Published in final edited form as:

Life Sci. 2008 November 7; 83(19-20): 671–680. doi:10.1016/j.lfs.2008.09.003.

The Role of MAP Kinase Phosphatase-1 in the Protective Mechanism of Dexamethasone against Endotoxemia

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Abstract

Aims: We have previously shown that glucocorticoids induce the expression of MAP kinase phosphatase (Mkp)^a-1 in innate immune cells. Since Mkp-1 is a critical negative regulator of the innate immune response, we hypothesize that Mkp-1 plays a significant role in the anti-inflammatory action of glucocorticoids. The specific aim of the present study is to understand the role of Mkp-1 in the anti-inflammatory function of glucocorticoids.

Main methods: Wild-type and *Mkp-1*^{-/-} mice were treated with different doses of dexamethasone and then challenged with different doses of lipopolysaccharide (LPS). The survival and blood cytokines were assessed. The effects of dexamethasone on cytokine production in wild-type and *Mkp-1*^{-/-} primary macrophages *ex vivo* were also examined.

Key findings: We found that dexamethasone induced the expression of *Mkp-1* *in vivo*. Dexamethasone treatment completely protected wild-type mice from the mortality caused by a relatively high-dose of LPS. However, dexamethasone treatment offered only a partial protection to *Mkp-1*^{-/-} mice. Dexamethasone attenuated TNF- α production in both wild-type and *Mkp-1*^{-/-} mice challenged with LPS, although TNF- α production in *Mkp-1*^{-/-} mice was significantly more robust than that in wild-type mice. Dexamethasone pretreatment shortened the duration of p38 and JNK activation in LPS-stimulated wild-type macrophages, but had little effect on p38 or JNK activation in similarly treated *Mkp-1*^{-/-} macrophages.

Significance: Our results indicate that the inhibition of p38 and JNK activities by glucocorticoids is mediated by enhanced *Mkp-1* expression. These results demonstrate that dexamethasone exerts its anti-inflammatory effects through both Mkp-1-dependent and Mkp-1-independent mechanisms.

Keywords

Septic shock; glucocorticoids; phosphatase; anti-inflammatory; cytokines; Mkp-1

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INTRODUCTION

Glucocorticoids, such as dexamethasone, are potent anti-inflammatory drugs frequently prescribed for the treatment of various inflammatory diseases, including asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome (Barnes 1998; Joyce et al. 2001; Rhen and Cidlowski 2005; Saklatvala 2002). In addition to these chronic inflammatory diseases, glucocorticoids have also been used for the treatment of severe sepsis and septic shock in patients in the intensive care unit (Annane 2005). Clinical trials have indicated that low dose glucocorticoids alleviate the systemic inflammatory response, reduce the duration of shock, and favorably affect survival in patients with septic shock (Annane et al. 2002). In animal models of endotoxic shock, prophylactic treatment with dexamethasone attenuates the production of inflammatory cytokines including TNF- α and IL-1 β , and prevents shock and mortality (Berry and Smythe 1964; Spink and Anderson 1954). Although glucocorticoids have been used in clinical medicine as anti-inflammatory drugs for more than half a century, the underlying anti-inflammatory mechanisms are still not fully understood (Rhen and Cidlowski 2005; Saklatvala 2002). Glucocorticoids can modulate the expression of genes involved in inflammation in both positive and negative manners (Saklatvala 2002). Although transcriptional repression of pro-inflammatory cytokine and chemokine genes by activation of nuclear glucocorticoid receptors is widely accepted to be the primary anti-inflammatory mechanism, induction of anti-inflammatory genes may also contribute to the therapeutic activity (Barnes 1998; Joyce et al. 2001; Rhen and Cidlowski 2005; Saklatvala 2002). Glucocorticoids can induce the expression of I κ B and IL-10 (Goulding 2004; Rhen and Cidlowski 2005). Both I κ B and IL-10 play an important role in antagonizing inflammatory cascades. I κ B inhibits the transcription factor NF- κ B by forming inactive heterotrimer with NF- κ B, a master regulator in the transcription of a variety of cytokine and chemokine genes. IL-10 can inhibit the production of inflammatory cytokines and promote the resolution of inflammation. Previously, we and several other laboratories have independently demonstrated that glucocorticoids induce MAP kinase phosphatase (MKP)-1 in macrophages, mast cells, and transformed epithelial cells (Chen et al. 2002; Kassel et al. 2001; Lasa et al. 2002). Moreover, we have reported that the capacity of a given synthetic glucocorticoid to induce *Mkp-1* in cultured immortalized macrophages is associated with the relative anti-inflammatory potency of the synthetic corticosteroid (Zhao et al. 2005). However, whether *Mkp-1* plays a significant role in the anti-inflammatory action of glucocorticoids *in vivo* has not been clearly defined. Very recently, a number of studies conducted using *Mkp-1* knockout mice have demonstrated that *Mkp-1* is responsible for the inactivation of p38 and JNK in innate immune cells, and is a critical negative regulator of inflammatory cytokine biosynthesis *in vivo* (Chi et al. 2006; Hammer et al. 2006; Salojin et al. 2006; Zhao et al. 2006). In this report, we examined the contribution of *Mkp-1* in the protective and anti-inflammatory action of glucocorticoids using both cultured macrophages and mice. We found that dexamethasone exerts its anti-inflammatory action through both *Mkp-1*-dependent and *Mkp-1*-independent mechanisms. *Mkp-1* is required for the optimal anti-inflammatory activity of dexamethasone. In the absence of *Mkp-1*, dexamethasone fails to accelerate the dephosphorylation of p38 and JNK, and is less effective in preventing endotoxic shock and mortality. These results indicate that *Mkp-1* induction constitutes a part of host-protective and anti-inflammatory mechanisms of glucocorticoids. Our results also support notion that full anti-inflammatory activity of glucocorticoids required both *Mkp-1*-dependent inhibition on MAP kinases and *Mkp-1*-independent mechanisms.

MATERIALS AND METHODS

Animals

The generation of *Mkp-1* knockout mice has been described previously (Dorfman et al. 1996). Cryopreserved embryos of *Mkp-1* knockout mice (*Mkp-1*^{+/-} and *Mkp-1*^{-/-}) were kindly provided by Bristol-Myers Squibb Pharmaceutical Research institute and were regenerated into mice in The Jackson Laboratory (Bar Harbor, ME). These mice were bred in-house to yield both wild-type and *Mkp-1*^{-/-} mice. These mice were maintained on Harlan Teklad irradiated diet (Harlan Sprague Dawley, Indianapolis, IN) at 24°C with relative humidity between 30 and 70% on a 12-h day-night cycle. All of the animals received humane care in accordance with the guidelines of the National Institutes of Health and were sacrificed by CO₂ inhalation. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Columbus Children's Research Institute.

LPS and dexamethasone administration

Wild-type and *Mkp-1*^{-/-} mice were injected intraperitoneally with the designated doses of LPS dissolved in phosphate-buffered saline (PBS). Dexamethasone was first dissolved in 2-Hydroxypropyl- β -cyclodextrin solution (Sigma, St Louis, MO), and then diluted in PBS prior to intraperitoneal injection. Mice were sacrificed, and blood was harvested by cardiac puncture for determination of serum TNF- α , IL-6, and IL-10 levels.

Isolation, culture, and treatment of peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were isolated from wild-type or *Mkp-1*^{-/-} mice by peritoneal lavage as described previously (Shepherd et al. 2004; Zhao et al. 2006). Briefly, each mouse was injected intraperitoneally with 2 ml of 3% Brewer Thioglycollate Medium (BD Diagnostic, Sparks, MD). Four days later cells in the peritoneum were harvested by lavage with cold RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 5% FBS (HyClone Laboratories, Logan, UT). Peritoneal cells were recovered by centrifugation, resuspended in RPMI 1640 medium containing 5% FBS, and plated onto tissue culture plates. Cells were allowed to adhere for 2 h, washed free of nonadherent cells, and maintained overnight in RPMI 1640 medium containing 5% FBS. The next day, macrophages were pretreated with either dexamethasone or vehicle (DMSO dissolved in PBS) for 15 min, and then stimulated with LPS (100 ng/ml) for the indicated periods of time. Cells were harvested in lysis buffer as previously described (Shepherd et al. 2004).

Western blotting and ELISA

Western blot analysis was conducted using ECL reagent (Amersham Biosciences) essentially as described previously (Chen et al. 2002). Mkp-1 protein levels were assessed using a rabbit polyclonal antibody (Catalog number: Sc-10199, Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated ERK, JNK, and p38 were detected using rabbit polyclonal phospho-specific antibodies purchased from Cell Signaling Technology (Beverly, MA). Total p38 was detected using a monoclonal antibody (BD Transduction Laboratories, San Jose, CA). β -Actin was detected using a monoclonal antibody purchased from Sigma. ELISA was performed as previously described using commercial kits according to manufacturers' recommendations (Zhao et al. 2006).

Northern blotting

Total RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX). Northern blot analysis was carried out using a full-length mouse *Mkp-1* cDNA as a probe as described previously (Barnes 1998). The membrane was stripped and reprobed with GAPDH cDNA to normalize for RNA loading.

Statistical analysis

Cytokine production was compared between wild-type and *Mkp-1*^{-/-} cells or mice using one-way analysis of variance (ANOVA) and a modified t-test. Differences in survival after LPS challenge between wild-type and *Mkp-1*^{-/-} mice with or without dexamethasone treatment were determined by Kaplan-Meier analysis. All the tests were performed using SPSS 13.01 software (SPSS Inc., Chicago, IL). A *P* value less than 0.05 was considered significant.

RESULTS

Effect of dexamethasone on LPS-induced mortality in wild-type and *Mkp-1*^{-/-} mice

To determine the role of Mkp-1 in the protective effect of dexamethasone against endotoxic shock, *Mkp-1*^{-/-} mice and their wild-type littermates were given either 30 mg/kg dexamethasone or vehicle intraperitoneally. Thirty minutes later these mice were given LPS (20 mg/kg body weight) intraperitoneally. Animal survival was monitored periodically, using death or a moribund state as end-point criteria. Wild-type mice, that were pretreated with vehicle, exhibited 100% mortality by 156 h after challenge with 20 mg/kg LPS (Fig. 1A). Prophylactic treatment with dexamethasone at a dose of 30 mg/kg completely prevented mortality (different from vehicle, *P*<0.001, *n*=12). All *Mkp-1*^{-/-} mice that were pretreated with vehicle died by 24 h after LPS challenge (*Mkp-1*^{-/-} different from wild-type, *P*<0.001, *n*=12). The median survival time of this group was 17 h. Prophylactic treatment of the *Mkp-1*^{-/-} mice with dexamethasone at the same dose (30 mg/kg body weight) delayed the onset of mortality (Median survival time, 28 h). However, dexamethasone treatment had little effect on final survival (Fig. 1A). These results clearly illustrate the contribution of Mkp-1 to the dexamethasone-associated protection in this endotoxic shock model.

Since a relatively high dose of dexamethasone (30 mg/kg) completely protected wild-type mice from LPS-induced mortality, we examined dose effects of dexamethasone on LPS-induced mortality at a dose range commonly used in patients. Wild-type and *Mkp-1*^{-/-} mice were treated intraperitoneally with either 0.3 or 3.0 mg/kg dexamethasone, and then challenged with LPS (20 mg/kg body weight) intraperitoneally 30 min later. Prophylactic treatment with dexamethasone at a dose of 3.0 mg/kg completely protected wild-type mice from LPS-induced mortality (Fig. 1B). At 156 h post LPS challenge, wild-type mice pretreated with vehicle exhibited 100% mortality, while wild-type mice pretreated with 0.3 mg/kg dexamethasone had a mortality rate of 40% (*P*<0.005). While both doses of dexamethasone had little effect on final survival of the LPS-stimulated *Mkp-1*^{-/-} mice (Fig. 1C), dexamethasone at a dose of 3 mg/kg significantly delayed mortality. The *Mkp-1*^{-/-} mice pretreated with 0.3 mg/kg dexamethasone exhibited 100% mortality at 28 h post LPS challenge, with a median survival time of 19 h. The *Mkp-1*^{-/-} mice pretreated with 3.0 mg/kg dexamethasone displayed 90% mortality at 30 h and 100% mortality at 62 h, with the median survival time of 28 h. Thus, dexamethasone had a dose-dependent protective effect against mortality in both wild-type and *Mkp-1*-deficient mice.

To determine whether dexamethasone protects against endotoxic shock caused by a smaller dose of LPS in *Mkp-1*^{-/-} mice, *Mkp-1*^{-/-} mice were first given 0, 0.3 or 3.0 mg/kg dexamethasone intraperitoneally then were given LPS (5 mg/kg body weight) 30 min later. *Mkp-1*^{-/-} mice that received vehicle exhibited 50% mortality at 21 h and 100% mortality by 39 h post LPS challenge. *Mkp-1*^{-/-} mice given 0.3 mg/kg dexamethasone had 50% mortality at 89 h, and those given 3.0 mg/kg dexamethasone had 50% mortality at 107 h post LPS challenge. At the end of the 192 h study period, mice that received 0.3 mg/kg dexamethasone had 70% mortality, while mice that received 3.0 mg/kg dexamethasone had a mortality rate of 56% (Fig. 2A). We further asked the question of whether the apparently less effective protection offered by dexamethasone to *Mkp-1*^{-/-} relative to wild-type mice was due to the more robust inflammatory responses in these *Mkp-1*^{-/-} mice. To answer this question, we challenged

Mkp-1^{-/-} mice with an even lower dose of LPS (1.5 mg/kg body weight), which caused mortality in *Mkp-1*^{-/-} mice that was less than the mortality caused by 20 mg/kg LPS in wild-type mice. To this end, *Mkp-1*^{-/-} mice were first pretreated with either vehicle or dexamethasone (3.0 mg/kg body weight), and 30 min later mice were challenged with LPS (1.5 mg/kg body weight). This dose was chosen because 1) it is a clinically relevant dose; and 2) it increased the survival rate of wild-type mice given 20 mg/kg LPS from 0% to 100%. For *Mkp-1*^{-/-} mice challenged with 1.5 mg/kg LPS, dexamethasone substantially prolonged the survival time (50% survival at 38 h for vehicle-treated mice vs. 50% survival at >168 h for dexamethasone-treated mice), although overall survival to 7 days was not different between the two groups (40% for vehicle-treated mice vs. 50% survival for dexamethasone-treated mice; Fig. 2B). Taken together, our results indicate that dexamethasone can protect mice against endotoxic shock in the absence of the *Mkp-1* gene, although the maximal protective effect of dexamethasone to mice challenged with endotoxin requires a functional *Mkp-1* gene.

Effects of dexamethasone on cytokine production *in vivo* in LPS-challenged wild-type and *Mkp-1*^{-/-} mice

To determine the role of *Mkp-1* on dexamethasone-mediated effects on cytokine production *in vivo*, wild-type mice and their *Mkp-1*^{-/-} littermates were given vehicle or dexamethasone (0.3, 3.0, or 30 mg/kg) intraperitoneally, and then challenged with LPS (20 mg/kg body weight) 30 min later. Mice were sacrificed at 1 and 3 h post LPS challenge, and blood was harvested by cardiac puncture for determination of serum inflammatory cytokine levels. The serum levels of TNF- α , IL-6, and IL-10 in mice pretreated with either vehicle or 30 mg/kg dexamethasone alone were barely detectable in both wild-type and *Mkp-1*^{-/-} mice at 1 and 3 h, indicating that pretreatment alone with either vehicle or dexamethasone did not induce cytokine production (Fig. 3). LPS challenge resulted in a dramatic increase in serum TNF- α concentrations in both wild-type and *Mkp-1*^{-/-} mice pretreated with vehicle, and as expected the TNF- α concentrations were markedly lower at 3 h than at 1 h post LPS challenge in both strains of mice. Importantly, serum levels of TNF- α were substantially greater in the LPS-treated *Mkp-1*^{-/-} mice than in the wild-type mice at both 1 and 3 h (Fig. 3A). Dexamethasone treatment attenuated TNF- α production in both the wild-type and the *Mkp-1*^{-/-} mice, although the serum TNF- α levels were significantly greater than those in the wild-type mice at every dose 1 hour after LPS challenge.

Treatment with LPS resulted in a significant increase in serum IL-6 levels in both wild-type and *Mkp-1*^{-/-} mice, however, the time-course of changes in serum IL-6 concentrations was markedly different in the two groups of mice. In the wild-type mice, serum IL-6 levels peaked at 1 h, while in *Mkp-1*^{-/-} mice serum IL-6 levels were substantially greater at 3 hours than at 1 hour post LPS challenge (Fig. 3B). There was no substantial difference in serum IL-6 levels between *Mkp-1*^{-/-} and wild-type mice at 1 h post LPS challenge. However, the serum IL-6 levels were 5-10-fold higher in the *Mkp-1*^{-/-} mice than in the wild-type mice 3 h post LPS challenge (Fig. 3B). Unlike the effect of dexamethasone on TNF- α production, LPS-induced IL-6 production *in vivo* was not significantly affected by dexamethasone pretreatment in wild-type mice. In *Mkp-1*^{-/-} mice, the effect of dexamethasone was more complex. At doses of 0.3 and 3 mg/kg, dexamethasone significantly inhibited IL-6 production, with the higher dose exhibiting a stronger inhibition. Although 10 mg/kg dexamethasone also appeared to inhibit IL-6 production, the difference in serum IL-6 levels was not statistically significant between vehicle and dexamethasone-pretreated *Mkp-1*^{-/-} mice.

IL-10 is a powerful anti-inflammatory cytokine that plays an important role in the resolution of inflammation (Gerard et al. 1993; Howard et al. 1993). To assess the effect of dexamethasone on production of IL-10, wild-type and *Mkp-1*^{-/-} mice were first pretreated with either vehicle or dexamethasone (30 mg/kg body weight) intraperitoneally, and 30 min later mice were

challenged with LPS (20 mg/kg body weight).. Administration of dexamethasone alone did not significantly alter the serum IL-10 levels in either wild-type or *Mkp-1*^{-/-} mice (Fig. 3C). LPS treatment resulted in a dramatic increase in serum IL-10 concentration in both wild-type and *Mkp-1*^{-/-} mice pretreated with vehicle. In vehicle-treated wild-type mice, IL-10 levels reached a peak at 1 h, and substantially decreased by 3 h post LPS challenge. Unlike what was observed in vehicle-treated wild-type mice, IL-10 levels in vehicle-treated *Mkp-1*^{-/-} mice continued to increase at least for 3 h after LPS challenge. IL-10 levels in LPS-challenged, vehicle-pretreated wild-type and *Mkp-1*^{-/-} mice were not significantly different at 1 h (Fig. 3C). Dexamethasone pretreatment significantly enhanced IL-10 production in *Mkp-1*^{-/-} mice at both 1 and 3 h post LPS challenge, but had little effect on IL-10 production in wild-type mice (Fig. 3C).

Effects of dexamethasone on cytokine biosynthesis in peritoneal macrophages from wild-type and *Mkp-1*^{-/-} mice

Peritoneal macrophages harvested from wild-type and *Mkp-1*^{-/-} mice were pretreated with dexamethasone at doses ranging from 0 to 100 μ M for 15 min, and then stimulated with LPS. The medium was harvested 6 h post LPS stimulation for analysis of TNF- α , IL-6, and IL-10 by ELISA. Dexamethasone inhibited the production of TNF- α by both wild-type and *Mkp-1*^{-/-} macrophages in a dose-dependent manner (Fig. 4A). Reflecting the critical role of Mkp-1 in the negative regulation of cytokine production, at every dexamethasone dose tested the macrophages isolated from *Mkp-1*^{-/-} mice produced substantially more TNF- α than did wild-type macrophages after LPS stimulation (Fig. 4A). Similarly, dexamethasone inhibited IL-6 production in both wild-type and *Mkp-1*^{-/-} macrophages in a dose-dependent manner. However, more IL-6 was produced by *Mkp-1*^{-/-} macrophages than by wild-type macrophages at every dose of dexamethasone studied (Fig. 4B).

The effect of dexamethasone on IL-10 production in both wild-type and *Mkp-1*^{-/-} macrophages after LPS stimulation was examined. In wild-type macrophages, dexamethasone up to 1 μ M did not affect IL-10 production; however, dexamethasone at 10 or 100 μ M modestly increased IL-10 production (Fig. 4C). Consistent with our previous report (Zhao et al. 2006), *Mkp-1*^{-/-} macrophages produced significantly greater quantities of IL-10 than did wild-type macrophages after LPS stimulation. Remarkably, even a very low dose of dexamethasone (10 nM) dramatically enhanced IL-10 production by *Mkp-1*^{-/-} macrophages (Fig. 4C). However, increasing the dexamethasone concentration up to 10 μ M did not result in further increases in IL-10 production, suggesting the stimulatory effect of dexamethasone was already saturated at 10 nM. At the highest concentration studied (100 μ M), dexamethasone actually attenuated IL-10 production in LPS-treated *Mkp-1*^{-/-} macrophages (Fig. 4C).

Effects of dexamethasone on Mkp-1 expression and MAP kinase activation in primary wild-type and *Mkp-1*-deficient macrophages after LPS stimulation

We examined the effects of dexamethasone on the kinetics of MAP kinase activation and Mkp-1 expression in wild-type and *Mkp-1*^{-/-} macrophages following LPS stimulation. Peritoneal macrophages were pretreated with vehicle or dexamethasone for 15 min, and then stimulated with LPS (100 ng/ml). Cells were harvested at various time points after LPS stimulation and analyzed by Western blotting. In the absence of dexamethasone, LPS stimulation resulted in a transient increase in Mkp-1 protein level in the wild-type macrophages (Fig. 5). Consistent with the knockout genotype, no Mkp-1 protein was detected in the *Mkp-1*^{-/-} macrophages. MAP kinases, including ERK, JNK and p38, were also transiently activated in response to LPS in both wild-type and *Mkp-1*^{-/-} macrophages. The kinetics of ERK activation did not differ significantly between the two groups of cells (Fig. 5). On the other hand, the activation of JNK and p38 was not only enhanced but also lasted much longer in LPS-stimulated *Mkp-1*^{-/-} macrophages than in wild-type cells, indicating that p38 and JNK

are the primary targets of Mkp-1 in this system. In the wild-type macrophages, dexamethasone pretreatment substantially augmented the induction of Mkp-1 by LPS, although dexamethasone treatment in the absence of LPS stimulation (time 0) did not result in appreciable changes in Mkp-1 protein levels (Fig. 5). Perhaps reflecting the increase in Mkp-1 protein level, the duration of Mkp-1 expression also appeared to be longer in wild-type macrophages pretreated with dexamethasone than in the vehicle-pretreated cells. The enhancement in Mkp-1 protein expression in dexamethasone-pretreated wild-type macrophages was also functionally reflected in the shorter duration of MAP kinase activation, including activation of ERK, JNK and p38 (Fig. 5). In contrast, pretreatment of *Mkp-1*^{-/-} macrophages with dexamethasone did not significantly alter the kinetics of MAP kinase deactivation. Surprisingly, dexamethasone pretreatment delayed the initial activation of the MAP kinases through a mechanism independent of Mkp-1. Specifically, in the absence of dexamethasone, all three MAP kinase subfamily members (ERK, JNK, and p38) became activated within 15 min following LPS stimulation. However, in the presence of dexamethasone, the activation of these kinases was significantly delayed, indicated by the absence of phospho-MAP kinases at 15 min post LPS stimulation (Fig. 5).

Comparing MAP kinase activation and Mkp-1 expression by Western blotting using different blots may result in unavoidable subtle experimental variations. To overcome this limitation, we compared MAP kinase phosphorylation and Mkp-1 expression on identical blots. To this end, we pretreated wild-type and *Mkp-1*^{-/-} macrophages with dexamethasone, or vehicle, for 30 min, and then stimulated these cells with LPS for either 45 or 60 min. As indicated in Figure 6A, in the presence of dexamethasone Mkp-1 expression induced by LPS was substantially enhanced in wild-type macrophages. While pretreatment of wild-type macrophages with dexamethasone consistently resulted in lower p38 and JNK activities, dexamethasone pretreatment had no effect on either p38 or JNK activities in *Mkp-1*^{-/-} macrophages (Fig. 6A). Interestingly, while dexamethasone had little effect on ERK activity at the 45 min time point, dexamethasone pretreatment resulted in a significant decrease in ERK activity at 60 min in wild-type macrophages. As seen for p38 and JNK, dexamethasone pretreatment did not alter ERK activity in the *Mkp-1*^{-/-} macrophages, suggesting that Mkp-1, particularly when expressed at high levels, participates in ERK inactivation. Taken together, these results indicate that the inhibitory effect of dexamethasone on the MAP kinases is mediated by Mkp-1.

To understand the induction of Mkp-1 by dexamethasone, the dose effect of dexamethasone on Mkp-1 protein expression was examined in primary peritoneal macrophages harvested from wild-type mice (Fig. 6B). The macrophages were pretreated with concentrations of dexamethasone ranging from 0 to 100 μ M, for 15 min, and then stimulated with LPS for 60 min. Mkp-1 protein in the lysates was detected by Western blotting. Treatment with LPS resulted in induction of Mkp-1, and pretreatment of wild-type macrophages with dexamethasone enhanced Mkp-1 induction in a dose-dependent manner. The effect of dexamethasone appeared to be saturable, with the maximal effect reached at 1 μ M concentration (Fig. 6B).

To evaluate the effect of dexamethasone on *Mkp-1* expression *in vivo*, wild-type (*Mkp-1*^{+/+}) mice were treated with dexamethasone (30 mg/kg body weight) for 1, 2, 4, or 6 h, or treated with vehicle for 6 h. Each treatment group consisted of two mice. Because the lung is a major organ where inflammation occurs during bacterial infection, we examined *Mkp-1* expression in this vital organ. Induction of *Mkp-1* mRNA in the lungs was detectable at 1 h post dexamethasone administration and elevated *Mkp-1* expression persisted for at least 6 h (Fig. 7A). Since *in vivo* studies often observe significant variations between individual animals, we increased the animal number to 5 in each treatment group to verify the effect of dexamethasone on *Mkp-1* expression. Mice were treated with dexamethasone (30 mg/kg body weight) or vehicle for 6 h. The lungs and the spleens were harvested, since both organs play an important

role in the innate immune responses (Raz 2007). Compared to vehicle, administration of dexamethasone resulted in significant increases in *Mkp-1* mRNA expression in both the lungs and the spleens (Fig. 7B). Our results indicate that dexamethasone enhances *Mkp-1* expression both *in vitro* and *in vivo*, and suggesting that *Mkp-1* induction contributes to the anti-inflammatory mechanisms of glucocorticoids.

DISCUSSION

Glucocorticoids are potent anti-inflammatory drugs often used to treat a wide-variety of acute and chronic inflammatory diseases (Goulding 2004). A number of mechanisms have been proposed for the anti-inflammatory actions of glucocorticoids, including repression of inflammatory cytokine genes by inhibition of transcriptions factors like NF- κ B and AP-1, as well as induction of anti-inflammatory cytokines such as IL-10 (Goulding 2004). We have previously reported that glucocorticoids induce *Mkp-1* in immortalized macrophages with their relative anti-inflammatory potency directly correlated with their capacity to induce *Mkp-1* expression (Chen et al. 2002; Zhao et al. 2005). Since *Mkp-1* is a critical negative regulator of MAP kinases, particularly p38 and JNK, and plays a pivotal role in the restraint of the inflammatory response in innate immune cells (Chi et al. 2006; Hammer et al. 2006; Salojin et al. 2006; Zhao et al. 2006), we postulated that induction of *Mkp-1* is part of the anti-inflammatory mechanism of glucocorticoids. In this study, we tested this hypothesis by using a murine model of endotoxic shock. We found that dexamethasone can substantially inhibit the production of TNF- α in the absence of *Mkp-1* gene (Figs. 3 and 4). Although in the absence of *Mkp-1* dexamethasone can increase survival and prolong median survival time (Fig. 2A), its maximal protective effects requires a functional *Mkp-1* gene. This is supported by following observations. First, dexamethasone, at the dose of 3 mg/kg, increased the survival rate of wild-type mice challenged with 20 mg/kg LPS from 0 to 100% (Fig. 1B). However, in the absence of *Mkp-1* gene, the same dose of dexamethasone only modestly increased the survival of *Mkp-1*^{-/-} challenged with 1.5 mg/kg LPS from 40% to 50% (Fig. 2B). Second, although dexamethasone can effectively inhibit the production of TNF- α in the *Mkp-1*^{-/-} mice, it cannot suppress TNF- α production to levels seen in the *Mkp-1*^{+/+} mice (Fig. 3A). This finding indicates that maximal inhibition of the production of TNF- α triggered by endotoxin requires a functional *Mkp-1* gene (Fig. 3). Finally, dexamethasone induces *Mkp-1* expression both *in vitro* and *in vivo* (Fig. 6 and 7). Pretreatment with dexamethasone augmented *Mkp-1* protein expression in wild-type primary peritoneal macrophages. Dexamethasone pretreatment resulted in an accelerated deactivation of the MAP kinases in wild-macrophages but not in *Mkp-1*-deficient macrophages following LPS stimulation (Figs. 5 and 6). Likewise, dexamethasone administration also significantly enhanced the expression of *Mkp-1* gene *in vivo* in organs including the lung and the spleen (Fig. 7), two important organs critical for immune defense. Since *Mkp-1* protein acts as a major inhibitory factor responsible for the restraint of the inflammatory responses to LPS, an increase in *Mkp-1* expression is bound to shorten the inflammatory signaling and attenuate the production of inflammatory mediators such as TNF- α (Zhao et al. 2005).

Although our results support the notion that the protective effect of dexamethasone on lethal endotoxic shock is partially dependent on a functional *Mkp-1* gene and that the induction of the *Mkp-1* gene contributes to the anti-inflammatory mechanism of glucocorticoids, our experimental designs clearly has a limitation. Because a total knockout strategy is utilized, it is impossible to distinguish the contribution of *Mkp-1* that is dexamethasone-induced from the contribution of *Mkp-1* that is not dexamethasone-dependent (Fig. 8). Perhaps such a limitation can only be overcome in the future by the utilization of knock-in mice harboring a mutant *Mkp-1* gene that is unresponsive to dexamethasone yet fully responsive to LPS. Based on the dose-dependent protection by dexamethasone observed in the two strains of mice under different doses of endotoxin, we propose a model that the ultimate outcome following

endotoxin will be determined by the balance between the inflammatory insult (LPS dose) and the combined anti-inflammatory activity of both Mkp-1 and glucocorticoids (Fig. 8). The contribution of Mkp-1 to this anti-inflammatory action can be classified into glucocorticoid-dependent and glucocorticoid-independent. Likewise, glucocorticoids exert anti-inflammatory action through Mkp-1-dependent and independent mechanisms. When wild-type mice were given a high dose of LPS, the anti-inflammatory activity of Mkp-1 alone is insufficient to resist the toxic effects of LPS. Therefore, the balance tilts to the inflammatory side, and as a result mortality occurs. Administration of dexamethasone not only boosted the Mkp-1-mediated anti-inflammatory effects, but also offered protection through an Mkp-1-independent mechanism (s). Therefore, it tilts the balance towards survival in a manner dependent on dexamethasone dose (Fig. 1). In the absence of Mkp-1, the only anti-inflammatory activity left is the Mkp-1-independent anti-inflammatory function of dexamethasone, which may not be sufficient to offset the toxic effects of high dose LPS. Thus, the balance tilts to the inflammatory side, and the animals succumb (Fig. 1C). When *Mkp-1*-deficient mice were challenged with a lower dose of LPS, the Mkp-1-independent anti-inflammatory effects may be sufficient to prolong survival, perhaps allowing time for the latent anti-inflammatory action to take effect. As a result, the balance would tilt in the anti-inflammatory direction, and more animals may survive the lower endotoxin dose (Fig. 2).

Interestingly, Battacharyya et al. found that mice deficient in the glucocorticoid receptor (GR) were markedly more susceptible to endotoxic shock (Bhattacharyya et al. 2007). They also found that LPS challenge resulted in a substantial increase in the serum cortisol level. Since *Mkp-1* expression was enhanced in the presence of corticosteroids and inhibition of p38 improved the survival of the GR-deficient animals, their studies suggest that a large portion of the anti-inflammatory effects of glucocorticoids are mediated through Mkp-1-dependent p38 inhibition. Their studies also suggest that the anti-inflammatory function of *Mkp-1* in the endotoxic shock model depends on endogenous cortisol production by the hypothalamic-pituitary-adrenal axis. Our results suggest that the Mkp-1-independent anti-inflammatory mechanism is at least as important as the Mkp-1-dependent anti-inflammatory mechanisms in the protection against endotoxic shock in the mouse (Fig. 2).

Mkp-1-dependent and independent anti-inflammatory actions of dexamethasone

Dexamethasone can inhibit the expression of pro-inflammatory cytokines through mechanisms independent of MAP kinases and Mkp-1, for example through interference with transcription factors such as NF- κ B (Goulding 2004). In the *Mkp-1*-deficient macrophages or mice, dexamethasone exhibited a potent inhibitory effect on TNF- α production. Our results are consistent with the observation of Meier et al. who demonstrated TNF- α production in Mkp-1-deficient mast cells is still sensitive to glucocorticoid inhibition (Maier et al. 2007). It is likely that this Mkp-1-independent inhibition on pro-inflammatory cytokine production contributes to the partial protection against the lower dose LPS challenge (Fig. 2). In addition to repression of pro-inflammatory cytokine biosynthesis, dexamethasone also enhances the expression of anti-inflammatory proteins, including IL-10 (Goulding 2004). We found that IL-10 expression in LPS-stimulated *Mkp-1*^{-/-} macrophages was robustly increased in the presence of low dose dexamethasone while IL-10 production in wild-type macrophages was only modestly enhanced by high dose dexamethasone (Fig. 4). Similar to what was observed in the tissue culture system, IL-10 production *in vivo* was also substantially enhanced *in vivo* in LPS-challenged *Mkp-1*^{-/-}, whereas the drug had no significant effect on IL-10 production in wild-type mice (Fig. 3C). Since IL-10 has powerful inhibitory effects on the production of pro-inflammatory cytokines (Gerard et al. 1993; Howard et al. 1993), the robust enhancement of IL-10 production by dexamethasone in response to LPS in *Mkp-1*^{-/-} macrophages may contribute to the inhibition of cytokine biosynthesis seen in *Mkp-1*^{-/-} mice. Previously, Hammer et al. have shown that IL-10 can cooperate with LPS and enhance the expression of Mkp-1 in cultured

macrophages. They suggested that IL-10 might, at least in part, exert its anti-inflammatory function through augmenting Mkp-1 expression (Hammer et al. 2005). The substantial increase in IL-10 production in *Mkp-1^{-/-}* mice relative to *Mkp-1^{+/+}* mice following LPS challenge may represent a defective cellular response due to the absence of a downstream effector.

In addition to anti-inflammatory effects, glucocorticoids also can have profound effects on the cardiovascular system (Annane and Cavaillon 2003). Glucocorticoids have been shown to increase mean arterial pressure and systemic vascular resistance and enhance pressor sensitivity. Severe hypotension due to myocardial dysfunction and decreased systemic vascular resistance is regarded as the direct cause of multi-organ failure syndrome that often leads to mortality of patients suffering from severe sepsis (Parrillo 1993). Thus, it is likely that the protective effects of glucocorticoids against endotoxemia are not only mediated by their anti-inflammatory actions, but are also mediated by their cardiovascular effects. It is tempting to speculate that some of the protective effects of dexamethasone on the cardiovascular system may also depend on Mkp-1. In the absence of Mkp-1, the substantial inhibitory effects of dexamethasone on the production of pro-inflammatory cytokines, such as TNF- α (Fig. 3A), may be not sufficient to prevent the development of septic shock and multi-organ failure. Interestingly, it has been shown that Mkp-1 is induced in rat aorta during acute hypertension caused by stress or vasoconstrictors, such as phenylephrine, vasopressin, and angiotensin II (Xu et al. 1997).

The effects of dexamethasone on MAP kinases

Previously, it has been reported that dexamethasone inhibits the activity of JNK and p38 and blocks the production of inflammatory cytokines in macrophages (Han et al. 1990; Kontoyiannis et al. 1999; Swantek et al. 1997). Here we show that dexamethasone induced the expression of *Mkp-1* and attenuated MAP kinase activation in primary macrophages in a dose-dependent manner, thus providing an additional explanation for the inhibitory effects of glucocorticoids on cytokine expression. Our results are consistent with a recent report suggesting that dexamethasone inhibits cytokine production through a mechanism partially mediated by Mkp-1 (Abraham et al. 2006). In addition to the observations first reported by Abraham and colleagues (Abraham et al. 2006), we have made several new observations. First, we found that dexamethasone inhibits MAP kinase signaling through two mechanisms: 1) acceleration of MAP kinase deactivation through enhanced expression of Mkp-1; and 2) a Mkp-1-independent early delay of MAP kinase activation. The latter observation suggests that dexamethasone may interfere with early signal transduction events either at or after TLR4 but prior to the divergence of the three separate MAP kinase cascades. Although the mechanism underlying this delay of signal transduction remains unclear, such a delay is likely to reduce the overall signal output, resulting in less cytokine biosynthesis.

Dexamethasone accelerated the deactivation of ERK in LPS-stimulated macrophages and this process appears to depend on Mkp-1 (Figs. 5 and 6). This finding was somewhat unexpected, since in the absence of dexamethasone pretreatment Mkp-1 does not appear to play a significant role in ERK deactivation (Chi et al. 2006; Hammer et al. 2006; Zhao et al. 2006). Perhaps, the simplest explanation is that Mkp-1 protein levels in cells treated with both LPS and dexamethasone were significantly higher than in cells treated with LPS alone (Figs. 5 and 6). In the absence of dexamethasone, the portion of ERK that was dephosphorylated by Mkp-1 might be relatively small, thereby making the contribution of Mkp-1 to ERK dephosphorylation less obvious. In cells pretreated with dexamethasone, the relatively large amounts of Mkp-1 protein may result in dephosphorylation of a larger portion of the activated ERK, thus making the contribution of Mkp-1 easily detectable.

In summary, we found that clinically relevant doses of dexamethasone protect the host against LPS-induced mortality via mechanisms mediated by both Mkp-1-dependent and Mkp-1-

independent pathways. Our findings strongly support a significant role for Mkp-1 in the anti-inflammatory and host-protective properties of dexamethasone, and suggest that Mkp-1 may represent a novel therapeutic target for patients with sepsis and other inflammatory diseases.

ACKNOWLEDGMENTS

We would like to thank Bristol-Myers Squibb Pharmaceutical Research Institute for providing *Mkp-1* knockout mice. This study was supported by grants from NIAID (AI 57798, AI 68956, and AI 57798 to YL) and from NHLBI (HL 75261 to LDN).

Abbreviations used are

MAP, mitogen-activated protein
Mkp, MAP kinase phosphatase
LPS, lipopolysaccharides
ERK, extracellular signal-regulated kinase
JNK, c-Jun N-terminal kinase
TNF, tumor necrosis factor
IL, interleukin
ELISA, enzyme-linked immunosorbent assay
FBS, fetal bovine serum
PBS, phosphate-buffered saline
ANOVA, analysis of variance
NF- κ B, nuclear factor- κ B
I κ B, inhibitor- κ B
GR, glucocorticoid receptor

REFERENCES

- Abraham SM, Lawrence T, Kleiman A, Warden P, Medghalchi M, Tuckermann J, Saklatvala J, Clark AR. Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *Journal of Experimental Medicine* 2006;203(8):1883–1889. [PubMed: 16880258]
- Anname D. Glucocorticoids in the treatment of severe sepsis and septic shock. *Current Opinion in Critical Care* 2005;11(5):449–453. [PubMed: 16175031]
- Anname D, Cavaillon JM. Corticosteroids in sepsis: from bench to bedside? *Shock* 2003;20(3):197–207. [PubMed: 12923489]
- Anname D, Seville V, Charpentier C, Bollaert PE, Francois B, Korach JM, Capellier G, Cohen Y, Azoulay E, Troche G, Chaumet-Riffaut P, Bellissant E. Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. *The Journal of the American Association* 2002;288(7):862–871.
- Barnes P. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clinical Science (London)* 1998;94(6):557–572.
- Berry LJ, Smythe DS. Effects of bacterial endotoxins on metabolism. Vii. Enzyme induction and cortisone protection. *Journal of Experimental Medicine* 1964;120:721–732. [PubMed: 14247715]
- Bhattacharyya S, Brown DE, Brewer JA, Vogt SK, Muglia LJ. Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase. *Blood* 2007;109(10):4313–4319. [PubMed: 17255352]
- Chen P, Li J, Barnes J, Kokkonen GC, Lee JC, Liu Y. Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *Journal of Immunology* 2002;169(11):6408–6416.
- Chi H, Barry SP, Roth RJ, Wu JJ, Jones EA, Bennett AM, Flavell RA. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proceedings of the National Academy of Sciences* 2006;103(7):2274–2279.

- Dorfman K, Carrasco D, Gruda M, Ryan C, Lira SA, Bravo R. Disruption of the *erp/mkp-1* gene does not affect mouse development: normal MAP kinase activity in ERP/MKP-1-deficient fibroblasts. *Oncogene* 1996;13(5):925–931. [PubMed: 8806681]
- Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, Fiers W, Goldman M, Velu T. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *Journal of Experimental Medicine* 1993;177(2):547–550. [PubMed: 8426124]
- Goulding N. The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus. *Current Opinion in Pharmacology* 2004;4(6):629–636. [PubMed: 15525555]
- Hammer M, Mages J, Dietrich H, Schmitz F, Striebel F, Murray PJ, Wagner H, Lang R. Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *European Journal of Immunology* 2005;35(10):2991–3001. [PubMed: 16184516]
- Hammer M, Mages J, Dietrich H, Servatius A, Howells N, Cato AC, Lang R. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *Journal of Experimental Medicine* 2006;203(1):15–20. [PubMed: 16380512]
- Han J, Thompson P, Beutler B. Dexamethasone and pentoxifylline inhibit endotoxin-induced cachectin/tumor necrosis factor synthesis at separate points in the signaling pathway. *Journal of Experimental Medicine* 1990;172(1):391–394. [PubMed: 2358784]
- Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. *Journal of Experimental Medicine* 1993;177(4):1205–1208. [PubMed: 8459215]
- Joyce DA, Gimblett G, Steer JH. Targets of glucocorticoid action on TNF- α release by macrophages. *Inflammation Research* 2001;50(7):337–340. [PubMed: 11506387]
- Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, Cato AC. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO Journal* 2001;20(24):7108–7116. [PubMed: 11742987]
- Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 1999;10(3):387–398. [PubMed: 10204494]
- Lasa M, Abraham SM, Boucheron C, Saklatvala J, Clark AR. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Molecular and Cellular Biology* 2002;22(22):7802–7811. [PubMed: 12391149]
- Maier JV, Brema S, Tuckermann J, Herzer U, Klein M, Stassen M, Moorthy A, Cato AC. Dual specificity phosphatase 1 knockout mice show enhanced susceptibility to anaphylaxis but are sensitive to glucocorticoids. *Molecular Endocrinology* 2007;21(11):2663–2671. [PubMed: 17636038]
- Parrillo J. Pathogenetic mechanisms of septic shock. *New England Journal of Medicine* 1993;328(20):1471–1477. [PubMed: 8479467]
- Raz E. Organ-specific regulation of innate immunity. *Nat. Immunol* 2007;8(1):3–4. [PubMed: 17179960]
- Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. *New England Journal of Medicine* 2005;353(16):1711–1723. [PubMed: 16236742]
- Saklatvala J. Glucocorticoids: do we know how they work? *Arthritis Research* 2002;4(3):146–150. [PubMed: 12010562]
- Salojin KV, Owusu IB, Millerchip KA, Potter M, Platt KA, Oravec T. Essential role of MAPK phosphatase-1 in the negative control of innate immune responses. *Journal of Immunology* 2006;176(3):1899–1907.
- Shepherd EG, Zhao Q, Welty SE, Hansen TN, Smith CV, Liu Y. The function of mitogen-activated protein kinase phosphatase-1 in peptidoglycan-stimulated macrophages. *Journal of Biological Chemistry* 2004;279(52):54023–54031. [PubMed: 15485842]
- Spink WW, Anderson D. Experimental studies on the significance of endotoxin in the pathogenesis of brucellosis. *Journal of Clinical Investigation* 1954;33(4):540–548. [PubMed: 13152193]
- Swantek JL, Cobb MH, Geppert TD. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor α (TNF- α) translation: glucocorticoids inhibit TNF- α translation by blocking JNK/SAPK. *Molecular and Cellular Biology* 1997;17(11):6274–6282. [PubMed: 9343388]

- Xu Q, Fawcett TW, Gorospe M, Guyton KZ, Liu Y, Holbrook NJ. Induction of mitogen-activated protein kinase phosphatase-1 during acute hypertension. *Hypertension* 1997;30(1 Pt 1):106–111. [PubMed: 9231829]
- Zhao Q, Shepherd EG, Manson ME, Nelin LD, Sorokin A, Liu Y. The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide: Attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. *Journal of Biological Chemistry* 2005;280(9):8101–8108. [PubMed: 15590669]
- Zhao Q, Wang X, Nelin LD, Yao Y, Matta R, Manson ME, Baliga RS, Meng X, Smith CV, Bauer JA, Chang CH, Liu Y. MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. *Journal of Experimental Medicine* 2006;203(1):131–140. [PubMed: 16380513]

Fig. 1.

The effect of dexamethasone on mortality of *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice after challenge with high-dose LPS. Sex- and age-matched *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were pretreated with vehicle or dexamethasone (DEX), and 30 min later were challenged with 20 mg/kg (body weight) LPS intraperitoneally. **A.** Survival curves of *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice after LPS challenge. Mice were pretreated with vehicle or dexamethasone (30 mg/kg). *, $P < 0.001$, $n = 11$. **B.** The effect of dexamethasone dose on the survival of wild-type mice. Wild-type mice were first pretreated with vehicle, 0.3, or 3.0 mg/kg dexamethasone, and then challenged with LPS. Both doses of dexamethasone yielded results significantly different from vehicle, $P < 0.005$, $n = 10$. **C.** The effect of dexamethasone dose on the survival of *Mkp-1*^{-/-} mice. *Mkp-1*^{-/-} mice

were first pretreated with vehicle, 0.3, or 3.0 mg/kg dexamethasone, and then challenged with LPS (n=10). *, $P<0.001$, comparing vehicle-treated group.

Fig. 2.

The effects of dexamethasone on mortality of *Mkp-1*-deficient mice challenged with lower doses of LPS. **A.** *Mkp-1*^{-/-} mice were first pretreated with either vehicle, 0.3 mg/kg, or 3.0 mg/kg dexamethasone intraperitoneally. Thirty min later mice were challenged with 5 mg/kg LPS intraperitoneally, and mortality was monitored. * different from mice pretreated with vehicle, $P < 0.05$, $n = 12$. **B.** *Mkp-1*^{-/-} mice were first pretreated with either vehicle or 3.0 mg/kg dexamethasone intraperitoneally, and 30 min later mice were challenged with 1.5 mg/kg body weight LPS intraperitoneally. The mortality was monitored for 7 days ($n = 10$). $P = 0.33$.

Fig. 3.

The differential effects of dexamethasone on the production of TNF-α, IL-6, and IL-10 in LPS-challenged wild-type and *Mkp-1*^{-/-} mice. *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were pretreated with either vehicle, 0.3, 3.0, or 30 mg/kg dexamethasone, and 30 min later these mice were given either PBS (-) or 20 mg/kg LPS (+) intraperitoneally. Blood samples were collected either 1 or 3 h post LPS challenge. TNF-α concentration in the sera was measured by ELISA. **A.** *Mkp-1* knockout blunted the inhibitory effect of dexamethasone on TNF-α production *in vivo*. **B.** The effect of *Mkp-1* knockout on the modulatory effect of dexamethasone on IL-6 production *in vivo*. **C.** The effect of dexamethasone on IL-10 production in LPS-challenged wild-type and *Mkp-1*^{-/-} mice. *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were first pretreated with either

vehicle or 30 mg/kg dexamethasone, and 30 min later mice were challenged with either PBS (–) or 20 mg/kg LPS intraperitoneally. Values represent the means $\pm$ SE from 5 independent experiments. ‡ dexamethasone treated different from vehicle-treated (dose = 0) in wild-type, $P < 0.05$; * dexamethasone treated different from vehicle-treated (dose = 0) in *Mkp-1*^{–/–}, $P < 0.05$; # *Mkp-1*^{–/–} different from wild-type mice for same treatment and dose of dexamethasone, $P < 0.05$.

Fig. 4.

Differential effects of dexamethasone on the production of pro-inflammatory and anti-inflammatory cytokines in wild-type and *Mkp-1*^{-/-} peritoneal macrophages *ex vivo*. Peritoneal macrophages isolated from wild-type and *Mkp-1*^{-/-} mice were pretreated with 0, 0.01, 0.1, 1, 10 or 100 μM dexamethasone for 15 min, and then stimulated with 100 ng/ml LPS for 6 h. The concentrations of TNF- α , IL-6 and IL-10 in the medium were measured by ELISA. **A.** Dose-dependent inhibition of TNF- α production by dexamethasone in both wild-type and *Mkp-1*^{-/-} macrophages, although TNF- α production was significantly greater in *Mkp-1*^{-/-} macrophages than in wild-type macrophages at all doses of dexamethasone studied, $P < 0.05$. **B.** Dose-dependent inhibition of LPS-stimulated IL-6 biosynthesis by dexamethasone, although IL-6 production was significantly greater in *Mkp-1*^{-/-} macrophages than in wild-type macrophages at all doses of dexamethasone, except the highest dose, $P < 0.05$. **C.** Differential effects of dexamethasone on IL-10 in wild-type and *Mkp-1*^{-/-} macrophages. IL-10 production is significantly greater in *Mkp-1*^{-/-} macrophages than in wild-type macrophages when pretreated with 0-10 μM dexamethasone, $P < 0.05$. Data are presented as fold increase relative to the mean concentrations of cytokines produced by wild-type macrophages in the absence of dexamethasone. Values represent the means $\pm$ SE from at least 3 independent experiments. ‡ dexamethasone different from vehicle (dose = 0) in wild-type, $P < 0.05$; * dexamethasone different from vehicle (dose = 0) in *Mkp-1*^{-/-}, $P < 0.05$; # *Mkp-1*^{-/-} different from wild-type for same dose of dexamethasone, $P < 0.05$.

Fig. 5.

The kinetics of Mkp-1 induction and MAP kinase activation in LPS-stimulated wild-type and *Mkp-1*^{-/-} macrophages in the absence and presence of dexamethasone. Peritoneal macrophages isolated from *Mkp-1*^{+/+} and *Mkp-1*^{-/-} mice were pretreated with vehicle or 10 μ M dexamethasone for 15 min. These cells were then stimulated with LPS (100 ng/ml) for different periods of time. Cell lysates were analyzed by Western blotting for Mkp-1 expression and MAP kinase activation. MAP kinase activation was detected using phospho-specific antibodies. As a control for sample loading, after blotting with phospho-p38 antibody the membrane was stripped and blotted again with a monoclonal antibody against total p38.

Fig. 6.

Acceleration of MAP kinase deactivation by dexamethasone through Mkp-1-mediated dephosphorylation in LPS-stimulated macrophages. **A.** Direct comparison of LPS-stimulated Mkp-1 expression and MAP kinase activation among wild-type and *Mkp-1*^{-/-} macrophages pretreated with vehicle or dexamethasone. Peritoneal macrophages isolated from wild-type or *Mkp-1*^{-/-} mice were pretreated with 10 μ M dexamethasone for 15 min prior to LPS stimulation (100 ng/ml). After 45 and 60 min, cells were harvested, and cell lysates subjected to Western blot analysis. The membranes were stripped and reprobed with a β -actin antibody as a control for sample loading. **B.** Dose-dependent enhancement of Mkp-1 expression by dexamethasone. Peritoneal macrophages isolated from wild-type mice were pretreated with indicated dose of dexamethasone for 15 min, then the cells were stimulated with LPS (100 ng/ml) for 60 min. Cell lysates were analyzed by Western blotting using Mkp-1 antibody. The membranes were stripped and reprobed with β -actin antibody as a loading control. Data shown are from a representative experiment.

Fig. 7. Induction of *Mkp-1* mRNA by dexamethasone in lungs and spleens from *Mkp-1*^{+/+} mice. **A.** Time course of *Mkp-1* mRNA induction by dexamethasone *in vivo*. *Mkp-1*^{+/+} mice were challenged with either vehicle for 6 h or 30 mg/kg dexamethasone for indicated time intraperitoneally, and the lungs were harvested. *Mkp-1* mRNA was detected by Northern blotting using a mouse *Mkp-1* cDNA fragment as a probe. RNA loading was controlled by hybridizing to GAPDH cDNA. Two mice were included in each treatment group. *Mkp-1* mRNA expression was normalized to GAPDH expression and presented underneath the panel as fold relative to the average signal of the two controls. **B.** Induction of *Mkp-1* mRNA in the lungs and the spleens by dexamethasone *in vivo*. *Mkp-1*^{+/+} mice were challenged with either vehicle or 30 mg/kg dexamethasone for 6 h. Lungs and spleen were harvested, and *Mkp-1* expression was examined. The graph depicts *Mkp-1* mRNA expression relative to the mean expression level in vehicle-treated controls. Values represent the mean $\pm$ SE of 5 mice. * dexamethasone-treated different from vehicle-treated, $P < 0.05$.

Fig. 8.

A model illustrating the balance between inflammatory insult and the combined anti-inflammatory capacity contributed by both Mkp-1 and glucocorticoids. The ultimate outcome, either death or survival, is determined by the balance between the strength of the inflammatory insult and the anti-inflammatory capacity. The anti-inflammatory activity of Mkp-1 relies on both glucocorticoid-independent and glucocorticoid-dependent effects. In the animal endotoxic shock model, a portion of the anti-inflammatory action of Mkp-1 is dependent on endogenous cortisol, even in the absence of therapeutic glucocorticoid administration. Glucocorticoids exert anti-inflammatory function through Mkp-1-dependent and Mkp-1-independent mechanisms. The contribution of glucocorticoids depends on dose.