,‡ Xiang-An Li,§ Xiaomei Meng,* Leif D. Nelin,*† Andrew C. B. Cato,¶ and Yusen Liu2*†‡

MAPKs are crucial for TNF-α and IL-6 production by innate immune cells in response to TLR ligands. MAPK phosphatase 1 (Mkp-1) deactivates p38 and JNK, abrogating the inflammatory response. We have previously demonstrated that Mkp-1−/− mice exhibit exacerbated inflammatory cytokine production and increased mortality in response to challenge with LPS and heat-killed Staphylococcus aureus. However, the function of Mkp-1 in host defense during live Gram-negative bacterial infection remains unclear. We challenged Mkp-1+/+ and Mkp-1−/− mice with live Escherichia coli i.v. to examine the effects of Mkp-1 deficiency on animal survival, bacterial clearance, metabolic activity, and cytokine production. We found that Mkp-1 deficiency predisposed animals to accelerated mortality and was associated with more robust production of TNF-α, IL-6 and IL-10, greater bacterial burden, altered cyclooxygenase-2 and iNOS expression, and substantial changes in the mobilization of energy stores. Likewise, knockout of Mkp-1 also sensitized mice to sepsis caused by cecal ligation and puncture. IL-10 inhibition by neutralizing Ab or genetic deletion alleviated increased bacterial burden. Treatment with the bactericidal antibiotic gentamicin, given 3 h after Escherichia coli infection, protected Mkp-1+/+ mice from septic shock but had no effect on Mkp-1−/− mice. Thus, during Gram-negative bacterial sepsis Mkp-1 not only plays a critical role in the regulation of cytokine production but also orchestrates the bactericidal activities of the innate immune system and controls the metabolic response to stress. The Journal of Immunology, 2009, 183: 7411–7419.

Severe sepsis and septic shock, the most dire manifestations of bacterial infection in humans, are major contributors to morbidity and mortality worldwide. Mortality from septic shock in adult patients approaches 50%, with survivors often facing serious disabilities (1, 2). Excessive production of inflammatory cytokines with resultant vascular collapse has been implicated in early mortality from septic shock and contributes to the development of multiorgan dysfunction syndrome (3). Dysregulation of innate immune responses has been implicated in a variety of human inflammatory diseases, including septic shock (4–6). During microbial infection, innate immune effector cells (e.g., mononuclear macrophages) recognize pathogens through highly conserved, constitutively expressed pattern recognition receptors. Interaction of these pattern recognition receptors, such as TLRs, with microbe-associated molecular patterns activates a variety of signal transduction pathways, leading to the production of inflammatory cytokines and initiation of downstream inflammatory cascades (6–11). Among the critical pathways mediating the inflammatory response is the MAPK system, including ERK, JNK, and p38 (8, 12, 13). These MAPKs regulate immune responses at multiple levels, including facilitation of Ag presentation and induction of cytokine expression (12). Inflammatory cytokines in turn mediate a number of physiologic changes characteristic of the inflammatory response, including recruitment and activation of monocytes and other phagocytes (7). In addition to influencing the local inflammatory milieu in the host, proinflammatory cytokines affect a variety of changes in organs and tissues remote from the site of infection, such as alterations in body temperature, capillary permeability, and activation of the adaptive immune system (3, 8, 9, 14). In addition to releasing cytokines to alter host physiology in response to infection, innate immune cells are responsible for the earliest phases of fend off infection through the killing and processing of microbes. Monocytes/macrophages engulf pathogens by phagocytosis. Intracellular killing of bacteria is accomplished through the generation of reactive oxygen and nitrogen species within the phagolysosome by enzymes such as myeloperoxidase (MPO) and inducible NO synthase (iNOS), which in turn lyse bacterial cell walls (15, 16). Pathogen-derived antigenic peptides are then presented by the monocyte/macrophage to elements of the adaptive immune system (7).

Inflammatory insults often cause a shift to catabolic pathways and mobilization of energy stores (17, 18). Catabolism is characterized by protein breakdown in the liver and skeletal muscle, increased glycogenolysis, and enhanced lipolysis to release free fatty acids and free fatty acid oxidation.
acids and triglycerides (18). Recent studies suggest that catabolic shifting both provides substrate for ongoing cellular metabolic processes and modulates host defense against invading pathogens (19, 20). Each of these responses is crucial for host survival during infection. Additionally, although the inflammatory response is essential for preventing life-threatening infection, excessive or unregulated inflammation produces pathologic systemic derangements with vascular compromise and widespread collateral damage (3, 8).

Previously, we and other investigators have shown that MAPK phosphatase 1 (Mkp-1) plays a pivotal role in regulation of the innate immune response (21–26). Mkp-1 protein inhibits proinflammatory cytokine production by dephosphorylating and thereby inactivating JNK and p38 after exposure to microbial products, such as the Gram-negative bacterial cell wall component LPS. In the absence of a functional Mkp-1 gene, mice produce substantially greater quantities of inflammatory cytokines and exhibit increased mortality from endotoxic shock (23). Although the role of Mkp-1 in regulating the immune response to purified bacterial components has been well characterized, its role in the host response to live Gram-negative bacteria has not been described. Since live bacteria contain multiple antigenic components and can actively proliferate in the host, Mkp-1 may have additional novel functions in the regulation of the immune response and metabolism during live bacterial infection.

To understand the role of Mkp-1 in host defense against Gram-negative bacterial infection, we studied the immune and metabolic responses of Mkp-1−/− and Mkp-1+/+ mice to both i.v. live Escherichia coli and mixed bacteria via cecal ligation and puncture (CLP). We found that Mkp-1 regulated cytokine production and was key to both bacterial killing and resource mobilization during Gram-negative bacterial infection. In addition, we demonstrated that antimicrobial therapy for Gram-negative sepsis was only effective in Mkp-1−/− mice, illustrating the critical role of Mkp-1 in determining the clinical outcome of such therapy.

Materials and Methods

Experimental animals

Mkp-1−/− mice have been described previously (27) and have no obvious phenotype before experimental use (22, 27). Mkp-1−/− and Mkp-1+/+ mice were provided by Bristol-Myers Squibb Pharmaceutical Research Institute and littermates on a C57/129 mixed background were used for all experiments except for studies specified using Mkp-1−/−/Il-10−/− double knockout mice. For those studies, Il-10−/− mice (28) on a pure 129 background were obtained from The Jackson Laboratory. These mice were crossed to Mkp-1−/− mice on a 129 background to yield Mkp-1−/−/Il-10−/− double knockout mice. All mice were housed with a 12-h alternating light-dark cycle at 25°C, with humidity between 30% and 70% and free access to food and water. All experiments were performed according to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children’s Hospital.

E. coli infection

A wild-type (smooth) strain of E. coli (O55:B5, ATCC 12014) was used for all experiments. This E. coli strain was the source of LPS used in previous studies in our laboratory (22, 23). Bacteria were grown in Luria broth for 18 h at 37°C and bacterial CFU of each culture was determined by comparing the specific OD600 value to a standard curve. Bacteria were washed twice with sterile PBS and adjusted to the appropriate final concentration. For bacterial injection, mice were placed in a clear plastic restraining device and tails cleaned with 70% ethanol. The bacterial suspension was injected into the tail vein using a new syringe and needle for each mouse; the dose was 2.5 × 10^8 CFU/g of body weight unless otherwise specified. To avoid bias, Mkp-1−/− and Mkp-1+/+ mice were injected in a blinded fashion.

Cecal ligation and puncture

CLP was performed as previously described (29). In brief, 8- to 12-wk-old Mkp-1−/− and Mkp-1+/+ mice (n = 15/group) were anesthetized using 2–5% isoflurane in 100% oxygen. A midline incision (1.0 cm) was made below the diaphragm. The cecum was isolated, ligated, punctured twice with a 23-gauge needle, and then gently compressed to express a small amount of cecal contents. The cecum was returned to the abdomen and the muscle and skin incisions were closed with 6–0 Ethilon suture material. Mice received 1 ml of PBS subcutaneously after surgery and survival was monitored for 5 days.

Survival

Male Mkp-1−/− and Mkp-1+/+ mice (8- to 12-weeks old; n = 10/group) were infected i.v. with live E. coli. Mice were observed every 3 to 4 h for death or moribund state through 72 h. Moribund animals and all mice alive at the end of the experiment were euthanized by CO2 inhalation. To examine the effect of gentamicin on survival, mice were injected with E. coli i.v. and received a 25 μg/mouse dose of gentamicin sulfate (Sigma-Aldrich) subcutaneously 3 h postinfection. The gentamicin dose is comparable to doses used clinically to treat Gram-negative bacterial infections in humans (10 mg/kg/day). Gentamicin was administered in 1 ml of PBS, providing fluid support similar to treatment levels for sepsis in humans (nearly 40 ml/kg) (30–32).

Histological and biochemical analyses

Animals were weighed and euthanized 18 h or 24 h after E. coli infection by overdose of pentobarbital i.p. Blood was collected by cardiac puncture for a complete blood biochemical report (clinical laboratory at The Veterinary Hospital of The Ohio State University). Lungs, spleens, thymus, and livers were harvested aseptically. The right lung was weighed and used to calculate lung weight/body weight ratio. The left lung was inflated, fixed in formalin, and routinely processed to produce H&E-stained sections for histological analysis. Livers and the right lung were snap frozen in liquid N2 and stored at −80°C until analysis.

The MPO activity in frozen lungs was assessed as previously described (21). To evaluate the expression levels of iNOS and cyclooxygenase 2 (COX-2), frozen lung and liver tissues were homogenized and sonicated in a lysis buffer containing protease inhibitors. Triton X-100 was added to the homogenates to a final concentration of 1%, and the homogenates were incubated on ice for 15 min. After centrifugation at 14,000 × g for 15 min at 4°C, the supernatants were subjected to Western blot analysis as previously described (33) using Abs against COX-2 and iNOS (Santa Cruz Biotechnology). Glycogen content in the livers was assayed according a procedure described by Suzuki et al. (34). In brief, frozen liver tissues (50–100 mg) were hydrolyzed in 0.3 ml of 30% (w/v) KOH solution in a boiling water bath for 30 min. To facilitate digestion, the tubes were vigorously shaken by vortex every 10 min. After cooling to room temperature, the tubes were boiled again for 5 min to precipitate glycogen. Precipitated glycogen was pelleted by centrifugation at 20,000 × g for 10 min, and then washed twice with 70% ethanol. The pellet was first hydrolyzed in 0.5 ml of 4 N H2SO4 for 10 min at 100°C, and then the acidic solution was neutralized with 0.5 ml of 4 N NaOH. To determine the glucose concentration, 5 μl of the diluted sample was added to 0.2 ml of the glucose (HK) assay solution (Sigma-Aldrich). The glycogen concentration was determined by measuring the absorbance at 340 nm and glycogen content expressed as micromoles of glucosyl units per gram (wet weight).

Cytokine and nitrate measurement

The concentrations of TNF-α, IL-6, and IL-10 in serum were determined using ELISA kits (eBioscience). Nitrate levels in the plasma were determined as previously described (23).

IL-10 neutralization

Mkp-1−/− mice received an i.v. injection of either 1 mg of monoclonal rat anti-mouse IL-10 Ab (JESS-2A5) or 1 mg of a monoclonal rat isotype control Ab (HRPN) (mAb Core of University of California, San Francisco) 60 min before i.v. challenge with live E. coli at 5 × 10^8 CFU/g of body weight. The blood and splenic bacterial loads were assessed 18 h postinfection. Separately, Mkp-1−/−/Il-10−/− and Mkp-1−/−/Il-10−/− double knockout mice were inoculated with 1 × 10^6 CFU/g and euthanized 18 h later to determine splenic bacterial load as described below.

Bacterial load determination

Mice were infected with E. coli and euthanized with i.p. pentobarbital 18 or 24 h postinfection. Blood and spleens were harvested aseptically. Each
spleen homogenized in 1 ml of sterile PBS. Spleen homogenates and blood were serially diluted in sterile PBS, plated onto separate Luria-Bertani agar plates, and the plates incubated for 18 h at 37°C. Colonies were counted separately for each sample. Spleen samples were normalized to organ weight, and blood samples were normalized to blood volume.

Statistical analyses
Survival differences between Mkp-1−/− and Mkp-1+/+ mice and between Mkp-1−/−/Il-10−/− and Mkp-1+/+/Il-10−/− double knockout mice were analyzed by Kaplan-Meier analysis with log-rank test using the on-line statistics program (http://department.obg.cuhk.edu.hk/researchsupport/Survival.ASP) (Dr. Allan Chang, Department of Obstetrics and Gynaecology, Chinese University of Hong Kong). Differences in cytokine production, inflammatory markers, bacterial load, organ function markers, and lipid levels between groups were compared using SPSS statistics software. Data were log-transformed when deemed appropriate. A value of p < 0.05 was considered statistically significant for all analyses.

Results
The effect of Mkp-1 knockout on mortality and cytokine production following infection with live E. coli and CLP
To determine the function of Mkp-1 in the host response to live Gram-negative infection, we infected Mkp-1+/+ and Mkp-1−/− mice i.v. with log-phase growth live E. coli at 2.5 × 10⁷ CFU/g of body weight, a dose that was the LD₅₀ for Mkp-1+/+ mice by 72 h. There was significant mortality in the Mkp-1+/+ group starting at 30 h postinfection, with a total mortality rate of 60%. In contrast, Mkp-1−/− mice began to die by 20 h postinfection and all Mkp-1−/− mice died during the observation period. Kaplan-Meier analysis revealed a significant difference in survival between the two groups (Fig. 1A).

To assess the impact of Mkp-1 deficiency during subacute abdominal sepsis, we subjected Mkp-1+/+ and Mkp-1−/− animals to CLP (Fig. 1B). As with live E. coli, onset of mortality in Mkp-1−/− mice was earlier than in Mkp-1+/+ mice. The overall survival rate of Mkp-1+/+ mice tended to be higher than that of Mkp-1−/− mice after CLP. However, this difference did not reach statistical significance (p = 0.08, n = 15/group).

To assess the role of Mkp-1 in cytokine production, Mkp-1+/+ and Mkp-1−/− animals were infected with E. coli and sacrificed 18 h postinfection. Similar to previous reports of LPS-challenged mice, Mkp-1−/− mice produced markedly higher levels of both TNF-α and IL-6 compared with Mkp-1+/+ animals. Interestingly, Mkp-1−/− mice also had increased IL-10 production (Fig. 1C).

Histopathological analyses
Since the lung is often compromised in cases of sepsis, we examined pulmonary histology in Mkp-1−/− and Mkp-1+/+ mice 24 h after E. coli infection. Mice of both genotypes had comparable alveolar neutrophil infiltration (Fig. 2A) and this finding correlated with similar pulmonary MPO activity in Mkp-1−/− and Mkp-1+/+ mice (Fig. 2B). In contrast Mkp-1−/− mice had a significantly higher lung/body weight ratio (Fig. 2C), indicating more severe pulmonary edema. Finally, the majority of Mkp-1−/− mice 24 h postinfection had dramatic pulmonary microvascular failure as evidenced by profuse hemocoagulation in the pulmonary venules (Fig. 2D). Similar signs of hemocoagulation were also seen in Mkp-1−/− livers, kidneys, and thyromes (data not shown). In contrast, hemocoagulation was not seen in Mkp-1+/+ mice (Fig. 2D).

Disruption of lipid and glycogen utilization in Mkp-1-deficient animals
Lipemic serum during sepsis has been documented both in animal models and human clinical cases, although the underlying mechanisms and clinical significance are not fully understood (19). Serum turbidity consistent with lipemia was consistently seen in Mkp-1−/− animals infected with 2.5 × 10⁷ CFU/g E. coli, but not in infected Mkp-1−/− mice (Table I). Uninfected Mkp-1+/+ and Mkp-1−/− mice did not have lipemia (data not shown). The infection-associated lipemia in wild-type animals did not appear to be due to increased dietary fat intake, since infected mice of both strains were lethargic and did not appear to eat despite free access to food. To determine the role of Mkp-1 in infection-associated lipemia, sera were examined from Mkp-1−/− and Mkp-1+/+ mice infected with a broad range of E. coli doses (Table I). Wild-type mice developed lipemia in a dose-dependent manner at doses higher than 1 × 10⁸ CFU/g. In contrast, lipemia was absent in Mkp-1−/− mice within the tested range (Table I).

To understand the role of Mkp-1 in lipid metabolism during Gram-negative bacterial infection, we measured serum lipid levels in Mkp-1−/− and Mkp-1+/+ mice 24 h after live E. coli infection. In uninfected animals, there were no significant differences between
groups with regard to total plasma cholesterol or triglycerides. Following infection, blood cholesterol levels increased modestly in wild-type mice from 116.0 ± 9.5 to 153.7 ± 5.1 mg/dl (Fig. 3A) and triglyceride levels increased dramatically from 203.2 ± 42.7 to 1,218 ± 246 mg/dl (Fig. 3B). In contrast, E. coli infection did not significantly alter blood cholesterol or triglyceride levels in Mkp-1−/− mice. These results suggest that the Mkp-1+/+ animals actively mobilized lipid stores in response to infection. The lack of triglyceride elevations above the uninfected baseline in Mkp-1−/− mice suggests a defect in mobilizing metabolic stores after infection.

In addition to lipid, glycogen is another key energy source that is rapidly mobilized during starvation and the stress response. We examined liver glycogen stores in Mkp-1+/+ and Mkp-1−/− mice following infection (Fig. 3C). Uninfected mice of both genotypes had similar hepatic glycogen levels. After infection, hepatic glycogen in the Mkp-1+/+ mice was substantially reduced, decreasing from 2.98 ± 0.99 to 0.20 ± 0.02 mg/g of liver tissue. In contrast, hepatic glycogen levels did not change significantly in Mkp-1−/− mice after infection. The defects in both triglyceride and glycogen metabolism are indicative of metabolic liver dysfunction in E. coli-infected Mkp-1−/− mice. These results also suggest that Mkp-1 is crucial for orchestrating a proper metabolic response during Gram-negative bacterial infection.

Organ function in Mkp-1-deficient mice

To understand the role of Mkp-1 in regulating organ function during E. coli infection, we measured organ biomarkers used clinically in human patients with sepsis (Table II). Although biomarker measurements were similar in the uninfected animals, several biomarkers were significantly different between E. coli-infected Mkp-1+/+ and Mkp-1−/− mice. Aspartate aminotransferase and alanine aminotransferase are markers of liver injury. Aspartate aminotransferase activity in the infected Mkp-1−/− mice was significantly higher than in the infected Mkp-1+/+ mice. Blood alanine aminotransferase activity in Mkp-1−/− mice tended to be higher than in the Mkp-1+/+ mice, although the difference did not reach statistical significance. These results suggest that Mkp-1−/− mice suffered more severe liver injury than Mkp-1+/+ mice after infection. Pancreatic amylase was reduced in infected Mkp-1−/− mice compared with uninfected whereas lipase enzyme activity was decreased in infected wild-type mice after infection. These enzymes are crucial for starch and lipid utilization, respectively. Moreover, creatinine kinase activity was significantly higher in the Mkp-1−/− mice than in the wild-type mice. This finding suggests that Mkp-1−/− mice suffered organ hyperperfusion secondary to reduced effective circulating volume, resulting in more severe myocardial and/or skeletal muscle injury. In addition, Mkp-1−/− mice had an elevated albumin/globulin ratio, indicating increased extravasation of plasma and small plasma proteins into the interstitial spaces. Surprisingly, Mkp-1+/+ mice had significantly higher levels of urea nitrogen than did Mkp-1−/− mice, indicating either more severe dehydration or protein catabolism in wild-type mice (Table II). In summary, these findings support the histological evidence of capillary leakage, decreased circulating blood volume, and impaired organ perfusion in Mkp-1−/− mice.

Table I. Development of lipemia in mice after E. coli infection

<table>
<thead>
<tr>
<th>Dose of Infection (CFU/g)</th>
<th>Portion of Lipemic Mice</th>
<th>Mkp-1+/+</th>
<th>Mkp-1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 × 10⁷</td>
<td>14/20</td>
<td>0/30</td>
<td></td>
</tr>
<tr>
<td>1 × 10⁷</td>
<td>12/19</td>
<td>0/20</td>
<td></td>
</tr>
<tr>
<td>9 × 10⁶</td>
<td>8/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>5 × 10⁵</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

*Mice were infected i.v. with E. coli and euthanized 24 h later. Blood was collected by cardiac puncture. Lipemia was defined as the appearance of cloudy serum and was confirmed by lipid panel analysis.*
for bacterial CFU at 24 h postinoculation. Mkp-1−/− blood samples produced more bacterial colonies than samples from Mkp-1+/+ mice (Fig. 4A). Mkp-1−/− splenic homogenates produced approximately 10-fold more bacterial colonies than wild-type samples. In summary, these data indicate that Mkp-1−/− mice have deficient bacterial clearance, which could contribute to the increased inflammatory activity and mortality seen after E. coli infection.

To determine potential mechanisms underlying the reduced antimicrobial activity in Mkp-1−/− mice, we examined several cytokines and antimicrobial factors. First, we evaluated the production of NO, a potent bactericidal substance critical for phagocyte killing of intracellular bacteria (35). Since NO is rapidly converted into nitrate, we measured blood nitrate levels as a surrogate for NO production (36). Since NO is a mediator of inflammation, we measured serum cytokines and antimicrobial factors. First, we evaluated the production of cytokines. The expression of COX-2, another important mediator of inflammation, was substantially higher in both lung and liver in E. coli-infected Mkp-1−/− mice than in the infected Mkp-1+/+ mice (Fig. 5B). Densitometry quantification of iNOS expression in the livers of infected Mkp-1−/− mice (Fig. 5B) suggests that iNOS expression may be reduced in additional organs of Mkp-1−/− mice. These results also support the idea that Mkp-1−/− mice have diminished bactericidal activity, since insufficient NO could reduce bacterial killing efficiency.

To determine whether elevated IL-10 after infection is responsible for the diminished antimicrobial responses in Mkp-1−/− animals, knockout mice were treated with a neutralizing mAb specific for mouse IL-10 and then challenged with E. coli. A lower

### Table II. Metabolic analyses of Mkp-1+/+ and Mkp-1−/− mice

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mkp-1+/+</td>
<td>Mkp-1−/−</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>36.4 ± 4.5</td>
<td>24.4 ± 2.4</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>391.8 ± 71.8</td>
<td>202.6 ± 32.8</td>
</tr>
<tr>
<td>Amylase (U/L)</td>
<td>2171 ± 164</td>
<td>2829 ± 273</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>256 ± 7.7</td>
<td>285 ± 0.11</td>
</tr>
<tr>
<td>Lipase (U/L)</td>
<td>281 ± 9</td>
<td>239 ± 21</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>850 ± 164</td>
<td>583 ± 96</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dL)</td>
<td>29.4 ± 2.2</td>
<td>25.6 ± 1.1</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Anion gap (mEq/l)</td>
<td>42.6 ± 0.87</td>
<td>41.4 ± 0.92</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>4.9 ± 0.13</td>
<td>5.06 ± 0.05</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.46 ± 0.06</td>
<td>3.56 ± 0.04</td>
</tr>
<tr>
<td>Albumin/globulin ratio</td>
<td>2.44 ± 0.11</td>
<td>2.4 ± 0.11</td>
</tr>
</tbody>
</table>

*Infected mice were injected i.v. with 2.5 × 10⁷ CFU/g of E. coli and euthanized 24 h later. Blood was collected by cardiac puncture. Serum was analyzed for complete biomarkers. Data are presented as mean ± SE (n = 7). †, p < 0.05 vs uninfected Mkp-1+/+ mice; ‡, p < 0.05 vs uninfected Mkp-1−/− mice; †, p < 0.05 vs infected Mkp-1+/+ mice.
bacterial dose ($5 \times 10^6$ CFU/g) was used since mice with markedly decreased IL-10 levels may have increased bacterial sensitivity. Mkp-1$^{-/-}$ mice treated with IL-10 neutralizing Ab exhibited significantly lower splenic bacterial load than mice administered control IgG1 Ab (Fig. 4B). Blood bacterial load was very low in both groups and not significantly different (data not shown). Finally, similar results were seen in Mkp-1$^{-/-}$/Il-10$^{-/-}$ double knockout mice infected with E. coli (1 $\times 10^7$ CFU/g), with splenic bacterial load significantly lower in double knockout mice than in Mkp-1$^{-/-}$/Il-10$^{-/-}$ mice (Fig. 4C).

**Antibiotic therapy, survival, and cytokine production**

Timely antibiotic treatment is a current standard of care in human sepsis cases (32). We therefore examined the effect of antibiotic therapy in E. coli-infected Mkp-1$^{+/+}$ and Mkp-1$^{-/-}$ mice. Gentamicin treatment 3 h after infection with live E. coli resulted in dramatic differences in 72 h survival between the groups. Gentamicin therapy completely prevented mortality in the Mkp-1$^{+/+}$ mice but had no impact on Mkp-1$^{-/-}$ survival, with all mice dying within 72 h (Fig. 6A). Although gentamicin treatment differentially affected the survival of the two groups of animals, it did not substantially alter the pattern of cytokine production in these mice (Fig. 6B).

**Discussion**

This is the first report to detail the responses of Mkp-1-deficient animals to live Gram-negative bacterial challenge, including mortality rate, inflammatory parameters, and clinical biomarkers of...
Given these divergent findings, it was not surprising that Mkp-1 does not appear to play an important role in determining the mortality after CLP (Fig. 1). Increased mortality in antimicrobial therapy depended on yet attenuated NO production (Fig. 5). There were also marked changes in inflammation, metabolic resource distribution, and bacterial clearance. The mortality rate in infected Mkp-1−/− mice was accompanied by higher bacterial burdens (Fig. 4), elevated cytokine production in E. coli-infected mice after gentamicin treatment. Mice were euthanized 18 h after the initial infection, and ELISA used to measured cytokines in the blood. Values in the graphs represent the means ± SE from five to eight mice. *, different from Mkp-1+/+ mice (p < 0.05).

Defects of Mkp-1-deficient mice in bactericidal mechanisms

Another key finding was the markedly attenuated bacterial clearance in Mkp-1−/− mice, evidenced by higher splenic and blood bacterial burdens. Since proinflammatory cytokines were higher in infected Mkp-1−/− mice (Fig. 1), these data suggest that Mkp-1−/− mice are not defective in the detection of E. coli. It is more likely that these mice have defects in bacterial capture or killing. Although the exact mechanism(s) underlying the defect(s) in Mkp-1−/− mice remains unclear, alterations in the innate immune response are most likely, since innate immunity is key to initial pathogen recognition, phagocytosis, and phagolysosomal killing (7). Altered production of several innate immune factors in Mkp-1−/− mice likely contributed to inhibited bacterial clearance.

First, levels of iNOS and nitrate (a reliable surrogate of NO) were lower in Mkp-1−/− mice (Fig. 5). NO is a potent bactericidal molecule (35) and decreased production in Mkp-1−/− mice may lead to attenuated bactericidal activity. Surprisingly, Mkp-1−/− mice exhibited elevated NO production after LPS challenge (21, 23, 36). These contrasting findings may be due to differences in the host immune responses to a purified bacterial component (LPS) vs intact bacteria. The combined responses to different E. coli components could produce a net reduction in iNOS expression in the Mkp-1−/− mouse. Alternatively, lower iNOS levels may be a compensatory host response to minimize blood pressure fluctuations.

A second factor that can potentially result in decreased bactericidal activity in Mkp-1−/− mice is the elevated IL-10 expression (Fig. 1). IL-10 is a pleotropic cytokine (37, 38) that can potently inhibit production of reactive oxygen and nitrogen species, including NO, in macrophages (39). In peritonitis models, excess IL-10 compromises bacterial clearance (40), whereas IL-10 knockout permits E. coli elimination (41). IL-10 can inhibit pulmonary defense during sepsis (42) and prevent apoptosis of cells infected with pathogenic bacteria (37). In our study, Mkp-1−/− mice pretreated with an IL-10-neutralizing Ab had significantly reduced splenic bacterial load. Similarly, infected Mkp-1−/−/Il-10−/− double knockout mice also had lower splenic bacterial counts compared with Mkp-1−/− mice. Together, these experiments suggest an important role for IL-10 in depressing bacterial clearance in Mkp-1−/− mice.

Finally, excessive levels of COX-2 and the potential production of downstream mediators like prostaglandin E2 may also be implicated in the compromised bactericidal activity of the Mkp-1−/− mice. It has been shown that prostaglandin E2 inhibits macrophage production of reactive oxygen species and that knockout of either COX-2 or prostaglandin E2 receptors enhance bacterial clearance in a pneumonia model (43). E. coli-infected Mkp-1 knockout mice expressed higher COX-2 levels than did Mkp-1+/+ mice (Fig. 5), suggesting that Mkp-1−/− mice might have elevated prostaglandin E2 levels. However, we believe that elevated COX-2 expression is not likely a significant contributor to the compromised bactericidal activity in Mkp-1−/− mice, since treatment with the COX-2 inhibitor aspirin did not alter splenic or blood bacterial load in Mkp-1−/− mice (data not shown).

organ function. Mkp-1−/− animals had accelerated mortality after i.v. infection with live E. coli and had similarly decreased survival after CLP (Fig. 1). Increased mortality in Mkp-1−/− mice was accompanied by higher bacterial burdens (Fig. 4), elevated cytokine production (Fig. 1), higher COX-2 expression (Fig. 5), and yet attenuated NO production (Fig. 5). There were also marked differences in energy utilization between the two genotypes (Fig. 3 and Table 1). Finally, we showed that the efficacy of bactericidal antimicrobial therapy depended on Mkp-1 gene function (Fig. 6). Overall, these findings support a role for Mkp-1 in modulating the host response during Gram-negative infection, including regulating inflammation, metabolic resource distribution, and bacterial clearance.
The function of Mkp-1 in the regulation of energy mobilization during sepsis

Systemic inflammation triggers release of energy stores to provide fuel for increased metabolic demands (17, 44) and can lead to elevations in plasma lipids (lipemia of sepsis) (19). LPS can trigger inflammatory cytokines and sympathetic output from the autonomic nervous system to increase plasma triglyceride levels (45, 46). Interestingly, new evidence shows that in addition to their role as an energy source, blood lipids may also function in the innate immune response. Infusion of chylomicrons, very low density lipoproteins, and triglyceride-rich lipids can sequester LPS, enhance LPS degradation in the liver, and dampen the host inflammatory response (47, 48). In the present study, we found that E. coli-infected Mkp-1−/− mice failed to mobilize key lipid and carbohydrates energy stores (Fig. 3). Whereas infected Mkp-1−/− mice had markedly elevated serum triglycerides after infection, there was no change in Mkp-1−/− triglyceride levels. The absence of lipemia in infected Mkp-1−/− mice is unlikely to be an epiphenomenon of hyperinflammation, as both low and high inoculation doses failed to induce lipemia (Table I). In addition to altered lipid metabolism, E. coli-infected Mkp-1−/− mice demonstrated dramatic hepatic glycogen depletions, whereas liver glycogen in Mkp-1−/− mice was unaltered. This finding was unexpected, as blood glucose levels in infected Mkp-1−/− and Mkp-1−/− mice were similar (Table II) and severe inflammation is known to perturb glucose homeostasis (49–51). Although the exact mechanism underlying defective lipid and glycogen mobilization in Mkp-1−/− mice remains unclear, these defects may be related to metabolic abnormalities previously reported by another laboratory. Wu et al. (52) have demonstrated that Mkp-1 deletion accelerates energy catabolism, resulting in leaner body mass, decreased adiposity, and accelerated hepatic lipid metabolism. Given these reported differences in body mass and adipose depots, it was surprising that blood baseline triglyceride and glycogen levels were comparable in Mkp-1−/− and Mkp-1−/− mice in the absence of infection (Fig. 3). These findings indicate that Mkp-1 may have divergent roles in maintaining blood and tissue energy stores. In other words, while Mkp-1 plays a significant role in maintaining the normal metabolism in the absence of infection, it becomes critical in mobilizing energy resources when the host must combat infectious pathogens. Since Mkp-1 is a negative regulator of MAPKs, the protein most likely serves as an upstream regulator of energy catabolism rather than having direct interactions with individual lipolysis and glycogenolytic pathways.

The importance of Mkp-1 in determining outcome after antibiotic therapy

Although gentamicin therapy was protective for 100% of Mkp-1−/− mice, it did not decrease mortality in Mkp-1−/− mice (Fig. 6). This phenomenon may be due to our use of a bacterialid antibiotic, which lyses bacteria and may cause the release of large quantities of endotoxins, thus mimicking an LPS challenge model. In such a case, high rates of mortality in Mkp-1−/− mice are not surprising, since these mice are exclusively sensitive to LPS challenges (22, 23). The absence of any protective response after antibiotic treatment suggests that excessive cytokine production in high-dose infected Mkp-1−/− mice is severe enough to mitigate any benefit from bacterial killing. In contrast, the potential reduction in mortality of Mkp-1−/− mice after antibiotic treatment indicates that decreased bacterial burden promotes survival, so long as the animals are not hypersensitive to endotoxin. Ultimately, bacteriostatic antibiotics such as protein synthesis inhibitors may decrease bacterial load with limited endotoxin release and potentially increase Mkp-1−/− mouse survival.

Although MKP-1 activity in septic human patients has not directly been studied, several findings indicate that the protein will be a clinically relevant factor. First, MKPs are highly conserved across species and appear to function similarly in humans. Second, reduced MKP-1 expression in the lung can be seen clinically, as has been shown for immune cells isolated from human asthma patients (53, 54). Finally, sepsis and multiple organ dysfunction syndrome in humans are systemic inflammatory diseases mechanistically similar to the animal models reported in this study. If MKP-1 in humans shows similar potent anti-inflammatory activity, supports bacterioidal activity of the immune system, and facilitates resource utilization, modulation of MKP-1 will be a valuable therapeutic target for patients suffering from septic shock and multiple organ dysfunction syndrome.

Acknowledgments

We are grateful to Bristol-Myers Squibb Pharmaceutical Research Institute for providing Mkp-1 knockout mice. We thank Dr. Y. J. Yin for assistance measuring serum nitrates. We also gratefully acknowledge Sylvia Tuzbikian, Joshua Kuhlman, and Nan Zhang for technical support. We thank Drs. James Cooper and Laura Goodchild for vivarium expertise and Dr. Richard Ye for thoughtful insight and discussion.

Disclosures

The authors have no financial conflict of interest.

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