Mitogen-Activated Protein Kinase-Activated Protein Kinase 2-Deficient Mice Show Increased Susceptibility to *Listeria monocytogenes* Infection

Martin D. Lehner, Frank Schwoebel, Alexey Kotlyarov, Marcel Leist, Matthias Gaestel and Thomas Hartung

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Mitogen-Activated Protein Kinase-Activated Protein Kinase 2-Deficient Mice Show Increased Susceptibility to *Listeria monocytogenes* Infection

Martin D. Lehner, Frank Schwoebel, Alexey Kotlyarov, Marcel Leist, Matthias Gaestel, and Thomas Hartung

Mitogen-activated protein kinase-activated protein kinase 2 (MK2) is one of several kinases activated through direct phosphorylation by p38 mitogen-activated protein kinase. MK2 regulates LPS-induced TNF mRNA translation, and targeted mutation of the MK2 gene renders mice more resistant to d-galactosamine plus LPS-induced liver damage. In the present study, we investigated the role of MK2 in immune defense against *Listeria monocytogenes* infection. MK2-deficient mice displayed diminished resistance to *L. monocytogenes* due to impaired control of bacterial growth. The increase in bacterial load in MK2−/− mice was associated with normal levels of IL-1β, IL-6, and IFN-γ, whereas TNF production was strongly attenuated. In line, MK2-deficient bone marrow-derived macrophages showed impaired release of TNF, but not of IL-1β, in response to various bacterial stimuli in addition to decreased phagocytosis of fluorescence-labeled bacteria. Furthermore, spleen cells from MK2−/− mice displayed diminished IFN-γ synthesis after stimulation with *L. monocytogenes*. In contrast, MK2 deficiency had no effect on macrophage generation of NO or on oxidative burst activity in response to *L. monocytogenes*. These results indicate an essential role of MK2 in host defense against intracellular bacteria probably via regulation of TNF and IFN-γ production required for activation of antibacterial effector mechanisms. *The Journal of Immunology*, 2002, 168: 4667–4673.

Inhibition of the p38 mitogen-activated protein kinase (MAPK) pathway that is involved in a number of cellular stress responses (1–3) has been used successfully to decrease cytokine production in vitro (4, 5) and inflammatory damage in a number of murine models of a dysregulated immune system in vivo (6–9). However, the central role of p38 MAPK, which is responsible for the activation of a number of downstream kinases (10–14) and transcription factors (15, 16), limits the use of p38 inhibitors as a selective anti-inflammatory strategy. This view has been supported recently by van den Blink et al. (17), who demonstrated contrasting effects of the p38 inhibitor SB203580 on cytokine production in vitro and in vivo, depending on the cell type and inflammatory model used. Thus, downstream substrates of the MAPK pathways could represent promising targets for a specific suppression of cytokine production. MK2-activated protein kinase 2 (MK2), a kinase phosphorylated by p38 MAPK and p42/44 MAPK (18), has been shown to be involved in posttranscriptional regulation of TNF and IFN-γ expression (19). Targeted disruption of the MK2 gene in mice results in strongly decreased TNF production associated with enhanced resistance to liver damage induced by concomitant injection of d-galactosamine plus LPS in vivo (19), suggesting that MK2 represents an interesting pharmacological target for specific anti-inflammatory therapy.

However, an intact cytokine response is essential for efficient host defense against invading pathogens (20–25) and global suppression of cytokine production via, for example, anti-inflammatory glucocorticoid therapy strongly increases susceptibility to microbial infection (26–29). These considerations prompted us to study the effect of MK2 deficiency on host defense in a murine model of bacterial infection in vivo and on leukocyte effector functions in vitro.

To this aim, we infected MK2−/− and control mice with a virulent strain of the facultative intracellular bacterium *Listeria monocytogenes* and determined the survival, bacterial load, and mediator production. Our results indicate that MK2 plays a critical role in resistance against intracellular bacteria probably via regulation of selected cytokines and phagocytosis.

**Materials and Methods**

**Mice**

Mice carrying a mutation in the catalytic domain of the MK2 gene (19) were kept at 24°C, 55% humidity, at a 12-h day-night rhythm on a diet of Altromin C 1310 (Altromin, Lage, Germany). Age- and sex-matched male and female MK2−/− and MK2+/+ littermates on a mixed background (S129 × C57BL/6) bred in the internal animal facility of the University of Konstanz were used for the experiments. All animals received humane care in accordance with the National Institutes of Health guidelines and the legal requirements in Germany.

**Bacteria**

The hemolytic *L. monocytogenes* EGD strain was generously provided by S. H. E. Kaufmann (Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany). After passaging in vivo, bacteria were grown on tryptic soy broth (Difco, Detroit, MI) at 37°C and gentle rotation. Aliquots of 3 × 10⁷ viable bacteria/ml in 25% glycerin were stored at −80°C. Just before use, stock aliquots were thawed and diluted in pyrogen-free saline. For in vitro experiments, aliquots were incubated for 1 h at 70°C to inactivate bacteria. These heat-killed *L. monocytogenes*
(HKLM) were opsonized by incubation with 10% normal mouse serum at 37°C for 30 min.

**Experimental infection**

*L. monocytogenes* infection was initiated by i.v. inoculation with 10⁴ or 1.5 × 10⁷ bacteria per kilogram of body weight, and survival was monitored for 14 days. Bacterial load and cytokine levels were analyzed 48 and 72 h after challenge in parallel experiments. In addition, leukocyte counts were determined at 72 h.

**Determination of CFU**

CFU were determined from serial dilutions of organ homogenates or blood plated on Columbia blood agar plates (Heipha, Heidelberg, Germany) and incubated at 37°C for 30 h.

**Leukocyte counts**

Blood was obtained by cardiac puncture under terminal pentobarbital anaesthesia (Narcoren; Merial, Hallbergmoos, Germany). White blood cell counts were determined microscopically in a Neubauer chamber after erythrocyte lysis with Türk’s solution (Merck, Darmstadt, Germany). Leukocyte differential counts were done on May-Grünwald/Giemsa-stained smears.

**Cytokine ELISA**

Cytokine levels in organ homogenates, plasma, and culture supernatants were determined by ELISA, using specific Abs purchased from PharMingen (Hamburg, Germany). For the detection of TNF in vivo samples, the OpEia kit from PharMingen was used. The detection limits were <25 pg/ml.

**Generation of BMDM**

Mice were killed by cervical dislocation, and bone marrow cells were isolated from the femurs. After erythrocyte lysis with 0.17 M ammonium chloride, bone marrow cells were cultured at an initial density of 10⁵ cells/ml on 94/16-mm microbiology plates (Greiner, Nuringen, Germany) in 20 ml RPMI 1640 with glutamine supplemented with 10% heat-inactivated FCS (Biochrom, Berlin, Germany), antibiotics (100 U/ml penicillin, 10 mg/ml streptomycin; Life Technologies, Eggenstein, Germany), and 20% M-CSF-containing L929 supernatant for 6–9 days. Differentiated bone marrow-derived macrophages (BMDM) were harvested with cold PBS without Ca²⁺ and Mg²⁺, resuspended in medium without L929 supernatant, and used at a density of 6.5 × 10⁵ cells/ml.

**Determination of cytokine production by BMDM**

BMDM (6.5 × 10⁵ cells/well) from MK2⁻/⁻ and MK2⁺/⁺ mice were added to 96-well microtiter plates (Cellstar; Greiner) and allowed to adhere for at least 3 h. Then, cells were stimulated by addition of LPS (Salmonella abortus equi; Bioclot, Wustenhofen, Germany), lipopolysaccharide (LTA; Life Technologies, Egggenstein, Germany), and 20% M-CSF-containing L929 supernatant for 6–9 days. Cytokine levels in supernatants were determined.

**IFN-γ production by spleen cells**

Spleens were removed aseptically from male MK2⁻/⁻ and MK2⁺/⁺ mice, and single cell suspensions were generated in 1 ml saline by pressing spleens through 50-µm nylon meshes (BD Biosciences, Heidelberg, Germany). After lysis of erythrocytes with 0.17 M ammonium chloride, cells were washed and resuspended in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 10% FCS (Boehringer Mannheim, Mannheim, Germany) and 100 U/ml penicillin/streptomycin (Biochrom). A total of 1 × 10⁵ cells/well were added to 96-well culture plates (Greiner) and stimulated with serum-opsonized HKLM for 24 h at 37°C. IFN-γ was determined in supernatants by ELISA.

**Determination of macrophage phagocytosis**

Determination of macrophage phagocytosis was performed essentially as described (31), with the following modifications. BMDM (6.5 × 10⁵ cells/well) were plated in 96-well microtiter plates and allowed to adhere for at least 3 h. At different time points, tetramethylrhodamine-conjugated fluorescent E. coli particles were added to a final concentration of 5 µg/ml. In some experiments, the E. coli particles had been opsonized with 10% normal mouse serum at 37°C for 30 min before use (serum-opsonized E. coli). Phagocytosis was stopped by washing the cells twice with PBS to remove nonphagocytosed bacteria. Then, cells were lysed by addition of 100 µl well PBS plus 0.1% Triton X-100. Fluorescence was determined at 530 nm excitation and 590 nm emission wavelengths using a fluorescence microplate reader (microplate fluorescence reader FL 600; DeLux Labotechnik, Goedeinster, Germany). Cells without bacteria were used to determine the background fluorescence. Inhibition by low temperature or cytochalasin D was used to distinguish between binding and phagocytosis of bacteria in preliminary experiments.

**Determination of NO production**

BMDM (6.5 × 10⁵ cells/well) were pretreated with saline or 10 ng/ml recombinant murine (rmu)IFN-γ (a generous gift from G. R. Adolf, Bender, Vienna, Austria) for 45 min. Then, cells were stimulated by addition of LPS, LTA, or HKLM for 24 h. NO production was determined indirectly by measuring nitrite levels in supernatant by the Griess reaction (32).

**Determination of oxidative burst**

For determination of luminol-ECL (33), 10⁵ peritoneal lavage cells from naive mice per well were cultured in white 96-well cell culture plates (Cellstar; Greiner) for 24 h in the presence of either 0.9% NaCl/0.1% human serum albumin (HSA) or a combination of 10 ng/ml mufILN-γ plus 10 ng/ml LPS. Then, cells were washed twice with PBS and resuspended in HBSS without Phenol Red (Life Technologies) containing 10% FCS (Boehringer Mannheim). Background luminescence was recorded after addition of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, Deisenhofen, Germany) dissolved in 0.04% triethylamin (Sigma) at a final concentration of 1 mg/ml. Then, cells were stimulated by addition of either 10⁷ serum-opsonized HKLM or 1 mg zymosan (Sigma) per milliliter, and luminescence was determined for 2 s every 3.2 min for a period of 70 min at 37°C in a luminometer (VICTOR² multilabel counter; Wallac Instruments, Turku, Finland).

**Statistics**

Data are presented as SEM. Statistical differences were determined by the two-sided, unpaired Student t test after testing for normality using the Kolmogorov-Smirnov test. In case of multiple comparisons, the Bonferroni’s multiple comparison test of selected data pairs was used. In case of unequal variances (Bartlett’s t, p < 0.05), data were log transformed before analysis. Survival curves were generated according to the method of Kaplan and Meier and compared using the log-rank test. A value of p < 0.05 was considered significant. All tests were performed with GraphPad Prism, version 3.0 for Windows (GraphPad, San Diego, CA).

**Results**

**MK2-deficient mice display increased susceptibility to L. monocytogenes infection**

It has been shown previously that MK2-deficient mice, probably as a result of decreased TNF synthesis, are more resistant to inflammatory liver damage induced by the concomitant injection of LPS plus α-galactosamine, suggesting a decisive role of MK2 in the inflammatory response to endotoxin (19). To study the involvement of MK2 in inflammation and host defense during Gram-positive bacterial infection, we determined the susceptibility of MK2⁻/⁻ mice to infection with L. monocytogenes. MK2⁻/⁻ and MK2⁺/⁺ mice were infected i.v. with 1 × LD₅₀ (1.5 × 10⁵ CFU/kg), and the survival was monitored for 14 days. MK2⁻/⁻ mice displayed significantly increased mortality as compared with wild-type (wt) mice (survival 1 of 7 (MK2⁻/⁻) vs 9 of 10 (wt), p < 0.001; Fig. 1). However, a residual capacity to clear bacteria was retained in MK2⁻/⁻ mice, because all survived infection with 10⁴ bacteria per kilogram of body weight (data not shown).

**Increased bacterial load in MK2-deficient mice during L. monocytogenes infection**

To test whether the increased susceptibility of MK2⁻/⁻ mice to L. monocytogenes challenge was due to impaired bacterial elimination, we determined the bacterial load in blood and organ homogenates following infection with L. monocytogenes. No significant differences in CFU numbers were found at 48 h postinfection (data not shown). However, at 72 h postinfection, MK2⁻/⁻ mice displayed higher bacterial numbers in all organs tested (liver, spleen,
lung, blood), although only the differences in CFU counts in spleen and lung reached statistical significance (Fig. 2). These results suggest that despite comparable initial host defense, MK2−/− mice failed to control bacterial replication at the later stage of L. monocytogenes infection. Notably, the numbers of total blood leukocytes did not differ significantly between the two genotypes at 72 h postinfection (2.7 ± 0.3 × 10⁶/ml MK2+/+ vs 2 ± 0.3 × 10⁶/ml MK2−/−), n = 5; p = 0.12).

Decreased TNF production in MK2-deficient mice during L. monocytogenes infection

Efficient host defense against the intracellular pathogen L. monocytogenes is dependent on an intact cytokine response (20–22, 34, 35). Because previous data demonstrated a regulatory role of MK2 in TNF and IFN-γ induction after exposure to the model inflammatory stimulus LPS (19), we hypothesized that decreased production of these cytokines could be responsible for deficient antibacterial defense in MK2−/− mice.

We determined the production of several proinflammatory cytokines in blood and homogenates of liver, spleen, and lung 48 and 72 h after infection with 1.5 × 10⁸ Listerial/kg. The tissue/blood levels of IL-1β, IL-6, and IFN-γ at either time point of analysis were not significantly affected by the genotype (data not shown). However, TNF concentrations were strongly reduced in L. monocytogenes-infected MK2−/− animals at late stage of infection (Fig. 3), despite similar or even increased CFU numbers (see above).

Impaired TNF production by MK2−/− macrophages is stimulus independent

To characterize the effect of MK2 on TNF production in more detail, we performed in vitro studies on TNF release by isolated macrophages from wt and MK2−/− mice. As an extension of previous studies using the Gram-negative model stimulus LPS (19), we used also the Gram-positive analog, LTA, and entire Gram-positive or Gram-negative bacteria.

MK2−/− BMDM displayed significantly reduced TNF release in response to all stimuli tested (LPS, LTA, E. coli, HKLM) (Fig. 4). This indicates a key role of MK2 in TNF production elicited by upstream signal transduction pathways involving different Toll-like receptors (TLR). In contrast to TNF, reduction of IL-6 production was confined to LPS challenge, and IL-1β release was even enhanced in MK2−/− macrophages stimulated with HKLM (Fig. 4). These results support the in vivo finding of a critical involvement of MK2 in TNF regulation, whereas production of IL-6 and especially of IL-1β appears to be largely independent of MK2.

Decreased IFN-γ production by spleen cells from MK2−/− mice

In addition to the posttranscriptional control of TNF synthesis, previous studies indicated a regulatory role of MK2 in the synthesis of IFN-γ after LPS stimulation of spleen cells in vitro (19). In contrast, IFN-γ levels in MK2−/− mice 48 and 72 h after infection with L. monocytogenes were not significantly different from those of wt animals (data not shown). However, because samples were taken at later phases of infection with significantly higher bacterial load in MK2−/− mice (72 h), we chose a different approach to study early IFN-γ production in response to defined numbers of pathogens. To this aim, we determined in vitro IFN-γ production by isolated spleen cells from MK2−/− and MK2−/− mice upon stimulation with increasing concentrations of HKLM. Indeed, in this in vitro model, IFN-γ levels were significantly lower in supernatants from MK2−/− spleen cells (Fig. 5), confirming and extending previous findings of a major regulatory role of MK2 in IFN-γ production.

Diminished phagocytosis of MK2−/− macrophages

Engulfment of blood-borne bacteria and subsequent intracellular killing by cytokine-activated macrophages is a key defense mechanism during the early phase of listeriosis, restricting bacterial replication rate before the establishment of a specific and sterilizing T cell response (36). To test whether phagocytic activity depended on functional MK2, we determined the uptake of fluorescence-labeled E. coli particles by BMDM in an optimized quantitative phagocytosis assay. Uptake of bacteria, as measured by specific fluorescence increase, was approximately linear over several hours and could be blocked by addition of cytochalasin D or low temperature (Fig. 6A). As shown in Fig. 6B, MK2−/− BMDM displayed functional, albeit reduced, phagocytic activity compared with wt cells. Prior opsonization of E. coli with 10% normal mouse serum enhanced bacterial phagocytosis by cells from either genotype by ~60% of the respective value for nonopsonized bacteria (Fig. 6C), suggesting that complement receptors were still fully functional in MK2−/− cells and that a general mechanism of phagocytosis was impaired in the absence of MK2.

Normal oxidative burst activity of MK2−/− macrophages

Generation of reactive oxygen intermediates (ROI) by the NADPH-dependent oxidase has been shown to be critical for resistance against L. monocytogenes (37–39). We determined ROI production by adherent peritoneal cells from wt and MK2−/− mice in response to HKLM. No difference in peak activity or overall oxidative burst (area under the curve) was observed between cells from either genotype (Fig. 7A). When cells were pretreated for 24 h with a combination of LPS and rmIFN-γ, oxidative burst activity in response to HKLM (Fig. 6B), but not to zymosan (data not shown), was strongly increased. Although peak levels and area

![FIGURE 1](image-url) Decreased resistance of MK2−/− to L. monocytogenes infection. Female MK2+/+ (n = 10) and MK2−/− mice (n = 7) were injected i.v. with 1.5 × 10⁸ virulent L. monocytogenes per kilogram of body weight, and survival was monitored for 14 days. ***, p < 0.001 based on the log-rank test.

![FIGURE 2](image-url) Increased bacterial load of MK2−/− mice during L. monocytogenes infection. Female MK2+/+ (filled bars, n = 9) and MK2−/− mice (open bars, n = 9) were injected i.v. with 1.5 × 10⁸ virulent L. monocytogenes per kilogram of body weight. Animals were sacrificed 72 h after infection for determination of CFU in spleen, liver, and lung homogenates as well as in blood samples. *, p < 0.05; **, p < 0.01 vs MK2+/+ based on two-sided unpaired Student’s t test.
under the curve were slightly reduced in macrophages from MK2−/− mice (Fig. 7B), functional MK2 was not essential for oxidative burst activity, arguing against a direct regulation of macrophage NADPH oxidase by MK2.

### Differential effect of MK2 deficiency on NO production in vitro

In addition to activation of the NADPH oxidase, production of NO has been suggested as an important antimicrobial killing mechanism of activated macrophages (39–41). We determined the ability of BMDM from MK2−/− and wt mice to generate NO (as measured by nitrite accumulation in the medium over 24 h) in response to stimulation with LPS, LTA, and HKLM. Nitrite production in response to LPS stimulation was significantly decreased in MK-2-deficient cells. LTA or HKLM alone was not sufficient to trigger NO production in vitro (Table I). IFN-γ has been shown to be a potent costimulus for expression of the inducible NO synthase (iNOS) (42, 43). Therefore, we examined NO production by BMDM also in the presence of exogenously added rmuIFN-γ. Indeed, besides increasing NO production by LPS stimulus, addition of this cytokine boosted NO production in response to LTA as well as HKLM to levels comparable with those induced by LPS/IFN-γ. However, NO release in response to IFN-γ plus LTA/HKLM was not affected by MK2−/− deficiency, indicating that TLR2-specific stimuli can induce iNOS independent of MK2, provided that enough IFN-γ is present.

### Discussion

MK2-deficient mice show decreased release of TNF and NO in response to LPS challenge and increased resistance to inflammatory liver damage induced by the concomitant injection of d-galactosamine plus LPS (19), suggesting that MK2 could represent a

### FIGURE 3.

Cytokine levels in *Listeria*-infected MK2−/− mice. Female MK2+/+ (n = 4–9; filled bars) and MK2−/− (n = 4–9; open bars) mice were injected i.v. with 1.5 × 10⁵ virulent *L. monocytogenes* per kilogram of body weight. Animals were sacrificed at 48 and 72 h after infection for determination of cytokine levels in homogenates of spleen (A), liver (B), lung (C), or in plasma (D). *, p < 0.05; **, p < 0.01 vs MK2+/+ based on the unpaired Student t test.

### FIGURE 4.

Differential effect of MK2 deficiency on cytokine production by BMDM. Cytokine release by MK2+/+ (filled bars) and MK2−/− (open bars) BMDM (6.5 × 10⁵ cells/well) was determined 6 h after stimulation with the indicated concentrations of LPS, LTA, *E. coli* particles, or HKLM. Cells from four cultivation plates per group were independently adjusted to cell number and plated in duplicates. Data are expressed as means ± SEM. *, p < 0.05; **, p < 0.01 vs the respective MK2+/+ group based on ANOVA, followed by Bonferroni’s multiple comparison test.

### FIGURE 5.

Defective IFN-γ production in MK2−/− spleen cells. A total of 10⁶ spleen cells from three MK2+/+ (filled bars) and MK2−/− mice (open bars) was plated in triplicates and incubated for 24 h in the presence of the indicated numbers of HKLM. IFN-γ release was determined in supernatants. Pooled data are expressed as means ± SD. **, p < 0.01 vs MK2+/+ according to unpaired Student’s t test.

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*INCREASED SUSCEPTIBILITY OF MK2 KNOCKOUT MICE TO LISTERIOSIS*
promising target for anti-inflammatory therapy. However, host defense against microbial infection depends on an intact cytokine response, and suppression of cytokine production via inhibition of MK2 could increase susceptibility to infection. We addressed the role of MK2 in cytokine production and antimicrobial defense during infection with the facultative intracellular, Gram-positive pathogen *L. monocytogenes*. MK2-deficient mice succumbed readily to infection with an LD_{50} before a T cell-mediated immunity could develop. This increase in susceptibility was associated with a relative inefficiency to curb bacterial growth in spleen, liver, and lung, which are the major sites of bacterial replication. Primary host defense against *L. monocytogenes* requires an intact cytokine response; TNF and IFN-γ, especially, have been shown to be essential for the activation of listericidal activity in infected macrophages (22, 39, 44–46). In line with previous findings for LPS challenge, TNF levels were decreased in MK2-deficient mice infected with *L. monocytogenes*, indicating that TNF induction by Gram-positive infection is regulated by the MK2. Although disruption of the MK2 gene significantly increased the susceptibility to *Listeria* infection, MK2−/− mice were still able to control infection with a lower inoculum (10^7/kg), suggesting a moderate effect of MK2 on resistance compared with the drastically increased susceptibility of TNFR or IFN-γ receptor knockout mice (20–22). This can be explained by the finding that MK2−/− mice were still able to produce lower, but still substantial amounts of TNF and normal levels of IFN-γ. Our results of an increased bacterial load in MK2−/− mice confirm and extend recent data from van den Blink et al. (17), who demonstrated deficient control of bacterial replication after administration of the p38 inhibitor SB203580 in murine models of pneumonia and tuberculosis. However, our data demonstrate reduced TNF release in MK2−/− mice despite an increase in bacterial numbers, which is in contrast to the enhanced TNF production observed by van den Blink et al. (17). This difference could be due to inefficient inhibition of p38 in macrophages in vivo when SB203580 was used. Alternatively, because *Listeria* infection has been shown to induce signaling via both the p38 and the p42/p44 MAPK pathway (47–49), impairment of additional macrophage activation via p42/44 MAPK in MK2−/− mice could be responsible for the diverging effects of p38 inhibition and MK2 deficiency. During the first 48 h of listeriosis, polymorphonuclear granulocytes (PMN) play a pivotal role by engulfing free bacteria, e.g., in the sinusoids of the liver (50). Thus, impaired ability of MK2−/− mice to recruit PMN from the bone marrow could be responsible for the higher bacterial load observed in the MK2 knockout animals. Our data do not support a major defect in leukocyte recruitment, because no differences in total number of circulating leukocytes or leukocyte composition were found at 72 h postinfection. However, further studies using quantitative determination of PMN organ infiltration at earlier time points postinfection will be necessary to definitely settle this point. Besides infiltration of neutrophils, phagocytosis and breakdown of bacteria by resident macrophages of the reticuloendothelial system is a key defense mechanism to restrict bacterial dissemination and infection of more permissive cells such as hepatocytes during the

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**FIGURE 6.** Impaired phagocytosis by MK2−/− macrophages. BMDM were incubated with rhodamine-labeled *E. coli* for the times indicated, before washing and measurement of fluorescence of phagocytosed bacteria. A, Specificity of the assay is shown by inhibition of uptake by 25 μM cytochalasin D (Cyto D) or low temperature (4°C). Differences were analyzed by ANOVA, followed by Dunnett’s test. ***, p < 0.01. B, Uptake of bacteria was compared between MK2+/+ (filled symbols) and MK2−/− (open symbols) BMDM at 37°C. C, Similar experiments were performed with serum-opsonized bacteria. Data are from three independently adjusted BMDM cultures per genotype, with each measurement performed in triplicate. Data were analyzed by two-way ANOVA according to genotype and time. ***, p (genotype) < 0.01 (F = 8.8); ***, p < 0.0001 (F = 19.8), respectively, for B or C.

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**FIGURE 7.** Normal oxidative burst in MK2−/− macrophages. Oxidative burst was induced by addition of 10^8 HKLM/ml to MK2+/+ (filled symbols) and MK2−/− (open symbols) peritoneal macrophages pretreated with NaCl/HSA (A) or 10 ng/ml muIFN-γ plus 10 ng/ml LPS (B) for 24 h. Luminol-ECL was determined for 2 s at intervals of 3.2 min and is expressed as counts per second (CPS). Chemiluminescence was determined in triplicate with cells isolated from three mice per genotype. Pooled data are expressed as mean values ± SEM.
early course of *L. monocytogenes* infection (51). In addition, infiltrating monocytes and macrophages contribute to granuloma formation and digestion of bacteria at the site of infection (52, 53). Due to this pivotal role of macrophages in early control of bacterial replication, we were interested in determining the effect of MK2 disruption on macrophage functions in vitro. When we studied the uptake of fluorescence-labeled *E. coli*, BMDM from MK2−/− mice showed a moderate decrease in the rate of phagocytosis as compared with wt cells. In addition, measurement of TNF levels in supernatants from the same experiments indicated a drastic reduction in the capacity of MK2−/− BMDM to produce TNF in response to *E. coli*. The early onset of phagocytosis suggested that the observed differences in bacterial uptake were independent of the defect in TNF production. Attenuation of TNF release in MK2−/− macrophages was neither restricted to a certain class of stimuli, nor TLR specific, because similar results were obtained with particular (*E. coli* and *L. monocytogenes*) as well as soluble stimuli (LPS and LTA) signaling either via TLR2 (*L. monocytogenes* (54), LTA (55)), or TLR4 (*E. coli*, LPS (56)). This observation indicates that intracellular signaling pathways of the different receptors converge upstream of MK2. Following uptake by cytokine-activated macrophages, bacteria in the phagososome are confronted with a battery of microbiocidal substances such as reactive oxygen and nitrogen species (37, 39, 40). Evidence has been provided that generation of NO is an essential host mechanism in antilisterial defense because inhibition of iNOS increases bacterial load and susceptibility of mice to *L. monocytogenes* infection (40). Because previous data indicated diminished NO production in MK2−/− mice in response to LPS stimulation (19), we hypothesized that defective generation of reactive nitrogen intermediates by MK2−/− macrophages could contribute to the impaired control of bacterial replication in vivo. However, no NO production at all was detectable upon stimulation of BMDM from either genotype with LTA or HKLM unless very high concentrations (10^6 HKLM/ml) were used or cells were costimulated with IFN-γ. The finding that HKLM plus IFN-γ-costimulated BMDM from MK2−/− mice produced comparable amounts of NO as wt cells argues against a direct involvement of MK2 in the expression of the iNOS during listeriosis. In addition to NO production, the generation of ROI, which is considered a key listericidal mechanism of activated macrophages, was not significantly impaired in macrophages from MK2−/− mice. This finding is in contrast to previous results showing MK2-dependent oxidative burst by human neutrophils in response to fMLP or phorbol ester stimulation (57). This discrepancy could be due to the different cell types (human PMN vs murine macrophages) or stimuli (fMLP or PMA vs HKLM) used in the different studies.

Our results derived from MK2-deficient macrophages indicate that the increased susceptibility of MK2−/− mice to *L. monocytogenes* infection did not result from an inherent deficiency of macrophage-killing mechanisms. However, NO production as well as oxidative burst induced by HKLM strongly depended on costimulation with IFN-γ. Thus, an attenuation of early IFN-γ production could result in insufficient macrophage activation. Our results on IFN-γ production by isolated spleen cells upon stimulation with HKLM (Fig. 4) support this hypothesis. In contrast to the strong reduction of IFN-γ release in vitro, IFN-γ levels in vivo in MK2−/− mice infected with viable *L. monocytogenes* were not significantly different from those of wt mice. Nevertheless, because IFN-γ production by spleen cells increased with higher concentrations of HKLM, a potentially more drastic defect in IFN-γ production in vivo could have been compensated by the increase in bacterial load in MK2−/− mice at the time of IFN-γ determination (72 h). Determination of early IFN-γ levels immediately after infection will be necessary to clarify this question.

Experiments in knockout mice suggested additional macrophage listericidal mechanisms independent of NO/ROI production that required macrophage activation via the p55 TNFR (44). MK2 deficiency most markedly impaired TNF production, whereas the effect on phagocytosis as well as on the production of other cytokines (IL-1, IL-6) or NO was much less pronounced. From these results, it can be speculated that the defect of MK2−/− mice to restrict early growth of *L. monocytogenes* could be due to a combination of inefficient activation of these yet unknown TNF-dependent listericidal activity and the TNF/IFN-γ-dependent stimulation of NO generation and oxidative burst. Taken together, our results showing a decisive role of MK2 in host defense against virulent Gram-positive bacteria emphasize that caution must be taken when addressing MK2 inhibition as an anti-inflammatory therapy during acute or chronic infection.

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**References**


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**Table I. Nitrite production by BMDM**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>NaCl</th>
<th>LPS (ng/ml)</th>
<th>LTA (µg/ml)</th>
<th>HKLM (cells/ml)</th>
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</thead>
<tbody>
<tr>
<td>MK2−/− NaCl/HSA</td>
<td>&lt;1</td>
<td>13 ± 5</td>
<td>21 ± 6</td>
<td>34 ± 4</td>
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<tr>
<td>MK2−/− NaCl/HSA</td>
<td>&lt;1</td>
<td>8 ± 4</td>
<td>12 ± 5</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>MK2−/− IFN-γ</td>
<td>4 ± 2</td>
<td>67 ± 5</td>
<td>60 ± 11</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>MK2−/− IFN-γ</td>
<td>2 ± 1</td>
<td>54 ± 7</td>
<td>52 ± 7</td>
<td>59 ± 8</td>
</tr>
</tbody>
</table>

*MK2−/− and MK2−/− BMDM were pretreated for 45 min with either NaCl/HSA (co) or rmuIFN-γ (10 ng/ml) prior to addition of the indicated stimuli. Nitrite levels in supernatant were determined after incubation for 24 h at 37°C. For each genotype, BMDM from three culture plates were independently adjusted to 3.25 × 10^6 cells/ml and plated in triplicate (6.5 × 10^4 cells/well), yielding a total of nine values per treatment. All data are expressed as means ± SD (micromoles of nitrite per liter). *p < 0.05 vs the respective MK2+/+ value.


