Research Article

Myeloid Heme Oxygenase-1 Regulates the Acute Inflammatory Response to Zymosan in the Mouse Air Pouch

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Heme oxygenase-1 (HO-1) is induced by many stimuli to modulate the activation and function of different cell types during innate immune responses. Although HO-1 has shown anti-inflammatory effects in different systems, there are few data on the contribution of myeloid HO-1 and its role in inflammatory processes is not well understood. To address this point, we have used HO-1M-KO mice with myeloid-restricted deletion of HO-1 to specifically investigate its influence on the acute inflammatory response to zymosan in vivo. In the mouse air pouch model, we have shown an exacerbated inflammation in HO-1M-KO mice with increased neutrophil infiltration accompanied by high levels of inflammatory mediators such as interleukin-1β, tumor necrosis factor-α, and prostaglandin E2. The expression of the degradative enzyme matrix metalloproteinase-3 (MMP-3) was also enhanced. In addition, we observed higher levels of serum MMP-3 in HO-1M-KO mice compared with control mice, suggesting the presence of systemic inflammation. Altogether, these findings demonstrate that myeloid HO-1 plays an anti-inflammatory role in the acute response to zymosan in vivo and suggest the interest of this target to regulate inflammatory processes.

1. Introduction

Heme oxygenase (HO) catalyzes the oxidative degradation of heme to carbon monoxide (CO), iron, and biliverdin which is converted to bilirubin by biliverdin reductase [1]. HO-2 is constitutively expressed in the testes, brain, and endothelium and would regulate normal physiological functions while HO-1 is highly inducible by a wide range of stimuli. HO-1 plays an important role in the antioxidant defence system and iron homeostasis. In addition, HO-1 is involved in the regulation of different cell functions such as proliferation, differentiation, and apoptosis (reviewed in [2]). A wide range of evidence indicate that HO-1 regulates the activation and function of different cell types driving the inflammatory process and innate and adaptive immune responses [3–8]. Therefore, HO-1 upregulation or administration of its metabolites results in anti-inflammatory and antioxidant effects in many disease models such as atherosclerosis [9], cardiac ischemia/reperfusion injury [10], diabetes [11], inflammatory bowel disease [12], or rheumatoid arthritis [4]. Conversely, the deletion of Hmox1 in mice increases the severity of many experimental diseases, and the incidence or severity of several human diseases are associated with polymorphisms in the Hmox1 promoter that regulates HO-1 expression [7, 13].

HO-1 knockout (Hmox1−/−) mice exhibit growth retardation, anemia, proteinuria, hepatic and renal iron accumulation, chronic inflammation, and reduced life span [14], which are similar to the important alterations observed in human HO-1 deficiency [15, 16]. Conditional deletion of HO-1 could allow a more specific approach to study the role of HO-1 in physiological and pathological processes. Previous works have shown that mice with selective deletion of myeloid HO-1 (HO-1M-KO) do not exhibit any relevant physiologic changes compared with wild-type animals, and unlike HO-1−/− mice, they do not have enlarged spleens or pathological abnormalities [17].

A great number of studies have been devoted to assess HO-1 biological effects, in contrast with the relatively few investigations using targeted approaches to specifically focus...
on myeloid HO-1. Previous reports have indicated that myeloid HO-1 may be a protective pathway against renal ischemia/reperfusion injury [18] or autoimmune central nervous system diseases. Therefore, genetic ablation of myeloid HO-1 enhances the infiltration of macrophages and Th17 cells into the central nervous system resulting in aggravation of experimental autoimmune encephalomyelitis [17]. In contrast, other works have shown that macrophage conditional HO-1 deletion evokes resistance to in aggravation of experimental autoimmune encephalomyelitis [17]. In contrast, other works have shown that macrophage conditional HO-1 deletion evokes resistance to in aggravation of experimental autoimmune encephalomyelitis [17].

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facilities and were routinely screened for health status. (Research Center, Vari, Greece, and crossed with were obtained from Alexander Fleming Biomedical Sciences Foundation, Munich, Germany). Genotyping of mice was performed using the KAPA Mouse Genotyping Kit (KAPA Biosystems Europe Ltd., Abingdon, UK), with sensitivity of 15.6 pg/ml. MMP-3 levels were measured by ELISA (R&D Systems, Minneapolis, MN, USA) with a sensitivity of 15.6 pg/ml. MMP-3 levels were measured by ELISA (R&D Systems, Minneapolis, MN, USA) with a sensitivity of 8.0 pg/ml. MMP-3 levels were measured by ELISA (R&D Systems Europe Ltd., Abingdon, UK), with sensitivity of 19.0 pg/ml. The eicosanoid prostaglandin E2 (PGE2) was determined by radioimmunoassay [23].
2.4. Western Blotting. Cell pellets from the air pouch exudates were resuspended in a buffer pH 7.46 containing 10 mM HEPES, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na3VO4, 1 μg/ml leupeptin, 0.1 μg/ml aprotinin, and 0.5 mM phenylmethyl sulfonyl fluoride and were sonicated (3 × 10 sec) and centrifuged at 10,000 × g for 10 min at 4°C. Supernatants were collected and protein concentration was evaluated by the DC protein reagent (Bio-Rad, Hercules, CA, USA). Proteins (30 μg) were separated by SDS/PAGE (12.5%) and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk and incubated with specific polyclonal antibody against HO-1 (1/1000) (Enzo Life Sciences Inc., Farmingdale, NY, USA) or β-actin (1/5000, Sigma-Aldrich) for 2 h at room temperature. Finally, membranes were incubated with peroxidase-conjugated polyclonal goat anti-rabbit IgG (1/5000, Dako, Glostrup, Denmark) and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL®, GE Healthcare, Buckinghamshire, UK) using an AutoChemil image analyser (UVP Inc., Upland, CA, USA). Band intensity was analysed by optical densitometry using ImageJ analysis software (NIH, USA). Band densities were corrected for background, and protein levels were normalized against β-actin.

2.5. Statistical Analysis. The data were presented as mean ± SD. Normal distribution of data was assessed by the Shapiro-Wilk normality test. Two-way analysis of variance (ANOVA) followed by Sidak’s multiple comparisons test, or unpaired t-test was performed. All statistical analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, USA). A P value lower than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Confirmation of HO-1 Deletion in Mouse Peritoneal Macrophages. Hmox1™FL/FL mice were crossed with LysMCre™ mice to delete HO-1 in myeloid cells. The LysMCre™ transgene mediates the excision oflox-P-flanked sequences in these cells [24]. We assessed HO-1 protein expression in mouse peritoneal macrophages by immunocytochemistry (Figure 1(a)) and flow cytometry (Figure 1(b)). HO-1 expression was observed in macrophages from Hmox1™FL/FL (control) but not in LysMCre™Hmox1™FL/FL (HO-1™KO) mice, thus confirming the efficient deletion of myeloid HO-1.

3.2. Cell Migration into the MAP. Zymosan induces cell activation and production of a wide range of proinflammatory mediators that are quickly released into the inflammatory milieu. As a consequence, there is an important leukocyte influx into the air pouch. We have previously reported that migrating cells are predominantly neutrophils. They represent about 88-89% of cells present in air pouch exudates [25]. The interaction between treatment (saline or zymosan) and genotype (control or HO-1™KO) was significant for cell migration (F = 10.67, P = 0.0039). Figure 2(a) shows that zymosan induced a significant cell migration into the air pouch compared with saline-injected air pouches in both control and HO-1™KO mice. It is interesting to note that inflammatory cell accumulation in air pouch exudate induced by zymosan was significantly higher in HO-1™KO mice compared with control mice (11.3 ± 3.9 versus 5.1 ± 1.7 × 106 cells/ml). In this experimental model, there is a time-dependent increase in HO-1 expression in the cells migrating to the air pouch exudate [26]. We assessed HO-1 protein expression in these migrating cells and observed a high level in cells from control animals whereas a very reduced expression was present in cells from HO-1™KO mice (Figure 2(b)). HO-1 protein was not completely suppressed in this last group as some non-myeloid cells are also present in exudates [25].

3.3. Proinflammatory Mediators in Air Pouch Exudate. There were no significant differences in levels of proinflammatory mediators between control and HO-1™KO mice in the absence of zymosan stimulation. The proinflammatory cytokines IL-1β and TNFα play an important role in the response induced by zymosan (Figures 3(a) and 3(b)). Our results indicate that IL-1β and TNFα production in response to zymosan depended on genotype (F = 19.36, P = 0.0003 and F = 5.27, P = 0.0472, resp.). Zymosan elicited a higher IL-1β level in HO-1™KO mice (1582.0 ± 40.3 pg/ml) compared with control mice (1185.0 ± 191.1 pg/ml). Similarly, TNFα levels in the presence of zymosan were enhanced in HO-1™KO mice (521.1 ± 175.0 pg/ml) with respect to control animals (290.8 ± 49.02 pg/ml). In addition, the interaction between treatment and genotype was significant for the production of the chemokine CXCL-1 (F = 8.13, P = 0.0102). The levels of CXCL-1 in inflammatory exudates were increased by zymosan administration in both control and HO-1™KO mice (Figure 4(a)) although there was no significant difference between them (9.83 ± 1.0 and 8.9 ± 1.0 pg/ml, resp.). PGE2 is an important proinflammatory mediator in this experimental model. We found that PGE2 production induced by zymosan depended on genotype (F = 12.11, P = 0.0083). Figure 4(b) shows that zymosan stimulation resulted in high levels of this eicosanoid in the inflammatory exudate in both control and HO-1™KO mice. The production of PGE2 in HO-1™KO mice (109.0 ± 16.1 ng/ml) was significantly higher than that of control animals (42.7 ± 21.9 ng/ml).

3.4. Levels of MMP-3 in Air Pouch Exudate and Serum. There was a significant interaction between treatment and genotype for MMP-3 levels in air pouch exudate and serum (F = 16.75, P = 0.0006 and F = 58.93, P < 0.0001, resp.). MMP-3 levels in the air pouch exudate were enhanced by the inflammatory response to zymosan (Figure 5(a)), and we observed a stronger effect in HO-1™KO mice (153.5 ± 54.8 ng/ml) compared with controls (61.3 ± 28.6 ng/ml). We also assessed MMP-3 levels in serum as a marker of systemic inflammation. As shown in Figure 5(b), in the absence of zymosan stimulation, serum levels of MMP-3 were significantly higher in HO-1™KO mice (50.1 ± 9.9 versus 11.6 ± 5.4 ng/ml). Zymosan injection did not increase serum MMP-3 in the control
group \((10.6 \pm 2.5 \text{ ng/ml})\) but exerted a dramatic effect in HO-1\(^{-/-}\) mice \((151.2 \pm 32.5 \text{ ng/ml})\).

### 4. Discussion

Myeloid cells have important roles in both innate and adaptive immune responses [27]. Studies in mice with specific deletion of HO-1 in myeloid cells have revealed the contribution of HO-1 to the maturation and differentiation of monocytes/macrophages. In addition, HO-1 is relevant for cell functions such as phagocytic activity and bacterial clearance. These actions of HO-1 can be reproduced by exogenously applied CO [28, 29] which exerts anti-inflammatory and proresolution effects [30]. We have demonstrated using HO-1\(^{-/-}\) mice that myeloid-restricted deletion of HO-1 potentiates the inflammatory response to zymosan. These results are consistent with our previous data showing that induction of HO-1 exerts anti-inflammatory effects in this experimental model [26]. In the current study, we have shown that HO-1\(^{-/-}\) mice exhibit increased neutrophil infiltrates in response to zymosan administration. This effect on cell migration was higher than that observed in another inflammatory model using HO-1\(^{+/+}\) and HO-1\(^{-/-}\) mice [13]. Our results thus indicate that myeloid HO-1 plays an important role in the regulation of neutrophil migration and the production of inflammatory and catabolic mediators during the acute response to zymosan in vivo. In particular, we observed an increased production of IL-1\(\beta\), TNF\(\alpha\), PGE\(2\), and MMP-3 in the inflammation focus. This is in contrast to the observed in vitro response of mouse macrophages to lipopolysaccharide, where myeloid HO-1 is not essential for TLR4-induced nuclear factor-\(\kappa\)B activation and proinflammatory cytokine production but is required for TLR4/TLR3/interferon regulatory factor 3-induced production of interferon-\(\beta\) [17].

Proinflammatory cytokines and elevated levels of PGE\(2\) promote the production of MMPs. These enzymes degrade extracellular matrix but they also contribute to the
regulation of proinflammatory mediators. In particular, MMP-3 (stromelysin-1) is able to cleave and activate other MMPs such as collagenases [31] and the cytokines TNFα and IL-1β. In addition, MMPs such as MMP-3 can induce macrophage secretion of TNFα leading to cyclooxygenase-2 induction and PGE2 production which in turn increases MMP expression. This nexus between MMPs and prostanoids may be relevant in the pathogenesis of chronic inflammatory diseases and cancer [32].

There are a number of ways in which MMP-3 participates in tissue destruction. Studies in MMP-3 knockout mice have shown the key role of this MMP in cartilage lesion and the contribution of synovial macrophages to MMP-mediated damage [33]. We have previously shown that HO-1 overexpression downregulates MMP-3 and other degradative enzymes in articular cells such as synoviocytes [5]. In the current study, we have demonstrated that deficiency in myeloid HO-1 enhances the induction of MMP-3 by zymosan in vivo, which may contribute to the inflammatory amplification process involving cytokines, eicosanoids, and proteases which results in tissue damage.

Interestingly, HO-1M-KO mice showed high levels of serum MMP-3 even in the absence of zymosan treatment, which indicates the presence of a systemic inflammatory response in these animals. In fact, serum MMP-3 levels are related to inflammation and tissue damage in conditions such as rheumatoid arthritis, where synovial macrophages are crucial in early MMP activity and serum MMP-3 may be a biomarker of disease activity and predictor of joint destruction [34, 35]. Therefore, our data suggest that myeloid HO-1 contributes to regulatory mechanisms in systemic inflammation.

**Figure 2:** Cell migration into the MAP. (a) Cell numbers in the exudate, mean ± SD (n = 6). (b) Immunoblot for HO-1 expression in cells of the exudate. Representative images and rate of HO-1/β-actin; **P < 0.01 and ++++P < 0.0001 with respect to saline-injected group; *P < 0.05 and ***P < 0.001, compared to control mice (a: two-way ANOVA followed by Sidak’s test; b: unpaired t-test).

**Figure 3:** Levels of proinflammatory cytokines in MAP exudate. IL-1β (a) and TNFα (b) were measured by ELISA. Results are shown as mean ± SD (n = 6); **P < 0.01, +++P < 0.001, and ++++P < 0.0001 with respect to saline-injected group; *P < 0.05 and ****P < 0.0001 compared to control mice (two-way ANOVA followed by Sidak’s test).
In conclusion, this study has revealed that myeloid HO-1 mediates protection against the inflammatory response to zymosan in vivo. These data point to new directions in therapeutic interventions that may potentially limit the deleterious effects of excessive inflammation.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Acknowledgments

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