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Tissue-resident Macrophages Protect the Liver From Ischemia Reperfusion Injury via a Heme Oxygenase-1-Dependent Mechanism

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Kupffer cells are the resident macrophage population of the liver and have previously been implicated in the pathogenesis of hepatic ischemia–reperfusion injury (IRI). Kupffer cells are the major site of expression of hepatic heme oxygenase-1 (HO-1), which has been shown to have anti-inflammatory actions and to protect animals and cells from oxidative injury. Kupffer cells and circulating monocytes were selectively ablated using liposomal clodronate (LC) in the CD11b DTR mouse before induction of hepatic ischemia. Kupffer cell depletion resulted in loss of HO-1 expression and increased susceptibility to hepatic IRI, whereas ablation of circulating monocytes did not affect IRI phenotype. Targeted deletion of HO-1 rendered mice highly susceptible to hepatic IRI. In vivo, HO-1 deletion resulted in pro-inflammatory Kupffer cell differentiation characterized by enhanced Ly6c and MARCO (macrophage receptor with collagenous structure) expression as well as decreased F4/80 expression, mirrored by an expansion in immature circulating monocytes. In vitro, HO-1 inhibition throughout macrophage differentiation led to increased cell numbers, and pro-inflammatory Ly6c+ CD11c– F4/80– phenotype. These data support a critical role for tissue-resident macrophages in homeostasis following ischemic injury, and a co-dependence of HO-1 expression and tissue-resident macrophage differentiation.

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INTRODUCTION

As key mediators of immune responses following hepatic injury, Kupffer cells have been implicated in the pathogenesis of ischemia–reperfusion injury (IRI). Antigen presenting cells (APCs) are at the heart of the pathway of immune activation described by the “danger model.” When activated by danger associated molecular patterns (DAMPs) released by injured parenchymal cells, APCs secrete pro-inflammatory cytokines, activate T cells, and initiate neutrophil chemotaxis. “Classical” activation is characterized by secretion of pro-inflammatory cytokines including IL-1β and TNF-α, while “alternate” activation is characterized by secretion of anti-inflammatory IL-10. Analogous to “classical” and “alternate” modes of macrophage activation, macrophage differentiation has been shown to proceed broadly down two divergent pathways. One population, termed “inflammatory monocytes” express CCR2 and are attracted to acutely inflamed sites by MIP-1 signaling: these cells also express the antigen Ly6c. The second group, termed “resident” monocytes, express CX3CR1, patrolling, and re-populating resting tissues. These cells also express the cell surface marker CD11c.

Modulation of immune activation at any stage has been shown to protect animals from IRI. Interruption of DAMPs, Toll-like receptors (TLRs), pro-inflammatory cytokines, T-cell receptors, co-stimulatory pathways, or T cells have all been shown to ameliorate IRI. Experimental modulation of Kupffer cell function has been attempted using gadolinium chloride and glycine. Although the mechanisms through which these reagents have their protective effects are incompletely characterized, both have been shown to suppress TNF-α secretion and to ameliorate IRI. This has been taken to imply that Kupffer cells have a broadly harmful effect following ischemic injury.

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme into biliverdin, free iron, and carbon monoxide. In the liver, it is expressed principally by Kupffer cells. HO-1 induction protects cells and animals from IRI by modulation of every stage of the immune activation pathway. It confers direct cytoprotection which may limit production of DAMPs, mediates the protected phenotype arising from interruption of TLR signaling, switches macrophages in vitro from “classical” to “alternate” activation, modulates T-cell activation, and enhances immunological tolerance.

In light of the anti-inflammatory role of HO-1, we hypothesized that inhibition or deletion of this enzyme might drive macrophage differentiation down an inflammatory pathway. We demonstrate in vivo and in vitro that HO-1 is required for differentiation of “resident” macrophages, which promote homeostasis, while suppressing expansion of inflammatory monocyte populations. By selectively depleting macrophage populations using...
liposomal clodronate (LC)\(^1\) and diphtheria toxin in the CD11b\(^{DTR}\) mouse,\(^4\) we show that Kupffer cells and not circulating monocytes are required for hepatocellular survival of IRI. We suggest that the effect of HO-1 upon macrophage differentiation is a potential avenue of investigation for the amelioration of injury in the wide range of human diseases caused by ischemia. Since HO-1 has been shown to modulate the immunopathological contribution to a wide range of disorders, our findings concerning its effect upon macrophage differentiation may have broad application.

RESULTS

Kupffer cells but not circulating monocytes are essential for hepatic protection from ischemic insults

First, to verify our model against others in the published literature, we treated animals with GdCl\(_3\) before 50’ hepatic ischemic insults and found them to be protected from injury (GdCl\(_3\)-treated alanine aminotransferase (ALT) 1929 ± 414 IU, untreated ALT 4467 ± 532 \(P = 0.006\)) (Figure 1a). Immunohistochemistry for F4/80 demonstrated no significant anatomical ablation of Kupffer cells (Figure 1b). Western blotting of liver lysates from GdCl\(_3\)-treated animals demonstrated HO-1 induction (Figure 1c), and pretreatment of bone marrow–derived monocytes (BMDMs) with GdCl\(_3\) before LPS stimulus resulted in decreased TNF-α secretion (Figure 1d). Thus, our findings were broadly in line with published data using this reagent, with the added observation that HO-1 was induced by GdCl\(_3\) without altering Kupffer cell number or spatial distribution. In light of these findings and previously published literature,\(^19,20\) it is likely that GdCl\(_3\) induces a functional change in macrophages, which accounts for its protective effect against ischemia.

As an alternative manipulation of macrophage populations, we used the CD11b\(^{DTR}\) mouse to selectively ablate tissue-resident and circulating macrophage populations before ischemia–reperfusion insults. As previously described,\(^4\) treatment of CD11b\(^{DTR}\) mice with diphtheria toxin (DT) 25 ng/g i.p. resulted in loss of circulating monocytes (DT 2.1 × 10\(^5\) monocytes/ml, PBS 7.2 × 10\(^5\) monocytes/ml \(P < 0.004\)) (Figure 2a) but sparing of Kupffer cells and splenic macrophages (Figure 2b). Administration of LC 100 μl i.v.\(^17\) resulted in complete loss of Kupffer cells from the liver, and substantial depletion of splenic macrophages (Figure 2b). Whereas the systemic stress caused by serial blood sampling led to a progressive monocytosis in PBS-treated mice, this was suppressed at 24 hours in LC-treated mice with recovery at 48 hours after injection (Figure 2a). In keeping with immunohistochemical findings that HO-1 expression is principally within Kupffer cells (Figure 2c), Western blotting demonstrated that Kupffer cell ablation arising from LC treatment led to complete loss of hepatic HO-1 expression, even in the ischemic liver lobe in which HO-1 is normally potently induced (Figure 2d).

Twenty-four hours following DT, LC, or PBS treatments, mice were subjected to 40-minute left hepatic ischemia–reperfusion insults. Following injury, circulating monocyte depleted and PBS-treated animals were not significantly injured in terms of ALT release or histological injury (PBS ALT 339 ± 78 IU, Hist inj 3 ± 0.6 arbitrary units, DT ALT 384 ± 67 IU, Hist inj 1.7 ± 1 arbitrary units) whereas Kupffer cell–depleted animals were highly susceptible to ischemia (ALT 4640 ± 790, \(P = 0.002\), Hist inj 6.5 ± 0.5 arbitrary units \(P = 0.019\)) (Figure 2e, f). These data imply a homeostatic role for Kupffer cells but not circulating monocytes following hepatic ischemic injury. The focus of HO-1 expression within Kupffer cells and its loss following Kupffer cell ablation suggests a key role for this enzyme in the protection afforded by Kupffer cells.

HO-1 expression is essential for protection from hepatic ischemic insults

To examine the influence of heme oxygenase in protection from liver IRI we studied response in wild-type, heterozygous and homozygous Hmox-1 deleted mice. In accordance with previously published pharmacological inhibitor studies, Hmox-1\(^{-/-}\) mice were more susceptible to hepatic ischemic insults than Hmox-1\(^{+/+}\) and \(^{+/+}\) animals in terms of ALT release and histological injury score (Figure 3a, b). Following ischemia, relative HO-1 expression in the nonischemic and ischemic lobes of Hmox-1\(^{-/-}\) animals was approximately half that of wild-type animals (Figure 3c, d) (nonischemic Hmox-1\(^{+/+}\) 1 ± 0.11x, Hmox-1\(^{-/-}\) 0.47 ± 0.16x, \(P = 0.04\); ischemic Hmox-1\(^{+/+}\) 2.1 ± 0.32x, Hmox-1\(^{-/-}\) 1.0 ± 0.25x \(P = 0.04\)). No HO-1 expression was seen in Hmox-1\(^{-/-}\) animals in control or ischemic lobes (data not shown).

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Figure 1 The effect of gadolinium chloride. (a) Wild-type animals treated with GdCl\(_2\) 20 mg/kg IP were protected from 50 minute hepatic ischemic insults compared with vehicle treated controls (n = 4 per group). (b) Immunohistochemistry demonstrated no significant loss of F4/80+ Kupffer cells following GdCl\(_3\) administration. (c) When taken from animals 24 hours after GdCl\(_3\) treatment, liver lysates demonstrated HO-1 induction on western blotting. (d) Pretreatment of BMDMs with GdCl\(_3\) prior to LPS stimulus decreased TNF-α secretion.

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Macrophages Protect Liver From Ischemic Injury

Tissue-resident macrophage differentiation is HO-1 dependent. Hmox-1−/− Kupffer cells have inflammatory immunophenotype

Kupffer cells in resting Hmox-1+/+, +/−, and −/− mice were phenotyped for F4/80, CD11b, CD68 (macрослalin), CD206 (mannose receptor), MARCO (macrophage receptor with collagenous structure), and Ly6c. Immunohistochemical findings are summarized in Table 1. CD68 staining was highly abnormal in Hmox-1−/− livers, revealing dysmorphic, multinucleate cells (Figure 4).

These data suggest that HO-1 may be required for normal “resident” macrophage/macrophage differentiation. Hmox-1−/− Kupffer cells had a distinctly inflammatory phenotype characterized by expression of Ly6c and MARCO (Figure 4). Ly6c, a component of the Gr1 epitope is associated with inflammatory monocytes.4 MARCO is associated with “classical” macrophage activation, being upregulated following LPS stimulus.23,24 CD68 has also been associated with macrophage activation.23,24 F4/80, which is associated with modulation of adaptive immune responses,25 was absent from Hmox-1−/− Kupffer cells (Figure 4).

Hmox-1−/− animals have larger numbers of immature circulating monocytes

Blood samples from Hmox-1−/− and Hmox-1+/+ mice were compared on flow cytometry with respect to monocyte (Figure 5a–c) and neutrophil counts (Figure 5d). Hmox-1−/− mice had 2.1 times more monocytes than wild-type controls (P = 0.02). This was made up of a large immature Gr1hi group of which there was a 2.5-fold increase compared with Hmox-1+/+ animals (P = 0.06).
of pro-inflammatory stimuli. Wild-type, Hmox-1+/+, and Hmox-1−/− bone marrow–derived hemopoietic progenitors were matured for 6 days into monocytes (BMDMs) in L929 conditioned medium. Wild-type cells were cultured throughout their maturation in the presence or absence of the highly specific HO-1 inhibitor Chromium Mesoporphyrin IX (CrMP).28

HO-1 inhibition and deletion led to expansion of cell numbers (Figure 6a) as well as pleomorphism in terms of differences in size and granularity (not shown) compared with the homogenous population of cells derived from HO-1 intact mice.

In common with our in vivo observations, HO-1 inhibition in vitro resulted in loss of F4/80 expression. In HO-1 intact cultures, 71 ± 1% of cells were F4/80+ whereas HO-1 inhibition reduced this proportion to 23.5 ± 4.6% (P = 0.002). Calculation of cell number indicated a trend toward a smaller absolute number of F4/80+ cells (untreated 17.1 ± 3.5 million cells, HO-1 inhibited 12.2 ± 4.2 million cells, P = 0.08), and expansion of a very large F4/80− population in HO-1 inhibited cultures (accounting for their increased cell numbers) (HO-1 intact 7.2 ± 1.6 million cells, HO-1 inhibited 38.6 ± 7.4 million cells, P = 0.01) (Figure 6b,c).

Flow cytometry dot plots indicated that “inflammatory” Ly6c+ and “resident” CD11c+ subsets were mutually exclusive indicating divergent differentiation (Figure 6d). As we saw in our in vivo investigations, the inflammatory monocyte marker Ly6c was upregulated in HO-1-intact BMDMs. In HO-1 intact cultures, 31.3 ± 2.2% of cells were Ly6c+ whereas in HO-1-inhibited cultures, 74.6 ± 1.8% cells were Ly6c+ (P = 0.0003). Calculation of absolute cell number indicated that the number of Ly6c+/Ly6c− cells in HO-1 intact cultures was 13.1 ± 1/16.5 ± 3 million cells, compared with 43.5 ± 7.1/12.7 ± 2.6 million cells in the HO-1-inhibited group, P = 0.02/P = 0.03 (Figure 6e). The opposite was true of the resident macrophage marker CD11c which was much more frequent in HO-1 intact cultures (41.1 ± 9%) compared with HO-1-inhibited cultures (12.3 ± 4%), giving absolute cell counts of CD11c+/CD11c− in HO-1 intact cultures of 11.3 ± 4.3/13.1 ± 1 million cells compared with 7.4 ± 43.5/7.1 million cells in HO-1-inhibited cultures P = 0.08/P = 0.02 (Figure 6f).

HO-1 inhibition by CrMP (5 µmol/l for 1 hour) increased TNF-α production (1.57 ± 0.17 times untreated, P < 0.00001) (Figure 6g) whereas HO-1 induction by hemin (50µmol/l overnight) reduced TNF-α production following LPS stimulus (0.4 ± 0.05 times untreated, P = 0.01) (Figure 6h).

These in vitro data confirm our in vivo observation that HO-1 activity is required for “resident” patterns of macrophage differentiation, and suppression of “inflammatory” macrophage populations. We also confirm in primary culture the previously published finding15 that HO-1 induction reduces inflammatory cytokine production by macrophages. Together, these data indicate that HO-1 has an anti-inflammatory role which extends from macrophage differentiation right through to modulation of pro-inflammatory cytokine release. In the context of ischemia–reperfusion insults, resident macrophages appear critical for the survival of surrounding parenchymal cells.

**DISCUSSION**

In this article, we have shown that Kupffer cells are essential for hepatic survival of ischemia–reperfusion insults, that hepatic...
HO-1 expression is Kupffer cell dependent, and conversely that macrophage differentiation critically depends upon HO-1 expression.

Numerous studies have employed gadolinium chloride and glycine to modulate Kupffer cell activity, and have shown these drugs to protect animals from hepatic ischemia. Such data give the impression that Kupffer cells have a harmful effect following ischemia. We found that GdCl₃ induced HO-1 and, as shown before in rats, reduced pro-inflammatory cytokine release. The effect of GdCl₃ is thus somewhat complex and incompletely elucidated: as such it provides limited information regarding the physiological role of macrophage populations during ischemic insults.

To unpick the relative importance of circulating monocytes and Kupffer cells during ischemia–reperfusion insults, we selectively ablated each population in CD11bDTR mice using diphtheria toxin or LC, respectively. Kupffer cell but not circulating monocyte ablation led to HO-1 deletion from the liver, and susceptibility to hepatic ischemic insults. Taken together with data showing the importance of TNF-α in causing IRI, and our own data showing HO-1 induction and reduction in TNF-α production from GdCl₃-treated macrophages, these data suggest that the differentiation state of tissue-resident macrophages is critical to the survival of surrounding parenchymal cells following ischemia.

Tissue-resident macrophages could have their protective effect by modulation of immune responses to ischemically injured tissue, by modulation of apoptosis induced directly by TNF-α or by release of carbon monoxide acting as a diffusible paracrine protective factor.

As the immune system contributes so heavily to IRI, the site of hepatic HO-1 expression is the Kupffer cell, and previously published

**Figure 4** The effect of Hmox-1 copy number on Kupffer cell phenotype. Staining for F4/80, CD11b, CD68, CD206 (mannose receptor), MARCO, and Ly6c demonstrated that Hmox-1⁻⁻ Kupffer cell F4/80 expression is decreased, and MARCO and Ly6c expression is increased.
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The ability of HO-1 to manipulate macrophage phenotype could be harnessed for therapeutic gain by infusion of exogenous “anti-inflammatory” macrophage populations, or by using agents such as heme arginate or curcumin to induce HO-1 in the patient to protect from ischemic injury.

The ability of HO-1 to dictate macrophage phenotype adds a further dimension to its apparently great therapeutic potential, offering new avenues for treatment of a range of common and deadly diseases with their pathophysiology rooted in IRI.

MATERIALS AND METHODS

Hmox-1−/− and Hmox-1+/− animals. All procedures and animal care were approved by institutional ethics committees and were in accordance with UK Home Office licensing regulations. C57Bl/6J animals with targeted deletion of the Hmox-1 by neomycin resistance gene insertion were supplied by A Agarwal (Birmingham, Alabama), rederived and bred by heterozygote to heterozygote mating in accordance with local regulations.

Operative procedure. Operated animals underwent clamping of the portal pedicle (hepatic artery and portal vein) supplying the left hepatic under isoflurane anesthesia, with core temperature maintained at 36 °C throughout. Preliminary experiments demonstrated optimal ischemic times of 40 minutes for the detection of susceptible phenotypes, and 50 minutes for demonstration of protection from ischemic injury.

ALT measurement. At 24 hours after ischemic insult, blood was taken via terminal cardiac puncture. ALT was measured on Olympus AU2700 (Olympus Optical, Watford, UK) utilizing methodology recommended by the International Federation of Clinical Chemistry.

Histological injury scoring. Each animal was scored for necrosis and cytoplasmic injury on standardized scales by an experienced liver pathologist (C.O.B.) who was blinded to the experimental group and conditions. The two scores were added to give a single index of liver injury (maximum score 8). Degree of severity for the two parameters was determined as follows: for necrosis, 1 = increased single cell death (apoptosis, necrosis) without confluent necrosis; 2 = microfoci of confluent necrosis in a minority of lobules, not encircling the full circumference of the central vein; 3 = confluent necrosis in a majority of lobules, or confluent necrosis around the full thickness of the central vein >4 hepatocyte layers deep; 4 = panlobular necrosis. For cytoplasmic injury, 1 = sparse and minor non-specific changes (minor microvesicular change), 2 = confluent perivenular hepatocyte cytoplasmic injury in a small minority of lobules (<25%), 3 = perivenular hepatocyte injury in 25–50% lobules, 4 = diffuse severe cytoplasmic injury in a majority (>50%) of lobules.

Western blot. Western blotting was performed as described previously: HO-1 was detected using rabbit polyclonal anti HO-1 (Stressgen SPA-896; Nventa, San Diego, CA) diluted 1:5,000 in 1% BSA TBS 0.05% Tween. Loading control was Abcam mouse anti β-Actin (ab6276, Abcam, Cambridge, UK) diluted 1:10,000 in 1% BSA TBS 0.05% Tween. All antibodies were used overnight at 4 °C.

Figure 5 Monocyte and neutrophil counts in Hmox-1+/+ and Hmox-1−/− animals. (A) Monocyte counts in resting Hmox-1−/− animals were increased by 2.1-fold. This arose from a 2.5-fold expansion in immature Gr1hi monocytes and there was also a trend toward increased numbers of (B) Gr1lo monocytes, (C) although this did not achieve statistical significance. There was a trend toward increased numbers of neutrophils which again did not achieve statistical significance.

Data has shown such profound effects of HO-1 upon macrophage cytokine profiles, we hypothesized that differences in APC differentiation might account for the susceptible phenotype of Hmox-1−/− mice. Indeed, Hmox-1−/− but not Hmox-1+/+ or +/+ Kupffer cells expressed Ly6c which is found on inflammatory monocytes and MARCO, which is associated with macrophage activation. Hmox-1−/− Kupffer cells also expressed less F4/80, which has been implicated in modulation of adaptive immunity. Finally, HO-1 induction decreased, and HO-1 inhibition increased, TNF-α.

Experimental groups. Preliminary experiments demonstrated that the sham effect of surgery was negligible. Wild-type, Hmox-1−/−, and Hmox-1−/− mice underwent 40 minutes of ischemia (n = 6 per group). Two groups of C57Bl/6J animals underwent 50 minutes of ischemia following injection of 20 mg/kg GdCl3 (Sigma-Aldrich, Poole, UK) or vehicle (n = 6 per group). Three groups (four per group) of CD11bDTR animals underwent 50 minutes of ischemia.

Operative procedure. Operated animals underwent clamping of the portal pedicle (hepatic artery and portal vein) supplying the left hepatic under isoflurane anesthesia, with core temperature maintained at 36 °C throughout. Preliminary experiments demonstrated optimal ischemic times of 40 minutes for the detection of susceptible phenotypes, and 50 minutes for demonstration of protection from ischemic injury.

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Preparation of bone marrow-derived monocytes. Femurs and tibias from 8–10-week-old Hmox-1+/+, +/−, and −/− mice were harvested and grown as described elsewhere. At the point of marrow aspiration, cells from the femurs of the same animal were placed into paired cultures with the highly specific HO-1 inhibitor chromium mesoporphyrin IX (CrMP, Frontier Scientific, Logan, Utah) or vehicle (PBS).

Flow cytometry. BMDMs were stained with biotinylated anti-mouse Gr1 (eBioscience RB6-8D5, eBioscience, San Diego, CA), PE-conjugated anti-F4/80 (eBioscience clone BM8), PerCP-Cy5.5-labeled anti-CD11b (eBioscience M1/70) and FITC-conjugated anti-CD68 FITC (Acris SM1550F, Acris Antibodies GmbH, Germany), following permeabilization with Cytoperm plus (BD 554715; Becton Dickinson, Franklin Lakes, NJ), or with biotinylated anti-mouse Ly6c (cat. no. 557359, clone AL21; Becton Dickinson, Oxford, UK; Pharmingen, Oxford, UK), or with biotinylated anti-mouse Ly6c (cat. no. 557359, clone AL21; Becton Dickinson, Oxford, UK; Pharmingen, Oxford, UK), or with biotinylated anti-CD11c (BD Pharmingen cat. no. 550993, clone M1/70). Experiments were conducted on cells from paired, age-, and sex-matched wild type, HO-1 inhibited, and HO-1 intact cultures. FIGURE 6 The effect of HO-1 function upon macrophage differentiation in vitro. Suspension cultures of Hmox-1+/+, +/−, and −/− bone marrow-derived stem cells were grown in M-CSF conditioned medium for 6 days. Wild-type cells were grown in the presence or absence of the highly specific HO-1 inhibitor CrMP throughout their maturation. Hmox-1−/− and wild-type HO-1 inhibited BMDMs were considerably more numerous than control wild-type BMDMs. Flow cytometry demonstrated development of a large F4/80− population in HO-1 inhibited cultures. Dot plots indicated that Ly6c and CD11c markers were seen on mutually exclusive populations. Calculation of cell numbers demonstrated that HO-1 inhibition led to expansion of a large pro-inflammatory Ly6c+ population. LPS stimulus of BMDMs in vitro led to increased TNF-α production in conditions of HO-1 inhibition and decreased TNF-α secretion when HO-1 was induced.

Immunohistochemistry. Paraffin-fixed tissue sections underwent antigen retrieval by boiling in citrate buffer before goat serum, peroxide, avidin/biotin blocking (Vector SP2001, Vector UK, Peterborough, UK) staining for HO-1 (Stressgen SPA 896). Secondary antibody was Dako goat anti-rat biotinylated (E0468). Immunohistochemistry for CD11b, CD68, Ly6c, CD206, and MARCO was performed on frozen sections. Primary antibodies were CD11b (Serotec MCA711G), CD68 (Serotec MCA1957GA), Ly6c (Serotec MCA2389GA), CD206 (Serotec MCA2235GA), MARCO (Serotec MCA1849). Blocking, secondary, and tertiary steps were as for F4/80.
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**Macrophage TNF-α production studies.** Day 7 BMDMs were plated at a density of 1 × 10^5^ cells per well in 24 well plates, 1 day before stimulus. Before the addition of LPS 500 ng/ml, cells were preincubated with medium containing CrMP (5μmol/l) or plain medium for 1 hour. Supernatants were collected after 2 hours and immediately frozen at −20°C until ELISA analysis. ELISA for mouse TNF-α was performed using a “Duoset” kit obtained from R&D systems (Minneapolis, MN) according to the manufacturer's instructions. Experiments were repeated three to five times with cells derived from separate animals.

**Statistical analysis.** Datasets were prepared using Microsoft Excel. Statistical analysis was performed using Minitab 13 for Windows XP. Comparison of means was performed using unpaired two-tailed, parametric t tests (in vivo data, in vitro “between plate” data) or paired two-tailed, parametric t tests (in vitro, same plate data). P values of ≤0.05 were taken as significant. Data are presented as mean ± SEM.

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