Heme Oxygenase-1 Ameliorates Dextran Sulfate Sodium-induced Acute Murine Colitis by Regulating Th17/Treg Cell Balance*

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Background: Heme oxygenase-1 (HO-1) is an inducible enzyme with pleiotropic immunomodulatory effects.

Results: Induction of HO-1 inhibits Th17-mediated responses in experimental colitis and switches the naive T cells to Tregs under Th17-skewing conditions in vitro.

Conclusion: HO-1 ameliorates dextran sulfate sodium (DSS)-induced colitis via blocking IL-6/IL-6R signaling to regulate Th17/Treg cell balance.

Significance: HO-1 may become a novel therapeutic target in IBD.

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease, is a group of autoimmune diseases characterized by nonspecific inflammation in the gastrointestinal tract. Recent investigations suggest that activation of Th17 cells and/or deficiency of regulatory T cells (Treg) is involved in the pathogenesis of IBD. Heme oxygenase (HO)-1 is a protein with a wide range of anti-inflammatory and immune regulatory function, which exerts significantly protective roles in various T cell-mediated diseases. In this study, we aim to explore the immunological regulation of HO-1 in the dextran sulfate sodium-induced model of experimental murine colitis. BALB/c mice were administered 4% dextran sulfate sodium orally; some mice were intraperitoneally pretreated with HO-1 inducer hemin or HO-1 inhibitor stannum protoporphyrin IX. The results show that hemin enhances the colonic expression of HO-1 and significantly ameliorates the symptoms of colitis with improved histological changes, accompanied by a decreased proportion of Th17 cells and increased number of Tregs in mesenteric lymph node and spleen. Moreover, induction of HO-1 down-regulates retinoic acid-related orphan receptor γt expression and IL-17A levels, while promoting Treg-related forkhead box p3 (Foxp3) expression and IL-10 levels in colon. Further study in vitro revealed that up-regulated HO-1 switched the naive T cells to Tregs when cultured under a Th17-inducing environment, which involved in IL-6R blockade. Therefore, HO-1 may exhibit anti-inflammatory activity in the murine model of acute experimental colitis via regulating the balance between Th17 and Treg cells, thus providing a possible novel therapeutic target in IBD.

Inflammatory bowel disease (IBD) is a group of autoimmune diseases including two major clinical subtypes, ulcerative colitis (UC) and Crohn disease, characterized by chronic nonspecific inflammation in gastrointestinal tracts (1). Although the pathogenesis of IBD is still unclear, evidence has indicated that IBD is induced by abnormal T cell immune responses triggered by an inflammatory response to intestinal microorganisms in individuals with susceptible genes (2, 3). The abnormal immune response results in excessive activation of effector T cell subsets and/or deficiency of regulatory T cells (Treg), leading to persistent immune disorders and uncontrolled intestinal inflammation (4, 5).

Recent studies suggest that Th17 cells and related cytokines are critical factors in the pathogenesis of UC and Crohn disease, which have been novel targets of therapy in IBD (6–8). Th17 cells, as a recently discovered T cell subset involved in the pathophysiology of inflammatory disease, require specific cytokines and transcription factors for their differentiation, in which IL-6 and TGF-β are recognized as crucial factors (9). Moreover, retinoic acid-related orphan receptor γt (RORγt) is the key transcription factor modulating the Th17 lineage (10, 11). It has been reported that Th17 cells expressing RORγt can be isolated from peripheral blood and colon accompanied by elevated Th17-derived cytokine levels in IBD patients (12–14). Additionally, various investigations indicate that the Th17/Treg cell balance is considered to be critical for host immunity and the preservation of tolerance and that the imbalance in the development and function of Th17 cells and Tregs plays a crucial role in autoimmune diseases, including IBD (4, 15, 16). As one of the defense mechanisms developing in our immune system, Tregs are responsible for maintaining immune homeosta-

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§ The abbreviations used are: IBD, inflammatory bowel disease; HO, heme oxygenase; UC, ulcerative colitis; DSS, dextran sulfate sodium; Th17, T helper cell 17; Treg, regulatory T cell; SnPP, stannum protoporphyrin IX; MLN, mesenteric lymph node; RORγt, retinoic acid-related orphan receptor γt; Foxp3, forkhead box p3; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; IL-6R, interleukin-6 receptor.
Heme oxygenase (HO)-1, as a rate-limiting enzyme for heme metabolism, catalyzes heme into carbon monoxide (CO), free iron, and biliverdin, and exerts a wide range of anti-inflammatory, anti-apoptosis, and immune regulatory effects in the body (26–28). Studies have demonstrated that hemin, a substrate of HO-1, significantly increases the expression of HO-1 protein as well as its activity and ameliorates inflammation in a variety of animal models (29–31). The competitive inhibitor Sn-protoporphyrin IX (SnPP) also induces the expression of HO-1 but blocks the enzymatic activity in both animal models and cultured cells, creating an opposite effect (32–35). Because HO-1 has pleiotropic immunomodulatory effects, there are already numerous illustrations of therapeutic applications of HO-1 in multiple experimental models of IBD mediated by various immune mechanisms (36–38). Additionally, our previous studies have suggested that induction of HO-1 attenuates the airway inflammation in a mouse asthma model through promoting Tregs in eosinophilic airway inflammation (39, 40) and inhibiting Th17 responses in non-eosinophilic airway inflammation (41). However, little is known about how HO-1 exerts immune regulatory effects under different inflammatory conditions. Thus, this study aims to further explore the protective effects of HO-1 and the underlying mechanisms involved in regulating the Th17/Treg cell balance in the dextran sulfate sodium (DSS)-induced murine model of acute experimental colitis.

**EXPERIMENTAL PROCEDURES**

**Mice**—Female BALB/c mice (6–8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and maintained in specific pathogen-free conditions in the Research Center for Experimental Medicine of Ruijin Hospital, affiliated with the Shanghai Jiao Tong University School of Medicine. All animal experiments were approved by and performed in compliance with the guidelines of the Ethics Committee of Ruijin Hospital (Shanghai Jiao Tong University School of Medicine).

**Induction of Colitis and Administration of Hemin or SnPP**—Mice were randomly divided into four groups, including the DSS, DSS+hemin, DSS+SnPP, and control groups (n = 6 in each group). Mice in the DSS, DSS+hemin, and DSS+SnPP groups were fed with 4% (w/v) DSS (MP Biochemicals) in drinking water and normal water in the control group from day 0. Mice were intraperitoneally administered 75 μmol/kg of hemin (Sigma-Aldrich) or 75 μmol/kg of SnPP (Porphyrin Products) on days −2 and −1 in the DSS+hemin and the DSS+SnPP groups, respectively. Hemin or SnPP was dissolved in 0.2 mol/liter NaOH, titrated to pH 7.4 with 0.2 mol/liter HCl, and then diluted with phosphate-buffered saline (PBS). We evaluated the severity of colitis by the daily monitoring of clinical manifestations such as weight loss, diarrhea, and rectal bleeding, and scoring disease activity index as described previously (Table 1) (38). Mice were anesthetized and sacrificed at the end of the experiment (day 7), and the colons, spleens, and mesenteric lymph node (MLN) were harvested for further assays.

**Histopathology**—Colons were fixed with 10% neutral buffered formalin and embedded in paraffin. Four-micrometer-thick sections were stained with H&E. Colonic inflammation was assessed under the light microscope (Olympus AX70) according to the degree of epithelial erosion, ulceration, vascular density, and leukocyte infiltration.

**Western Blot Analysis**—Colons were homogenized with ice-cold radioimmune precipitation assay buffer (Beyotime, Shanghai, China) containing protease inhibitors. The extracts containing 30 μg of proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membrane was blocked with Tris-buffered saline Tween 20 (TBST) buffer containing 5% skim milk and incubated with the following primary antibodies: rabbit anti-mouse RORγt IgG and rabbit anti-mouse Foxp3 IgG (1/1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-mouse HO-1 IgG (1/1000 dilution, Cell Signaling). The samples were incubated overnight followed by addition of horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1/5000 dilution, Cell Signaling). The signals were visualized via enhanced chemiluminescence using a Thermo ECL kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions.

**HO-1 Activity Assay**—HO-1 enzyme activity was assessed by measuring bilirubin production as described previously with modifications (36). The colons were homogenized in 10 mmol/liter HEPES, 32 mmol/liter sucrose, 1 mmol/liter dithiothreitol (DTT), 0.1 mmol/liter EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin and centrifuged at 18,000 × g for 30 min at 4 °C. The supernatant was used to measure HO activity. The reaction mixture, consisting of 200 μl of sample homogenate, 100 μl of normal liver cytosol (source of biliverdin reductase), 20 μmol/liter hemin, and 0.8 mmol/liter NADPH, was incubated at 37 °C for 1 h. The optical density

| TABLE 1  |
|-----------|
| **The DAI scores** |
| Weight loss (%) | Stool consistency | Occult blood | Score |
| 0 | Normal | Negative | 0 |
| 1–5 | Loose | Occult blood-positive | 1 |
| 5–10 | Looser | Diarrhea | 2 |
| 10–20 | >20 | Gross bleeding | 3 |
| >20 | Diarrhea | Gross bleeding | 4 |

* Five grades of weight loss (0, no loss or weight gain; 1, 1–5% loss; 2, 5–10% loss; 3, 10–20% loss; 4, >20% loss).

* Three grades of stool consistency (0, normal; 2, loose; and 4, diarrhea).

* Three grades of occult blood (0, negative; 2, occult blood-positive; and 4, gross bleeding).

**Additional Information**

- **Hematoxylin and Eosin (H&E) Staining**
- **Western Blot Analysis**
- **HO-1 Activity Assay**
- **Histopathology**

For more detailed information and experimental procedures, please refer to the original article.
with TGF-β and anti-cytokines. For Th17 differentiation, cells were stimulated with neutral conditions (Th0) included no exogenous cytokines or sodium Na2EDTA. Cells were washed twice with PBS and incubated in RPMI 1640 medium (HyClone) supplemented with 1% l-glutamine (0.2 mol/liter), 10% FCS, 100 international units/ml penicillin/streptomycin, 1% HEPES (1 mol/liter), 1% sodium hydrogen carbonate (100 mmol/liter), and 0.1% 2-mercaptoethanol (50 mmol/liter, Invitrogen) in the presence of anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego, CA) for 48 h. Cells were collected and analyzed using flow cytometry (FCM). Cytokine production in culture supernatants was determined using enzyme-linked immunosorbent assay (ELISA).

Th17 Cell Differentiation in Vitro—Spleens from 8-week-old female BALB/c mice were removed and minced with a nylon mesh (70-μm pore size). After the cells were pelleted, erythrocytes were lysed with hypotonic buffer (0.15 mol/liter NH4Cl, 10 mmol/liter KHCO3, 0.1 mmol/liter Na2EDTA). Cells were washed twice with PBS and incubated in RPMI 1640 medium (HyClone) supplemented with 1% l-glutamine (0.2 mol/liter), 10% FCS, 100 international units/ml penicillin/streptomycin, 1% HEPES (1 mol/liter), 1% sodium hydrogen carbonate (100 mmol/liter), and 0.1% 2-mercaptoethanol (50 mmol/liter, Invitrogen) in the presence of anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego, CA) for 48 h. Cells were collected and analyzed using flow cytometry (FCM). Cytokine production in culture supernatants was determined using enzyme-linked immunosorbent assay (ELISA).

RESULTS

Colon HO-1 Expression and Activity in DSS-induced Colitis—HO-1 is the inducible one of the three HO isozymes (42). The expression of HO-1 is up-regulated in response to cellular stress and by several factors, such as pro-oxidative stimuli, UV light, LPS, and heavy metals (28). We sought to determine whether DSS challenge could increase HO-1 expression in colons. After mice were sacrificed on day 7, the colons were taken for RNA and protein extraction. qRT-PCR and Western blot assays confirmed that the HO-1 mRNA expression and protein level were enhanced in the DSS group as compared with the control group (Fig. 1, A and B), accompanying the increase of HO-1 enzyme activity (Fig. 1C). Furthermore, the levels of HO-1 mRNA and protein in colon were significantly increased after administration of hemin and SnPP (Fig. 1, A and B). HO-1 activity was enhanced in the DSS+hemin group, but inhibited in the DSS+SnPP group (Fig. 1C).

Induction of HO-1 by Hemin Ameliorates DSS-induced Colitis—The initial body weights did not differ among the four groups. Intake of DSS solution was monitored during the
experiments to maintain the same dose in each group. Mice receiving DSS developed colitis, characterized by sustained weight loss, diarrhea, and rectal bleeding. Disease activity index scores evaluated by these symptoms were lower in the DSS/hemin group than in the DSS group, but were not reduced after treatment with SnPP (Fig. 1D). Histological study confirmed that administration of 4% DSS induced pathological changes in colons, including epithelial crypt loss, ulceration, prominent monocytic infiltration throughout the mucosa, and mucosal bleeding (Fig. 1E). In contrast, mice treated with hemin intraperitoneally for 2 days before DSS challenge showed less inflammatory cell infiltration, smaller erosions and mucosal integrity, thus remarkably improving the histological changes and ameliorating DSS-induced colitis, but SnPP administration did not exert any protective effects (Fig. 1E).

Hemin Treatment Inhibits Th17 Cell-mediated Responses in Vivo—Next, we evaluated the effect of HO-1 on Th17 response in DSS-induced colitis. The MLNs and spleens were isolated to prepare single-cell suspensions. Cells were cultured with anti-CD3 and anti-CD28 antibodies for 48 h. FCM results revealed that the proportions of CD4+ IL-17A+ T cells (Th17 cells) in MLNs and spleens were increased in the DSS and the DSS+SnPP groups in comparison with those in the control group (Fig. 2A). However, the percentage of Th17 cells in the

FIGURE 1. Induction of colonic HO-1 by hemin ameliorates DSS-induced colitis. Colitis was induced in BALB/c mice using DSS as described under “Experimental Procedures.” A, qRT-PCR analysis of HO-1 mRNA in colons isolated from four groups. Data are presented as mean ± S.D. B, Western blot analysis of HO-1 protein in colons extracted from four groups. β-Actin was used as loading control. C, the analysis of HO-1 activity in colons extracted from four groups. Data are presented as mean ± S.D. D, disease activity index scores of each group. Data are presented as mean ± S.D. E, histological analysis of colons isolated from each group. Paraffin-embedded colonic sections were stained with hematoxylin and eosin to observe inflammation (original magnification, × 200). Each symbol in the graph represents an individual mouse (n = 6; *, compared with control group; *, p < 0.05; #, compared with DSS group; #, p < 0.05). All results shown are representative of three independent experiments.
DSS + hemin group was significantly lower than that in the DSS group (Fig. 2A). The levels of IL-17A and IL-6 in the cell culture supernatant, cytokines related to Th17 cells, were analyzed by ELISA. As shown in Fig. 2, B–E, IL-17A and IL-6 production in MLNs and spleens isolated from the DSS group exhibited higher levels than did those from the control group but were lower in mice treated with hemin. There was no significant difference in IL-17A or IL-6 levels between DSS and DSS + SnPP groups (Fig. 2, B–E).

We further determined the expression of RORγt, the key transcription factor that regulates Th17 cell differentiation as well as IL-17A and IL-6 levels in colons. Western blot and qRT-PCR assays showed that the levels of RORγt protein and mRNA expression were significantly up-regulated in the DSS and the DSS + SnPP groups, but declined in the DSS + hemin group (Fig. 3A and B). Meanwhile, the levels of IL-17A and IL-6 protein and mRNA expression in colons were also determined by ELISA and qRT-PCR. Consistent with the results of RORγt protein and mRNA expression, the levels of both IL-17A and IL-6 and mRNA expression were higher in the DSS and the DSS + SnPP groups than those in the control group, but decreased in mice after hemin treatment (Fig. 3, C–F).

**Hemin Treatment Promotes Treg Cell Development in Vivo**—Tregs are considered important for immune homeostasis (15), and the imbalance of Th17 cells and Tregs plays a crucial role in IBD development (4, 16). We have previously reported that induction of HO-1 by hemin suppressed airway inflammation via up-regulating Tregs and IL-10 production (39, 40). Thus, we
also investigated the changes in Tregs in DSS-induced colitis. As shown in Fig. 4A, FCM analysis indicated that DSS challenge slightly increased the percentages of CD4⁺CD25⁺Foxp3⁺ T cells (Tregs) in MLNs and spleens. There was a significant increase in Treg populations in the DSS+hemin group versus the DSS group, whereas inhibition of HO-1 enzymatic activity by SnPP led to a decline in Tregs (Fig. 4A). Foxp3 protein and mRNA levels in colon were further determined by Western blot and qRT-PCR, respectively. The results were in accordance with FCM, showing that the levels of Foxp3 protein and mRNA expression were significantly up-regulated by hemin treatment and were decreased in the presence of SnPP (Fig. 4B and C). In addition, the mRNA expression of anti-inflammatory cytokine IL-10 in colon was also measured by qRT-PCR. The results demonstrated that IL-10 mRNA expression was markedly elevated in the DSS+hemin group but decreased in the DSS+SnPP group as compared with the DSS group (Fig. 4D).

Induction of HO-1 Switched Naive T Cells to Treg Differentiation in Vitro—Because we observed that induction of HO-1 reduced Th17 cell populations but promoted Tregs in vivo, we further investigated the effect of HO-1 on Th17 cell differentiation in vitro. Purified naive CD4⁺ T cells from normal BALB/c mouse spleens were cultured under Th17-skewing conditions with or without hemin or SnPP. IL-17A and Foxp3 expression was determined by intracellular labeling, and the percentages of CD4⁺IL-17A⁺ T cells (Th17 cells) and CD4⁺Foxp3⁺ T cells (Tregs) were determined in CD4⁺ T cells by FCM. As shown in Fig. 5A, there was a significant inhibitory effect of hemin on Th17 cell differentiation at a concentration of 30 nmol/ml. Moreover, the population of CD4⁺Foxp3⁺ T cells was increased, resulting in switching naive T cells to Treg differentiation. However, Th17 cell differentiation was not affected when 30 nmol/ml SnPP was added to the medium, a result quite opposite to the hemin effect (Fig. 5A).
RORγt, IL-17A, and IL-6 mRNA, as well as IL-17A level in supernatants were determined by qRT-PCR and ELISA, respectively. Consistent with the results from FCM analysis, hemin treatment reduced IL-17A mRNA expression and protein level (Fig. 5B and C) and down-regulated RORγt mRNA expression (Fig. 5D). In addition, hemin also inhibited IL-6 mRNA expression (Fig. 5E) but increased IL-10 mRNA expression in cultured cells (Fig. 5F). However, SnPP did not exert the similar effect as hemin.

**Hemin Treatment Down-regulated IL-6R Expression**—IL-6 binds to a cognate membrane-bound receptor (IL-6R) to mediate many biological activities, which is necessary for the expression of multiple transcription factors involved in Th17 differentiation and directly regulates the RORγt and IL-17 expression. We supposed that HO-1 could blockade Th17 differentiation via affecting the expression of IL-6R on CD4+ T cells. Therefore, we detected IL-6R expression on CD4+ T cells and the level of IL-6R mRNA. FCM results showed that the expression of IL-6R on CD4+ T cells in MLNs and spleens was up-regulated in the DSS and the DSS+SnPP groups in comparison with the control group. But hemin treatment significantly down-regulated the expression of IL-6R on CD4+ T cells (Fig. 6A). Furthermore, splenocytes were isolated from BALB/c mice, and CD4+ T cells were activated in the presence of anti-CD3 and anti-CD28 in vitro for 72 h. The results also confirmed that the expression of IL-6R mRNA was significantly down-regulated by hemin at 24 and 48 h (Fig. 6B). We infer from these studies that HO-1 inhibits IL-6R expression and governs IL-6/IL-6R signaling to block Th17 cell differentiation.

**DISCUSSION**

In the present study, we use BALB/c mice to explore the consequences of DSS exposure on systemic and local inflam-
HO-1 Transforms Th17 to Tregs through IL-6/IL-6R Blockade

A

|        | Th0   | Th17  | Th17+Hemin | Th17+SnPP |
|--------|-------|-------|------------|-----------|
| IL-17A| 0.364%| 13.9% | 4.66%      | 15.1%     |
| Foxp3 | 0.00% | 1.87% | 8.45%      | 1.13%     |

B

\[ \text{IL-17A mRNA (Fold increased)} \]

C

\[ \text{IL-17A in supernatants (pg/ml)} \]

D

\[ \text{RORγt mRNA in cultured cells in vitro} \]

E

\[ \text{IL-6 mRNA (Fold increased)} \]

F

\[ \text{IL-10 mRNA (Fold increased)} \]

Figure 5. Induction of HO-1 switched naive T cell differentiation from Th17 to Treg cells in vitro. A, flow cytometric analysis of magnetically purified naive T cells from spleens of BALB/c mice cultured under Th17-skewing conditions with or without hemin or SnPP (30 nmol/ml) for 5 days. Numbers in upper left quadrants indicate the percentages of Th17 (CD4^+ IL-17^+ ) cells gated on CD4^+ T cells. Numbers in lower right quadrants indicate the percentages of Treg (CD4^+ Foxp3^+ ) gated on CD4^+ T cells. B, qRT-PCR analysis of IL-17A mRNA in cells cultured in vitro. Data are presented as mean ± S.D. C, ELISA analysis of IL-17A in supernatants of cultured cells. D, qRT-PCR analysis of RORγt mRNA in cultured cells in vitro. E, qRT-PCR analysis of IL-6 mRNA in cultured cells in vitro. F, qRT-PCR analysis of IL-10 mRNA in cultured cells in vitro. Data are presented as mean ± S.D. (*, compared with Th0 group, *, p < 0.05; #, with Th17 group, #, p < 0.05). All results shown are representative of three independent experiments.

HO-1 is a widely used chemical to induce a murine model of IBD, the clinical and pathological manifestations of which are found to resemble human UC (43–45). Thus, we fed the mice with 4% DSS for 7 days on the basis of pretreatment with or without hemin and SnPP according to our previous study (46) to observe the effect of HO-1 induction on DSS-induced colitis. The results demonstrate that the DSS-induced acute experimental colitis model increased the Th17 cell population and IL-17 production level. Furthermore, induction of HO-1 by hemin significantly ameliorates colitis in mice through inhibiting Th17 responses and down-regulating Th17-related transcription factors and cytokines, while increasing Treg numbers and promoting IL-10 production, to restore the Th17/Treg cell balance. These data suggest a possible role of HO-1 as a novel regulator of Th17/Treg cell balance to benefit the prognosis of colitis and improve the outcome of intestinal inflammation.

Although IBD is a multifactorial disease, involving genetic, immunological and environmental factors, the onset is characterized by an autoimmune inflammation that causes excessive
production of proinflammatory cytokines to damage intestinal mucosa (47). Notably, it has been determined that HO-1 is prominently up-regulated in inflamed colon in the DSS-induced model of experimental colitis (46, 48, 49), corresponding with our observations in this study. As a rate-limiting enzyme of heme metabolism, HO-1 has been considered an attractive target for the prevention and treatment of a variety of diseases (50). It catalyzes heme into CO, free iron, and biliverdin and is inducible by several factors, including pro-oxidative stimuli, UV light, LPS, heat shock, and heavy metals (42). As an anti-inflammatory and immunoregulatory protein, HO-1 has been well studied in several autoimmune disease models involving different T cell subsets (26, 51). Pharmacological induction of HO-1 has a potential therapeutic role in IBD, partly attributable to the beneficial effects of its products such as biliverdin and CO (52, 53). As a newly categorized T cell subset, Th17 cells are capable of producing IL-17A, which increases IL-6 and IL-8 levels, evoking recruitment of neutrophils and T cell proliferation and thus promoting inflammatory responses (54). IL-6, derived from several cell types such as T cells, dendritic cells, or macrophages activates the STAT3 (signal transducer and activator of the transcription 3) signal pathway to induce RORγt expression and determines Th17 cell differentiation (55). Th17 cells and the related cytokines are important factors in the pathogenesis of IBD, as IL-17A, IL-6 and IL-23 have been observed to increase remarkably in UC and Crohn disease patients. This is seen especially in UC, associating positively with disease activity and clinical grading (8). Research using animal models also has shown that IL-17/IL-23 is critical for IBD development (56, 57). Several studies have demonstrated that up-regulation of HO-1 has been found to ameliorate the symptoms of Th17 cell-mediated autoimmune disorders (58–60). In this experiment, our data show that both IL-17A and IL-6 production in spleen and MLN cell culture supernatants and colon were decreased by hemin administration. Furthermore, hemin suppressed RORγt expression and decreased the Th17 cell population. Thus, we suppose that the protective effect of HO-1 in DSS-induced colitis could be due to its inhibition of Th17 cell-mediated responses.

FIGURE 6. Hemin treatment inhibits IL-6R expression on CD4+ T cells. A, flow cytometric analysis of MLN and spleen cells isolated from BALB/c mice cultured in the presence of anti-CD3 and anti-CD28 antibodies for 48 h. Numbers in lower right quadrants indicate the percentages of IL-10+ IL-6R+ cells gated on CD4+ T cells. Each symbol in the graph represents an individual mouse (n = 6). B, qRT-PCR analysis of IL-6R mRNA in cultured cells with or without hemin or SnPP (30 nmol/ml) for 5 days in vitro. Data are presented as mean ± S.D. (*, compared with Th17 group, *, p < 0.05). All results shown are representative of three independent experiments.
HO-1 Transforms Th17 to Tregs through IL-6/IL-6R Blockade

In contrast to Th17 cells, Tregs are indispensable for immune homeostasis. They inhibit autoimmunity and protect against tissue injury, and Foxp3 is regarded as the marker of active Tregs (17, 18). As an important anti-inflammatory cytokine, IL-10 can be secreted by Tregs and be involved in intestinal homeostasis, a defect of which causes spontaneous colitis in mice (61). In addition, the beneficial effect of Tregs is dependent on IL-10 in animal models of colitis, and IL-10^+ Tregs are mainly observed in colonic lamina propria (62, 63). According to previous reports, HO-1 can exert an immune regulatory effect through modulating Tregs and IL-10 (39, 64). It has been proved that Tregs from humans or mice express HO-1 and that the function of Tregs is significantly inhibited when chemicals are added to suppress HO-1 activity in vitro (65, 66). Thus, we consider that the changes in the proportion of Tregs and expression of IL-10 in our current study suggest that HO-1 induction is implicated in promoting Foxp3 expression, enhancing Treg number and secreting IL-10 production to attenuate colitis.

There is a dynamic balance between the development of Th17 and Treg cells, which is driven by the cytokine milieu. TGF-β and IL-6 are required for activating STAT3 and RORγt and initiating Th17 cell differentiation, whereas TGF-β also directs Foxp3 expression and induces the generation of Tregs and IL-10 (22, 55). Furthermore, Treg cell differentiation can be suppressed by IL-6 via inhibition of the expression of Foxp3 in a STAT3-dependent pathway, leading to immune pathology. Therefore, IL-6 is a key modulator of the STAT3–RORγt/Foxp3 signaling pathway which drives Th17 and Treg cell differentiation (67). An in vitro study suggests a regulatory role of HO-1 and its products in controlling T cell differentiation (68). CO produced by HO-1 suppresses T cell proliferation via inhibition of IL-2 production (69); biliverdin, as the ligand of the aryl hydrocarbon receptor, directs the development of CD4^+ T cells toward Tregs or Th17 cells (70). Based on the above observations in vivo, we further explored the regulation of Th17/Treg cell balance by HO-1 through in vitro study, culturing naive T cells from spleens of BALB/c mice under Th17-skewing conditions with or without hemin or SnPP. Surprisingly, hemin interfered with the development of CD4^+ T cells while decreasing in Th17 cells and increasing in Tregs. In particular, TGF-β plus IL-6-induced Th17 cell differentiation is selectively inhibited by HO-1 via blockading IL-6R and switching Th17 cell differentiation toward Treg development. Although further studies are warranted to determine the mechanisms involved in this phenomenon in more detail, HO-1 may have great potential as a novel therapeutic target, offering a promising alternative to our current approaches to management of IBD. Development of new agents that are capable of inducing HO-1 with more specific anti-inflammatory effects and less compromise of host defense might be of therapeutic value for treatment of additional autoimmune disorders.

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