

Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1–dependent pathway

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Heme oxygenase (HO)-1 and its metabolic product carbon monoxide (CO) play regulatory roles in acute inflammatory states. In this study, we demonstrate that CO administration is effective as a therapeutic modality in mice with established chronic colitis. CO administration ameliorates chronic intestinal inflammation in a T helper (Th)1-mediated model of murine colitis, interleukin (IL)-10-deficient (*IL-10*^{-/-}) mice. In Th1-mediated inflammation, CO abrogates the synergistic effect of interferon (IFN)- γ on lipopolysaccharide-induced IL-12 p40 in murine macrophages and alters IFN- γ signaling by inhibiting a member of the IFN regulatory factor (IRF) family of transcription factors, IRF-8. A specific signaling pathway, not previously identified, is delineated that involves an obligatory role for HO-1 induction in the protection afforded by CO. Moreover, CO antagonizes the inhibitory effect of IFN- γ on HO-1 expression in macrophages. In macrophages and in Th1-mediated colitis, pharmacologic induction of HO-1 recapitulates the immunosuppressive effects of CO. In conclusion, this study begins to elucidate potential etiologic and therapeutic implications of CO and the HO-1 pathway in chronic inflammatory bowel diseases.

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Abbreviations used: CD, Crohn's disease; CO, carbon monoxide; CoPP, cobalt protoporphyrin; HO, heme oxygenase; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; NO, nitric oxide; SnPP, tin protoporphyrin; UC, ulcerative colitis.

The pathogenesis of the human inflammatory bowel diseases (IBDs) Crohn's disease (CD) and ulcerative colitis (UC) is complex. Genetic, immunologic, and environmental factors are involved in the initiation and perpetuation of chronic intestinal inflammation (1, 2). The role of environment in the pathogenesis of IBD is clearly demonstrated by the epidemiological observation that cigarette smoking is protective against the development of UC (3, 4). However, the etiology of this protective effect remains unclear. In this study, we address one component of cigarette smoke that may explain the clinical observation of protection against chronic intestinal inflammation, the gaseous molecule carbon monoxide (CO).

CO has antiinflammatory effects in murine models of sepsis, postoperative ileus, and organ xenotransplantation (5–7). In contrast to the accumulating evidence of a regulatory role for CO in acute tissue injury, little is known regarding the physiological role of CO in mucosal immunity and chronic inflammation. Mammalian cells generate CO endogenously as a product

of heme degradation by the heme oxygenase (HO) enzymes (8). Two primary isoforms of HO exist: HO-1 is ubiquitously expressed and highly inducible, whereas HO-2 is constitutively expressed primarily in the central nervous system and vascular beds (9). HO-1 is induced by a variety of agents causing oxidative stress and inflammation (10). HO-1 knockout (*hmox*^{-/-}) mice as well as one reported case of an HO-1-deficient human patient exhibited increased inflammatory indices, including leukocytosis and thrombocytosis (11, 12).

In acute inflammation, CO-mediated protection is associated with inhibition of the inflammatory cytokine response. For example, macrophages exposed to LPS in the presence of CO produced significantly less TNF (13). In addition, CO significantly increased LPS-induced accumulation of the antiinflammatory cytokine IL-10. Imbalances in proinflammatory and antiinflammatory cytokines play a major role in the pathogenesis of IBD (14). Recent studies have emphasized the importance of the cytokine subunit IL-12 p40. IL-12 p40, ex-

pressed in macrophages and dendritic cells, plays a central role in bridging innate and adaptive immune responses (15, 16). IL-12 and IL-23 are heterodimeric cytokines composed of this common p40 subunit and the p35 and p19 subunit, respectively. IL-12 and IL-23 are involved in the induction and maintenance of Th1 responses and chronic inflammation (17, 18). Anti-IL-12 p40 antibodies ameliorate colitis in Th1-mediated murine models, such as *IL-10*^{-/-} mice (19), and are currently under clinical evaluation in CD (20). Accordingly, akin to acute tissue injury, changes in cytokine profile induced by CO may be beneficial in chronic inflammation.

In this study, we demonstrate that CO administration ameliorates chronic intestinal inflammation in a Th1-mediated model of murine colitis, *IL-10*^{-/-} mice. Mechanistically, CO abrogates the synergistic effect of IFN- γ on LPS-induced IL-12 p40 in murine macrophages and alters IFN- γ signaling by inhibiting the IFN regulatory factor (IRF), IRF-8. A specific signaling pathway is delineated that involves an obligatory role for HO-1 induction in the protection afforded by CO. Accordingly, pharmacologic induction of HO-1 recapitulates the immunosuppressive effects of CO in macrophages and in vivo. Therefore, CO and HO-1 have immunomodulatory roles in chronic intestinal inflammation, and this pathway may have relevance in the human IBDs.

RESULTS

CO exposure ameliorates Th1-mediated chronic murine colitis

The immunomodulatory effects of CO were studied in a murine model of Th1-mediated chronic intestinal inflammation, *IL-10*^{-/-} mice. Mice were exposed to 250 ppm CO from 8 to 12 wk of age ($n = 12$) and compared with a control group exposed to ambient air ($n = 8$). Because hypoxia can become a confounding factor at carboxyhemoglobin levels of $\geq 20\%$ (13, 21), carboxyhemoglobin levels of CO-exposed mice were determined and were uniformly $< 14\%$ (as well as in all subsequent in vivo experiments). Assessment of histologic improvement in *IL-10*^{-/-} mice can be problematic because of incomplete penetrance and the segmental, patchy pattern of colitis. Therefore, histological results are depicted in two different ways. First, 20 random fields distributed over the entire length of colon for each mouse were graded by a pathologist blinded to the treatment group using a standard scoring system (22). Results are presented as the sum total of four averaged scores from five regions of the large intestine. CO-exposed mice demonstrate a 60% improvement in histological scores compared with air-exposed mice (Fig. 1 B, left). To represent the spectrum of histological changes in individual mice, results are also presented as the percentage of 20 fields per mouse that demonstrate no histological inflammation (colitis score of 0), mild to moderate inflammatory changes (colitis score of 1 and 2), and severe inflammation (colitis score of 3 and 4). In the CO-exposed group, compared with air-exposed *IL-10*^{-/-} mice, significantly more colonic fields demonstrate no evidence

of histologic inflammation, and likewise significantly fewer fields demonstrated inflammatory changes (Fig. 1 B, right). Furthermore, CO-exposed *IL-10*^{-/-} mice show less weight loss compared with air-exposed controls (1.48 ± 1.27 vs. 0.22 ± 1.35 grams; $P < 0.05$).

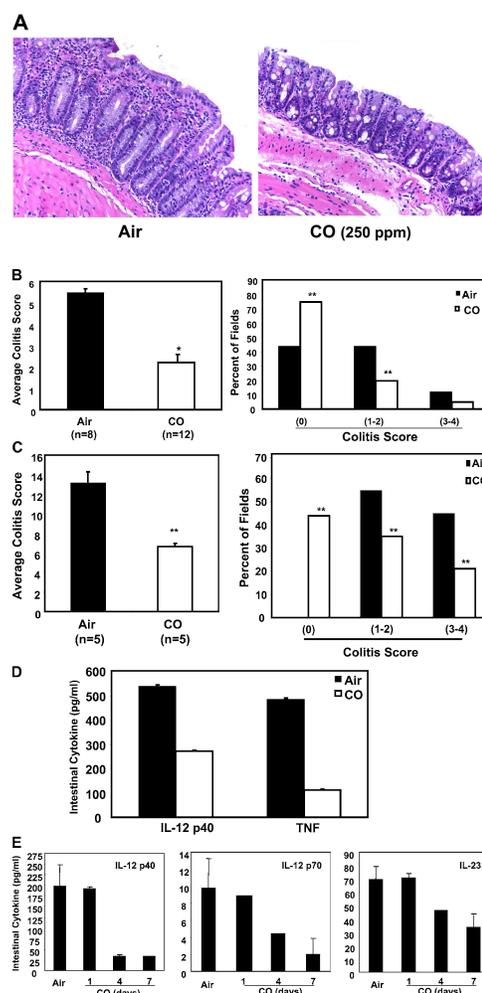


Figure 1. CO abrogates colitis in *IL-10*^{-/-} mice. *IL-10*^{-/-} mice were housed in ambient air ($n = 8$) or a chamber maintaining a constant concentration of 250 ppm CO ($n = 12$) from 8 through 12 wk of life. (A) Representative hematoxylin and eosin staining and histologic colitis of *IL-10*^{-/-} colonic tissue. Left, ambient air; right, CO-treated. (B) Colitis scores were significantly decreased in CO-exposed mice compared with control mice. *, $P < 0.01$; **, $P < 0.001$. (C) Colitis scores of 8-wk-old *IL-10*^{-/-} mice fed piroxicam-supplemented chow for 5 d and then exposed to ambient air or 250 ppm CO for 2 wk. *, $P < 0.01$; **, $P < 0.001$. (D) Spontaneous IL-12 p40 and TNF protein secretion was determined in 24-h supernatants from intestinal explants from CO- and air-exposed *IL-10*^{-/-} mice. Each result represents the mean \pm SD of triplicate assays and is representative of three independent experiments. (E) 10-wk-old *IL-10*^{-/-} mice were exposed to 250 ppm CO or ambient air for 1, 4, or 7 d (four mice per group). Intestinal explants were cultured for 24 h, and spontaneous IL-12 p40 and p70 as well as IL-23 protein secretion was determined. Results represent the mean of triplicate samples of pooled intestinal explant cultures from each group.

Importantly, these results suggest that CO exposure ameliorates preexisting colitis. To conclusively demonstrate this point, we tested the therapeutic effects of CO administration in a model of severe, chronic intestinal inflammation with 100% penetrance. Severe colitis was induced in 8-wk-old *IL-10*^{-/-} mice by feeding 200 ppm piroxicam-supplemented chow for 5 d. In the original description of this model (23), piroxicam administration for 10 d to 4–6-wk-old *IL-10*^{-/-} mice resulted in severe colitis in 100% of mice that persisted upon withdrawal of piroxicam from the diet. Due to 100% mortality within 7 d of piroxicam feeding in our facility (not depicted), the protocol was accordingly modified. After removal of piroxicam from the diet, mice were exposed to either CO or ambient air (*n* = 5 mice each) for 2 wk. CO exposure significantly ameliorated established, severe colitis as determined by improved histologic parameters (Fig. 1 C). In this model, piroxicam represents an initiating factor for chronic inflammation rather than the cause per se. Severe inflammation persists after withdrawal of piroxicam, and although piroxicam may cause acute gastrointestinal injury, 8-wk-old wild-type C57Bl/6 mice treated with piroxicam for 5 d do not demonstrate intestinal inflammation 2 wk after withdrawal (not depicted; reference 23).

To determine whether CO exposure affects mucosal inflammatory cytokine production in *IL-10*^{-/-} mice, spontaneous IL-12 p40 and TNF levels were measured in supernatants from colonic mucosal tissue. Intestinal explants of *IL-10*^{-/-} mice exposed to CO in vivo for 4 wk express significantly less IL-12 p40 and TNF compared with explants from air-exposed mice (Fig. 1 D). Kinetics of inhibition of intestinal IL-12 p40 by CO was next determined. *IL-10*^{-/-} mice were exposed to 250 ppm CO for 1, 4, and 7 d. Intestinal secretion of IL-12 p40 was measured in intestinal explant culture and compared with values obtained from air-exposed *IL-10*^{-/-} mice. Intestinal IL-12 p40 is inhibited after 4 and 7 d of CO exposure, but not after 1 d (Fig. 1 E). Importantly, at 4 and 7 d, significant histological improvement was not demonstrated between the groups (not depicted), suggesting that CO inhibits IL-12 p40 expression before resolution of intestinal inflammatory infiltrates. IL-12 p40 heterodimerizes with p35 or p19 to produce biologically active IL-12 p70 or IL-23, respectively. Intestinal secretion of IL-12 p70 and IL-23 was measured in CO-treated and air-exposed *IL-10*^{-/-} mice. CO strongly inhibits IL-12 p70 at days 4 and 7, whereas a less significant inhibitory effect on intestinal IL-23 secretion is demonstrated (Fig. 1 E).

CO alters IFN- γ signaling in macrophages

CO inhibits synergistic activation of IL-12 p40 protein secretion and mRNA expression by LPS/IFN- γ in murine macrophages. As IL-12 p40 protein secretion was reduced in intestinal explants of CO-exposed *IL-10*^{-/-} mice, the molecular effects of CO on IL-12 p40 expression were investigated in murine BM-derived macrophages. Because

IFN- γ is important for optimal expression of LPS-induced IL-12 p40 secretion, macrophages were stimulated with LPS or LPS/IFN- γ . Exposure of cells to CO at 250 ppm inhibits synergistic activation of IL-12 p40 protein production (Fig. 2 A) and mRNA expression (Fig. 2 B) by LPS/IFN- γ . A dose-dependent inhibition of LPS/IFN- γ -induced IL-12 p40 mRNA and protein at CO concentrations of 0, 50, 100, and 250 ppm was observed (not depicted). Furthermore, CO inhibits LPS/IFN- γ -induced IL-12 p40 protein and mRNA expression across a range of LPS concentrations (10–1,000 ng/ml; not depicted). Next, the effects of CO on biologically active IL-12 p70 and IL-23 were studied. Again, the main effect of CO was inhibition of the synergistic induction of IL-12 p70 by LPS/IFN- γ . IFN- γ does not augment LPS-induced IL-23 secretion, and CO did not inhibit IL-23 activation (Fig. 2 C). Similar results were obtained using murine splenocytes and peritoneal macrophages (not depicted).

CO inhibits LPS/IFN- γ -induced IL-12 p40 promoter activity and activates an IFN-stimulated response element (ISRE). IL-12 p40 is transcriptionally regulated through a number of cis-acting elements that are responsive to bacterial

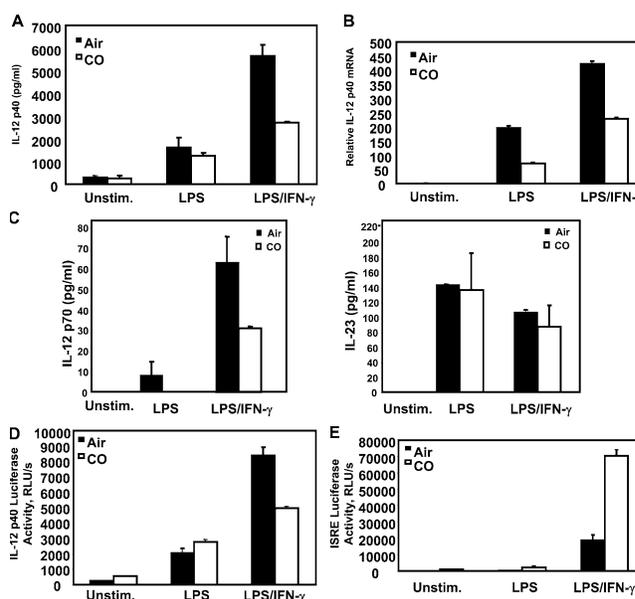


Figure 2. CO inhibits LPS/IFN- γ -induced IL-12 p40 expression in murine macrophages and alters IFN- γ signaling. (A) BM-derived murine macrophages were cultured in 250 ppm CO or ambient air. After activation with 1 μ g/ml LPS and/or 10 ng/ml IFN- γ , IL-12 p40 protein was assayed from supernatants at 24 h. (B) IL-12 p40 mRNA expression was determined at 4 h by real-time RT-PCR. (C) IL-12 p70 and IL-23 protein secretion was assayed by ELISA at 24 h. (D) J774 murine macrophages were transiently transfected with an IL-12 p40 promoter-luciferase reporter construct or (E) a multimerized ISRE-luciferase reporter plasmid. Cells were cultured in 250 ppm CO after the addition of 1 μ g/ml LPS or 1 μ g/ml LPS plus 10 ng/ml IFN- γ for 18 h. Results are expressed as relative light units normalized to activity from a cotransfected constitutively active β -galactosidase reporter plasmid.

products and IFN- γ (15). To study inhibition of LPS/IFN- γ -induced IL-12 p40 transcription by CO, IL-12 p40 promoter activity was determined in the J774 murine macrophage cell line. J774 cells were transiently transfected with a murine IL-12 p40 promoter fused to a luciferase reporter gene and then activated with IFN- γ plus LPS in the presence or absence of CO at 250 ppm for 24 h. CO selectively inhibits IL-12 p40 promoter activity synergistically induced by LPS/IFN- γ (Fig. 2 D).

IFN- γ signaling induces gene transcription through DNA motifs, designated ISRE, found in promoters of IFN- γ -inducible genes that bind the IRF family of transcription factors. Therefore, we determined whether CO modulates LPS/IFN- γ activation of a promoter that contains a consensus ISRE. J774 cells were transiently transfected with a plasmid containing a multimerized consensus ISRE fused to a luciferase reporter gene. Cells exposed to 250 ppm CO and activated with LPS/IFN- γ showed significantly augmented ISRE reporter activity (Fig. 2 E).

CO selectively inhibits IRF-8 protein expression. To summarize, for IL-12 p40, CO antagonizes IFN- γ responses, whereas for an ISRE, CO augments IFN- γ -induced activity. Accordingly, we hypothesized that CO affects gene transcription through IRF family members. A logical target is IRF-8. IRF-8 is an important transcription factor for IL-12 p40 regulation and promoter activation (16). Moreover, IRF-8 is a transcriptional repressor through an ISRE. J774 cells were stimulated with LPS/IFN- γ and cultured either in ambient air or 250 ppm CO for 4, 6, and 12 h. As determined by Western immunoblot in nuclear extracts, CO inhibits LPS/IFN- γ -activated IRF-8 protein expression compared with cells exposed to air (Fig. 3 A). Similar results are observed in murine splenocytes (Fig. 3 B). To determine whether CO inhibition of IRF-8 was specific, other transcription factors activated by IFN- γ were evaluated. CO exposure did not inhibit IRF-1, IRF-4, and phosphorylated STAT3 in LPS/IFN- γ -activated J774 cells (Fig. 3 A) or murine splenocytes (Fig. 3 B). Similarly, activated STAT1 (phosphorylated STAT1) and IRF-2 expression, induced by LPS/IFN- γ in murine macrophages, were not affected by CO (not depicted).

To provide evidence that IL-12 p40 inhibition by CO is directly mediated through IRF-8, IRF-8 was expressed in BM-derived macrophages by retroviral transduction using a derivative of the Moloney murine leukemia virus vector, pMMP412. 48 h after retroviral transduction, cells were exposed to 250 ppm CO or ambient air and activated with LPS with or without IFN- γ for 24 h. As expected, IRF-8-transduced macrophages produce higher levels of LPS-stimulated IL-12 p40 protein (Fig. 3 C). Importantly, in IRF-8-transduced BM-derived macrophages, the inhibitory effects of CO on LPS/IFN- γ -stimulated IL-12 p40 is abrogated, compared with control virus-transduced cells (Fig. 3 C). Collectively, these data demonstrate that CO inhibits LPS/IFN- γ -induced IL-12 p40 expression through IRF-8.

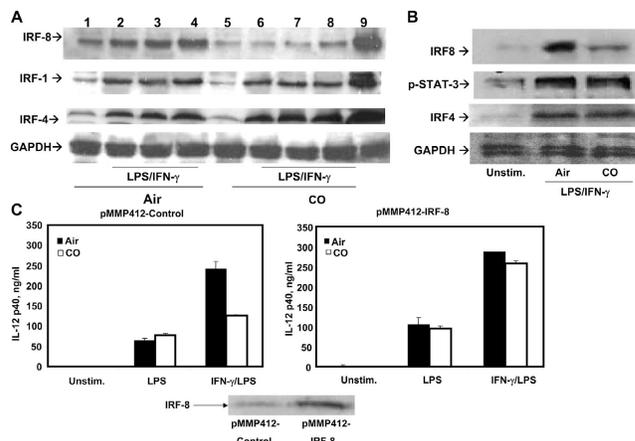


Figure 3. CO selectively inhibits LPS/IFN- γ -induced IRF-8 in murine macrophages. (A) Western blot analysis of IRF family members in J774 murine macrophages. Lanes 1–4 represent ambient air-exposed cells, and lanes 5–8 represent cells exposed to CO. Lanes 1 and 5, unstimulated; lanes 2 and 6, LPS/IFN- γ stimulated for 4 h; lanes 3 and 7, LPS/IFN- γ stimulated for 6 h; lanes 4 and 8, LPS/IFN- γ stimulated for 12 h; lane 9, nuclear extracts of RAW264.7 cells stimulated with LPS/IFN- γ . (B) Western blot analysis of IRF family members or phospho-STAT-3 in murine splenocytes. Lane 1, unstimulated; lanes 2 and 3 stimulated with LPS/IFN- γ for 8 h in the absence or presence of 250 ppm CO, respectively. (C) BM-derived macrophages were transduced with retrovirus IRF-8 or empty virus as control. 48 h after infection, cells were seeded in 24-well plates at 10^6 cells/ml, stimulated with 1 μ g/ml LPS or LPS and 10 ng/ml IFN- γ , and incubated for 24 h in the absence or presence of 250 ppm CO. Supernatants were then collected, and IL-12 p40 protein secretion was measured by ELISA. IRF-8 protein expression was determined by Western blot in nuclear extracts from unstimulated retrovirus IRF-8-infected macrophages and control virus-infected cells (inset). Each result is representative of three independent experiments.

CO inhibition of IL-12 p40 is independent of IL-10, MKK3, and nitric oxide (NO). The effects of CO on inflammatory cytokine signaling may encompass multiple pathways (13, 24). IL-10 has numerous antiinflammatory activities, perhaps the most important of which is its ability to strongly inhibit IL-12 p40 (25). Therefore, increases in LPS-induced IL-10 expression in CO-exposed macrophages may explain inhibition of IL-12 p40 production. To test this possibility, *IL-10*^{-/-} BM-derived macrophages were activated with LPS/IFN- γ in the presence or absence of CO. CO exposure inhibits IL-12 p40 protein production (Fig. 4 A) and mRNA expression (Fig. 4 B) induced by LPS and LPS/IFN- γ . Similar results were found in *IL-10*^{-/-} splenocytes, and CO also inhibits bioactive IL-12 p70 secretion (not depicted). CO also inhibits IRF-8 protein expression in *IL-10*^{-/-} macrophages (Fig. 4 C).

In a sepsis model, induction of IL-10 by CO was mediated by the MAPK signaling pathway MKK3/p38. p38 activation has been correlated with increased IL-12 p40 expression (13). Therefore, we investigated whether the inhibitory effects of CO on IL-12 is mediated through the MKK3/p38

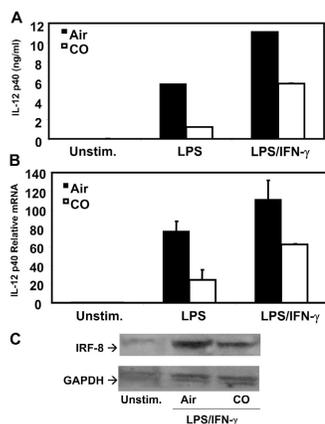


Figure 4. CO inhibits LPS/IFN- γ -induced IL-12 p40 expression independent of IL-10. (A) BM-derived macrophages from *IL-10*^{-/-} mice were cultured in 250 ppm CO or ambient air before activation with 1 μ g/ml LPS or 1 μ g/ml LPS plus 10 ng/ml IFN- γ . IL-12 p40 protein was assayed from supernatants at 24 h. (B) IL-12 p40 mRNA expression was determined at 4 h by real-time RT-PCR. Each result represents the mean \pm SD of triplicate assays and is representative of three independent experiments. (C) *IL-10*^{-/-} BM-derived macrophages were either unstimulated or activated with LPS/IFN- γ for 8 h in the absence or presence of 250 ppm CO. IRF-8 protein expression was determined in nuclear extracts by Western blot (representative of three experiments).

axis. In BM-derived macrophages from *MKK3*^{-/-} mice, CO inhibits LPS/IFN- γ -activated IL-12 p40 and p70 secretion (not depicted). Therefore, CO inhibition of LPS/IFN- γ -stimulated IL-12 p40 expression is independent of IL-10 and the p38 MAPK pathway.

We have previously shown that NO inhibits IL-12 p40 transcription (26). Furthermore, CO induces NO in hepatocytes (27). To test whether NO mediates inhibition of IL-12 p40 by CO, BM-derived macrophages from *inos*^{-/-} mice were incubated in 250 ppm CO and stimulated with LPS or LPS/IFN- γ . CO inhibits LPS/IFN- γ -stimulated IL-12 p40 and p70 secretion in *inos*^{-/-} macrophages (not depicted), suggesting that the inhibition of IL-12 expression by CO is inducible NO synthase (iNOS) independent.

CO-mediated modulation of IFN- γ signaling requires HO-1

In hepatocytes, CO up-regulates HO-1 activity, and inhibition of HO-1 abrogates the protective effects of CO in a model of acute liver failure (27). To determine whether HO-1 is required for downstream effects of CO on IFN- γ signaling, BM-derived macrophages were obtained from *hmox*^{-/-} mice. Interestingly, in *hmox*^{-/-} macrophages, inhibition of LPS/IFN- γ -induced IL-12 p40 production by exogenous CO exposure is completely abrogated (Fig. 5 A), fostering the hypothesis that the immunomodulatory effects of CO are mediated by HO-1 or one of its other metabolic products. In support of this hypothesis, in BM-derived macrophages, the selective HO-1 inhibitor tin protoporphyrin (SnPP) abrogates the inhibitory effect of exogenous CO on

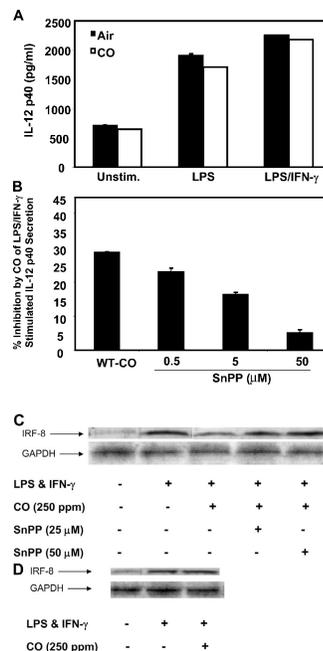


Figure 5. Inhibition of LPS/IFN- γ -induced IL-12 p40 production by CO is HO-1 dependent. (A) BM-derived macrophages from *hmox*^{-/-} mice were activated with 1 μ g/ml LPS and 10 ng/ml IFN- γ in the presence or absence of 250 ppm CO. IL-12 p40 protein was measured in supernatants at 24 h. Each result represents the mean \pm SD of triplicate assays and is representative of three independent experiments. (B) BM-derived murine macrophages were cultured with or without an HO-1 inhibitor (SnPP) for 1 h before stimulation with LPS/IFN- γ in the presence or absence of 250 ppm CO. Results are presented as percent inhibition of LPS/IFN- γ -stimulated IL-12 p40 secretion by CO at 24 h. (C) BM-derived macrophages were incubated with 25 and 50 μ M of the HO-1 inhibitor SnPP for 1 h before stimulation with 1 μ g/ml LPS plus 10 ng/ml IFN- γ in the absence or presence of 250 ppm CO for 8 h. IRF-8 protein expression in nuclear extracts was determined by Western blot. This result is representative of three independent experiments. (D) BM-derived macrophages from *hmox*^{-/-} mice were stimulated with 1 μ g/ml LPS plus 10 ng/ml IFN- γ in the absence or presence of 250 ppm CO for 8 h. IRF-8 in nuclear extracts was determined by Western blot. This result is representative of three independent experiments.

LPS/IFN- γ -stimulated IL-12 p40 secretion in a concentration-dependent manner (Fig. 5 B).

Expression of iNOS is up-regulated in macrophages by LPS/IFN- γ (28). Importantly, similar to IL-12 p40, the murine iNOS promoter is regulated by IRF-8 (28). Therefore, we studied whether CO would alter IFN- γ -induced accumulation of the end product of iNOS activity, NO. In murine BM-derived macrophages, CO inhibits LPS/IFN- γ -stimulated NO production (measured by nitrite production; not depicted). CO also inhibits LPS/IFN- γ -stimulated iNOS mRNA expression (not depicted). These results lend further credence to the observation that CO alters IFN- γ signaling through specific inhibition of IRF-8. Furthermore, pharmacologic inhibition of HO-1 by SnPP abrogates the inhibitory effect of CO on LPS/IFN- γ -stimulated NO production (not depicted), suggesting

that, similar to IL-12 p40, CO inhibits iNOS activity and/or expression through an HO-1–dependent pathway.

To demonstrate that IRF-8 is downstream of HO-1 in this CO inhibitory pathway, IRF-8 expression was evaluated in the presence of an HO-1 inhibitor and in *hmxo*^{-/-} macrophages. Pharmacologic inhibition of HO-1 with SnPP (Fig. 5 C) abrogates the inhibitory effect of CO on LPS/IFN- γ –stimulated IRF-8 protein expression in BM-derived macrophages. Likewise, in BM-derived *hmxo*^{-/-} macrophages, CO does not inhibit LPS/IFN- γ –stimulated IRF-8 expression (Fig. 5 D). These results further substantiate that CO alters IFN- γ signaling and abrogates IL-12 p40 expression through IRF-8, and that these effects require HO-1.

To begin to determine mechanistically how HO-1 mediates the described actions of CO, we tested whether CO alters HO-1 expression. HO-1 protein expression was determined in cellular extracts from murine BM-derived macrophages stimulated with LPS, IFN- γ , or both in the presence or absence of CO. IFN- γ inhibits basal and LPS-induced HO-1 protein expression in murine macrophages, and CO antagonizes the inhibitory effects of IFN- γ on HO-1 expression (Fig. 6 A). Likewise, HO-1 mRNA expression is inhibited by IFN- γ , and the inhibitory effect is abrogated by CO (not depicted). To determine whether CO alters HO-1 expression in vivo, intestinal sections from CO-exposed *IL-10*^{-/-} mice and air-exposed *IL-10*^{-/-} mice, matched for histologic colitis severity, were evaluated for HO-1 expression. Increased immunoreactivity for HO-1 in the intestinal epithelia and increased numbers of lamina propria cells with HO-1 immunoreactivity are observed in CO-exposed mice (Fig. 6 B). Thus, CO abrogates the inhibitory effect of IFN- γ on HO-1 expression in macrophages and induces intestinal HO-1 in vivo in *IL-10*^{-/-} mice.

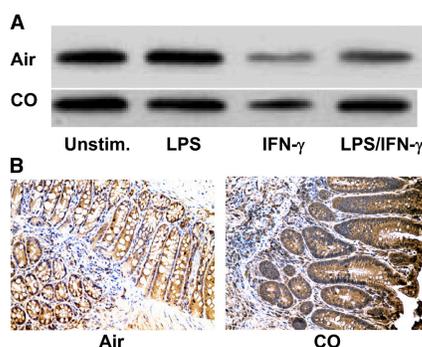


Figure 6. CO abrogates the inhibitory effect of IFN- γ on basal and LPS-induced HO-1 protein expression in macrophages and up-regulates intestinal HO-1 expression in *IL-10*^{-/-} mice. (A) BM-derived macrophages were stimulated with 1 μ g/ml LPS, 10 ng/ml IFN- γ , or both for 8 h and cultured in the presence or absence of CO. Western blotting was performed on whole cell extracts for HO-1 protein expression. Results are representative of three independent experiments (B) Immunohistochemical analysis of intestinal HO-1 protein expression in CO-treated and control air-exposed *IL-10*^{-/-} mice (exposed from 8 to 12 wk of age). Depicted intestinal sections were matched for similar histologic colitis activity.

Therefore, genetic and pharmacologic experiments demonstrate that CO acts through an HO-1–dependent pathway, and we hypothesized that another metabolic product of HO-1 may mediate the immunomodulatory effects of CO. Biliverdin has been recently described to alter T cell signaling and decrease IFN- γ production (29). BM-derived murine macrophages were treated with biliverdin at concentrations of 10 to 100 μ M for 2 h before activation with LPS or LPS/IFN- γ . Biliverdin does not inhibit IL-12 p40 mRNA expression or protein production (not depicted). Therefore, the effects of CO and HO-1 on IL-12 expression are not mediated by biliverdin in this experimental system.

HO-1 induction recapitulates the immunomodulatory effects of CO

BM-derived macrophages were treated with the HO-1 inducer hemin and then stimulated with LPS or LPS/IFN- γ . Pretreatment with hemin inhibits LPS/IFN- γ –stimulated IL-12 p40 secretion in a concentration-dependent manner (Fig. 7 A). Furthermore, hemin dose dependently induces HO-1 and inhibits IRF-8 protein expression (Fig. 7 B). To study HO-1 induction in vivo, hemin was administered by i.p. injection (25 mg/kg) twice weekly for 2 wk to 10-wk-old *IL-10*^{-/-} mice. In vivo administration of hemin increased intestinal HO-1 protein expression (Fig. 8 A). Histo-

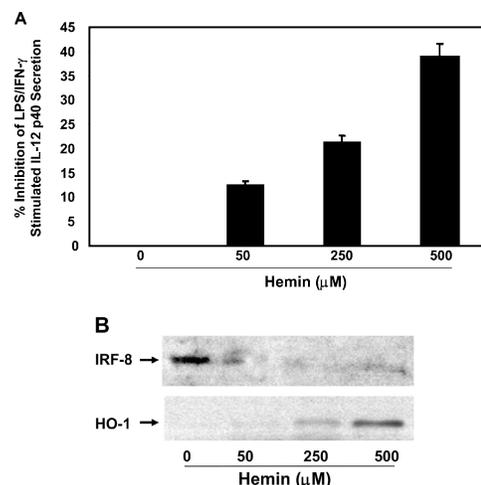


Figure 7. HO-1 induction inhibits LPS/IFN- γ –activated IRF-8 and IL-12 p40 expression in macrophages. (A) BM-derived macrophages were cultured with or without the HO-1 inducer hemin for 3 h before stimulation with 1 μ g/ml LPS or 1 μ g/ml LPS plus 10 ng/ml IFN- γ . IL-12 p40 protein was measured in 24-h supernatants. Results are presented as percent inhibition of LPS/IFN- γ –stimulated IL-12 p40 secretion by hemin and represent the mean \pm SD from three independent experiments. (B) BM-derived macrophages were cultured with or without hemin for 3 h before stimulation with LPS/IFN- γ for 8 h. IRF-8 and HO-1 protein expression was measured in 20 μ g of nuclear extract by Western blot. Equal loading of lanes was confirmed by equivalent expression of GAPDH between lanes (not depicted). Results are representative of three independent experiments.

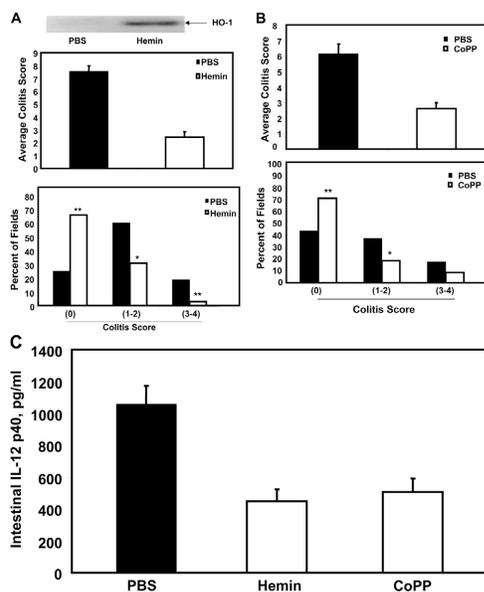


Figure 8. HO-1 induction ameliorates colitis in *IL-10*^{-/-} mice.

(A) 10-wk-old *IL-10*^{-/-} mice were treated with i.p. injection of hemin (25 mg/kg twice weekly for 2 wk), and control mice were treated i.p. with PBS vehicle ($n = 7$ per group). Induction of intestinal HO-1 in hemin-treated mice was detected by Western blot (top). Colitis scores were significantly lower in the hemin-treated mice. *, $P < 0.01$; **, $P < 0.001$. Histologic improvement in colitis is presented as average colitis scores (middle) and percent of histologic field that demonstrate no inflammation, mild to moderate inflammation, and severe inflammation as described in Results (bottom). (B) 10-wk-old *IL-10*^{-/-} mice were treated with i.p. injection of CoPP (5 mg/kg twice weekly for 2 wk), and control mice were treated i.p. with PBS vehicle ($n = 7$ per group). Colitis scores, presented as in A, were significantly lower in CoPP-treated mice. *, $P < 0.01$; **, $P < 0.001$. (C) Intestinal explants from vehicle-, hemin-, or CoPP-treated *IL-10*^{-/-} mice were cultured for 24 h for measurement of spontaneous IL-12 p40 secretion by ELISA. Results represent mean \pm SD of triplicate assays of pooled intestinal explants ($n = 7$ mice per group).

logic evaluation of colitis was compared with a control group treated i.p. with vehicle alone ($n = 7$ per group). Hemin-treated *IL-10*^{-/-} mice demonstrate improved histology (Fig. 8 B) and less weight loss (not depicted) than vehicle-treated controls. Similar histological improvement was observed in *IL-10*^{-/-} mice treated with another HO-1 inducer, cobalt protoporphyrin (CoPP; 5 mg/kg i.p. twice weekly for 2 wk; $n = 7$ per group; Fig. 8 C). Furthermore, decreased IL-12 p40 expression is demonstrated in intestinal explant cultures from hemin- and CoPP-treated *IL-10*^{-/-} mice compared with vehicle treated mice (Fig. 8 D). These results suggest a novel pathway where HO-1 mediates the antiinflammatory properties of CO. Collectively, cell culture and in vivo data demonstrate a potent immunomodulatory role for CO and HO-1 in chronic mucosal inflammation.

DISCUSSION

In summary, CO exposure at low concentrations ameliorates Th1-mediated chronic murine colitis. Mechanistically, the

effects of CO upon macrophage activation were studied, focusing on the regulation of the IL-12 p40 gene, important in the pathogenesis of Th1-mediated colitis. In murine macrophages, CO inhibits the synergistic activation of IL-12 p40 and iNOS by LPS/IFN- γ through selective inhibition of IRF-8. Furthermore, the immunologic effects of CO require HO-1. CO induces HO-1 expression, again, through antagonism of IFN- γ signaling. In the absence of functional HO-1, the inhibitory action of CO on IRF-8 and IL-12 p40 expression is lost. As proof of this concept, pharmacologic HO-1 induction with hemin and CoPP recapitulates the effects of CO in cells and in vivo. In macrophages, HO-1 inducers inhibit LPS/IFN- γ -induced IRF-8 and IL-12 p40 expression, and in *IL-10*^{-/-} mice, HO-1 inducers ameliorate colitis.

Despite the classification of CO as a toxic entity, lethal to aerobic life and one of the primary pollutants in industrial society, CO has recently emerged as a potent immunomodulatory entity. In the gastrointestinal tract, CO has been studied as a gaseous enteric neurotransmitter and as such is critically involved in smooth muscle membrane potential and ileal contractility (30). Recently, in a murine model of acute mucosal injury (dextran sodium sulfate-induced colitis), the HO-1 inducer CoPP modestly ameliorated inflammatory changes, but CO had no effect (29). In addition, pharmacologic inhibition of HO-1 exacerbated TNBS-induced acute colitis in rats (31). This study is the first to suggest an antiinflammatory role for CO in chronic intestinal inflammation and the first to investigate the HO-1 pathway in established chronic intestinal inflammation.

We directed our mechanistic analyses toward the observation that CO ameliorated Th1-mediated chronic intestinal inflammation in *IL-10*^{-/-} mice. In murine macrophages, CO affects IFN- γ signaling and IL-12 p40 expression. The IRF family member IRF-8 is specifically expressed in hematopoietic cells and is induced by IFN- γ (as well as LPS) at the protein and mRNA level in macrophages (32). *IRF-8*^{-/-} macrophages display selective impairment of IL-12 p40 expression. We have recently demonstrated a functional composite DNA binding element in the murine IL-12 p40 promoter that binds IRF family members, most notably IRF-8 (16). In different biologic situations, IRFs may serve as transcriptional activators or repressors (33). IRF-8 and IRF-2 have been demonstrated to inhibit IRF-1-mediated activation of promoters with a consensus ISRE, but IRF-8 is clearly involved in activation of the IL-12 p40 promoter. Therefore, our results suggest that CO exerts immunomodulatory effects through inhibition of IRF-8, thereby altering the ratio of IRF family members induced by IFN- γ in macrophages. Our finding that CO inhibits the synergistic activation of NO production by IFN- γ and LPS supports this hypothesis. The iNOS promoter contains a novel ISRE where IRF-8 plays an activating role (28).

In *hmox*^{-/-} macrophages, CO does not inhibit IL-12 p40 and IRF-8 expression. In addition, pharmacologic induction of HO-1 inhibits LPS/IFN- γ -induced IL-12 p40 and IRF-8

in wild-type macrophages. Therefore, CO may act primarily through induction of HO-1. Our findings using pharmacologic inducers and inhibitors of HO-1 parallel observations in a model of liver failure where inhibition of HO-1 activity in hepatocytes resulted in a loss of the protective effects conferred by CO (27). The precise mechanism(s) by which HO-1 confers protection against inflammation remains poorly understood, but more recently its products have emerged as the potential mechanisms of action. CO and the bile pigments biliverdin and bilirubin when administered exogenously may exert potent cytoprotective effects. Moreover, they exert their effects via distinctly different modes of action. For example, CO has effects on vasomotor tone, increasing organ perfusion after transplantation, whereas biliverdin does not (34). Biliverdin prevented the up-regulation of adhesion molecules and infiltrating leukocytes, whereas CO did not (35). In two recent studies of acute intestinal inflammation induced by dextran sodium sulfate in mice, HO-1 induction reduced mucosal injury (29, 36). In contrast to our findings, biliverdin recapitulated the protective effect of HO-1 induction in vivo, whereas CO did not (29). In our study, although CO and pharmacologic HO-1 inducers alter IFN- γ signaling in murine macrophages, biliverdin does not (not depicted). These results suggest that the products of the HO-1 pathway impart antiinflammatory effects via different modes of action. Furthermore, HO-1 may subserve different functions in acute versus chronic models of inflammation. Finally, we hypothesize that HO-1 may have a cytoprotective role independent of its two major metabolic byproducts, CO and biliverdin. Other possibilities include that ferritin expression resulting from iron produced through HO-1 mediates protective effects (37). Alternatively, perhaps endogenously produced CO directs additional protective mechanisms over that administered exogenously.

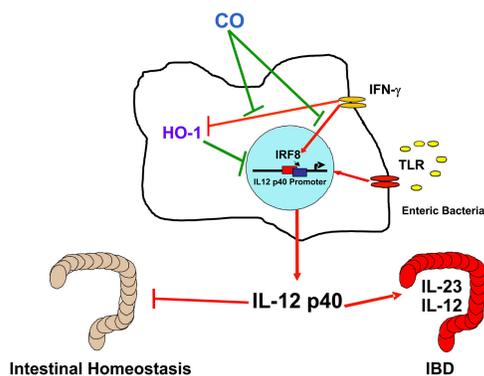


Figure 9. Model for the regulation of chronic intestinal inflammation by CO and HO-1. During chronic inflammation upon exposure to enteric bacteria and IFN- γ , intestinal macrophages secrete IL-12 p40. CO antagonizes this synergism through inhibition of IRF-8. IFN- γ also inhibits both basal and LPS-induced HO-1 expression, and CO abrogates this inhibitory effect. Therefore, HO-1 acts downstream of CO to ameliorate Th1-mediated chronic intestinal inflammation.

Collectively, these results show that CO alters IFN- γ signaling, suggesting that CO and HO-1 deviate an immune response from a Th1 profile toward an antiinflammatory response (Fig. 9). In Th1-mediated chronic mucosal inflammation, IFN- γ is a central mediator through induction of IRF-8 and IL-12 p40 as well as through direct inhibition of HO-1. Exogenous CO or HO-1 induction, likely through inhibition of IRF-8, reverses the mucosal inflammatory process. This concept is supported by a recent study (38) in which *hmox*^{-/-} splenocytes were found to express increased amounts of Th1 cytokines. This Th1 phenotype shift in *hmox*^{-/-} mice may be attributable to lack of appropriate control of IFN- γ signaling through the HO-1 pathway. Another prediction of this model that requires testing is that IFN- γ blockade in *IL-10*^{-/-} mice should be associated with induction of HO-1. Not surprisingly, antibodies against IFN- γ ameliorate Th1-mediated murine colitis (39) and may be an effective therapy in human CD (40).

This study may have important implications in human IBD. First, therapeutic strategies designed to induce HO-1 may eliminate toxicity concerns associated with direct administration of CO. Future experiments will further characterize metabolic products and enzymes in the HO-1 pathway as well as HO-1-inducing agents for immunologic actions in IBD models. Furthermore, we had initially pursued this line of investigation to study mechanisms of an important epidemiological observation in human IBD: Cigarette smoking is protective in UC (41). The “active” immunomodulatory component(s) in cigarette smoke that may explain this phenomenon remains ill defined. Cigarette smoke is a complex mix of more than 500 characterized compounds, each of which may exert independent immunologic effects. Despite this complexity, immunologic and clinical studies to address this issue in IBD have virtually all focused on nicotine. Therapeutic trial experience in UC patients treated with nicotine gum and transdermal nicotine has been inconclusive (42, 43). Interestingly, two recent reports demonstrated that cigarette smoke suppresses Th1 cytokines and enhances the production of IL-4 by human dendritic cell-primed T cells (44, 45). Based on our current findings, the immunomodulatory effects of cigarette smoke may in part be explained by CO (46). Blood carboxyhemoglobin level, a measure of systemic exposure to CO, has been reported to range from 1 to 18% in active smokers (47). The association between cigarette smoking, carboxyhemoglobin levels, and disease activity in patients with IBD warrants further investigation.

In conclusion, we demonstrate that CO administration ameliorates established chronic mucosal inflammation in an immunologic model of IBD. In Th1-mediated inflammatory responses, CO alters IFN- γ signaling in macrophages, resulting in decreased IRF-8 and IL-12 p40 expression. Immunologic and therapeutic effects of CO are dependent on HO-1. We have focused this analysis on how CO may alter macrophage activation, but CO may affect chronic inflammation

at many levels, including inhibiting T cell proliferation (48), decreasing epithelial cell apoptosis (36), altering cell adhesion and trafficking (49), and increasing intestinal blood flow (50). Elucidation of these phenomena is likely to clarify the pathogenesis of human IBD and may form the basis for new therapeutic approaches.

MATERIALS AND METHODS

Mice. Male C57/Bl6, *IL-10*^{-/-} and *inos*^{-/-} mice were obtained from The Jackson Laboratory, and *mkk3*^{-/-} and *hmox*^{-/-} mice were generated by R. Flavell (Yale University School of Medicine, New Haven, CT) and S. Yet (Harvard Medical School, Boston, MA), respectively. All wild-type and genetically deficient mice used in this study were on the C57/Bl6 background and matched for age and sex in all experiments. *IL-10*^{-/-} mice were fed a chow mixed with 200 ppm piroxicam (Sigma-Aldrich) for 5 d as indicated in Results. Pelleted diets were prepared by Dyets, Inc. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. At the end of the study period, animals were killed using excess CO₂ inhalation. Immediately afterward, blood by cardiac puncture (carboxyhemoglobin determination), spleens, intestinal tissue samples, and femurs were collected. BM-derived macrophages and splenocytes were cultured as described previously (26).

CO exposure. Mice or macrophages were exposed to compressed air or CO at a concentration of 250 ppm as described previously (13). In brief, CO at a concentration of 1% (10,000 ppm) in compressed air was mixed with compressed air before being delivered into the exposure chamber. Flow into animal chamber was maintained at rate of 12 liters/min and into the cell culture chamber at a rate of 2 liters/min. The cell culture chamber was humidified and maintained at 37°C. A CO analyzer (Interscan) was used to measure CO levels continuously in the chambers. Cardiac blood samples (0.2 ml) were taken immediately after the mice were killed to measure carboxyhemoglobin using a hemoximeter (OSM3; Radiometer Copenhagen).

Pharmacologic inhibition and induction of HO-1. SnPP, hemin, and CoPP (Frontier Scientific Porphyrin Products) were dissolved in sodium hydroxide, and the final pH of 7.4 was achieved by adding hydrochloric acid and further dilution with PBS. Because of light sensitivity, SnPP, CoPP, and hemin were prepared in dim light, light protected, and freshly made before injection.

Cytokine ELISAs. Murine IL-12 p40, IL-12 p70, IL-10, and TNF immunoassay kits (R&D Systems) and IL-23 (eBioscience) were used according to the manufacturers' instructions.

Western blot analysis. Western blot analyses were performed on nuclear or whole cell extracts as noted in Results (Figs. 3–8) and described previously (26). Anti-IRF-1, anti-IRF-2, anti-IRF-4, anti-IRF-8, anti-STAT-1, or anti-STAT-3 antibodies were from Santa Cruz Biotechnology, Inc., and anti-HO-1 was from StressGen Biotechnologies.

IRF-8 retroviral transduction. For retroviral transduction, we used a derivative of the Moloney murine leukemia virus vector pMMP412 (provided by H. Xiong, Mount Sinai School of Medicine, New York, NY; reference 28). To prepare pseudotyped virus, human 293 EbnaT cells were seeded at a density of 4 × 10⁶ cells in a 10-cm dish. The next day, cells were transfected using Superfect (QIAGEN) with a mixture of 2.5 μg of plasmid pMD.G encoding vesicular stomatitis virus G protein, 7.5 μg of plasmid pMD.OGP encoding gag-pol, and 10 μg of the IRF-8 retroviral expression construct. 48 h after transfection, the viral supernatant was collected, centrifuged at 1,500 rpm, and used to infect adherent BM-derived

macrophages in the presence of 4 μg/ml polybrene for 6 h. 48 h after infection, macrophages were seeded in 24-well plates at 10⁶ cells/ml, stimulated with LPS or LPS and IFN-γ, and incubated for 24 h in the absence or presence of 250 ppm CO.

Nitrite determination. Nitrite concentration, a measure of NO synthesis, was assayed by a standard Griess reaction adapted to a microplate system as described previously (51). NaNO₂ was used as a standard, and the data are presented as a concentration of nitrite.

RNA extraction and real-time RT-PCR analysis. Total RNA was isolated using TRIZOL reagent (Life Technologies). cDNA was derived from 1 μg total RNA by RT using an RT system (Promega). Real-time RT-PCR was performed on RT products with specific primers.

Fluorophore-labeled LUX primers (52) and their unlabeled counterparts were purchased from Invitrogen. LUX primers were designed using a software program (LUX Designer). LUX primers were designed to produce amplicons ranging in size between 69 and 145 basepairs. Primer pairs for IL-12 p40 and GAPDH were selected to span an intron. The primer sequences for IL-12 p40 are: LUX: 5'-GAACTTGTCAAAGGCTTCATCTGCAAG5TC and its unlabeled counterpart: 5'-GGAAGCACGGCAGCA-GAATA; for GAPDH: LUX: 5'-GACATACAGGCCGGTGCTGAG-TATG5C and its unlabeled counterpart: 5'-GCGGAGATGATGACCC-GTTT. Plots of fluorescence versus PCR cycle were generated by the ABI PRISM 7700 SDS software. Each 25-μl PCR reaction mix contained 2 μl cDNA, 1 μl of each gene-specific primer, and 12.5 μl 2X Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 1 μl 1X ROX reference dye, and 7.5 μl DEPC-treated water. Reactions were incubated at 50°C for 2 min, 95°C for 2 min, and then cycled (45 times) at 95°C for 15 s and 60°C for 30 s. Quantitation of IL-12 p40 mRNA was expressed as relative fold-increase in transcript level with respect to unstimulated cells. Real-time RT-PCR for iNOS and HO-1 were performed as described previously (53).

Transient transfections. J774 murine macrophages were transiently transfected using SuperFect Transfection Reagent (QIAGEN) by the described protocol with modifications. For each transfection, 2.5 μg of plasmid were mixed with 100 μl DMEM (without serum and antibiotics) and 10 μl of SuperFect reagent. The mixture was incubated at room temperature for 10 min, and then 600 μl DMEM complete medium was added and immediately placed onto the cells in six-well plates. After incubation for 3–4 h at 37°C, the cells were washed with PBS and split equally into two plates. 12–16 h later, one six-well plate was treated with CO. Cells were either unactivated or activated with 1 μg/ml LPS, 10 ng/ml IFN-γ, or both. 16–18 h after activation, the cells were harvested by using 1X Reporter Lysis Buffer (Promega). Luciferase activity was determined from 20 μl of cell extract as described previously (15). Cells were cotransfected with a constitutively active HSP promoter that expresses β-galactosidase to monitor transfection efficiency.

Intestinal tissue explant cultures. Sections of the transverse colon from individual mice were washed with PBS to remove fecal contents, shaken at 280 rpm at room temperature for 30 min in RPMI 1640 plus 50 μg/ml gentamicin, and cut into small fragments. Tissue fragments (0.5 g dry weight) were incubated in 1.0 ml RPMI 1640 supplemented with 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone (GIBCO BRL), and 5% heat-inactivated fetal calf serum. Tissue fragment supernatants were collected after 24 h for cytokine ELISAs.

Histology. Colons were removed after mice were killed and rinsed with cold PBS to remove fecal material. Tissue sections were fixed in 10% buffered formalin and embedded in paraffin. 5-μm-thick sections were stained with hematoxylin and eosin. Colitis scores (0–4) were determined by a staff pathologist who was blinded to the experimental protocol using the criteria reported by Berg et al. (22). 20 separate microscopic fields (magnification of

100) were evaluated for each mouse by a pathologist (A.R. Sepulveda) blinded to the treatment groups.

Data analysis. Statistical analysis was performed using Student's *t* test. A probability level of $P < 0.05$ was considered statistically significant.

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