Immunobiology

Macrophage glucocorticoid receptors regulate Toll-like receptor-4-mediated inflammatory responses by selective inhibition of p38 MAP kinase

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Running Title: Glucocorticoid receptor and TLR4 signaling

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Abstract

To explore the role of glucocorticoids in regulation of kinase pathways during innate immune responses, we generated mice with conditional deletion of glucocorticoid receptor (GR) in macrophages (MGRKO). Activation of toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) caused greater mortality and cytokine production in MGRKO mice than controls. Ex vivo, treatment with dexamethasone (Dex) markedly inhibited LPS-mediated induction of inflammatory genes in control but not GR-deficient macrophages. We show that Dex inhibits p38 MAPK, but not PI3K/Akt, ERK or JNK, in control macrophages. Associated with p38 inhibition, Dex induced MAP kinase phosphatase-1 (MKP-1) in control, but not MGRKO, macrophages. Consistent with the ex vivo studies, treatment with a p38 MAPK-specific inhibitor resulted in rescue of MGRKO mice from LPS-induced lethality. Taken together, we identify p38 MAPK and its downstream targets as essential for GR-mediated immunosuppression in macrophages.
Introduction

Pathogen invasion, mechanical trauma, and chemical irritation activate the host innate immune system to maintain physiological homeostasis. This inflammatory response is characterized by a temporally restricted cascade of cytokines and other signaling molecules that limit ongoing infection or compartmentalize damaged tissue. Excessive activation or failure to efficiently terminate these innate responses, however, contributes to common acute human disease states such as septic shock and chronic disorders such as arthritis, asthma, inflammatory bowel disease, osteolysis in periodontal disease and otitis media, and atherosclerosis. Members of the nuclear-receptor superfamily of transcription factors have emerged as important modulators of innate immunity through their effects on macrophage gene expression.

Amongst nuclear-receptor superfamily members, the glucocorticoid receptor (GR), peroxisome-proliferator-activated receptors (PPARs), and liver X receptors (LXRs) have been most robustly associated with regulation of macrophage function. Recent evidence suggests combinatorial interaction of these receptors to provide integration of responses. The relative contribution of or requirement for each of these factors is likely to depend upon the nature of the macrophage-activating stimulus. However, for no single mechanism of macrophage activation have the key molecular interactions of these nuclear receptors with the complex network of proinflammatory intracellular signaling pathways been completely elucidated.

Toll-like receptors (TLRs) on macrophages recognize pathogen-associated molecular patterns (PAMPs) to generate immunologically relevant responses. Recently, it has been shown that glucocorticoids acting through GR efficiently suppress cytokine induction downstream of toll-like receptor 4 (TLR4) and MyD88 activation by lipopolysaccharide (LPS). Normally, engagement of TLR4 triggers a cascade of signaling events leading to induction of mitogen-activated protein kinases (MAPKs), e.g., ERK, JNK, and p38, phospha...
hydroxykinase (PI3K)/Akt, (activator protein-1) AP-1, and NFκB resulting in enhanced transcription and stabilization of the mRNAs for several proinflammatory cytokines including IL-6, IL-1β, TNF-α and IL-12. Particular attention has been given to glucocorticoids acting through GR to impair NFκB-mediated cytokine induction, with recent evidence implicating an important role for GR mitigation of IRF-3-p65 interaction. GR has also been suggested to block activation of MAPK/Akt-mediated gene induction, but the relative importance of specific kinase pathways in GR-mediated suppression macrophage of cytokine production remains unclear.

Further, ligand-dependent GR action can result in transactivation of transcription by binding to glucocorticoid response elements and recruitment of coactivators, transrepression of transcription by protein-protein interaction with other components of transcription complexes either directly or through recruitment of co-repressors, or non-genomic effects with action outside the cell nucleus. Mice harboring a dimerization-deficient GR (GR\textsuperscript{dim}) retain the capacity to efficiently repress cytokine responses, suggesting that DNA binding and classic transactivation of new gene expression are not essential for GR-mediated suppression of inflammation. However, the GR\textsuperscript{dim} mutation engineered has been suggested to not completely abrogate GR transactivation. Global deletion of one novel target of GR transactivation, MKP-1, has recently shown this molecule to be important for survival after TLR4 expression by limiting p38 and JNK signaling. A recent report demonstrates that MKP-1-deficient macrophages also show resistance to dexamethasone-mediated cytokine suppression. Interestingly, GR\textsuperscript{dim} mice normally upregulate MKP-1 expression with glucocorticoid treatment.

Here, we test the hypothesis that macrophage glucocorticoid receptors are essential to limit cytokine production and morbidity after TLR4 activation in vivo. Further, using in vivo and ex vivo analyses of macrophages from control and macrophage GR deficient (MGRKO) mice, we find that glucocorticoids acting through the glucocorticoid receptor specifically impact upon p38 MAPK, but not ERK, JNK, or Akt, activation. This effect on p38 MAPK is associated with
induction of MKP-1 in control but not MGRKO macrophages. Specific inhibition of p38 MAPK results in substantial down-regulation of cytokine production in LPS-stimulated macrophages, though not to the extent of dexamethasone in GR-containing cells. The importance of p38 MAPK induction was confirmed in vivo, as MGRKO mice are rescued from LPS-induced mortality by pharmacological p38 MAPK inhibition.
Methods

Animal handling. Mouse protocols were approved by the Animal Care and Use Committee of Washington University. Mice were housed on a 12 h light and 12 h dark cycle. Blood was collected by retro-orbital phlebotomy into heparinized capillary tubes, with the time from first handling the animal to completion of the bleeding not exceeding 30 sec. Mice used for the experimentation were 6-10 weeks old and were of C57BL/6 X 129/Sv background.

Generation of MGRKO mice. Mice with conditional deletion of GR in macrophages (MGRKO) were generated using LysM promoter-driven, Cre recombinase-mediated excision of exons 1C and 2 of the GR gene 36,37. Southern blot analysis was performed as previously described 36. After germline transmission, we mated heterozygous mice harboring the floxed exon1C and 2 allele to LysM-Cre transgenic mice. LysM-Cre negative homozygous floxed GR littermates were used as controls. Experiments were done with sex-matched littermates as controls.

Isolation and culture of mouse peritoneal macrophages. Thioglycollate-elicited macrophages were isolated by peritoneal lavage 3 days following intraperitoneal (i.p.) injection of 2.0 ml of sterile 4% thioglycollate (Becton & Dickinson, Sparks, MD). Cells were plated in complete medium containing DMEM (Cambrex BioSciences, Walkersville, MD) with 10% fetal bovine serum (Invitrogen Corporation, Grand Island, NY) and penicillin/streptomycin (Invitrogen Corporation, Grand Island, NY). After 3 h of attachment, medium was aspirated and the monolayer rinsed twice with serum free DMEM. Fresh complete medium was then added to each well, and the cells were cultured under a humidified atmosphere of 5% CO2 in air at 37°C.

Cytokine Measurements. For in vivo cytokine studies, mice were injected i.p. with PBS (Cambrex BioSciences, Walkersville, MD) or LPS (Escherichia coli 0111:B4) (Sigma, St. Louis,
MO) at a dose of 10 mg/kg body weight. Plasma samples were collected after the indicated periods of time and stored at -80 °C until assay. Serum proinflammatory cytokine concentrations were measured by enzyme-linked immunosorbent assay (ELISA) for TNF-α and IL-6 according to the manufacturer’s instructions (BD Biosciences PharMingen, San Diego, CA).

For in vivo experiments with the p38 MAPK inhibitor, MGRKO mice were injected i.p. with vehicle or SB 203580 hydrochloride (559395) (Calbiochem, EMD Biosciences, La Jolla, CA) at a concentration of 2 mg/kg body weight and, 1 h later injected i.p. with LPS at a concentration of 10 mg/kg body weight. Plasma samples were collected after 6 h of LPS injection and accessed for TNF-α and IL-6 as described earlier.

For in vitro experiments cells were plated at 10⁶ cells/ml in a 24-well plate (Becton Dickinson Labware, Franklin Lakes, NJ) with 1.0 ml of complete medium. Cells were treated with dexamethasone (Dex) (Amtech, St. Joseph, MO) or corticosterone (Cort) (Sigma, St. Louis, MO) for 3 h at a concentration of 100 nM (except as indicated) followed by LPS (100 ng/ml) for 18 h or as indicated. Experiments with MKP-1 inhibitor triptolide (TP) (Calbiochem, EMD Biosciences, La Jolla, CA) were performed by treating cells at 0.5 – 2 µM concentrations 3 h before LPS exposure, with cell harvest 30 min after LPS treatment. For the study with p38 MAPK inhibitors cells were treated with 20 µM of SB 203580 (559389) or PD 169316 (513030) (Calbiochem, EMD Biosciences, La Jolla, CA) for 1 h followed by LPS stimulation. Supernatants were collected and accessed for TNF-α and IL-6 as described earlier.

**Survival Curves.** Control and MGRKO mice were injected i.p. with LPS at a dose of 10 mg/kg body weight and observed for 5 days. To study the effect of p38 MAPK inhibitor, MGRKO mice were injected i.p. with vehicle or SB 203580 hydrochloride at a concentration of 2 mg/kg body weight and 1 h later injected i.p. with LPS at a dose of 10 mg/kg body weight and observed for 5 days. Experiments were done with age and sex-matched littermate mice.
Western Blot Analysis. For Western blot analysis, whole cell extracts were resolved through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4-12% separating gel (Invitrogen, Carlsbad, CA). Proteins were transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using a semi-dry transfer system (BIO-RAD, Hercules, CA) and blocked with 5% dried milk in PBS and 0.1% Tween-20 (Sigma, St. Louis, MO). Blots were probed with anti-phospho-HSP27 (07-646) (Upstate, Lake Placid, NY), anti-GR (sc-1004), anti-ERK2 (sc-154), anti-MKP-1 (sc-1199), (Santa Cruz Biotechnology, Santa Cruz, CA); anti-COX-2 (160126) (Cayman, Ann Arbor, MI); anti-actin (A 5060) (Sigma, St. Louis, MO); anti-phospho-SAPK/JNK (9251), anti-SAPK/JNK (9252), anti-phospho-p38 MAPK (9211), anti-p38 MAPK (9212), anti-phospho-p44/42 MAPK (9101), anti-phospho-Akt (9271), anti-Akt (9272), and anti-HSP27 (2402) (Cell Signaling Technology, Beverly, MA) antibodies. Binding of horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (sc-2004) or goat anti-mouse antibody (sc-2005) (Santa Cruz Biotechnology, Santa Cruz, CA) was determined using SuperSignalWest Chemiluminescent Substrate (Pierce, Rockford, IL). Blots were stripped with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) and re-probed with different antibodies.

Statistical Analysis. Data are expressed as the mean ± S.E.M. Statistical significance was tested by unpaired two-tailed Student t-test. The differences were considered to be significant for P < 0.05.
Results

**GR in macrophages is essential for survival after TLR4 activation**

To define the role of GR in the regulation of innate immune responses, we developed mice with conditional disruption of GR in macrophages. Southern blot analysis showed efficient recombination of the loxP-flanked GR allele in thioglycollate-elicited macrophages isolated from MGRKO mice, and evidence for disruption in spleen, but not kidney, liver, or thymus (Supplementary Fig. 1 and data not shown). Western blot analysis with an anti-GR antibody demonstrated no GR immunoreactivity in thioglycollate-elicited peritoneal macrophages isolated from MGRKO mice (Supplementary Fig. 1). MGRKO mice where grossly indistinguishable from control littermates. However, after TLR4 activation by lipopolysaccharide (LPS) injection, 88% of the MGRKO mice died as opposed to only 13% of control mice (Fig. 1A). Thus, GR action in macrophages is required to limit LPS-induced lethality.

**Deficiency of GR in macrophages alters systemic secretion of proinflammatory cytokines**

To evaluate the involvement of GR in regulating inflammatory responses *in vivo*, we measured TNF-α and IL-6 after LPS treatment. We found no difference in cytokine secretion between genotypes 1 h after LPS injection (Fig. 1B). After 3 or 6 h of LPS treatment, we found 2-2.5-fold increases in TNF-α and IL-6 secretion in MGRKO as compared to the control mice (Fig. 1B). Further, we measured a 5-fold increase in TNF-α in MGRKO mice in comparison to control mice 12 h after LPS treatment (Fig. 1B). A large and equivalent rise in serum corticosterone occurred in both genotypes at 3 and 24 h after LPS administration (data not shown), implicating the differential cytokine secretion between genotypes resulted from impaired glucocorticoid-mediated inhibition of cytokine production in the MGRKO mice.
Dex suppresses LPS mediated inflammatory responses

To analyze direct actions of glucocorticoids on GR in macrophages, we exploited thioglycollate-elicited peritoneal macrophages. To examine the effect of GR deficiency on the secretion of inflammatory cytokines, macrophages from both genotypes were treated LPS with or without Dex. Treatment with Dex (100 and 1000 nM) suppressed TNF-α and IL-6 by 80% and 90% in control macrophages (Fig. 2A and Supplementary Fig. 2 for time course). GR-deficient macrophages demonstrated no significant change in cytokine secretion with Dex (Fig. 2A), consistent with their loss of GR immunoreactivity.

Induction of COX-2 accounts for elevated levels of prostanoids in chronic inflammatory disorders, and has been found to be glucocorticoid-regulated. Indeed, augmented prostanoid production following TLR4 engagement results in many of the systematic manifestations associated with endotoxemia. Similar to cytokine production, pretreatment with Dex abrogated LPS-mediated induction of COX-2 in control macrophages (Fig. 2B). Macrophages from MGRKO mice showed complete resistance to Dex (Fig. 2B).

GR selectively impairs LPS-mediated activation of p38 MAPK

Members of MAPK family play crucial roles in multiple aspects of inflammatory responses. Kinetic analysis revealed that LPS-mediated p38 MAPK phosphorylation follows a bi-phasic pattern. Induction of MAPK was found as early as 15 min after LPS stimulation and persisted for 30 min (Fig. 3A). Phospho-p38 MAPK was undetectable in between 1 h and 3 h after LPS treatment, however the abundance of total p38 MAPK was comparable throughout the time course. We observed a second phase of phospho-p38 MAPK induction after 6 h of LPS treatment, which persisted for until the 12 h time point. To study the effect of Dex on p38 MAPK phosphorylation, we chose the early (15 and 30 min) and the late phase (6 h and 12 h) of kinase induction. We observed that pretreatment with Dex suppressed p38 MAPK phosphorylation in the control macrophages during both phases (Fig. 3B). Densitometric analysis revealed that
Dex-mediated inhibition of p38 MAPK phosphorylation after 15 and 30 min of LPS stimulation was 2.2 and 4.8-fold, respectively. At the late phase of induction, phosphorylation of p38 MAPK was completely inhibited in Dex pretreated control macrophages (Fig. 3B). No detectable change in p38 MAPK phosphorylation was observed in MGRKO macrophages by Dex pretreatment (Fig. 3B). Similar experiments with corticosterone, the endogenous murine glucocorticoid, showed marked suppression of p38 MAPK phosphorylation in control macrophages and no detectable change in GR-deficient macrophages (Supplementary Fig. 3A). Temporal analysis showed a similar bi-phasic activation of JNK (Supplementary Fig. 3B). In contrast to p38 MAPK, phosphorylation of JNK was not altered with Dex treatment (Fig. 3C). A similar study with ERK1/2 showed that Dex does not alter ERK-phosphorylation (Fig. 3D). Additionally, Dex had no effect on Akt phosphorylation in the control macrophages (Fig. 3E).

**Effect of p38 MAPK inhibitors on proinflammatory gene expression**

To address the importance of p38 MAPK in proinflammatory gene expression, we used the p38 MAPK-specific inhibitors SB 203580 or PD 169316. Treatment with these inhibitors significantly inhibited LPS-induced secretion of TNF-α and IL-6 in control and GR-deficient macrophages by approximately 60% and 50%, respectively (Fig. 4A and Supplementary Fig. 4).

HSP27 is a downstream target of p38 MAPK. To examine the efficacy of p38 MAPK inhibitors, the phosphorylation of HSP27 at Ser^{82} was examined. Treatment with SB 203580 caused 90% reduction in stimulated phospho-HSP-27 (Fig. 4B), suggesting the differential attenuation of cytokine production between Dex and the p38 inhibitors was not due to inadequate p38 inhibition. Furthermore, treatment with SB 203580 markedly inhibited LPS-mediated COX-2 induction in control and GR-deficient macrophages, consistent with previous studies that have found both transcriptional and post-transcriptional stimulatory actions of p38 activity for COX-2 induction^{40-42}. We observed 12-fold suppression of COX-2 6 h after LPS treatment by SB 203580 (Fig. 4C). A similar experiment with GR-deficient macrophages showed
a comparable inhibition of COX-2 induction by SB 203580 pretreatment (Supplementary Fig. 5).

**Dex induces MKP-1 in control macrophages**

MKP-1, a phosphatase induced by ligand-activated GR, suppresses MAPK activation \(^{24,25,43,44}\). Without Dex treatment, weak induction of MKP-1 was observed at 1-3 h of LPS treatment, and thereafter declined to the basal levels in both genotypes (Fig. 5A). Treatment with Dex induced MKP-1 18-fold in control macrophages and resulted in prolonged expression (Fig. 5A). Dex did not increase expression of MKP-1 in GR-deficient macrophages (Fig. 5A).

To assess the contribution of GR-mediated induction of MKP-1 to attenuation of p38 MAPK phosphorylation we used triptolide, a pharmacological inhibitor, to block MKP-1 induction \(^{45-47}\). Dex in the presence of triptolide suppressed LPS-induced p38 MAPK phosphorylation by only 10% compared to triptolide treatment alone, and p38 MAPK phosphorylation in the presence of Dex plus triptolide remained elevated above LPS-alone levels (Fig. 5B). Without triptolide co-treatment, Dex again potently suppressed p38 phosphorylation (Fig. 5B).

**p38 MAPK inhibition rescues MGRKO mice from LPS-mediated lethality**

To assess the relative importance of p38 MAPK regulation by GR in vivo, we treated MGRKO mice with LPS and SB 203580 or vehicle. We observed 88% mortality in the vehicle treated MGRKO mice, while 89% of SB 203580-treated MGRKO mice survived (Fig. 6A). In accord with this improved survival, SB 203580 treatment decreased plasma TNF-α and IL-6 concentrations by 2-2.5 fold (Fig. 6B), resulting in concentrations comparable to control wild type mice after LPS treatment.
Discussion

These studies utilize in vivo and ex vivo methods to evaluate the importance of GR modulation of signaling down-stream of activated TLR4, and further, to elucidate the mechanisms by which GR attenuates pro-inflammatory activity in macrophages. We find that MGRKO mice are highly susceptible to LPS-induced mortality. This finding is consistent with the increased susceptibility of glucocorticoid-deficient, adrenalectomized animals to LPS administration, and highlights the macrophage as a key site of action for glucocorticoid enhancement of survival.

Recent efforts in understanding signaling down-stream of TLR4 have revealed an important role of disruption IRF-3-NFκB interaction by GR in inhibiting pro-inflammatory cytokine production. Activation of TLR4 in macrophages results not only in induction of NFκB activity, but also stimulates several kinases that increase proinflammatory gene expression. Here we demonstrate robust phosphorylation of p38 MAPK, JNK, ERK1/2, and Akt after TLR4 engagement by LPS. Both p38 MAPK and JNK display biphasic patterns of induction, with early (up to 30 min) and late (after 6 h) increases in phosphorylation. This biphasic time course corresponds to the transient induction of MKP-1 that occurs in LPS-stimulated macrophages in the absence of glucocorticoid exposure. Only p38 MAPK amongst this group of kinases, however, appears to have sensitivity to dexamethasone-mediated suppression in control macrophages. Based upon the inability of Dex to suppress of LPS-induced p38 MAPK phosphorylation in the presence of triptolide, an MKP-1 synthesis inhibitor, MKP-1 appears to be the primary target for GR-associated inhibition of p38 MAPK. It is somewhat surprising that dexamethasone-induced MKP-1 does not down-regulate JNK phosphorylation in control macrophages despite up-regulation of JNK phosphorylation in MKP-1 deficient macrophages.
suggests subcellular compartmentalization of MKP-1 action with steroid treatment or different affinity of MKP-1 for phosphorylated forms of p38 and JNK.

Given the wealth of data regarding GR-mediated down-regulation of the NFκB pathway, how important are actions of GR in modulating p38 MAPK? p38 was originally identified as a tyrosine-phosphorylated protein in macrophages after stimulation with LPS \(^{(49)}\), and more recently after a more diverse repertoire of stimuli \(^{(50)}\). Studies in humans have suggested a key role for p38 signaling after LPS exposure as healthy volunteers treated with p38 inhibitors displayed reduced ex vivo cytokine production with LPS stimulation \(^{(51)}\). In addition, clinical symptoms and inflammatory markers in humans after LPS exposure are improved with p38 inhibitor treatment in vivo as well \(^{(52,53)}\). Our ex vivo studies on primary macrophages demonstrate approximately 50-60% suppression of cytokine production by p38 inhibitors, whereas dexamethasone inhibits cytokine production 70-80%. These findings indicate that p38 inactivation accounts for a large part, but not the entirety of GR effects, and is in accord with an additional role for NFκB. The relative importance of p38-GR interaction for mediating glucocorticoid immunosuppression is highlighted further by the survival of p38-inhibitor treated MGRKO mice exposed to LPS described in this report, and the impaired glucocorticoid-mediated cytokine suppression in MKP-1-deficient macrophages reported by others.

The relative importance of transactivation properties of GR, that is induction of gene expression at the transcriptional level by DNA binding, versus its transrepression of gene expression by protein-protein interaction has been an area of active debate \(^{(5)}\). Previous studies have shown that induction of MKP-1 by the glucocorticoid-GR complex occurs at the transcriptional level, with GR transactivation mutants lacking the ability to activate the MKP-1 promoter in reporter assays \(^{(24)}\). Studies in GR\(^{\text{dim}}\) mice suggested that DNA-binding, transactivation-dependent effects may have less importance the GR protein-protein interactions \(^{(12)}\). Our evidence that MGRKO macrophages fail to induce MKP-1 with dexamethasone treatment, while GR\(^{\text{dim}}\) macrophages are capable of MKP-1 induction \(^{(35)}\) together implicate
significant residual transactivation properties with the GR$^{\text{dim}}$ mutation, as suggested in transfection studies $^{32}$.

**Clinical Implications**

The notion of pharmacological inhibition of p38 MAPK activity as a possible substitute for glucocorticoid-mediated immunosuppression has been an active area for drug discovery $^{52,54-57}$. Our data suggests that glucocorticoids normally impact upon p38 activity for a substantial portion of their therapeutic anti-inflammatory actions, and thus targeting p38 directly is a logical goal. Recent efforts to bring p38 inhibitors into clinical practice have had limited success. While efficacious, these compounds lead to heptocellular toxicity, which has impeded their use. The induction of MKP-1 by glucocorticoids in LPS-stimulated macrophages provides one important target for GR action, and may serve as a new target for drug development. MKP-1 is a threonine/tyrosine dual-specificity phosphatase that has been shown to limit cytokine hyper-responsiveness in rodents in vivo and macrophages ex vivo through the analysis of MKP-1-deficient mice $^{33,34}$. We find that without glucocorticoid stimulation, MKP-1 is only transiently induced with TLR4 activation, but GR activation induces higher levels of MKP-1 expression, for longer durations, ex vivo. This effect would be anticipated in vivo with activation of the hypothalamic-pituitary-adrenal axis and production of glucocorticoids with sustained inflammation. One attractive mechanism, then, by which anti-inflammatory glucocorticoid effects could be accomplished without detrimental glucocorticoid sequelae or p38 inhibitor toxicity is through pharmacological augmentation of MKP-1 action.
Acknowledgements

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References


Figure Legends

Fig. 1 Deletion of GR in macrophages augments LPS-induced lethality and proinflammatory responses. (A) Kaplan-Meyer plot of control (n=8) and MGRKO (n=8) mice treated with LPS (10 mg/kg). P<0.01 for MGRKO vs. control. Plasma concentrations of (B) TNF-α and IL-6 in control (n=7-10) and MGRKO (n=6-10) mice treated with LPS for the indicated periods of time.

Fig. 2 Dex suppresses LPS-mediated inflammatory gene expression. (A) Effect of Dex on LPS mediated induction of TNF-α and IL-6 in control (■) and MGRKO (□) macrophages. Data are shown as mean ± S.E.M.; n=5; *, P<0.01 compared to treatment group without Dex in control macrophages. (B) Effect of Dex on LPS mediated induction of COX-2. Representative of 3 independent experiments.

Fig. 3 Dex selectively suppresses LPS-mediated activation of p38 MAPK. (A) Time-dependent effect of LPS (100 ng/ml) on p38 MAPK phosphorylation. Representative of 2 independent experiments. Effect of Dex (100 nM) on LPS mediated phosphorylation of (B) p38 MAPK in control and MGRKO macrophages and (C) JNK (p46/p54) (D) ERK 1/2 (E) Akt in the control macrophages. Representatives of 3-4 independent experiments.

Fig. 4 p38 MAPK inhibitors impair proinflammatory gene expression and effect of Dex on MKP-1. (A) Effect of SB (20 μM) and PD (20 μM) on LPS mediated induction of TNF-α and IL-6 in control macrophages. n=3; **, P<0.01 and *, P<0.02 compared to treatment group with LPS. Effect of SB (20 μM) on LPS mediated (B) phosphorylation of HSP27 and (C) induction of COX-
2 for the indicated period of time in control macrophages. Representatives of 2-3 independent experiments.

**Fig. 5** Dex induction of MKP-1 expression is the major GR target for suppression of p38 MAPK phosphorylation. (A) Effect of Dex on MKP-1 induction in control and MGRKO macrophages treated with LPS for the indicated periods of time. (B) Effect of triptolide on p38 MAPK phosphorylation in the presence and absence of Dex. Representative of 2-3 independent experiments.

**Fig. 6** SB suppresses LPS induced systemic secretion of proinflammatory cytokines and lethality in MGRKO mice. (a) Effect of SB (■) or vehicle (□) on LPS-mediated mortality in MGRKO mice. For the SB-treated group, mice were injected i.p. with SB (2 mg/kg) 1 h before LPS (10 mg/kg) treatment. p<0.01 for SB treated mice (n=9) compared to vehicle treated mice (n=8). (b) Effect of SB (■) or vehicle (□) on LPS-mediated induction of TNF-α and IL-6 in MGRKO mice. Mice were injected i.p. with SB or vehicle followed by treatment with LPS for 6 h. *, P<0.05 for SB-treated group (n=5) compared with vehicle-treated group (n=11).

**Fig. 7** GR attenuates proinflammatory responses in macrophages by impairment of p38 MAPK. While TLR4 engagement activates PI3 kinase (PI3K) and multiple MAP kinase kinases (MKK) and their downstream kinases, p38 MAPK is selectively glucocorticoid (GC) sensitive by transcriptional activation of MKP-1 through GR.
Figure 1

A. Survival (%) over time (d) for Control and MGRKO groups.

B. Comparison of TNF-α (pg/ml) and IL-6 (pg/ml) levels between Control and MGRKO groups at 1h, 3h, 6h, and 12h with statistical significance indicated (p<0.05).
Figure 3

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Figure 4

A. Bar graphs showing concentrations of TNF-α (ng/ml) and IL-6 (ng/ml) with different treatments.

B. Western blots showing P-HSP 27 and Total HSP 27 levels in various conditions.

C. Western blots showing COX-2 and β-Actin expression in different treatments.
Figure 5

A. 

Control

MGRKO

MKP-1

β-Actin

MKP-1

β-Actin

B. 

LPS-30'

Media

DMSO

TP-0.5 µM

TP-1.0 µM

TP-2.0 µM

Dex

Dex+TP-0.5 µM

Dex+TP-1.0 µM

Dex+TP-2.0 µM

P-p38 MAPK

Total p38 MAPK
Figure 6

a.

Survival (%) vs. Time (d)

- SB
- Vehicle

b.

Bar charts showing:
- TNF-α (pg/ml)
- IL-6 (pg/ml)

- Vehicle
- SB

* indicates statistical significance.
Macrophage glucocorticoid receptors regulate Toll-like receptor-4-mediated inflammatory responses by selective inhibition of p38 MAP kinase

Sandip Bhattacharyya, Diane E. Brown, Judson A. Brewer, Sherri K. Vogt and Louis J. Muglia