Heme oxygenase-1 derived carbon monoxide permits maturation of myeloid cells

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Heme oxygenase-1 derived carbon monoxide permits maturation of myeloid cells

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Critical functions of the immune system are maintained by the ability of myeloid progenitors to differentiate and mature into macrophages. We hypothesized that the cytoprotective gas molecule carbon monoxide (CO), generated endogenously by heme oxygenases (HO), promotes differentiation of progenitors into functional macrophages. Deletion of HO-1, specifically in the myeloid lineage (Lyz-Cre:Hmox1fl/fl), attenuated the ability of myeloid progenitors to differentiate toward macrophages and decreased the expression of macrophage markers, CD14 and macrophage colony-stimulating factor receptor (MCSFR). We showed that HO-1 and CO induced CD14 expression and efficiently increased expansion and differentiation of myeloid cells into macrophages. Further, CO sensitized myeloid cells to treatment with MCSF at low doses by increasing MCSFR expression, mediated partially through a PI3K-Akt-dependent mechanism. Exposure of mice to CO in a model of marginal bone marrow transplantation significantly improved donor myeloid cell engraftment efficiency, expansion and differentiation, which corresponded to increased serum levels of GM-CSF, IL-1x and MCP-1. Collectively, we conclude that HO-1 and CO in part are critical for myeloid cell differentiation. CO may prove to be a novel therapeutic agent to improve functional recovery of bone marrow cells in patients undergoing irradiation, chemotherapy and/or bone marrow transplantation.

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Myeloid cells have a critical role in the innate and adaptive immune system responses to danger signals and pathogens.1 Upon recognition of pathogens or danger-associated molecular patterns (DAMPs), myeloid cells mature into macrophages, secrete proinflammatory cytokines, and provide appropriate inflammatory responses to infection and injury.2 As such, the differentiation status of myeloid cells is critical for proper phagocytic function and elimination of pathogens. The process by which myeloid progenitor cells differentiate into monocytes and eventually into fully mature macrophages requires the concerted activation and repression of several lineage-specific genes such as PU.1, GATA2, and c-myb, among others. Functional maturation of myeloid cells is of particular importance in situations requiring acute responses such as during chemotherapy, bone marrow transplantation or sepsis.

Numerous reports have shown that the heme degradation pathway regulates immune system responses. It has been reported that HO-1 and CO induce erythroid differentiation leading to increased production of hemoglobin and survival of differentiated cells under low oxygen tension.3 HO-1 has also been shown to inhibit myoblast differentiation through downregulation of Lin-28, DGC8 and heme synthesis.4 In addition, HO-1 favors development of mesenchymal stem cells toward adipocytes by blocking lipid accumulation, PPARγ and CD13 expression through the Wnt pathway.5 In contrast, HO-1 has been shown to accelerate mesenchymal stem cell differentiation toward an osteoblast lineage.5 Bone marrow (BM)-derived stem cells expressing HO-1 are important for improved myocardial repair following reperfusion injury in the heart.6

We have previously shown that CO induces differentiation and mobilization of BM progenitors toward an endothelial cell phenotype in response to vascular injury to accelerate healing of an injured vessel.7 HO-1 and CO are now recognized as salutary molecules that reestablish homeostasis following acute and chronic inflammation such as that associated with ischemia–reperfusion injury, hemorrhagic and septic shock, autoimmune disease, inflammatory bowel disease and acute lung injury.8,9 Many of these pathological conditions require rapid response of the bone marrow and mobilization of progenitor cells that contribute to tissue repair. While several studies have linked HO-1 with stem cell differentiation, we hypothesized that heme degradation may...
be important in early expansion, differentiation, and maturation of myeloid cells into macrophages. Using a model of marginal bone marrow transplant, which leads to tissue damage, we find that HO-1-derived and exogenously delivered CO modulates in part the fate of myeloid progenitors and mediates the ability of these cells to differentiate into functional mature cells.

Results

Lack of HO-1 in myeloid cells results in poor maturation of macrophages. Comparative immunostaining with antibodies against the residential macrophage marker F4.80 was performed on the spleens, lungs, and livers from Hmox1−/− and Hmox1+/+ mice. We observed significantly lower F4.80-positive counts in Hmox1−/− spleens versus Hmox1+/+ with no differences in expression in either the lung or liver (Figures 1a and b). The enlarged spleens with abnormal white and red pulp typically seen in Hmox1−/− may explain in part the difference in the macrophage counts and phenotype. We therefore next focused on deletion of HO-1, specifically in myeloid cells. Analysis of spleens from Lyz-Cre:Hmox1flfl mice showed no significant difference in surface expression of F4,80 in the liver or brain or any of the lymphoid organs including the spleen, thymus, and lymph nodes (Supplementary Figures 1b and 2). In contrast, we observed lower expression of CD14, Mac3, and MCSFR in the spleens from Lyz-Cre:Hmox1flfl mice as compared with Hmox1flfl mice (Figure 1c and d). Further, lymph nodes and thymus from Lyz-Cre:Hmox1flfl mice showed lower total expression of CD14 as measured by immunoblotting and immunostaining, and a significant difference in surface CD14 expression was seen in the lymph nodes as measured by FACS (see also Figure 1d and Supplementary Figure 3 versus Supplementary Figure 1). These observations, in addition to decreased circulating CD14+ cell numbers in Lyz-Cre:Hmox1flfl mice support the hypothesis that HO-1 is important in maturation of myeloid progenitor cells into a monocyte/macrophage lineage (Figures 1e and f). Moreover, the differential expression patterns of CD14 observed in the thymus, lymph nodes, and spleen suggest that CD14 in

![Figure 1](https://example.com/figure1.png)

**Figure 1** Lack of HO-1 in myeloid cells results in lower number of mature macrophages. (a) Immunohistochemistry with antibody against F4,80 in the spleens, lungs, and livers from Hmox1−/− and Hmox1+/+ mice. Number of F4,80-positive cells per field of view (n = 4–6) was evaluated. Inset: Representative staining is shown. n = 3 per group. **P < 0.001 Hmox1−/− versus Hmox1+/+. (b) Immunohistochemistry of the spleens from Hmox1−/− and Hmox1+/+ mice with staining against CD14. Representative sections are shown. (c) Immunostaining with antibodies against CD14 (Alexa-488) was performed on the spleen sections from Hmox1flfl and CreLyz:Hmox1flfl mice. n = 3–4 per group. (d) Immunoblotting in the lysates of the spleens from Hmox1flfl and CreLyz:Hmox1flfl mice. Antibodies against CD14, Mac3, and MCSFR were applied to test for macrophages maturation. β-Actin was used as loading control. n = 4 mice per group. (e, f) Blood cells were isolated by Ficol extraction from Hmox1flfl and CreLyz:Hmox1flfl mice (n = 2–3 per group) and stained against CD14 conjugated with FITC. Appropriate IgG-FITC control antibodies were used. Representative flow cytometry plots are shown in e, and quantitation of fluorescent mean is shown in f. ANOVA, P < 0.0026; Tukey’s test: **P < 0.01 Hmox1flfl versus IgG and *P < 0.05 Hmox1flfl versus CreLyz:Hmox1flfl
macrophage is differentially regulated by HO-1 at the total protein level (in thymus and spleen) versus surface expression (lymph node and blood).

**HO-1 regulates macrophage differentiation.** HO-1 is expressed in various hematopoietic cells in the BM, including Lin−/Sca1+ and Lin−/Sca1−, and Lin+ populations (Figures 2a–c). The highest level of HO-1 expression was observed in Sca1+/Lin− populations, suggesting that HO-1 might have a role in maintaining the stemness features of hematopoietic stem and progenitor cells by contributing to their commitment and differentiation into various lineages (Figures 2b and c). To test the role of HO-1 during myeloid cell differentiation and maturation, we quantitated the number of colonies in methylcellulose medium (MethoCult) derived from myeloid cells harvested from Lyz-Cre:Hmox1flfl versus Hmox1flfl mice. No significant differences were observed in colony numbers between genotypes suggesting that the ability of BM cells to form colonies was not altered in the absence of HO-1 (Figure 2d). However, we did observe a significant decrease in CD14 expression in colony-derived cells lacking HO-1 when cultured in methylcellulose medium for 9 days (Figure 2e). These data suggest HO-1 is important for phenotypic maturation of myeloid cells into a subset of functional macrophages and support our *in vivo* observations, in which monocytes isolated from Lyz-Cre:Hmox1flfl mice present with low CD14 expression compared with control Hmox1flfl cells in the spleen and peripheral blood (Figure 1).

Treatment with macrophage colony-stimulating factor (MCSF) over 5 days induced differentiation of myeloid cells characterized by increased expression of HO-1 and macrophage maturation markers Mac3 and CD14 (Figure 2f). Myeloid progenitors from Lyz-Cre:Hmox1flfl treated with MCSF confirmed that CD14 expression was reduced as compared to progenitors from Hmox1flfl mice (Figure 2g).

We next evaluated whether the absence of maturation markers such as CD14 translated to alterations in macrophage function. Treatment of macrophages with bacterial lipopolysaccharide (LPS) induced rapid degradation of IκB regulated by CD14 and toll-like receptor activation. Bone marrow-derived macrophages (BMDM) from Lyz-Cre:Hmox1flfl showed low CD14 expression, and a delayed response to LPS treatment as compared with control Hmox1flfl macrophages (Supplementary Figure 4).

**CO induces myeloid cell expansion and differentiation in vitro.** The effects of HO-1 on maturation and differentiation prompted us to next test the effects of CO, a product of HO-1 activity, on CD14 expression in response to MCSF. CO

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**Figure 2** HO-1 dictates bone marrow differentiation toward macrophage lineage. (a) Immunohistochemistry with antibody against HO-1 in bone marrow of wild-type mice. Note the single positive cells in the bone marrow progenitor fraction. (b) Immunofluorescent staining of HO-1 on enriched progenitor cells from the bone marrow in a C57/BL6 mouse. Isolated splenocytes (positive control for HO-1 staining), total bone marrow cells, sca1+/lin− and sca1−/lin− populations were subjected to cytopsin and stained with antibody against HO-1. Representative pictures from 2 to 3 experiments are shown. (c) Real time PCR was performed in the cell fractions as in (b). HO-1 expression per β-Actin is presented as fold change over total BM levels of HO-1. (d) Total BM cells from Hmox1flfl and CreLyz:Hmox1flmice were isolated and cultured in methylcellulose medium for 9 days following manufacturer’s protocol. Colony counts in methylcellulose medium were performed on day 9. P > 0.05, NS (e). Total BM cells from Hmox1flfl and CreLyz:Hmox1flmice were isolated and cultured in methylcellulose medium for 9 days following manufacturer’s protocol. Immunoblotting with antibody against CD14 and HO-1 was performed on cell lysates. Data are representative for 2–3 experiments. (f) BM cells were induced toward macrophage differentiation using MCSF (20 ng/ml). Detection of HO-1, Mac3, and CD14 was performed by immunoblotting. Cells were harvested on days 0–5 during differentiation. Data are representative for at least three experiments. (g) BM cells isolated from Hmox1flfl and CreLyz:Hmox1flmice were subjected to MCSF-induced differentiation as in f, and the levels of CD14 were measured by immunoblotting. Note that the levels of CD14 are lower in BM from CreLyz:Hmox1flmice as early as day 1. Two different exposures of the same CD14 blot are presented to better appreciate the changes in early and later time points.
enhanced CD14 and Mac3 expression compared with air controls in macrophages treated with MCSF to stimulate differentiation for 6–8 days in culture (Figures 3a and b). To evaluate the mechanism by which CO influenced CD14 expression, we measured levels of macrophage growth factor receptor (MCSFR) and tested the effects of CO exposure on MCSF-induced differentiation. Exposure to CO resulted in a significant increase in expression of both surface and total MCSFR expression (Figures 3c and d) while lack of endogenous CO production in myeloid cells from Lyz-Cre:Hmox1flfl mice resulted in attenuation of MCSFR expression. Of note, under these treatment conditions, CO had no effect on HIF1α expression or cytokine release measured in vitro in macrophages or in vivo in the lymph nodes (Supplementary Figure 5).

Next, we investigated whether CO might effectively induce differentiation of myeloid progenitors into macrophages with lower doses of MCSF. CO significantly increased the number of fully differentiated macrophage as measured by morphology (Figures 3e and f). Further, CO amplified

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**Figure 3** CO accelerates differentiation and maturation of macrophages. (a) BM was isolated from wild-type mice and stimulated with MCSF in the presence or absence of CO (250 p.p.m.) for 6 and 8 days. Immunoblotting with antibody against Mac3 and CD14 was performed in the lysates of differentiated macrophages. (b) Immunostaining with antibody against CD14 in CO differentiated cells cultured in methylcellulose for 9 days. Data are representative for two independent experiments performed in triplicates. (c) Flow cytometry of BMDM treated with CO for 24 h and cell surface MCSFR was detected. n = 3 in duplicates, **P < 0.01. (d) Immunoblotting and immunostaining with antibodies against MCSFR and Mac3 in total BM cells from Hmox1flfl and CreLyz:Hmox1flfl mice differentiated with MCSF (20 ng/ml) for 2, 4, and 5 days. Data are representative for three independent experiments. Densitometric quantitation is shown in Supplementary Figure 6. (e, f) Macrophage colony formation was tested at suboptimal levels of MCSF (1 ng/ml) in the presence or absence of CO (250 p.p.m.). Significant increase in the number of CO-induced BMDM was observed at day 3. Data are representative for three independent experiments. *P < 0.05. (g, h) U937 cells were stimulated with PMA (1, 5, or 10 ng/ml) to induce differentiation toward macrophages. Representative data are shown in g and quantitation is presented in h. n = 3 experiments in triplicates. *P < 0.05. CO versus Air at each time point.
macrophage colony counts in response to PMA, an agent that acts through protein kinase C (PKC) to induce macrophage differentiation (Figures 3g and h). These findings define a cellular mechanism by which CO, as a bioactive product of heme catabolism, influences macrophage differentiation.

**CO increases myeloid cell expansion and differentiation after marginal bone marrow transplantation.** To correlate our *in vitro* findings with a relevant *in vivo* model, we performed a marginal bone marrow transplantation. Lethally irradiated mice were reconstituted with an insufficient number of bone marrow-derived myeloid cells. Poor survival following irradiation is associated with organ damage and bacterial sepsis largely due to increased gut permeability in combination with a lack of properly differentiated hematopoietic cells. We know this effect to be abrogated with CO treatment. Treatment of mice with CO for 1 h prior to marginal bone marrow transplant and then daily thereafter for 1 h resulted in 95–100% survival with increased early bone marrow engraftment versus controls (Figures 5a–c). Of the cells that engrafted, a significant number were positive for CD133, a myeloid progenitor surface marker (Figure 5d). In addition, lethally irradiated BM recipient mice treated with CO showed a higher number of colony-forming units in the spleen on day 7 in culture as compared with air-treated control mice (Figure 6a). The spleens isolated from mice treated with CO after BM transplantation also showed an increased number of Ki67 positive cells and a significant expansion of F4.80-positive myeloid cells (Figure 6b).

**Discussion**

The role of HO-1 and its active products on macrophage function are well established with numerous reports describing HO-1 as critical in macrophage activation, proliferation, and survival. The majority of these reports, however, focused on how HO-1 and CO modulated mature macrophage function *versus* understanding their role on myeloid maturation. Recently, it has become more evident that HO-1 is important in regulating reprogramming and differentiation of other cell lineages including endothelial progenitors, which are mobilized from bone marrow to the site of vessel injury. Bone marrow cells isolated from *Hmox1*−/− mice generate fewer endothelial colony-forming cells and respond poorly in models of angiogenesis and ischemic injury of the heart and hindlimb. Importantly, lack of HO-1 was rescued with CO treatment that enhanced endothelial cell recruitment and vessel development. While the mode of action of HO-1 is mediated to a large extent by CO, there is in all likelihood a contribution of the other HO-1 bioactive products, iron and the bile pigments to bone marrow physiology and end-organ tissue function.

We describe here that lack of HO-1 specifically in myeloid cells reduced their ability to undergo maturation into differentiated and functional macrophages classified as such by CD14 and MCSFR expression, two well-accepted markers of macrophage maturation. These macrophage-specific markers are not only critical for responses to the growth factor M-CSF (MCSFR) but are important for innate responses to bacterial endotoxins (CD14). CD14 is a glycoprotein that binds bacterial LPS and activates a proinflammatory phenotype essential for phagocytosis...
Figure 5  CO treatment enhances survival and bone marrow engraftment in marginal bone marrow transplant. (a–c) Representative section of bones and flow cytometry differential counts of bone marrow cells at day 7 are shown; n = 3–4 per group. *P < 0.05. (d) Immunohistochemical staining with antibodies against CD133 in the group of CO and Air-treated mice after marginal bone marrow transplantation at day 7.

Figure 6  CO induces early engraftment and expansion of myeloid progenitors in the spleen during marginal bone marrow transplant. (a) Number of colony-forming units in the spleen in the mice after marginal BM transplant as in Figure 5. *P < 0.05 Air versus Naive and #P < 0.05 CO versus Air. (b) Immunohistochemistry with antibody against CD133 (progenitor marker), P-Histone-H3 (proliferation marker), F4.80 (macrophage marker) as well as TUNEL staining (apoptosis) was performed in the spleens as in a.
of pathogens. Macrophages from CD14\(^{-/-}\) mice are incapable of clearing apoptotic cells suggesting a broader role of CD14 in tissue repair following injury.\(^2^0\) Low CD14 levels may explain in part the poor responses of HO-1 knockout mice against bacterial infection and greater organ damage in models of sepsis.\(^1^3,^2^1\) Others and we have shown that mice lacking HO-1 in macrophages have a low phagocytic index whereas exposure to CO can rescue and augment bacterial recognition and clearance.\(^1^3,^2^2\)

We observed significantly less CD14 and MCSFR expression in mononuclear cells isolated from the spleen and in peripheral leukocytes from Lyz-Cre:Hmox1\(^{fl/fl}\) mice. This phenotype might explain in large part the inability of these mononuclear cells to remove apoptotic bodies and dead erythrocytes due to impaired phagocytic activity, in combination with an inability to process heme. However, in contrast to low numbers of F4.80-positive cells in Hmox1\(^{-/-}\) mice as compared with Hmox1\(^{+/+}\) mice, we observed no difference in F4.80 levels when comparing macrophages harvested from Hmox1\(^{fl/fl}\) versus Lyz-Cre:Hmox1\(^{fl/fl}\) mice. One explanation may be uncontrolled expansion of the T-cell zone in Hmox1\(^{-/-}\) mice with less infiltration and recruitment of macrophages from the bone marrow due to abnormal white pulp distribution.

Unlike Hmox1\(^{-/-}\) mice, Lyz-Cre:Hmox1\(^{fl/fl}\) mice do not present with enlarged spleens likely because deletion of HO-1 in these mice is limited to myeloid lineage.

Hematopoietic stem cells (HSC) from Hmox1\(^{-/-}\) mice repopulated lethally irradiated recipients, but were ineffective in affording radioprotection in part due to decreased p38 MAPK activation in HSC in response to oxidative stress.\(^2^3\) Our studies here show that exogenously applied CO promoted engraftment and radioprotection in a marginal bone marrow transplant model. CO augmented expansion of CD133\(^+\) progenitors in secondary myelopoietic organs and early differentiation of myeloid cells. Perhaps one of the principal mechanisms of CO action in myeloid progenitors may be to increase reactive oxygen species (ROS) and modulate the overall redox status of the cell. Stem cell niche and progenitor expansion and differentiation are highly regulated by oxygen tension.\(^2^4–^2^6\) Hypoxia is a general stimulus for bone marrow activation\(^2^6\) and regulates hematopoiesis in the marrow. The generation of CO under stress conditions likely reduces oxygen tension locally in the bone marrow that in turn influences cellular oxygen sensor signaling and upregulation of CO-responsive transcription factors such as Bach1 and NPAS2.
These signals activate survival pathways, compelling mobilization and differentiation of precursor cells into those necessary to befit the requirements of the body during development and in response to cellular and tissue stress. The release of specific mediators, chemokines, and growth factors initiate the directionality of the ultimate cell type, and we posit that HO-1, necessary for heme turnover, is ideally situated to regulate and direct successful transition of an immature progenitor into a mature and functional differentiated cell ultimately mediated by CO. Indeed, CO specifically increased expression of the growth factors and cytokines GM-CSF, IP-10, IL1α, as well as the chemokine MCP-1. Increased mobilization of progenitors from the bone marrow in response to these factors may be an explanation for improved repair that has been observed in models of wound healing, cardiac myocyte injury, and endothelial denudation.

In summary, we have shown that HO-1 and CO are important in differentiation of myeloid progenitors into a macrophage phenotype. The precise cellular target remains elusive and whether these protective molecules influence all progenitor cell differentiation and mobilization remains to be determined. Collectively, these data and published reports support substantial dependence on the milieu of growth factors, chemokines, and cytokines present as well as the presence of other bioactive gases including nitric oxide and hydrogen sulfide. Collectively, CO serves to intensify and increase sensitivity to these mediators to instill specificity and a hierarchy of differentiation of predisposed cell types. Data presented here would support the conclusion that CO: (i) promotes recovery from infection associated with radioablation and loss of the intestinal barrier, (ii) enhances reconstitution of bone marrow, and (iii) induces clearance of apoptotic cells more efficiently in response to radiation so as to allow a more rapid repopulation of bone marrow. These attributes of CO are supported by work by others and us showing that CO decreases cell death in part by increased DNA repair mechanisms. Inhaled CO is currently in multiple clinical trials (http://www.clinicaltrials.gov) and thus, the potential therapeutic opportunities continue to increase. The use of CO in patients undergoing bone marrow transplantation or in patients treated with chemoablative therapies may show great beneficial effects with a CO treatment regimens that enables efficient and appropriate regeneration and differentiation of myeloid cells to maintain barrier integrity of the host and ensure robust defense systems.

Materials and Methods

Materials and Methods

Immuno blotting. Cells or tissues were homogenized and lysed by brief sonication in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 10 mM NaF, 1% SDS, 1 mM Na-EDTA pH 8.0, 0.5 mM NaDOC and protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland) as previously described. Samples were centrifuged for 20 min at 13 500 × g and separated by 12% SDS-PAGE, followed by transfer to PVDF Hybond-P membranes (Amersham, GE Healthcare, Pittsburgh, PA, USA). Membranes were probed with appropriate primary and secondary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, Danvers, MA, USA) or alkaline phosphatase (AP)-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA). The following primary antibodies were used: CD14 (BD Biosciences, San Jose, CA, USA) diluted 1 : 500 and CD133 (Sigma-Aldrich), HO-1 (Epitomics), and HIF1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemistry, TUNEL, and immunofluorescence. The spleens, lungs, and livers were obtained from Hmox1−/− and CreLyzyHmox1−/− mice and either snap frozen or fixed in formalin following fixation by subcutaneous inoculation and processed for paraffin embedding and antigen retrieval using citrate buffer as previously described. Bones were fixed, decalcified, and paraffin-embedded. After fixation with 2% PFA, sections were permeabilized with 0.5% Triton X-100, blocked with horse serum, and respective primary antibodies were applied overnight. The following day, secondary antibodies conjugated with Alexa-488 were applied for 1 h at room temperature. Nuclear staining was done with Hoechst, and slides mounted on gelvatol. Pictures were taken at 20 or 40 magnification under confocal microscopy. The following primary antibodies were used: CD14 (BD Biosciences, San Jose, CA, USA), CD133 (Sigma-Aldrich), HO-1 (Epitomics), P-Histone-H3 (Cell Signaling), F4/80 (Serotec).

Flow cytometry. Blood cells were isolated from Ficoll as described and stained with CD14-FITC (Biolegend, San Diego, CA, USA) or M5C-APC (Biolegend) or appropriate vehicle controls were used. BM isolation: Bone marrow cells were isolated from wild type, or CreLyzyHmox1−/−, Hmox1−/− mice and differentiated toward macrophages in RPMI medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 1–20 ng/ml MCSF and 15% fetal bovine serum (FBS) and antibiotics. All cultures were maintained at 37 °C in a 5% CO2 atmosphere. CO treatment: CO administration was done in 21% O2, 5% CO2, and 250 p.p.m. CO in gas exposure chambers with constant monitoring. Both the incubator and CO chambers were kept at 37 °C.

Colony-forming assay. Bone marrow differentiation was assessed by using a methylcellulose-based colony-forming assay according to the manufacturer’s description (StemCell Technologies, Vancouver, BC, Canada). Isolated bone marrow was separated for different lineages and counted. Bone marrow cells were mixed with 3.5 ml of semisolid methylcellulose-based medium (Metrocult GF M3434) (StemCell Technologies) containing Stem Cell Factor, IL-3, IL-6, and erythropoietin, and triplicate 1.1 ml aliquots of the cell-containing medium was dispensed into 39-mm dishes (StemCell Technologies). Colony plates were cultured in incubators at 37 °C, 5% CO2, and 95% humidity in the presence or absence of CO (250 p.p.m.). Colonies were counted at day 3, 6, 8, and 9 and cells were harvested for protein analysis.

Mouse bone marrow lineage separation: Bone marrow was isolated by crushing mice tibias and femurs. The bone marrow cells were thoroughly separated by repeated pipetting in PBS with 2 mM EDTA and 5% FBS, referred to as recommended medium by the manufacturer (StemCell Technologies). Cells were filtered through a 70-μm strainer (BD Biosciences, San Jose, CA, USA) to obtain single-cell suspensions. Lineage-negative cells were separated from mature cells by using EasySep Mouse Hematopoietic Progenitor Enrichment Kit (StemCell Technologies, cat# 19756) according to manufacturer’s protocol. Briefly, lineage positive cells (Lin+) were labeled with magnetic microparticles with biotinylated antibodies against cell surface antibodies, including anti-CD5, CD11b, CD145R, Ly-6G, and TER119. The magnetically labeled Lin− cells were then separated from unlabeled lineage-negative (Lin−) cells using EasySep Magnetic column.

Cell culture and treatment. U937 cells were purchased from ATCC and were cultured in RPMI medium supplemented with 10% FBS. PMA (0.1, 1, or 10 ng/ml, Sigma, St. Louis, MO, USA), LY24009 (10 μM), and ODQ (20 nM);
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F4-80-FITC (Biologend) antibodies at RT for 30 min. After washing with PBS, samples were subjected to flow analysis using Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). Peritoneal cells and bone marrow cells were harvested, and differential counts were performed by flow cytometry.

Statistical analysis. Statistical analyses were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA). Data are presented as the mean ± S.D. and are representative for at least three independent experiments. Student’s t, ANOVA, Wilcoxon tests were used for estimation of statistical significance for the experiments (P < 0.05). Densitometric analysis was performed using ImageJ software (http://image.nih.gov/ij/).

Conflict of Interest
The authors declare no conflict of interest.

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