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An Anti-Inflammatory Role for Carbon Monoxide and Heme Oxygenase-1 in Chronic Th2-Mediated Murine Colitis

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Abstract

Cigarette smoking is a significant environmental factor in the human inflammatory bowel diseases, remarkably, conferring protection in ulcerative colitis. We previously demonstrated that a prominent component of cigarette smoke, CO, suppresses Th17-mediated experimental colitis in IL-10$^{-/-}$ mice through a heme oxygenase (HO)-1–dependent pathway. In this study, homeostatic and therapeutic effects of CO and HO-1 were determined in chronic colonic inflammation in TCR-α–deficient ($^{-/-}$) mice, in which colitis is mediated by Th2 cytokines, similar to the cytokine milieu described in human ulcerative colitis. TCRα$^{-/-}$ mice exposed to CO or treated with the pharmacologic HO-1 inducer cobalt protoporphyrin demonstrated amelioration of active colitis. CO and cobalt protoporphyrin suppressed colonic IL-1β, TNF, and IL-4 production, whereas IL-10 protein secretion was increased. CO induced IL-10 expression in macrophages and in vivo through an HO-1–dependent pathway. Bacterial products regulate HO-1 expression in macrophages through MyD88- and IL-10–dependent pathways. CO exposure and pharmacologic HO-1 induction in vivo resulted in increased expression of HO-1 and IL-10 in CD11b^{+} lamina propria mononuclear cells. Moreover, induction of the IL-10 family member IL-22 was demonstrated in CD11b^{-} lamina propria mononuclear cells. In conclusion, CO and HO-1 induction ameliorated active colitis in TCRα$^{-/-}$ mice, and therapeutic effects correlated with induction of IL-10. This study provides further evidence that HO-1 mediates an important homeostatic pathway with pleiotropic anti-inflammatory effects in different experimental models of colitis and that targeting HO-1, therefore, is a potential therapeutic strategy in human inflammatory bowel diseases.

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Cigarette smoking is one of the most significant environmental risk factors identified in the human inflammatory bowel diseases (IBD): Crohn's disease (CD) and ulcerative colitis (UC). Meta-analyses showed that the risk for developing UC in current smokers is ~40% that of nonsmokers (1). Former smokers have ~1.7 times increased risk for developing UC (2). Some studies even suggested a dose response, with heavier smokers having greater protection (1). Based on these compelling epidemiological observations, one of the important unanswered questions in IBD is how does cigarette smoking mechanistically mediate this protective effect?

The gaseous molecule CO is one candidate that may contribute to the beneficial association between smoking and UC. CO is a prominent component of cigarette smoke: blood carboxyhemoglobin levels, a measure of systemic exposure to CO, were reported to range from 1–18% in active smokers (3). CO, best known as a toxic compound, is also produced endogenously during normal physiology by the heme oxygenase (HO) enzymes, which mediate the degradation of heme into equimolar quantities of CO, iron, and biliverdin. In particular, the enzyme HO-1 and its metabolic products regulate immune responses, tissue injury, and repair (4). We previously showed that CO ameliorates active inflammation in an experimental model of chronic IBD, IL-10–deficient ($^{-/-}$) mice, through induction of HO-1 (5).

$CD4^+$ Th cells play a key role in the regulation of immune responses in the intestine. $CD4^+$ T cells have been divided into functionally important subsets based on the cytokines that they produce (6). Although these subdivisions represent a reduction of complex biology, most applicable to the mouse, they provide a framework to understand mucosal T cell responses in human IBD. Th1 cells produce the cytokines IFN-γ and IL-2. Th1 cells are a hallmark of cell-mediated immunity, necessary for the eradication of intracellular pathogens and the development of long-term immunity against infectious agents (6). Numerous mouse models of IBD are characterized by an overabundance of intestinal Th1 cytokines. CD was initially described as a prototype Th1-mediated chronic inflammatory disorder, characterized by mucosal granulomas (the histological hallmark of a Th1 response), increased expression of IFN-γ, as well as increased IL-12, a cytokine necessary for Th1 development (7). However, the discovery of the cytokine IL-23, which shares a common p40 subunit with IL-12, led to a paradigm shift in our understanding of inflammatory responses in IBD (8). IL-23, unlike IL-12, promotes a distinct $CD4^+$ T cell activation profile, the Th17 cell, characterized by the production of the cytokine IL-17. A pivotal role for IL-23 and Th17 cells was demonstrated in experimental IBD models, such as the IL-$10^{-/-}$ mouse, and recent genetic and immunologic findings highlight the importance of this pathway in human IBD (9).

Th2 cells produce the cytokines IL-4, IL-5, and IL-13. These cytokines provide help for B cell Ab production, and are involved in host defense against extracellular helminthic parasites in the mucosal immune system (10). Inflammation in human UC has been characterized as mediated by Th2 cytokines. Lamina propria T cells from UC patients produce IL-13 and IL-5 and little IFN-γ (11). Although multiple experimental models of chronic Th1/17-driven intestinal inflammation have been elucidated, few have been described in which chronic disease occurs in a Th2 cytokine milieu. Mice with targeted disruption of the TCRα gene (TCR$\alpha^{-/-}$) perhaps most closely recapitulate the colonic Th2 signature in human UC. IL-4 and IL-1β play important roles in the development of colitis in TCR$\alpha^{-/-}$ mice (12).

Our previous work showed that CO and HO-1 induction ameliorates Th17-mediated colonic inflammation in IL-$10^{-/-}$ mice (5). To model the protective effects of cigarette smoking in human UC, CO and the HO-1 pathway were studied in a murine model with immunologic

similarities to UC. We demonstrate the anti-inflammatory effects of CO and HO-1 induction in spontaneous Th2-mediated colitis in TCR$\alpha^{-/-}$ mice.

Materials and Methods

Mice

Wild-type (WT), TCR$\alpha^{-/-}$, and IL-10$^{-/-}$ mice were obtained from The Jackson Laboratory. *Hmox1*$^{-/-}$ mice were provided by Leo Otterbein (Harvard Medical School, Boston, MA). All mice used in this study were on the C57BL/6 background and matched for age and sex in all experiments. Animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and by the Institutional Animal Care and Use Committee of the University of Pittsburgh and the University of North Carolina Schools of Medicine.

CO exposure

Mice or macrophages were exposed to compressed air or CO at a concentration of 250 ppm, as previously described (5). Briefly, CO at a concentration of 1% (10,000 ppm) was mixed with compressed air before delivery into the exposure chamber. Flow into the animal chamber was maintained at a rate of 12 l/min and into the cell-culture chamber at a rate of 2 l/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. The CO-releasing molecule-186 (ALF186) was a generous gift from Alfama (Porto Salvo, Portugal) and was injected i.p. twice a week for 2 wk. Control mice were injected with an inactive form of ALF186 (iALF186) that does not possess CO-releasing capacity. At the end of CO exposure, cardiac blood samples (0.2 ml) were taken immediately after mice were sacrificed to measure carboxyhemoglobin (HbCO) using a hemoximeter (OSM3; Radiometer Copenhagen). Doses of ALF186 were extensively optimized because hypoxia can become a confounding factor at HbCO levels ≥20% (13). HbCO levels were uniformly <20% in all experiments using CO gas and ALF186.

Pharmacologic inhibition and induction of HO-1

Tin protoporphyrin (SnPP) and cobalt protoporphyrin (CoPP) (Frontier Scientific Porphyrin Products, Logan, UT) were dissolved in sodium hydroxide; a final pH of 7.4 was achieved by adding hydrochloric acid and further dilution with PBS. Because of light sensitivity, SnPP and CoPP were prepared in dim light, light protected, and freshly made before injection.

TLR ligands and cytokines

Ultrapure TLR ligands (Invivogen, San Diego, CA), LPS, synthetic bacterial lipopeptide (sBLP), flagellin, and CpG oligodeoxynucleotides (CpG) were dissolved in endotoxin-free water at concentrations described in the *Results*. IL-10 (PeproTech, Rocky Hill, NJ) was dissolved in endotoxin-free water.

Cytokine ELISAs

Linco Cytokine-16 plex Mouse ELISA was performed for IL-4, IL-1β, IL-10, TNF, and IL-17 ((Millipore, Billerica, MA), per the manufacturer's instructions. Murine IL-10 was measure with cytokine-specific immuno-assay kits (R&D Systems, Minneapolis, MN).

Real-time RT-PCR analysis

Quantitative real-time PCR, using SYBR Green Master Mix (Applied Biosystems, Bedford, MA), was performed on an HT-7900 (Applied Biosystems, Bedford, MA), as previously

published (14). The following primer sequences were used: *Hmox1*: forward, 5′-CCAGAGTTTCCGCATACAACC-3′, reverse, 5′-TCTCTGGACACCTGACCCTTCG-3′; *β-actin*: forward, 5′-AGCCATGTACGTAGCCATCCAG-3′, reverse, 5′-TGGCGTGAGGGAGAGCATAG-3′; *Il10*: forward, 5′-GTCATCGATTTCTCCCCTGTG-3′, reverse, 5′-CCTTGTAGACACCTTGGTCTTGG-3′; and *Il22*: forward, 5′-GGTGCCTTTCCTGACCAAAC-3′, reverse, 5′-TTTCACTGTCTCCTTCAGCCTTC-3′.

Western immunoblots

Western blots were performed on whole-cell extracts, as described (15). HO-1 Abs were from Stressgen (Plymouth Meeting, PA); NF-E2–related factor 2 (Nrf2) and β-actin Abs were from Abcam (Cambridge, MA).

Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMMs) were cultured as described previously (5).

Colonic tissue explant cultures

Colonic tissue fragments (0.5 g dry weight) were processed, as previously described (14). Tissue-fragment supernatants were collected after 24 h for cytokine ELISAs.

Isolation of colonic macrophages

Lamina propria mononuclear cells (LPMCs) were isolated from mouse colon by an enzymatic method, followed by Percoll (GE Healthcare, Piscataway, NJ) density-gradient centrifugation, as previously described (16). LPMCs were further separated into $CD11b^+$ cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Purity was >90% by flow cytometric analysis.

Flow cytometry

Splenic, mesenteric lymph node, and colonic cells were isolated, as previously described (17), and stained with the B cell marker FITC anti-mouse B220 and cell surface marker PE anti-mouse CD1d (1B1) (eBioscience, San Diego, CA). In other experiments, cells were stained extracellularly with FITC-conjugated anti-$CD4^+$ (RM4-5), fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, San Jose, CA), and stained intracellularly with allophycocyanin-conjugated anti-Foxp3 (FJK-16s). Colonic LPMCs from $TCR^{-/-}$ mice exposed to CO or injected with ALF186, as well as control mice (air-exposed and iALF186-treated mice), were labeled with Abs against macrophage lineage and activation markers (CD14, F4/80, CD86, and CD80; eBioscience, San Diego, CA). Dead cells were excluded with propidium iodide staining. Samples were acquired on a FACSCalibur (Becton Dickinson), and data were analyzed with Cell-Quest Pro software (BD Biosciences, San Jose, CA).

Histology

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. (18). Twenty separate microscopic fields (original magnification ×100) were evaluated for each mouse by a pathologist (A.R.S.) blinded to the treatment groups.

Data analysis

Statistical significance for data subsets from experiments performed in cells was assessed by the two-tailed Student *t* test. Statistical significance for in vivo data subsets was assessed by the Mann–Whitney *U* test (SPSS, Chicago, IL) with Bonferroni correction.

Results

CO exposure ameliorates active colitis in TCRα$^{-/-}$ mice

TCRα$^{-/-}$ mice were exposed to 250 ppm of CO from 12–16 wk of age (n = 10) and compared with a control group (n = 10) exposed to ambient air. Mice in both treatment groups were matched for age, sex, and initial body weight. CO-exposed mice showed an increase in body weight compared with mice housed in ambient air (Fig. 1A). Assessment of histological improvement was performed by a pathologist blinded to treatment groups. CO-exposed mice demonstrated significantly reduced histologic inflammation (Fig. 1B, Supplemental Fig. 1).

We next determined whether CO exposure affects colonic cytokine expression in TCRα$^{-/-}$ mice. Colonic explant cultures from TCRα$^{-/-}$ mice exposed to CO in vivo for 4 wk produced less IL-1β, IL-4, TNF, and IL-17 (Fig. 1C, 1D), correlating with histological improvement. CO treatment in vivo also resulted in increased colonic IL-10 secretion compared with explant cultures from air-exposed TCRα$^{-/-}$ mice (Fig. 1C).

Because IL-10 is an important regulatory cytokine, correlations between CO exposure and colonic IL-10 induction were further explored. Ten-week-old TCRα$^{-/-}$ mice were divided into three groups: group 1 was exposed to CO (250 ppm) for 4 wk, group 2 was exposed to air for 4 wk, and group 3 was exposed to CO for 2 wk and then transferred to ambient air for 2 wk. Mice exposed to CO demonstrated increased secretion of IL-10 in colonic explants. Mice transferred from CO exposure to air after 2 wk showed intermediate IL-10 secretion, with more colonic IL-10 compared with air-exposed mice but less than mice continually exposed to CO (Fig. 1E). These findings suggested that CO may ameliorate inflammation through induction of IL-10. Furthermore, because IL-10 secretion was still increased 2 wk after removing mice from CO, CO may induce a durable change in a cell population that secretes IL-10 in the colon.

CO induces IL-10 and HO-1 in colonic CD11b^{+} LPMCs

To elucidate potential mechanisms through which CO ameliorates experimental colitis, TCRα$^{-/-}$ mice were treated i.p. with ALF186 (30 mg/kg) and iALF186 (30 mg/kg) twice weekly for 2 wk. Colonic CD11b^{+} LPMCs, predominantly representing a macrophage cell population (14), were isolated from both groups, and *Hmox1* and *Il10* expression were analyzed. *Hmox1* was induced in CD11b^{+} and CD11b^{-} LPMCs in ALF186-treated TCRα$^{-/-}$ mice compared with iALF186-treated mice (Fig. 2A), with the most significant induction in CD11b^{+} cells. Interestingly, markedly increased *Il10* expression was demonstrated in CD11b^{+} LPMCs, but not in CD11b^{-} LPMCs, from ALF186-treated mice compared with iALF186-treated mice (Fig. 2B). No differences were observed in expression of surface markers (F4/80, CD80, CD86, and CD14) in CD11b^{+} LPMCs from TCRα$^{-/-}$ mice treated with ALF186 or iALF186 (Supplemental Fig. 2A). Similar results were obtained for *Hmox1* and *Il10* expression in LPMCs from ALF186-treated WT mice, with the exception that *Il10* induction was also demonstrated in the CD11b^{-} LPMC population (Supplemental Fig. 3), possibly reflecting increased numbers of CD4^{+} Foxp3^{+} lamina propria T regulatory cells in WT mice compared with TCRα$^{-/-}$ mice (see later discussion; Supplemental Fig. 1C).

Another member of the IL-10 family of cytokines, IL-22, was shown to dampen innate mucosal inflammatory responses and attenuate colitis in TCRα$^{-/-}$ mice (19). LPMCs from ALF186-treated TCRα$^{-/-}$ mice demonstrated significantly more IL-22 (*Il22*) expression compared with iALF186-treated mice, predominantly in the CD11b^{-} LPMC population, but in CD11b^{+} cells as well (Fig. 2C).

Regulatory CD4^{+} Foxp3^{+} T and CD11d^{+} B cells were demonstrated to be a source of intestinal IL-10 production and have important anti-inflammatory roles in murine IBD (19). However, no differences in the numbers of CD1d^{+} B cells in LPMCs were found between iALF186- and ALF186-treated TCRα$^{-/-}$ mice (Supplemental Fig. 2B). Interestingly, a marked decrease in the numbers of splenic CD4^{+} Foxp3^{+} T cells was observed in TCRα$^{-/-}$ mice compared with WT mice (Supplemental Fig. 2C), and Foxp3^{+} cells were undetectable in LPMCs from TCRα$^{-/-}$ mice (data not shown). These results suggested that CO may be protective in experimental colitis through induction of IL-10 and HO-1, specifically in CD11b^{+} LPMCs.

CO induces IL-10 in macrophages through induction of HO-1

To further elucidate regulation of IL-10 by CO in macrophages, WT and TCRα$^{-/-}$ BMMs were stimulated with LPS in CO (250 ppm) or ambient air for 24 h. As previously described (13), CO-augmented LPS stimulated IL-10 secretion in WT BMMs (Fig. 3A) (5). CO also augmented IL-10 secretion from LPS-stimulated TCRα$^{-/-}$ BMMs (Fig. 3B). However, in LPS-activated BMMs from HO-1–deficient (*Hmox1*$^{-/-}$) mice, CO failed to induce IL-10 secretion (Fig. 3C), suggesting that CO augments IL-10 secretion through an HO-1–dependent signaling pathway. Moreover, WT BMMs incubated with ALF186 (100 μg/ml) for 3 h demonstrated significantly increased basal *Hmox1* and *Il10* expression compared with iALF186 BMMs (Fig. 3D, 3E).

HO-1 induction recapitulates immunomodulatory effects of CO in vivo

To understand the role of HO-1 in the anti-inflammatory effects of CO in vivo, TCRα$^{-/-}$ mice were treated with a pharmacological inducer of HO-1, CoPP (5 mg/kg i.p.), twice a week for 2 wk and compared with vehicle (DMSO)-treated controls. CoPP treatment resulted in improved histological scores compared with vehicle treatment (Fig. 4A). Moreover, colonic explant cultures revealed decreased IL-4 (Fig. 4B), IL-1β, TNF, and IL-17 (Fig. 4C) secretion in CoPP-treated TCRα$^{-/-}$ mice. CoPP treatment also resulted in robust induction of *Hmox1* expression in colonic CD11b^{-} and CD11b^{+} LPMC populations (Fig. 4D). Colonic CD11b^{+} LPMCs were the primary source of IL-10 secretion from CoPP-treated TCRα$^{-/-}$ mice, because less IL-10 expression was observed in CD11b^{-} cells (Fig. 4E). These results strongly implicated CD11b^{+} LPMCs as the primary source of IL-10 in TCRα$^{-/-}$ mice and important targets for the immunomodulatory effects of CO and HO-1.

Next, to address whether immunomodulatory effects of CO are mediated by HO-1 in vivo, TCRα$^{-/-}$ mice were treated i.p. with ALF186, with or without the HO-1 inhibitor SnPP. ALF186-treated mice demonstrated reduced histologic inflammation compared with ALF186+SnPP-treated mice (Fig. 5A). Importantly, CD11b^{+} LPMCs from ALF186+SnPP-treated TCRα$^{-/-}$ mice expressed significantly less *Il10* than did CD11b^{+} LPMCs from ALF186-treated mice (Fig. 5B). Likewise, CD11b^{-} and CD11b^{+} LPMCs from ALF186+SnPP-treated TCRα$^{-/-}$ mice demonstrated lower Il22 expression than did LPMCs from ALF186-treated mice (Fig. 5C). These findings demonstrated that the anti-inflammatory effects of CO are abrogated in the presence of an HO-1 inhibitor.

LPS and IL-10 regulate HO-1 expression in macrophages

Because HO-1 is required for the protective effects of CO, we next studied HO-1 (*Hmox1*) regulation in macrophages. BMMs from WT and IL-10$^{-/-}$ mice were stimulated with LPS, with or without IL-10, and expression of *Hmox1* mRNA and HO-1 protein was determined. IL-10$^{-/-}$ BMMs demonstrated decreased expression of *Hmox1* compared with WT BMMs. Addition of rIL-10 restored *Hmox1* mRNA and protein expression in LPS-activated IL-10$^{-/-}$ BMMs and augmented *Hmox1* expression in WT BMMs (Fig. 6A, 6B). Moreover, incubation of WT BMMs with an IL-10 Ab inhibited LPS-induced expression of HO-1. These results demonstrated that LPS and IL-10 are regulators of HO-1 in macrophages.

TLRs recognize specific molecular patterns present in a broad range of microbial pathogens. TLR activation uses a common signal-transduction pathway initiated by the adaptor protein MyD88. To further elucidate TLR-mediated induction of HO-1, WT and MyD88$^{-/-}$ BMMs were stimulated with MyD88-dependent (sBLP, LPS, flagellin, and CpG DNA) bacterial ligands. Interestingly, sBLP, LPS, flagellin, and CpG DNA induced HO-1 expression in WT BMMs but not in MyD88$^{-/-}$ BMMs (Fig. 6C). Addition of rIL-10 restored HO-1 expression in MyD88$^{-/-}$ BMMs, suggesting that IL-10–induced expression of HO-1 in macrophages is independent of TLR/MyD88-signaling pathways. As previously reported, TLR-mediated IL-10 expression, another MyD88-dependent gene, was abrogated in MyD88$^{-/-}$ BMMs (Supplemental Fig. 4).

The transcription factor Nrf2 is a critical regulator of HO-1 through binding to antioxidant response elements (20). In the absence of MyD88, Nrf2 protein expression in macrophages was also markedly reduced (Fig. 6C). These results elucidated a novel regulatory circuit, with MyD88-dependent *Hmox1* expression by bacterial products, in part through Nrf2, and MyD88-independent regulation by IL-10.

Discussion

In summary, CO exposure ameliorates chronic Th2-mediated colitis in TCRα$^{-/-}$ mice. Immunomodulatory effects of CO were recapitulated by pharmacologic HO-1 induction. Moreover, pharmacologic inhibition of HO-1 blocked the protective effects of CO on colitis, suggesting that in vivo, CO mechanistically requires HO-1 function. CO and HO-1 induction resulted in increased colonic IL-10 expression prominently in CD11b^{+} LPMCs, with consequent inhibition of inflammatory cytokines. We previously demonstrated that CO ameliorated colitis in IL-10$^{-/-}$ mice (5). IL-10$^{-/-}$ mice exhibit a Th17-mediated immune pathology. The protective effects of CO in IL-10$^{-/-}$ mice were attributed, in part, to inhibition of the common p40 subunit of the inflammatory cytokines IL-12 and IL-23 (5). Our current study elucidated the anti-inflammatory effects of CO and the HO-1 pathway in TCRα$^{-/-}$ mice characterized by a distinctly different immunopathogenesis, with increased colonic Th2 cytokine expression that, to some extent, recapitulates the colonic inflammatory cytokine milieu in human UC (12). This study further elucidated pleiotropic immunomodulatory effects of CO and the HO-1 pathway. There are now illustrations of therapeutic applications of this pathway (5, 21, 22) in multiple experimental models of IBD mediated by divergent immune mechanisms. Given the genetic, immunologic, and clinical heterogeneity of the human IBDs, the therapeutic benefit of CO and HO-1 in numerous preclinical models suggests potentially broad applications in patients.

Notably, in TCRα$^{-/-}$ mice, CO- and HO-1–mediated inhibition of inflammation correlated with increased levels of the anti-inflammatory cytokine IL-10. Cross-talk between CO/HO-1 and IL-10 regulation may underlie the homeostatic function of each modality. Lee and Chau (23) demonstrated that IL-10 induced the expression of HO-1 via a p38 MAPK-dependent pathway. IL-10–induced expression of HO-1 also requires activation of STAT-3 (24).

Moreover, HO-1 may be an important downstream mediator of the anti-inflammatory effects of IL-10 in macrophages. HO-1 activity and the generation of endogenous CO were necessary for IL-10–dependent inhibition of TNF expression (25). Likewise, LPS-activated macrophages overexpressing HO-1 or exposed to CO demonstrated reduced TNF production, whereas IL-10 secretion was enhanced (26). Interestingly, we demonstrated that LPS stimulated HO-1 expression in WT BMMs, but not IL-10$^{-/-}$ BMMs, and blocking IL-10 diminished LPS-activated HO-1 expression. Hence, IL-10 is a cofactor for HO-1 induction by TLR ligands. Moreover, *Hmox1* induction occurred through MyD88-dependent (bacterial products) and -independent (IL-10) pathways. LPS and inflammatory cytokines (IL-1β and TNF) are well described as potent inducers of HO-1, and several studies linked NF-κB and AP-1 transcription factors in this response (27, 28). However, given the absence of a clearly identified functional NF-κB element, how NF-κB promotes *Hmox1* gene transcription is a matter of speculation (29–31). Nrf2, a basic leucine zipper transcription factor, is involved in cellular protection against oxidative stress through antioxidant response element-directed induction of multiple detoxifying and antioxidant enzymes, including HO-1 (20). We demonstrated defective induction of Nrf2 in MyD88$^{-/-}$ BMMs, suggesting a mechanism for how TLR signaling may affect *Hmox1* transcription.

HO-1 was also shown to exert its protective effect in experimental asthma through a mechanism mediated by IL-10 expression in CD4^{+}CD25^{+}Foxp3^{+} T regulatory cells (32). In vivo CO exposure ameliorated intestinal injury induced by LPS or ischemia–reperfusion. Mucosal levels of IL-10 were shown to be increased in CO-exposed mice (33). Similarly, CO-releasing molecules promoted resolution of acute pancreatic inflammation in rats, which correlated with increases in local IL-10 expression (34). CO was also shown to augment local IL-10 and afford protection in other models of inflammation, including sepsis, renal injury, and diabetes (35–37).

Activated macrophages are an abundant source of IL-10 (38). CO augments basal and LPS-induced IL-10 secretion from WT and TCRα$^{-/-}$ BMMs. Specifically, TCRα$^{-/-}$ mice treated with CO and CoPP demonstrated a specific increase in IL-10 secretion exclusively from CD11b^{+} LPMCs, which include a predominant resident macrophage population. These findings expand upon an important homeostatic role for IL-10–producing colonic macrophages, which acted on T regulatory cells to maintain expression of Foxp3 in a T cell adoptive-transfer model of murine colitis (39).

Several regulatory B cell populations have been characterized in TCRα$^{-/-}$ mice. A subset of regulatory B cells was identified as an important source of IL-10 and was responsible for inhibiting IL-1β and ameliorating colitis (40). However, we could not discern any difference in the numbers of CD1d^{+} MLNs from ALF186-treated TCRα$^{-/-}$ mice compared with iALF186-treated mice. An IL-12–producing regulatory B cell subset that develops in the presence of IL-10 was also shown to be involved in the regulation of colonic inflammation in this model (41). During CO exposure and pharmacologic HO-1 induction in vivo, IL-10 is almost exclusively detected in the CD11b^{+} LPMC fraction. These findings suggested that CO and HO-1 induction, mediated in part through IL-10, has anti-inflammatory effects that extend beyond the induction of previously described regulatory B cell populations in this model.

Colonic Foxp3^{+} cells were not detected in ALF186- or iALF186-treated TCRα$^{-/-}$ mice. Moreover, a significant deficiency of splenic CD4^{+}Foxp3^{+} T cells was observed in TCRα$^{-/-}$ mice compared with WT mice. The TCR was shown to be involved in the development of CD4^{+}Foxp3^{+} T cells (42); however, the influence of TCRα-chain repertoire on the development of CD4^{+} Foxp3^{+} T cells has not been analyzed. TCRα-chain expression is not essential for CD4^{+}CD25^{+} T cell development, but its effect on Foxp3 expression remains

unknown (43). Although the purpose of our study was not to discern T regulatory cell development in TCRα$^{-/-}$ mice, taken as a whole, our results suggested that the predominant source of IL-10 in LPMCs from TCRα$^{-/-}$ mice, and therefore a target for CO and HO-1 induction, resides in the CD11b^{+} population and not Foxp3^{+} T cells.

We unexpectedly detected a Th17-cytokine signature in colonic explants from TCRα$^{-/-}$ mice with abundant levels of IL-17. The IL-17–producing cell population(s) remain(s) to be determined. Given recent reports, it is interesting to speculate that γδ T cells may be a source of IL-17 (44). Interestingly, IL-17 levels decreased following CO exposure or HO-1 induction, correlating with histologic improvement. Recently, the IL-10 family member IL-22, expressed by Th17 cells, was demonstrated to ameliorate colitis in TCRα$^{-/-}$ mice (19). CO-treated TCRα$^{-/-}$ mice demonstrated significant increases in IL-22 mRNA expression in CD11b^{-} LPMCs, consistent with previous studies suggesting that that nonmacrophage-derived IL-22 may also be involved in the protective effects of CO/HO-1. Notably, IL-22 is a potent inducer of IL-10 (19). The description of a Th17 signature in TCRα$^{-/-}$ mice also substantiated this as a model for human UC, in which the same genetic associations within the IL-23/Th17 pathway confer susceptibility to CD and UC (45). Likewise, current biological interventions that inhibit TNF are approved for the treatment of moderate to severe UC (46). CO and pharmacological induction of HO-1 resulted in a significant decrease in TNF secretion in TCRα$^{-/-}$ colons, which may also mediate therapeutic effects.

To our knowledge, these results are the first to characterize anti-inflammatory properties of CO and HO-1 in a Th2-mediated model of chronic colonic inflammation. The anti-inflammatory effects of CO are attributed to the induction of HO-1 and highlight the broad impact of these pathways in intestinal inflammation. HO-1 induction correlated with increased IL-10 and IL-22 expression in vivo, which may be relevant anti-inflammatory mechanisms of this pathway, because both cytokines were previously determined to have a protective role in colonic inflammation in TCRα$^{-/-}$ mice (19, 41). It remains to be determined whether HO-1 induction mediates downstream anti-inflammatory effects in colitis models through increased enzymatic activity and production of endogenous metabolic products, including CO, or through other mechanisms. Nonetheless, these experiments demonstrated that HO-1 is a central regulator of intestinal homeostasis through pleiotropic mechanisms and that understanding these pathways are of mechanistic and therapeutic relevance in human IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

ALF186	CO-releasing molecule-186
BMM	bone marrow-derived macrophage
CD	Crohn's disease
CoPP	cobalt protoporphyrin
CpG	CpG oligodeoxynucleotides
HbCO	carboxyhemoglobin
HO	heme oxygenase
iALF186	inactive form of ALF186
IBD	inflammatory bowel disease
LPMC	lamina propria mononuclear cell
Nrf2	NF-E2–related factor 2
sBLP	synthetic bacterial lipopeptide
SnPP	tin protoporphyrin
UC	ulcerative colitis
WT	wild-type

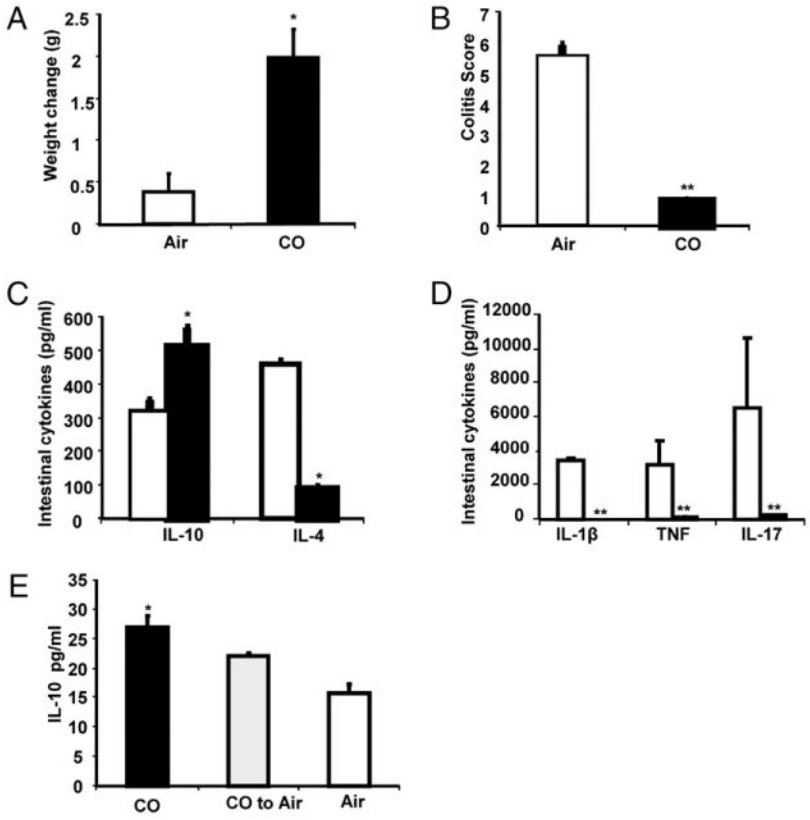

FIGURE 1.

CO ameliorates Th2-mediated colitis in TCRα$^{-/-}$ mice. TCRα$^{-/-}$ mice were housed in ambient air or a chamber maintaining a constant concentration of CO at 250 ppm (n = 10 each) from 12–16 wk of age. *A*, CO-exposed mice gained more weight than did air-exposed mice. *B*, Colitis scores were significantly decreased in CO-exposed mice compared with control mice. Results are presented as the sum total of four averaged scores from five regions of the large intestine graded by a pathologist blinded to the groups using a standard scoring system. Bars represent mean ± SEM of 10 mice/group. *C*, Spontaneous protein secretion determined in 24-h supernatants from colonic explants from CO-exposed (black bars) and air-exposed (white bars) *TCRα*$^{-/-}$ mice. Spontaneous IL-10 and IL-4 were measured by cytokine-specific ELISA. *D*, IL-1β, TNF, and IL-17 secretion were assessed by Linco 16-multiplex cytokine assay. Each result represents the mean ± SEM of triplicate assays. *E*, Ten-week-old *TCRα*$^{-/-}$ mice were divided into three groups: exposed to CO (250 ppm; black bar) for 4 wk, exposed to air for 4 wk (white bar), or exposed to CO for 2 wk and then transferred to ambient air housing conditions for 2 wk (gray bar). Spontaneous IL-10 secretion was measured in full-length colonic cell-free supernatants using cytokine-specific IL-10 ELISA. Results represent mean ± SEM from three experiments (n = 3 mice/group). *$p < 0.05$, **$p < 0.01$ versus air-exposed mice.

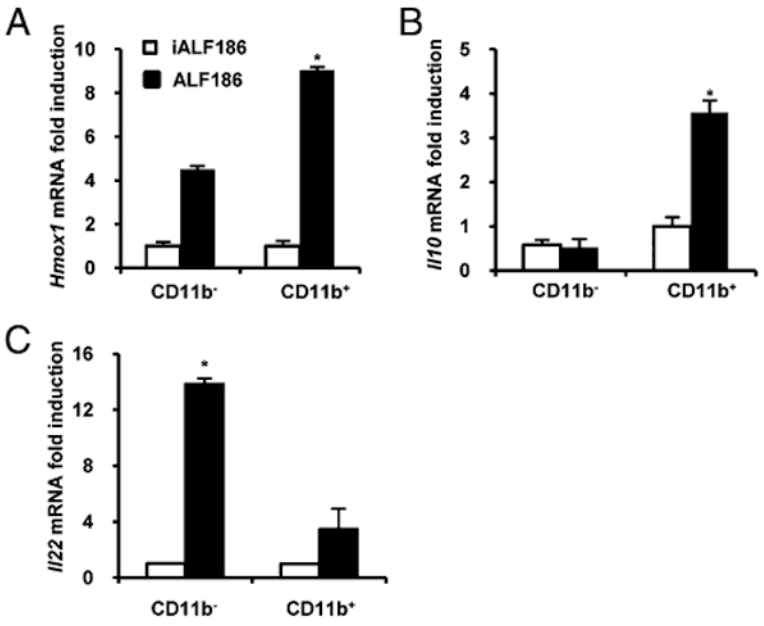

FIGURE 2.

CO upregulates *Hmox1* and *Il10* expression in colonic CD11b$^+$ LPMCs from TCRα$^{-/-}$ mice. LPMCs were isolated from colons of TCRα$^{-/-}$ mice treated with iALF186 (n = 4) or ALF186 (n = 4). LPMCs were further separated into CD11b$^-$ and CD11b$^+$ cells and analyzed for *Hmox1* (*A*), *Il10* (*B*), and *Il22* (*C*) expression by real-time RT-PCR. Results were normalized to β-actin. Bars represent mean ± SEM of triplicate cultures from pooled LPMCs from four mice per group. *$p < 0.05$ versus iALF186-treated CD11b$^+$ LPMCs.

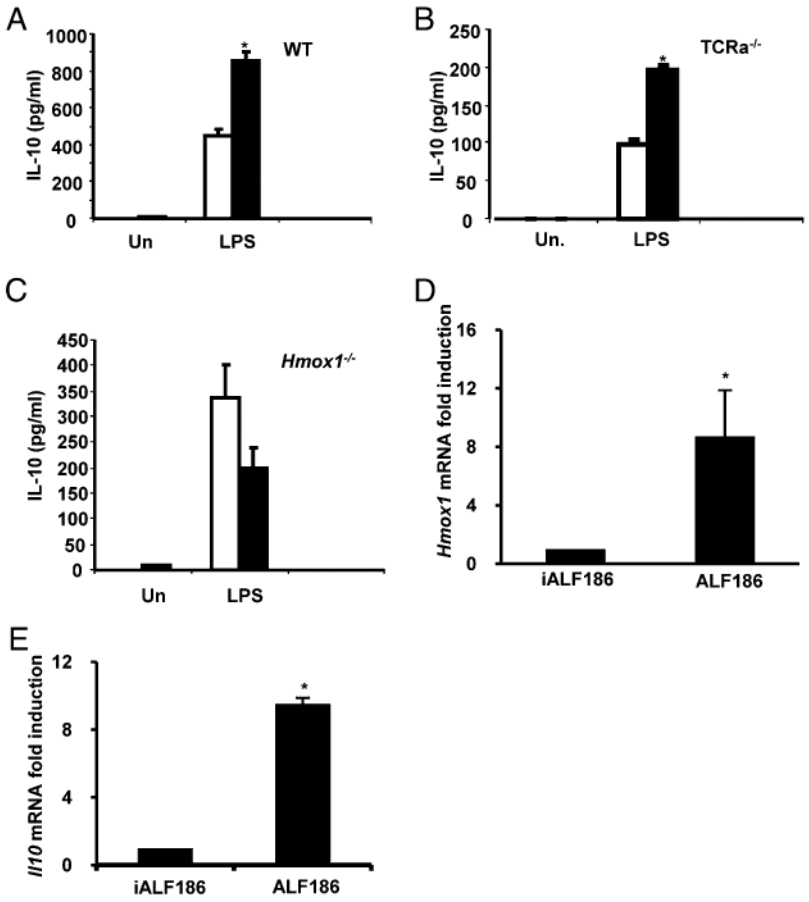

FIGURE 3.

CO induces IL-10 in murine macrophages via the HO-1 pathway. BMMs from WT (*A*), TCRα$^{-/-}$ (*B*), or *Hmox1*$^{-/-}$ (*C*) mice were cultured in CO (250 ppm, black bars) or ambient air (white bars). Following activation with LPS (1 μg/ml), IL-10 protein secretion was assayed in 24-h supernatants by ELISA. WT BMMs were incubated with iALF186 (100 μg/ml) or ALF186 (100 μg/ml) for 3 h, and *Hmox1* (*D*) and *Il10* (*E*) expression was analyzed by real-time RT-PCR. Results were normalized to β-actin and represent the mean ± SEM of triplicate assays from three independent experiments. *$p < 0.05$ versus air-exposed or iALF186-treated BMMs.

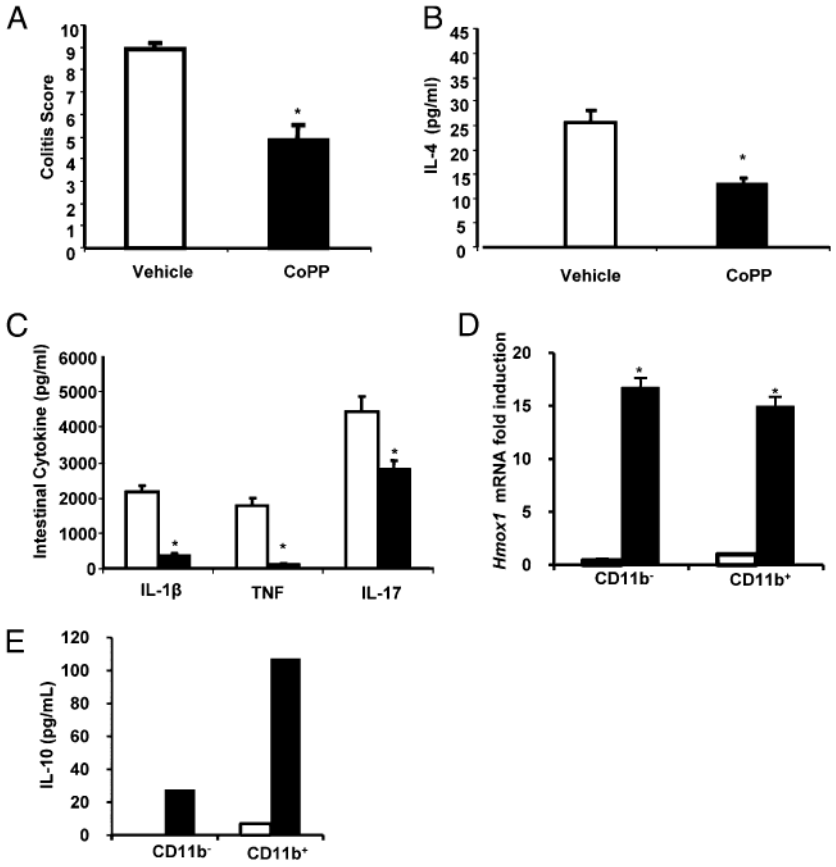

FIGURE 4.

The HO-1 inducer CoPP ameliorates colitis in TCRα$^{-/-}$ mice. Twelve-week-old TCRα$^{-/-}$ mice were treated with i.p. injection of CoPP (5 mg/kg, twice a week for 2 wk) (n = 8), and control mice were treated with DMSO vehicle i.p. (n = 12). *A*, CoPP-injected mice had less severe colitis. *B*, Spontaneous protein secretion determined in 24-h supernatants from colonic explants from CoPP-treated (5 mg/kg) and vehicle-treated (DMSO) *TCRα*$^{-/-}$ mice. *C*, Spontaneous IL-4 was determined by cytokine-specific ELISA, and IL-1β, TNF, and IL-17 secretion were determined by Linco 16-multiplex cytokine assay. Bars represent mean ± SEM from 12 mice per group. *D*, LPMCs were isolated from colons of TCRα$^{-/-}$ mice treated with CoPP (black bars) and vehicle (white bars), separated into CD11b^{-} and CD11b^{+} cells, and analyzed for *Hmox1* expression by real-time RT-PCR, with results normalized to β-actin. *E*, IL-10 secretion was determined by ELISA. Each result represents the mean ± SEM of triplicate assays from four mice/treatment group. *$p < 0.05$ versus vehicle-treated mice.

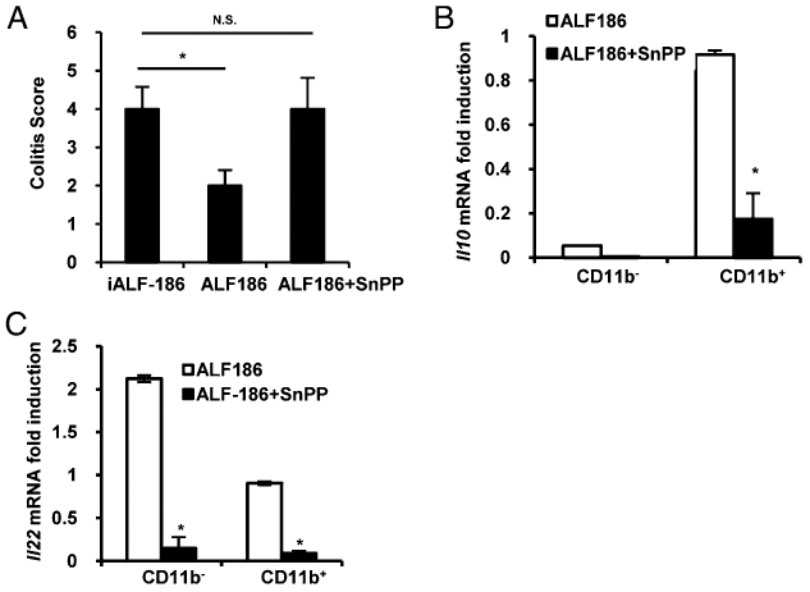

FIGURE 5.

Hmox1 function is required for amelioration of colitis and *Il10* and *Il22* upregulation by CO in TCRα$^{-/-}$ mice. *A*, Colitis scores were significantly higher in iALF-186 ($n = 6$) ALF186^{+} SnPP (50 μM/kg twice/weekly for 2 wk) ($n = 6$) treated mice compared with mice treated with ALF186 alone ($n = 6$). *B* and *C*, LPMCs were isolated from colons of TCRα$^{-/-}$ mice treated with ALF186 ($n = 6$) or ALF186+SnPP ($n = 6$). They were separated into CD11b^{-} and CD11b^{+} cells and analyzed for *Il10* (*B*) and *Il22* (*C*) expression by real-time RT-PCR. Results were normalized to β-actin. Bars represent mean ± SEM triplicate cultures from pooled LPMCs from six mice per group. *$p < 0.05$ versus iALF186-treated TCRα$^{-/-}$ mice and CD11b^{+} LPMCs.

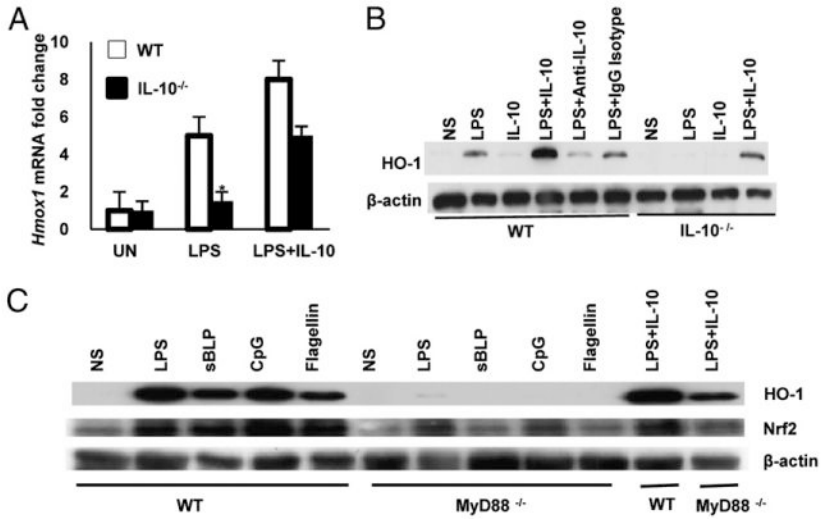

FIGURE 6.

Regulation of HO-1 in macrophages is IL-10 and MyD88 dependent. *A*, WT and IL-10^{-} BMMs were stimulated with LPS alone (100 ng/ml) or with LPS plus IL-10 (10 ng/ml) for 12 h. Total RNA was isolated and analyzed for *Hmox1*, and β-actin mRNA expression was detected by real-time RT-PCR. Results are expressed as mean ± SEM from three independent experiments. *$p < 0.05$ versus LPS-treated WT BMMs. *B*, WT and IL-10$^{-/-}$ BMMs were stimulated with LPS (100 ng/ml) in the presence of IL-10 (10 ng/ml) for 24 h after initial preincubation with anti–IL-10 Ab (10 μg/ml) for 1 h. HO-1 protein was analyzed by Western blotting. Data are representative of five independent experiments with similar results. *C*, WT and MYD88$^{-/-}$ BMMs were stimulated with LPS (100 ng/ml), CpG (1 μM), sBLP (100 ng/ml), flagellin (10 ng/ml), or IL-10 (10 ng/ml) for 24 h. HO-1 and Nrf2 protein was analyzed by Western blotting. Data are representative of three independent experiments.