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Knockout of *Mkp-1* Enhances the Host Inflammatory Responses to Gram-Positive Bacteria¹

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MAPK phosphatase (MKP)-1 is an archetypal member of the dual specificity protein phosphatase family that dephosphorylates MAPK. We have previously demonstrated that MKP-1 acts as a negative regulator of p38 and JNK in immortalized macrophages after stimulation with peptidoglycan isolated from Gram-positive bacteria. To define the physiological function of MKP-1 during Gram-positive bacterial infection, we studied the innate immune responses to Gram-positive bacteria using *Mkp-1* knockout (KO) mice. We found that *Mkp-1*^{-/-} macrophages exhibited prolonged activation of p38 and JNK, but not of ERK, following exposure to either peptidoglycan or lipoteichoic acid. Compared with wild-type (WT) macrophages, *Mkp-1*^{-/-} macrophages produced more proinflammatory cytokines such as TNF- α and IL-6. Moreover, after challenge with peptidoglycan, lipoteichoic acid, live or heat-killed *Staphylococcus aureus* bacteria, *Mkp-1* KO mice also mounted a more robust production of cytokines and chemokines, including TNF- α , IL-6, IL-10, and MIP-1 α , than did WT mice. Accordingly, *Mkp-1* KO mice also exhibited greater NO production, more robust neutrophil infiltration, and more severe organ damage than did WT mice. Surprisingly, WT and *Mkp-1* KO mice exhibited no significant difference in either bacterial load or survival rates when infected with live *S. aureus*. However, in response to challenge with heat-killed *S. aureus*, *Mkp-1* KO mice exhibited a substantially higher mortality rate compared with WT mice. Our studies indicate that MKP-1 plays a critical role in the inflammatory response to Gram-positive bacterial infection. MKP-1 serves to limit the inflammatory reaction by inactivating JNK and p38, thus preventing multiorgan failure caused by exaggerated inflammatory responses. *The Journal of Immunology*, 2007, 178: 5312–5320.

Sepsis represents a major challenge to the health care system. According to a recent report, in the United States ~751,000 people each year are hospitalized due to microbial infection, with ~215,000 deaths (1). Despite the advancements in infection prevention and treatment, the incidence of sepsis continues to rise at an astonishing annual rate of ~8.7% (1, 2). Among the microbial organisms that cause sepsis, Gram-positive bacteria are becoming increasingly prevalent. Since 1987, Gram-positive bacteria have become the predominant organisms isolated in cases of sepsis. It has been reported that >52% of all cases of sepsis were caused by Gram-positive bacteria in 2000 (1). *Staphylococcus aureus* is a leading cause of nosocomial pneumonia and wound infections and represents one of the bacteria most commonly isolated from patients with sepsis (1, 2). *S. aureus* is also the major cause of toxic shock syndrome and scalded skin syndrome (3, 4). During the past decade, community acquired, methicillin-resistant strains of *S. aureus* (CA-

MRSA)³ have caused a marked increase in serious infections in many regions of the United States (5–9).

Sepsis often leads to vasodilatory shock and multiorgan failure as the result of severe hypotension caused by an exaggerated host immune response to the invading pathogens (10). Once microbial pathogens penetrate the epithelial barrier, the microbial components are recognized by the pattern-recognition receptors of the innate immune cells, particularly macrophages (11). Peptidoglycan (PepG) and lipoteichoic acid (LTA) are considered to be the major Gram-positive bacterial cell wall components responsible for the intense inflammatory response during Gram-positive bacterial infection (3, 12–15). Although LTA is recognized by TLR-2 on the cell surface, PepG is recognized by nucleotide oligomerization domain (NOD) 1 and NOD2 that belong to a different class of pattern-recognition receptors localized inside of the cells (16). Interaction of TLR-2 and/or NOD proteins with these bacterial components triggers a cascade of signaling events including the activation of the transcription factor NF- κ B and the MAPK pathways, ultimately leading to the production of a variety of proinflammatory cytokines, including TNF- α , IL-1 β , and IL-6 (17–19). These inflammatory cytokines are crucial for the host immune response against bacterial infection (20). An important host response to bacterial infection is the increase of inducible NO synthase (iNOS) expression and the resultant robust production of NO, which has a potent bactericidal activity. In addition to its

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³ Abbreviations used in this paper: CA-MRSA, community acquired, methicillin-resistant strains of *S. aureus*; PepG, peptidoglycan; LTA, lipoteichoic acid; NOD, nucleotide oligomerization domain; iNOS, inducible NO synthase; eNOS, endothelial NO synthase; MKP, MAP kinase phosphatase; BUN, blood urea nitrogen; MPO, myeloperoxidase; WT, wild type; KO, knockout.

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antimicrobial properties, NO is also a powerful vasodilator which also facilitates the infiltration of leukocytes such as neutrophils and T lymphocytes into the site of infection. Furthermore, the proinflammatory cytokines can enhance host immunity by inducing acute-phase proteins and promoting the production of inflammatory lipid mediators, reactive oxygen species, and various effector cytokines that direct the development of adaptive immunity (21). By inducing the expression of cell adhesion molecules and chemokines, the proinflammatory cytokines also facilitate the migration of leukocytes to the infection site to combat the pathogen (20). Although these proinflammatory cytokines are crucial for host defense, excessive production of these inflammatory cytokines may result in septic shock, multiorgan failure, and mortality (22). Elucidation of the mechanisms that negatively regulate the production of the proinflammatory cytokines will facilitate the understanding of the immune response to Gram-positive bacterial pathogens. More importantly, such studies may reveal potential therapeutic targets for sepsis and other inflammatory diseases caused by exaggerated inflammatory responses.

The MAPK family, which includes the ERK, JNK, and p38 subfamilies, plays a pivotal role in the biosynthesis of proinflammatory cytokines (23–26). MAPK phosphatases (MKPs) are dual specificity protein phosphatases that dephosphorylate and thereby inactivate MAPKs (27). We have previously found that MKP-1, the archetypical member of the MKP family, appears to negatively regulate the production of proinflammatory cytokines by inactivating p38 and JNK in immortalized macrophages stimulated with PepG (28). Very recently, we and several other groups have independently demonstrated that MKP-1 limits the innate immune responses and protects the host from hemodynamic shock after challenge with LPS, a Gram-negative bacterial endotoxin (29–32). However, the physiological role that MKP-1 plays in the host immune response against Gram-positive pathogens remains unclear. We hypothesized that MKP-1 restrains the inflammatory response following Gram-positive bacterial infection and thereby prevents septic shock and multiorgan failure. In the present study, we examined the physiological function of MKP-1 in innate immune responses to the Gram-positive bacteria, *S. aureus*, using *Mkp-1* knockout (KO) mice. Our results indicate that MKP-1 plays a critical role in vivo in controlling the host inflammatory response during Gram-positive bacterial infection.

Materials and Methods

Animals

Mkp-1^{+/-} and *Mkp-1*^{-/-} mice were provided by Bristol-Myers Squibb Pharmaceutical Research Institute and bred in-house to yield *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice. The mice are maintained on a mixed C57/129 background. All animals received humane care and all animal-related work was performed in accordance with National Institutes of Health guidelines. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Columbus Children's Research Institute.

Reagents

PepG and LTA, isolated from *S. aureus*, were purchased from Sigma-Aldrich and dissolved in sterile PBS. LTA was free of nucleic acid and contained <3% protein.

S. aureus preparation

Two strains of *S. aureus* were used in this study. *S. aureus* FDA 209P was purchased from American Type Culture Collection (ATCC) and grown at 37°C for 18 h in ATCC medium 117. The second strain of *S. aureus* was a virulent strain of CA-MRSA isolated from a pediatric patient with vertebral osteomyelitis (33). This strain of bacteria was cultured for 18 h in tryptic soy broth at 37°C. Bacteria were harvested by centrifugation at 800 × g for 10 min, washed twice with sterile PBS, and then resuspended in PBS for administration to the animals. The CFU of the bacterial sus-

pensions were determined through serial dilution and culture on dishes containing solid medium for 18 h.

To prepare heat-killed bacteria, *S. aureus* FDA 209P was grown at 37°C for 18 h in ATCC medium 117. The bacteria were washed twice with sterile PBS, suspended in sterile PBS, and then heated at 60°C for 45 min. The viability of the heat-killed bacteria was estimated to be ~0.002% by colony formation assays.

Isolation and culture of peritoneal macrophages

Peritoneal macrophages were elicited from mice using thioglycolate (28). Peritoneal macrophages were isolated from *Mkp-1*^{+/+} or *Mkp-1*^{-/-} mice by peritoneal lavage, and cultured as described previously (28, 29).

Challenge of mice with PepG and LTA or *S. aureus*

Mice were between 6 and 12 wk of age at the time of study. Age- and gender-matched mice were used in all experiments. PepG and LTA, dissolved in PBS, were injected i.p. into mice. Mice were weighed and sacrificed, and blood was harvested by cardiac puncture. The right lung and the liver were removed, weighed, snap-frozen in liquid nitrogen, and stored at -70°C for later analysis. For histological analysis, the left lung was inflated with 10% formalin at a constant distending pressure of 25 cm H₂O for 10 min, then excised from the animals, and placed in 10% formalin overnight at 4°C. Livers were fixed in 10% formalin overnight at 4°C. The tissues were embedded in paraffin and cut into 4-μm-thick sections and then stained with H&E.

To challenge mice with live bacteria, the FDA 209P strain or the CA-MRSA (clinical isolate) strain of *S. aureus* bacteria were injected into the tail vein at designated doses. To challenge mice with dead bacteria, heat-killed *S. aureus* FDA 209P bacteria were injected into the tail vein at a dose equivalent to 2.5 × 10⁸ CFU/g body weight. Blood and organs were harvested and processed as described above. Animal survival was monitored over 10 days (after inoculated with live bacteria) or 7 days (after challenged with dead bacteria) using death or moribund state as an endpoint criterion. LD₅₀ values were calculated based the method described by Reed and Muench (34).

Quantification of bacterial burden

To determine the bacterial burden, mice challenged with live *S. aureus* FDA209P (7.5 × 10⁶ CFU/g body weight) were euthanized 24 h postexposure. Blood was collected by cardiac puncture and the spleens were harvested under sterile conditions. The spleens were homogenized in 1 ml of sterile PBS. Bacterial loads in the blood and spleen were determined by spreading serial dilutions of blood or spleen homogenates onto triplicate plates containing solid culture medium and culture at 37°C for 18 h. Bacterial burden in each animal was calculated based on the means of the numbers of bacterial colonies on the triplicate plates.

Western blot analysis and ELISA

Western blot analysis was performed essentially as previously described (35), using cell lysates or tissue homogenates. The mouse mAbs against iNOS and endothelial NO synthase (eNOS) were purchased from BD Biosciences. The mouse mAb against β-actin was purchased from Sigma-Aldrich.

The concentrations of TNF-α, IL-6, and IL-10 in either the culture medium or serum were determined as previously described (29). The concentration of MIP-1α was assessed using an ELISA kit purchased from R&D Systems.

Myeloperoxidase (MPO) assay

To measure MPO activity, frozen lung tissues were homogenized and sonicated in 50 mM potassium phosphate (pH 6.0) buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). After centrifugation at 14,000 × g for 15 min at 4°C, the supernatants were incubated at 25°C in 50 mM potassium phosphate (pH 6.0) buffer containing 0.0005% H₂O₂ and 167 μg/ml *o*-dianisidine hydrochloride (Sigma-Aldrich). MPO activity was determined spectrophotometrically by measuring the change in absorbance at 450 nm over 2 min using a 96-well plate reader. One unit of MPO is defined as the activity that converts 1 μM H₂O₂ to H₂O in 1 min at 25°C.

Determination of nitrate and blood nitrogen urea (BUN)

The nitrate and BUN levels in the plasma were determined as previously described (29).

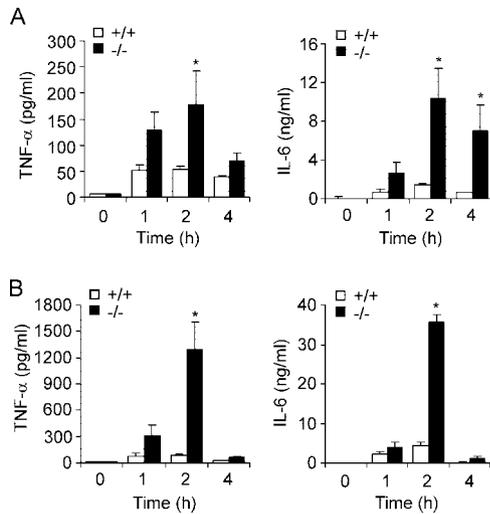


FIGURE 1. *Mkp-1* KO mice produce greater amounts of TNF- α and IL-6 than WT mice *in vivo* after challenge with either PepG or LTA. *Mkp-1* KO and WT mice were challenged i.p. with either PepG (5 mg/kg body weight) or LTA (5 mg/kg body weight). Blood was harvested via cardiac puncture at different times, and cytokines were measured by ELISA. **A**, Cytokine production in WT and *Mkp-1* KO mice after PepG challenge. **B**, Cytokine production in WT and *Mkp-1* KO mice after LTA challenge. The data shown in the graphs represent the means \pm SE of four independent experiments. *, $p < 0.05$, compared with similarly treated WT mice.

Statistical analysis

The biochemical and physiological parameters between the groups were compared using one-way ANOVA with a modified *t* test. All tests were performed using SPSS 13.01 software. A value of $p < 0.05$ was considered significant.

Results

Mkp-1-deficient primary macrophages exhibit prolonged p38 and JNK activation and enhanced cytokine production in response to cell wall components of Gram-positive bacteria

To investigate the physiological function of MKP-1 during Gram-positive bacterial infection, thioglycolate-elicited peritoneal macrophages from *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were stimulated with PepG or LTA; MAPK activation was examined as previously described (28, 36). Similar to what were observed using immortalized macrophages stimulated with PepG (28), stimulation of wild-type (WT) macrophages with either PepG or LTA resulted in transient activation of ERK, JNK, and p38 (data not shown). Compared with WT macrophages, *Mkp-1*^{-/-} macrophages exhibited prolonged p38 and JNK activation upon stimulation with either PepG or LTA (data not shown). However, the kinetics of ERK activation upon these stimulations did not significantly change (data not shown). These results indicate that p38 and JNK, but not ERK, are the physiological substrates in activated macrophages.

To assess the role of MKP-1 in the regulation of cytokine production, thioglycolate-elicited peritoneal macrophages isolated from both WT and *Mkp-1*^{-/-} mice were treated with PepG or LTA. Cytokines secreted into the medium were quantitated by ELISA. Similar to what we observed in immortalized macrophages stimulated with PepG (28), both PepG and LTA stimulated more robust biosynthesis of proinflammatory cytokines in *Mkp-1*^{-/-} macrophages than in WT cells, including both TNF- α and IL-6 (data not shown). These results indicate that MKP-1 negatively regulates the biosynthesis of proinflammatory cytokines through dephosphorylating p38 and JNK during

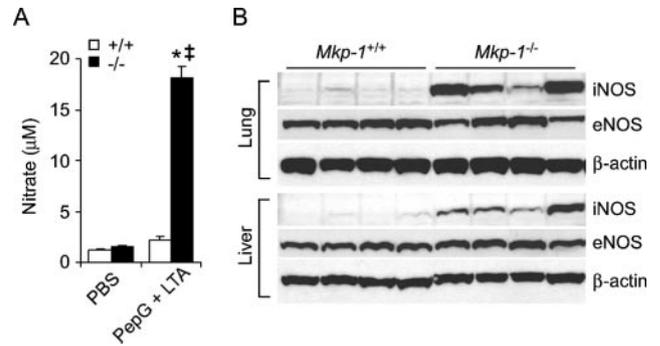


FIGURE 2. *Mkp-1* KO mice exhibit enhanced iNOS protein expression in the lungs and livers and elevated NO production. *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were challenged i.p. with a combination of PepG (40 mg/kg body weight) and LTA (40 mg/kg body weight) or vehicle (PBS). Twenty-four hours later, mice were sacrificed to harvest blood and vital organs. **A**, Plasma nitrate levels, as determined by chemiluminescence. Data represent the means \pm SE of five independent experiments. *, $p < 0.05$, compared with similarly treated WT mice. ‡, $p < 0.05$, compared to *Mkp-1* KO mice treated with PBS. **B**, Expression of iNOS and eNOS proteins in lungs and livers. The organs were homogenized and soluble proteins were analyzed by Western blotting using mAbs against iNOS or eNOS. The membranes were stripped and blotted with a β -actin Ab to control for sample loading. Data shown are from a representative experiment. Each sample represents an individual animal.

the innate immune response to Gram-positive bacterial components.

Gram-positive bacterial cell wall components elicit a more robust inflammatory response in Mkp-1 KO mice than in WT mice

To investigate the role of MKP-1 in innate immune response *in vivo*, *Mkp-1*^{-/-} mice and their WT counterparts were challenged i.p. with PepG and LTA. Plasma cytokine levels were measured by ELISA. Administration of PepG resulted in a profound increase in the serum concentrations of TNF- α and IL-6 in both *Mkp-1*^{-/-} and *Mkp-1*^{+/+} mice, although these cytokines were barely detectable in those mice injected with PBS (Fig. 1A). In WT mice, TNF- α levels reached maximum at 1–2 h and slightly decreased by 4 h post-PepG challenge. Compared with WT mice, *Mkp-1*^{-/-} mice produced substantially more TNF- α (Fig. 1A). Likewise, PepG also induced a marked increase in serum IL-6 levels in both strains of mice, although serum IL-6 levels were remarkably higher in *Mkp-1*^{-/-} mice than in WT mice (Fig. 1A).

Challenge of both WT and *Mkp-1*^{-/-} mice with LTA also resulted in significant increases in serum TNF- α and IL-6 levels (Fig. 1B). Both TNF- α and IL-6 became detectable in the serum at 1 h, reached maximal levels at 2 h, and declined markedly by 4 h post-LTA injection. Both serum TNF- α and IL-6 levels in the *Mkp-1*^{-/-} mice were dramatically higher than in WT mice. Although the peak serum TNF- α and IL-6 levels in the WT mice were 90 ± 13 pg/ml and 4.4 ± 0.8 ng/ml, respectively, the peak serum TNF- α and IL-6 levels in the *Mkp-1*^{-/-} mice were 1299 ± 298 pg/ml and 35.5 ± 2.0 ng/ml, respectively.

NO is a potent microbicidal substance and a powerful vasodilator produced by the host in response to microbial infection (37). We measured the plasma nitrate levels in WT and *Mkp-1*^{-/-} mice after i.p. challenge with a combination of PepG and LTA (Fig. 2A). Although challenge of WT mice with PepG plus LTA resulted in a relatively small increase in plasma nitrate levels, PepG plus LTA

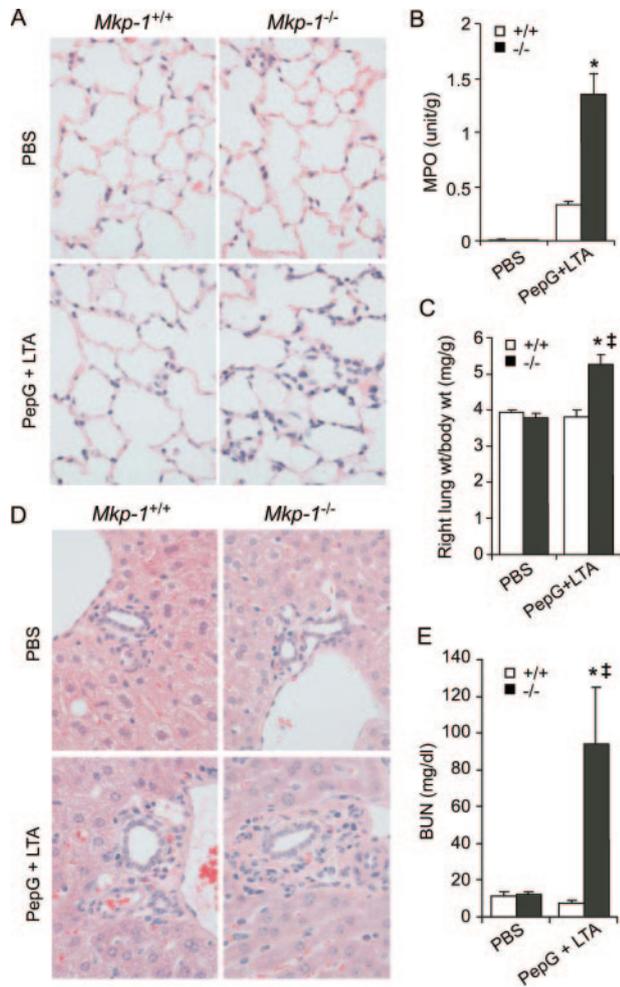


FIGURE 3. Upon challenge with PepG and LTA *Mkp-1* KO mice exhibit signs of multiorgan dysfunction syndrome. *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were subjected to i.p. challenge with either PBS or a combination of PepG (40 mg/kg) and LTA (40 mg/kg). Twenty-four hours later, the mice were sacrificed. The left lungs were fixed and stained with H&E (A). The right lungs were weighed and homogenized. MPO activity in the lung homogenates was determined and normalized to tissue weight (B). The ratio of wet right lung weight to body weight was used as an index of pulmonary edema (C). The livers were fixed, sectioned, and stained with H&E (D). The plasma BUN levels were measured (E). Results presented in A and D are representative lung and liver histological images. Results presented in B, C, and E are means \pm SE of five independent experiments. *, $p < 0.05$, compared with WT mice. ‡, $p < 0.05$, compared with PBS-treated *Mkp-1* KO mice.

given to *Mkp-1*^{-/-} mice resulted in a dramatic increase in plasma nitrate concentrations (Fig. 2A). We also assessed NOS protein levels in lung and liver tissues. Although iNOS was not detectable in either the lung or liver tissues harvested from PBS-treated mice, eNOS was present in abundant quantity in these tissues (data not shown). Upon treatment with PepG plus LTA, iNOS protein levels were dramatically increased in both the lung and liver tissues in *Mkp-1*^{-/-} mice, whereas in WT mice increases in iNOS protein levels were barely detectable (Fig. 2B). In contrast, eNOS protein levels in both organs did not change upon challenge with PepG and LTA (data not shown) and there was no difference between the two strains of mice in the eNOS protein levels in either the lung or the liver tissues (Fig. 2B). These results clearly indicate that MKP-1 negatively regulates iNOS induction and NO synthesis in vivo during the host response to Gram-positive bacterial components.

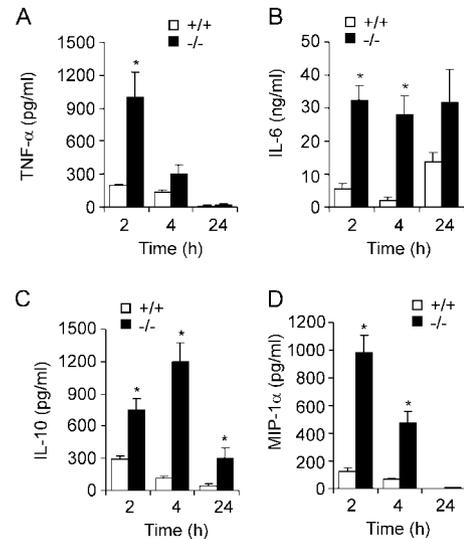


FIGURE 4. *Mkp-1*^{-/-} mice mount an enhanced inflammatory response upon infection with an ATCC strain of *S. aureus*. *Mkp-1*^{-/-} and *Mkp-1*^{+/+} mice were inoculated *via* the tail vein with live *S. aureus* bacteria at a dose of 1.75×10^7 CFU/g body weight. Blood was harvested at the indicated times. The concentrations of TNF- α (A), IL-6 (B), IL-10 (C), and MIP-1 α (D) in the serum were assessed by ELISA. Data represent the means \pm SE of six independent experiments. *, $p < 0.05$, compared with WT mice.

These results also suggest that iNOS but not eNOS is primarily responsible for the dramatic increase in circulatory NO levels in *Mkp-1*-deficient mice.

Deletion of *Mkp-1* predisposes mice to organ dysfunction following challenge with Gram-positive bacterial components

Mkp-1^{-/-} and *Mkp-1*^{+/+} mice were challenged with PBS or PepG plus LTA i.p., and after 24 h the mice were sacrificed to harvest blood, lungs, and livers. The lung tissues were sectioned, stained with H&E, and examined microscopically. The lungs isolated from the mice injected with PBS were normal and no neutrophils were detected in the lungs of either WT or *Mkp-1*^{-/-} mice (Fig. 3A). Although WT mice injected with PepG plus LTA showed only a mild pulmonary inflammation indicated by the presence of a small number of neutrophils in the alveolar septa, these agents caused more severe lung inflammation in *Mkp-1*^{-/-} mice. After PepG and LTA challenge, massive neutrophil infiltration, thickening of the alveolar septa, and accumulation of fluid in the alveolar spaces were observed in the lungs from *Mkp-1*^{-/-} mice but not in those from the WT mice (Fig. 3A). The infiltration of neutrophils into the lung tissues following PepG and LTA challenge was further confirmed by increased MPO activity in lung homogenates (Fig. 3B). MPO is a marker of inflammatory leukocytes, particularly neutrophils (38). MPO activity in the lung tissues isolated from *Mkp-1*^{-/-} mice treated with PepG and LTA was \sim 4-fold higher than that in the lungs harvested from similarly treated WT mice. The lack of MPO activity in the PBS-treated WT or *Mkp-1*^{-/-} mice indicate that PBS did not cause lung inflammation in either strain of mice. Relative lung weight, defined as wet right lung weight/body weight, is an index of fluid accumulation in the lung. The relative lung weights of the WT and *Mkp-1*^{-/-} mice treated with PBS were similar (Fig. 3C). Although challenge of WT mice with PepG plus LTA did not result in an appreciable change in relative lung weight, PepG plus LTA challenged *Mkp-1*^{-/-} mice had a 38% increase in relative lung weight, indicating pulmonary edema in these mice.

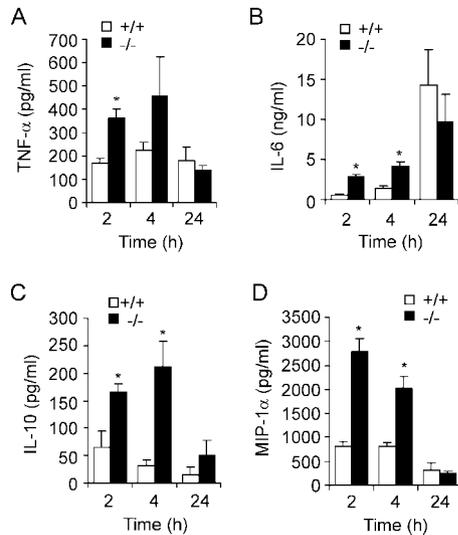


FIGURE 5. *Mkp-1* KO mice exhibit a more robust inflammatory response than WT mice upon infection with a clinically isolated strain of *S. aureus*. *Mkp-1*^{-/-} and *Mkp-1*^{+/+} mice were exposed to live *S. aureus* bacteria isolated from a patient with vertebral osteomyelitis at a dose of 3.5×10^6 CFU/g body weight via the tail vein. Blood was harvested at the indicated time, and the serum concentrations of TNF- α (A), IL-6 (B), IL-10 (C), and MIP-1 α (D) were determined by ELISA. Data represent the means \pm SE of six independent experiments. *, $p < 0.05$, compared with WT mice.

Liver and kidney are vital organs that play an important role in host defense against bacterial infection (20, 37). To examine whether MKP-1 plays a significant role in liver function during the host response to Gram-positive bacterial infection, we analyzed the histology of livers isolated from mice treated with PBS or PepG plus LTA. Histological examination of the livers from WT and *Mkp-1*^{-/-} mice treated with PBS revealed similar architecture and cell populations (Fig. 3D). Administration of PepG and LTA together resulted in a significant neutrophil infiltration in the vicinity of the bile ducts in the livers from WT mice (Fig. 3D). Compared with the WT livers, there were significantly more neutrophils in the areas surrounding the bile ducts in the livers of *Mkp-1*^{-/-} mice. Furthermore, marked sinusoidal dilation also occurred in the livers of *Mkp-1*^{-/-} mice after challenge with PepG and LTA, whereas the livers of WT mice treated with PepG and LTA appeared to have normal architecture (Fig. 3D). Additionally, depletion of glycogen stores were observed in *Mkp-1*^{-/-} mice treated with PepG

and LTA but not in similarly treated WT mice or in PBS-treated *Mkp-1*-deficient mice. Glycogen is the main form of energy stored in hepatocytes and glycogen is lost during the processing of liver sections resulting in the appearance of widespread vacuolated and “pale” spaces in hepatocytes. As shown in Fig. 3D, glycogen stores were present in the hepatocytes of the WT mice, regardless of whether these mice were treated with PBS or PepG and LTA. The hepatocytes in the PBS-treated *Mkp-1*^{-/-} mice appeared to contain a comparable amount of glycogen, suggesting that without challenge energy stores in the livers of *Mkp-1*^{-/-} were normal. However, upon PepG plus LTA challenge, glycogen stores disappeared from the hepatocytes in *Mkp-1*^{-/-} mice, as indicated by the complete disappearance of vacuolated and “pale” spaces. These findings suggest that *Mkp-1* KO mice either had difficulty with energy intake and/or accelerated energy consumption when challenged with PepG and LTA.

Kidney function was examined by measuring BUN concentrations (Fig. 3E). BUN concentrations did not differ between the WT and *Mkp-1*^{-/-} mice injected with PBS (11.4 ± 2.1 vs 11.7 ± 2.0 mg/dl, respectively). Although challenge with PepG and LTA did not result in a significant change in BUN levels in WT mice, such challenge caused an 8-fold increase in BUN levels in the *Mkp-1*^{-/-} mice, increasing BUN levels from 11.7 ± 2.0 to 94.0 ± 31.0 mg/dl. Taken together, these results indicate that deletion of the *Mkp-1* gene results in the rapid development of multiorgan dysfunction syndrome upon exposure to Gram-positive bacterial components.

S. aureus infection results in greater cytokine production in *Mkp-1*^{-/-} mice than in WT mice

To investigate the role of MKP-1 in host defense during Gram-positive bacterial infection, we infected *Mkp-1*^{-/-} and *Mkp-1*^{+/+} mice with two strains of *S. aureus*. First, *S. aureus* FDA 209P was cultured to logarithmic phase and injected into WT and *Mkp-1*^{-/-} mice via the tail vein at a dose of 1.75×10^7 CFU/g body weight. This dose was chosen because it did not cause death within 48 h in either WT or *Mkp-1*^{-/-} mice. Peripheral blood was collected 2, 4, or 24 h postbacterial infection to assay for blood cytokines (Fig. 4). Compared with WT mice infected with *S. aureus*, infected *Mkp-1*^{-/-} mice had markedly greater serum concentrations of TNF- α . In both WT and *Mkp-1*^{-/-} mice, TNF- α reached the highest serum concentrations at 2 h and declined to nearly basal levels by 24 h postinfection (Fig. 4A). IL-6 levels were higher in infected *Mkp-1*^{-/-} mice than in infected WT mice at all time points studied (Fig. 4B). *S. aureus* infection also resulted in increased serum IL-10

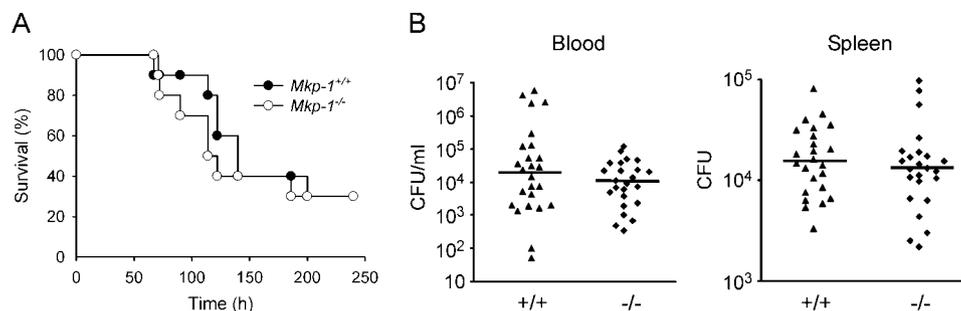


FIGURE 6. WT and *Mkp-1* KO mice exhibit similar survival and bacterial burden after infection with live *S. aureus* bacteria. Mice were injected i.v. with live *S. aureus* FDA209P bacteria at a dose of 7.5×10^6 CFU/g body weight. Animal survival was monitored for 10 days. To determine bacterial burden, mice infected with live bacteria at a dose of 7.5×10^6 CFU/g body weight were sacrificed 24 h postinfection. Blood and spleens were harvested and bacterial burden was determined by bacterial colony formation assays. A, Survival curves of WT and *Mkp-1* KO mice. Each group consisted of 10 mice. B, Bacterial burden in WT and *Mkp-1* KO mice. Horizontal lines represent median bacterial burden in 24 WT and 24 *Mkp-1* KO mice, with each symbol representing an individual animal.

Table I. Survival of WT and *Mkp-1*^{-/-} mice 10 days after inoculation with live *S. aureus* bacteria^a

Genotype	Dose of Inoculated <i>S. aureus</i> (×10 ⁷ CFU/g body weight)	Death at 10 Days
<i>Mkp-1</i> ^{+/+}	0.25	3/10
	0.75	7/10
	2.50	10/10
<i>Mkp-1</i> ^{-/-}	0.25	1/10
	0.75	7/10
	2.50	10/10

^a LD₅₀ was estimated to be 1.00 × 10⁸ CFU for WT mice and 1.15 × 10⁸ CFU for *Mkp-1*^{-/-} mice, assuming the body weight of every mouse was 20 g.

levels (Fig. 4C). Although serum IL-10 levels in WT mice reached maximal levels 2 h after bacterial infection and then declined gradually, infected *Mkp-1*^{-/-} mice exhibited the highest serum IL-10 levels at 4 h. Similarly, IL-10 levels were substantially higher in *Mkp-1*^{-/-} mice than in WT mice throughout the 24 h experimental period. Interestingly, serum MIP-1α levels were also greater in infected *Mkp-1*^{-/-} mice than in WT mice (Fig. 4D). The overall kinetics of serum MIP-1α accumulation in both WT and *Mkp-1*^{-/-} mice were similar to those of serum TNF-α levels, suggesting that TNF-α and MIP-1α may be regulated by similar mechanisms.

To mimic clinical Gram-positive bacterial infection, we infected WT and *Mkp-1*^{-/-} mice with a virulent strain of CA-MRSA isolated from a pediatric patient with vertebral osteomyelitis (33). Because this isolate is more virulent than *S. aureus* FDA 209P, we injected mice via the tail vein with a lower dose of bacteria (3.5 × 10⁶ CFU/g body weight). Like *S. aureus* FDA 209P, infection of *Mkp-1*^{-/-} mice with this clinical isolate of *S. aureus* resulted in a more robust inflammatory response than that seen in similarly infected WT mice. Serum TNF-α levels in the *Mkp-1*^{-/-} mice were 113% higher than those in the WT mice at 2 h postinfection (Fig. 5A). Likewise, serum IL-6 levels in the infected *Mkp-1*^{-/-} mice were higher than those in the infected WT mice at both 2 and 4 h postinfection, although IL-6 levels were no longer different between the two groups at 24 h (Fig. 5B). Similarly, serum IL-10 and MIP-1α levels in the infected *Mkp-1*^{-/-} mice were significantly higher than those in the WT mice at 2 and 4 h (Fig. 5, C and D). Taken together, these results indicate that the lack of *Mkp-1* results in a more robust inflammatory response following infection with this important Gram-positive bacterium.

To understand the effect of *Mkp-1* deficiency on host survival, we infected WT and *Mkp-1*^{-/-} mice with three doses of live *S. aureus* FDA 209P bacteria, and monitored animal survival over 10

days (Fig. 6A). Despite the observed substantial difference in cytokine production between the two groups of mice, no significant differences in survival were observed between WT and *Mkp-1* KO mice (Table I). The LD₅₀ values of the two groups of mice were very similar (1.0 × 10⁸ CFU for WT mice and 1.15 × 10⁸ CFU for *Mkp-1*^{-/-} mice). Likewise, there was no significant difference in survival between the two groups of mice following inoculation with the clinical CA-MRSA bacterial isolate (data not shown).

To assess the role of MKP-1 in bacterial clearance, we infected the two groups of mice with live *S. aureus* FDA 209P bacteria, and measured bacterial loads in both blood and spleen 24 h later. In both blood and spleen, bacterial burden varied widely within both groups of mice. There was no significant difference in bacterial loads between the two groups of mice in either blood or spleen (Fig. 6B). The tremendous variation in bacterial burden highlights the need for precise control of bacterial challenge in determining the importance of MKP-1 in Gram-positive bacterial sepsis. Because we were concerned that the variation in bacterial burden within each group may have masked an effect of MKP-1 deficiency on mortality in these mice, we next examined the response of these animals to heat-killed staphylococci.

Mkp-1 KO mice exhibit a higher rate of mortality upon challenge with heat-killed *S. aureus*

To better control bacterial burden, and more importantly, to more closely mimic the clinical course in patients with Gram-positive bacterial sepsis who have been treated with bactericidal antibiotics, we challenged mice with dead *S. aureus* and monitored mortality. *S. aureus* FDA 209P bacteria were heat-killed and administered to WT and *Mkp-1*^{-/-} mice at a dose equivalent to 2.5 × 10⁸ CFU/g body weight. Compared with WT mice, *Mkp-1*^{-/-} mice exhibited a markedly higher rate of mortality after challenge with heat-killed bacteria (Fig. 7A). Although the mortality among *Mkp-1*^{-/-} mice was 82% by 7 days, mortality among similarly treated WT mice was only 29% (Fig. 7A). To understand the basis for the difference in mortality between the two groups of mice, cytokine production after challenge with heat-killed bacteria was examined. *Mkp-1*-deficient mice produced greater amounts of cytokines, including TNF-α, IL-6, and IL-10, than did WT mice (Fig. 7B).

Mice challenged with heat-killed bacteria or vehicle (PBS) were sacrificed 24 h posttreatment to assess the function of vital organs. Histological analysis indicated severe neutrophil infiltration and edema in the lungs of *Mkp-1*^{-/-} mice challenged with heat-killed bacteria (Fig. 8A). Although neutrophil infiltration was also observed in the lungs of similarly challenged WT mice, the severity

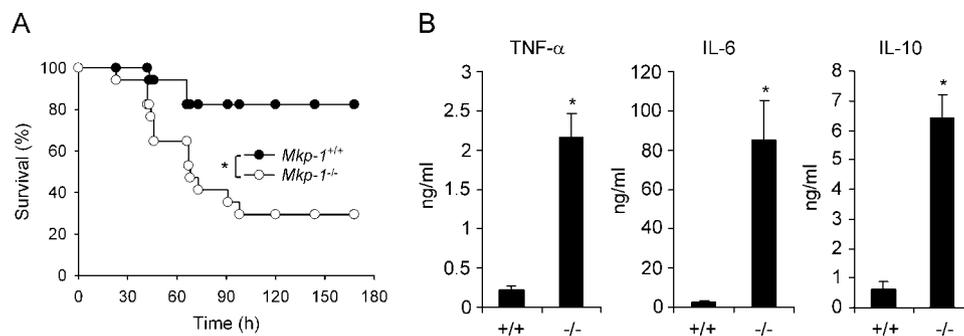


FIGURE 7. *Mkp-1* KO mice are more vulnerable to challenge with heat-killed Gram-positive bacteria and produced greater amounts of proinflammatory cytokines than WT mice. Mice were challenged with heat-killed *S. aureus* FDA 209P i.v. at a dose equivalent to 2.5 × 10⁸ CFU/g body weight. Survival was monitored over 7 days after bacterial challenge. **A**, Survival curves of WT and *Mkp-1* KO mice. Each group consisted of 17 animals. **B**, Cytokine production in WT and *Mkp-1* KO mice after challenge with heat-killed bacteria. Mice were sacrificed 3 h postbacterial challenge, and cytokines in the serum were measured by ELISA. Values represent mean ± SE from four individual animals. *, *p* < 0.05, compared with WT mice.

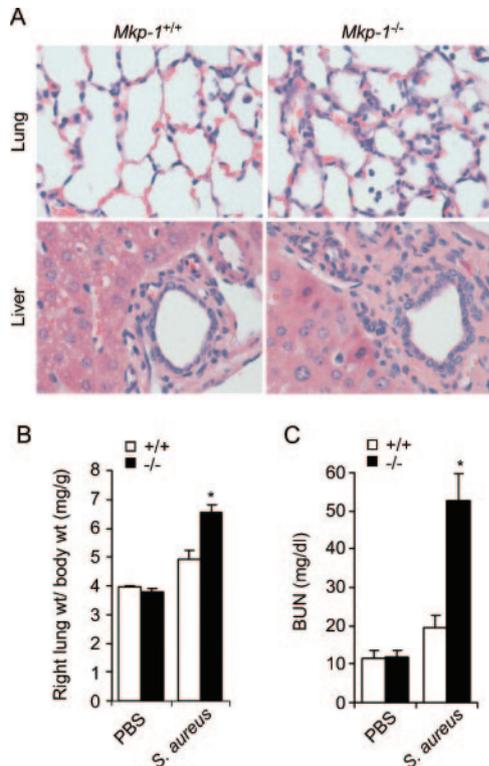


FIGURE 8. Heat-killed *S. aureus* causes more severe damage to vital organs in *Mkp-1* KO mice than in WT mice. WT and *Mkp-1*^{-/-} mice were challenged with heat-killed *S. aureus* FDA 209P i.v. at a dose equivalent to 2.5×10^8 CFU/g body weight. These animals were weighted and sacrificed 24 h later. The livers and left lungs were fixed and tissue sections were stained with H&E (A). The right lungs were weighed to evaluate pulmonary edema using the ratio of wet right lung weight-to-body weight (B). Blood was harvested by cardiac puncture to measure BUN (C). Results presented in B and C are means \pm SE of 5 independent experiments. *, $p < 0.05$, compared with similarly treated WT mice. Pictures presented in A are representative histological images.

was substantially less than their KO counterparts (Fig. 8A). Confirming the severe pulmonary edema observed in the bacteria-challenged *Mkp-1*^{-/-} mice, the relative lung weights, indicated by the ratio of right lung weight-to-body weight, were significantly higher in bacteria-challenged *Mkp-1*^{-/-} mice than in similarly treated WT mice (Fig. 8B). Greater neutrophil infiltration in the vicinity of bile ducts and more profound glycogen depletion were observed in the livers of bacteria-challenged *Mkp-1*^{-/-} mice compared with similarly treated WT mice (Fig. 8A). *Mkp-1*^{-/-} mice challenged with heat-killed bacteria also had higher amounts of BUN than similarly treated WT mice or PBS-treated mice (Fig. 8C). There were no significant differences in organ histology between the two groups of mice injected with PBS (data not shown). Taken together, these results clearly indicate that MKP-1 acts as a critical negative regulator in the innate immune response to Gram-positive bacteria. Our studies suggest that *Mkp-1* is a key factor determining the vulnerability to shock and mortality during Gram-positive bacterial infection.

Discussion

In this study, we demonstrated that MKP-1 is critical in the host inflammatory response to the virulent Gram-positive bacterium, *S. aureus*. We found that MKP-1 is the primary phosphatase responsible for the inactivation of both p38 and JNK in primary macrophages stimulated with either the Gram-positive bacterial wall

components, PepG or LTA (data not shown). We observed that deletion of *Mkp-1* gene resulted in an augmented inflammatory response to either PepG or LTA challenge as evidenced by the biosyntheses of TNF- α and IL-6 in innate immune cells (data not shown). When challenged with PepG and LTA, *Mkp-1* KO mice, but not WT mice, exhibited a substantial increase in the expression of iNOS in both the lungs and livers (Fig. 2). Only the *Mkp-1*-deficient mice exposed to Gram-positive bacterial products developed severe abnormalities in lung, liver, and kidney function (Fig. 3). Finally, we found that *Mkp-1* KO mice not only exhibited more robust inflammatory responses to cell wall components isolated from *S. aureus* (Figs. 1–3), but also to live or heat-killed *S. aureus* bacteria (Figs. 4, 5, 7, and 8). Consequently, upon challenge with heat-killed bacteria *Mkp-1* KO mice exhibited increased incidences of multiorgan failure and mortality compared with WT mice (Figs. 7 and 8). Our results presented in this report clearly establish that MKP-1 is an important negative regulator of the host inflammatory response during Gram-positive bacterial infection. By restraining the inflammatory response to bacterial infection, MKP-1 serves to prevent septic shock and maintain organ function.

Although MKP-1 was initially defined as a phosphatase specific for ERK MAPK (39, 40), more recent studies have provided compelling evidence supporting the notion that MKP-1 prefers p38 and JNK as substrates (41). We and other laboratories have recently shown that in primary macrophages stimulated with LPS, MKP-1 is the primary phosphatase responsible for the dephosphorylation of p38 and JNK (29, 30, 32). The fact that *Mkp-1*-deficient macrophages stimulated with Gram-positive bacterial components exhibited prolonged p38 and JNK activation also support the notion that MKP-1 is primarily responsible for the inactivation of p38 and JNK during Gram-positive bacterial infection (data not shown). Nonetheless, it should be pointed out that even in the absence of MKP-1 both p38 and JNK were eventually inactivated, albeit at much slower rates than in the presence of MKP-1 (data not shown), suggesting that other phosphatases may cooperate with MKP-1 in the feedback control of these MAPK pathways. So far 10 MKPs have been identified from mammalian cells (27) and at least 5 MKPs are expressed in macrophages, including MKP-1, MKP-2, MKP-5, MKP-M, and PAC-1 (35, 42–44). Both MKP-5 and PAC-1 have been shown to inactivate JNK in vivo in macrophages (42, 44). Thus, it is likely that MKP-1 cooperates with other MKP family members such as MKP-5 and PAC-1 in the dephosphorylation of p38 and JNK in innate immune cells during Gram-positive bacterial infection.

An interesting finding from this study is that the *Mkp-1* KO mice exhausted their glycogen storage in hepatocytes after challenge with PepG and LTA or heat-killed *S. aureus* (Figs. 3 and 8). In addition to PepG and LTA or heat-killed *S. aureus*, challenge of *Mkp-1* KO mice with LPS also resulted in glycogen depletion in the livers (data not shown). Although the exact mechanism underlying this dramatic difference in liver glycogen stores between the WT and *Mkp-1* KO mice is unclear, there are a number of possible explanations. The simplest explanation for this phenomenon may be that after challenge with bacterial products or heat-killed bacteria *Mkp-1* KO mice experience more severe distress than WT mice due to greater blood cytokines levels, such as TNF- α and IL-6. This is consistent with the experimental observation that after challenge with PepG and LTA or with heat-killed bacteria, most *Mkp-1* KO mice became lethargic and ceased to consume food within hours while almost all WT mice continued to demonstrate normal activity and appetite. In addition to the decreased food intake, the loss of glycogen stores in the livers of *Mkp-1* mice may also be partially due to greater energy expenditure. Because of the

higher blood TNF- α and IL-6 levels in *Mkp-1*-deficient mice (Figs. 1 and 7), it is tempting to speculate that the *Mkp-1* KO mice might have developed a more severe acute phase response, thus consuming more energy than the WT mice.

Although we observed increased incidences of organ dysfunction in *Mkp-1* KO mice following challenge with PepG and LTA (Fig. 3), we were unable to determine whether *Mkp-1* KO mice experience a higher mortality rate than WT mice due to the prohibitively high cost of these bacterial components. Unlike LPS that elicits a striking mortality at doses of 1.5–5 mg/kg body weight (29), substantially higher (>10-fold) doses of PepG or LTA are required to induce a robust inflammatory response. To overcome this limitation, and perhaps more importantly to complete our studies using an animal model of Gram-positive bacterial sepsis that is relevant to clinical settings, we challenged mice with live or heat-killed whole *S. aureus* bacteria to investigate the function of MKP-1 in Gram-positive bacterial sepsis (Figs. 4–8). However, we were unable to detect a significant difference in survival between WT and *Mkp-1* KO mice after infection with live *S. aureus* (Table I and Fig. 6A), despite significant differences in cytokine production between the two groups of mice (Figs. 4 and 5). In contrast, we documented greatly increased mortality in *Mkp-1* KO mice challenged with heat-killed *S. aureus* (Fig. 7).

It is possible that we failed to detect a difference in survival after infection with live bacteria between WT and *Mkp-1* KO mice because of the large variations in bacterial burden within each group of mice. We found that either within the *Mkp-1*^{+/+} group or within the *Mkp-1*^{-/-} group, the bacterial loads in the blood and spleens varied by as much as thousand-folds (Fig. 6B). The lack of differences in bacterial clearance between WT and *Mkp-1*-deficient mice strongly suggests that the inflammatory response in WT mice is already optimal for the required antibacterial mechanisms. The huge variation in bacterial loads is probably due to the dynamic nature of the host defense against bacteria rather than variation in the inoculum. This speculation is based on two observations: 1) the variations of the cytokine responses shortly (2–4 h) after injection were relatively small in both groups (Figs. 4 and 5) and 2) mice challenged with heat-killed bacteria exhibited rather uniform cytokine production and a clear difference in mortality (Fig. 7). The mixed C57/129 genetic background of the mice used, however, could also be a reason for the large variation in bacterial loads within one genotype.

It is also possible that the exaggerated inflammatory response in mice lacking *Mkp-1* is a double-edged sword, helping control bacterial replication in early stages of the infection but then resulting in damage to host tissues later in the course of the infection. This would parallel the observations made regarding the role of cytokines such as TNF- α in the host response to infections caused by another Gram-positive pathogen, *Streptococcus pneumoniae* (45, 46). Clearly, overproduction of TNF- α and other inflammatory mediators contributes to the pathogenesis of septic shock and multiorgan failure during overwhelming pneumococcal infection. However, interfering with the ability of mice to produce TNF- α leads to more severe pneumococcal pneumonia, indicating a key role for TNF- α in the early innate immune response to this organism (45, 46). Interestingly, we found that the eventual clearance of the staphylococcal infection was not significantly affected by knockout of *Mkp-1* in these mice (Fig. 6B), suggesting that timely induction of MKP-1 could be a promising treatment for sepsis patients by reducing inflammatory injury.

To control the bacterial challenge precisely, we used the i.v. injection of heat-killed *S. aureus* bacteria in a series of experiments (Figs. 7 and 8). In many ways, this model simulates the inflammatory response during clinical sepsis. Patients admitted to hos-

pital presenting with signs of sepsis are usually treated promptly with bactericidal antibiotics that rapidly reduce the bacterial burden. Despite this, morbidity and mortality related to sepsis remains high, in large part because of harmful inflammatory response that persists after antibiotic therapy is begun. In fact, considerable evidence suggests that rapid killing of the organisms by bactericidal antibiotics leads to an augmented, potentially harmful inflammatory response (47). We have recently reported that exposure of macrophages to *S. aureus* isolates in the presence of bactericidal antibiotics triggers the release of large amounts of TNF- α and NO (33). Using heat-killed *S. aureus* bacteria, we confirmed many observations made with purified bacterial components and demonstrated that *Mkp-1* KO mice are more vulnerable to septic shock and multiorgan failure during Gram-positive bacterial infection (Figs. 7 and 8). Given the importance of MKP-1 in host protection during Gram-positive bacterial infection, modulation of MKP-1 expression could represent a novel approach to treat severe bacterial sepsis and septic shock syndrome.

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Disclosures

The authors have no financial conflict of interest.

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