Heme Oxygenase-1 Attenuates Ovalbumin-Induced Airway Inflammation by Up-Regulation of Foxp3 T-Regulatory Cells, Interleukin-10, and Membrane-Bound Transforming Growth Factor- β 1

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Cumulative evidence suggests the up-regulation of interleukin (IL)-10 and T-regulatory (Treg) cells is implicated in anti-inflammatory effect of heme oxygenase-1 (HO-1). Thus, we postulated that induction of HO-1 could augment IL-10 and transforming growth factor (TGF)- β production and foxp3⁺CD4⁺CD25⁺ Treg cell function, thereby leading to attenuation of airway inflammation. In this study, CD4⁺CD25⁺ Treg cells isolated from mouse spleen were either transfected with a HO-1 expression vector (pcDNA3HO-1) or treated with a HO-1 inducer (hemin). Up-regulation of HO-1 enhanced foxp3 expression and IL-10 secretion in the Treg cells *in vitro*. Next, BALB/c, C57/B6.129, and IL-10-deficient B6.129P2-IL10^{tm1Cgn}/J mice were challenged by ovalbumin to induce airway inflammation. Consistent with *in vitro* findings, hemin treatment resulted in induction of HO-1 and foxp3 and production of IL-10 and membrane-bound TGF- β 1 *in vivo*. This was further correlated with decrease of ovalbumin-specific immunoglobulin E level and eosinophil infiltration in bronchial alveolar lavage fluid from the asthmatic mice. Furthermore, hemin significantly enhanced the biological activity of CD4⁺CD25⁺ Treg cells. This protective effect was specifically blocked by Sn-protoporphyrin, a HO-1 enzymatic inhibitor. Finally, hemin failed to up-regulate the function of CD4⁺CD25⁺ Treg cells from IL-10-deficient mice. Our study indicates that HO-1 exerts its protective effect on asthma through a mechanism mediated by foxp3⁺CD4⁺CD25⁺ Treg cells, IL-10, and membrane-

bound TGF- β 1. (Am J Pathol 2007, 171:1904–1914; DOI: 10.2353/ajpath.2007.070096)

Asthma is an airway disease characterized by recurrent inflammation. It occurs in all age groups and is ranked as one of the most prevalent noninfectious diseases. It is estimated that 160 million people worldwide suffer with asthma. Despite 20 years of effort, the morbidity and mortality rates of asthma have been rising all throughout the world. As a result, our society bears significant health and economic burdens for the disease. Therefore, it is imperative to understand fully the pathogenesis of asthma and develop novel preventive/therapeutic strategy.

Heme oxygenase (HO) is a rate-limiting enzyme for heme metabolism. It catalyzes heme into equivalent amounts of biliverdin, carbon monoxide (CO), and free iron. At present, there are three known isoenzymes of HO: inducible HO-1, constitutive HO-2, and a newly found isomer HO-3.^{1–3} Numerous studies have demonstrated that HO-1 is implicated in diverse biological processes such as anti-oxidation, anti-inflammation, anti-apoptosis, and anti-proliferation of smooth muscles.^{4,5} Furthermore, HO-1 has been shown to alleviate asthma and prolong transplant organ survival.^{6,7} These effects are in part mediated by HO-1 catalyzes such as biliverdin, bilirubin, and CO.^{8–10}

It is documented that T-regulatory (Treg) cells regulate the functions of other CD4⁺CD25[−] effector cells. Treg cells play an important role in balancing Th1/Th2/Th17 cell differentiation. Currently, five types of Treg cells have been identified, including Th3, Tr1, Tr, CD4⁺CD25⁺, and

Supported by the National Natural Science Foundation of China (grants 30170988 and 30570798), the Shanghai Municipal Science and Technology Commission Foundation (grant 044119662), and the Shanghai Municipal Education Commission Foundation (grant 03BZ04).

Z.-W.X., L.-Q.X., and W.-W.Z. contributed equally to this study.

Accepted for publication August 21, 2007.

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natural killer (NK) T cells; however, the specific mechanism of their action remains to be fully understood.¹¹ Among them, CD4⁺CD25⁺ cells are a subgroup of Treg cells generated in the thymus, and they comprise 5 ~ 10% of peripheral CD4⁺ T cells. These cells constitutively express α chain of interleukin (IL)-2 receptor and glucocorticoid-induced tumor necrosis factor receptor (GITR). The lack of CD4⁺CD25⁺ cells results in a variety of autoimmune diseases.^{12,13} In addition, CD4⁺CD25⁺ Treg cells can inhibit the infiltration of eosinophils in the airway.¹⁴

Transcription factor foxp3 is instrumental for CD4⁺CD25⁺ Treg cell development and function.^{15,16} It has been shown that transfection of foxp3 cDNA can convert CD4⁺CD25⁻ cells to CD25-positive ones.¹⁷ Conversely, genetic mutation of foxp3 results in extensive autoimmune and inflammatory diseases similar to the pathology displayed in the mice with CD4⁺CD25⁺ cell deficiency.¹⁸

IL-10 and transforming growth factor- β 1 (TGF- β 1) are anti-inflammatory cytokines secreted by a variety of cells including Treg cells. They control inflammation, thereby protecting tissues and organs from inflammatory injury.¹⁹ In this study, we tested whether HO-1 could up-regulate foxp3 expression, enhance CD4⁺CD25⁺ Treg cell function, and facilitate IL-10 or TGF- β 1 production, leading to attenuation of airway inflammation in an asthmatic model.

Materials and Methods

Animal Groups

Six- to eight-week-old female BALB/c, C57/B6.129, and B6.129P2-*Il10^{tm1Cgn}/J* mice were purchased from the Shanghai Laboratory Animal Company (Shanghai, China) and housed in the Experimental Animal Science Center of our institute. All studies were performed under approval of the experimental animal committee. BALB/c or C57/B6.129 mice were randomly divided into five different experimental groups ($n = 30$) including control mice, mice treated with ovalbumin (OVA) (Calbiochem, San Diego, CA), hemin (Sigma-Aldrich, St. Louis, MO), Sn-protoporphyrin (SnPP) (Porphyrin Products, London, UK), and combination of hemin and SnPP. B6.129P2-*Il10^{tm1Cgn}/J* mice were randomly divided into three groups ($n = 6$). They were control and OVA-challenged mice with and without hemin treatment.

OVA Sensitization and Challenge

OVA-induced mouse asthmatic model was established as described previously.^{20,21} Briefly, mice received an intraperitoneal injection of 100 μ g of OVA conjugated with alum (Sigma-Aldrich) in 200 μ l of normal saline on days 0 and 14. Then, the mice were intranasally challenged with 100 μ g of OVA in 50 μ l of normal saline on day 14, and 50 μ g of OVA in 50 μ l of normal saline on days 25, 26, and 27. Control animals received the same volume of vehicle solution intraperitoneally on days 0 and 14 and normal saline intranasally on days 14, 25, 26, and 27. All animals were sacrificed on day 28.

Administration of Hemin or SnPP

Mice were intraperitoneally administered 75 μ mol/kg of hemin and/or 75 μ mol/kg of SnPP on days -2, -1, 12, 13, 23, 24, and 27 of OVA challenge. Hemin and SnPP were dissolved in 0.1 mol/L NaOH and then diluted with phosphate-buffered saline (PBS) to adjust the pH to 7.4.

Isolation of Splenocytes

The spleens were removed by dissection and grounded over a wire mesh screen. Red blood cells were lysed in 0.85% NH₄ in Tris-HCl buffer. Then, the splenocyte suspension was centrifuged at $600 \times g$ for 5 minutes and resuspended in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 50 μ g/ml streptomycin (Hyclone, Logan, UT). These cells were checked for their viability using trypan blue and adjusted to the density of 2×10^6 cells/ml for further experiments.

Isolation of Splenic T Lymphocytes

Splenocyte suspension was adjusted to 4×10^7 cells/ml. The splenocytes in 750 μ l of RPMI 1640 with 10% fetal bovine serum were incubated in a nylon wool column at 37°C under an atmosphere of 5% CO₂ for 1.5 ~ 2 hours. Five ml of prewarmed normal saline were passed through the column to elute T cells. The purity of T-cell population was examined by flow cytometry, and it usually reached more than 90%.

Isolation of CD4⁺CD25⁺ Treg Cells

CD4⁺CD25⁺ Treg cells were isolated from 12×10^7 cells/ml splenocytes from BALB/c or B6.129P2-*Il10^{tm1Cgn}/J* by magnetic bead separation using a CD4⁺CD25⁺ T-cell-negative isolation kit (Miltenyi Biotec, Auburn, CA). Briefly, CD4⁺ cells were purified by negative selection using a LD depletion column, and then CD4⁺CD25⁺ T cells were passed through a MS column. The purity of CD4⁺CD25⁺ lymphocytes was determined by flow cytometry and reached greater than 90%.

Experimental Treatment of Splenic T Lymphocytes

Isolated splenic T cells were treated with hemin at various concentrations or a certain concentration for different time periods. Then, the cells were examined for the expression of HO-1 and foxp3. In addition, isolated splenic CD4⁺CD25⁺ Treg cells were incubated with 50 ng/ml of hemin or 50 ng/ml of SnPP in a 24-well plate at 37°C for up to 96 hours. The cells were harvested at different time points for real-time polymerase chain reaction (PCR) analysis of HO-1 and foxp3.

Plasmid Transfection

pcDNA3HO-1 was constructed as described previously.²² One $\mu\text{g}/\text{ml}$ of pcDNA3HO-1 and pcDNA3 (Invitrogen) were incubated with Lipofectamine 2000 (Invitrogen) diluted in OPTI-MEM I reduced serum medium at room temperature for 30 minutes to form lipofectamine-plasmid complex. The transfection complex was then added to the media containing 1×10^6 CD4⁺CD25⁺ Treg cells. Six hours later, serum was added for additional culture up to 72 hours.

Real-Time PCR

Reverse transcription of RNA was performed using the RNeasy mini kit and the Sensiscript RT kit (Qiagen, Valencia, CA) according to manufacturers' instructions. Real-time PCR was performed in an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green PCR master mix. The primer sequences were the following: β -actin (forward, 5'-CTAAGGCCAACCGT-GAAAAG-3', and reverse, 5'-AGCCTGGATGGCTACGTACAT-3'); HO-1 (forward, 5'-CAGCCCCACCAAGTTCAAAC-3', and reverse, 5'-AGGCGGTCTTAGCCTCTTCTG-3'); foxp3 (forward, 5'-CCCAGGAAAGACAGCAACCTT3', and reverse, 5'-TTCTCCAACCAGGCCACTTG-3'); IL-10 (forward, 5'-GACCAGCTGGACAACATACTGCTAA-3', and reverse, 5'-GATAAGGCTTGGCAACCCAAGTAA-3'); TGF- β 1 (forward, 5'-ATCCTGTCCAAACTAAGGCTCG-3', and reverse, 5'-ACCTCTTTAGCATAGTAGTCCGC-3'). All primers were synthesized by Shanghai Shengon Biotech Company (Shanghai, China). The thermal cycling conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and 50°C for 15 seconds, finally 60°C for 15 seconds, 95°C for 10 minutes.

Western Blot Analysis

Cellular lysates were boiled in 2 $\times$ sodium dodecyl sulfate loading buffer for 5 minutes. Protein concentration was determined using the method of Bradford. The samples containing 50 μg of proteins were separated by a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose filter. The membrane was blocked with Tris-buffered saline Tween-20 (TBST) buffer containing 5% skimmed milk and then incubated with rabbit anti-mouse HO-1 IgG at 1:500 dilution (Calbiochem, Cambridge, MA) or goat anti-mouse foxp3 at 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. This was followed by addition of appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The bands of HO-1 and foxp3 were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Cell Count in Bronchoalveolar Lavage

After mice underwent tracheotomy, the lungs were lavaged using 300 μl of cold sterile saline. Each lung was instilled and washed three times. Bronchial alveolar la-

vage fluid was centrifuged at $400 \times g$ at 4°C for 5 minutes. The numbers of total cells and eosinophils were determined by counting 500 cells stained with Wright-Giemsa solution.

Immunohistochemical Analysis

Right lungs of mice were fixed in 10% formalin and embedded in paraffin. Four- μm tissue sections were mounted on poly-L-lysine-coated microscope slides. After deparaffinization, each specimen was treated with 3% hydrogen peroxide for 5 minutes, and then incubated with rabbit anti-human HO-1 or goat anti-mouse foxp3 antibody (Ab) (Sigma-Aldrich) followed by HRP-goat anti-rabbit and HRP-rabbit anti-goat IgG (DAKO Corp., Carpinteria, CA), respectively, for 1 hour. The antibody reaction was visualized using diaminobenzidine solution (DAKO Corp.). The sections were counterstained with hematoxylin. All images were captured and analyzed by Image-Pro Plus 5.0 (Media Cybernetics, Inc., Silver Spring, MD).

HO-1 Enzyme Activity

HO enzyme activity in the mouse lung was quantified by assessing bilirubin generation. Briefly, the lungs were homogenized on ice in 100 mmol/L phosphate buffer with 2 mmol/L magnesium chloride (MgCl_2) and centrifuged for 15 minutes at $18,800 \times g$. The supernatant was used to measure HO activity. Ten g of fresh Sprague-Dawley rat liver tissue were added into 20 ml of 0.1 mol/L potassium phosphate buffer (pH 7.4), homogenized, and centrifuged at $40,000 \times g$ for 1 hour at 4°C. The middle level aqueous phase containing biliverdin reductase was collected, and the protein concentration was measured using BAC kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Enzyme-catalyzed system included 10 nmol/L hemin, 20 nmol/L β -nicotinamide adenine dinucleotide phosphate hydrogenase (β -NADPH) (Sigma-Aldrich), 1 U/ μl glucose-6-phosphate dehydrogenase (G-6-PD) (Sigma-Aldrich), 1.17 mol/L glucose-6-phosphate (G-6-P) (Sigma-Aldrich), 25 nmol/L MgCl_2 , 100 μl of normal liver cytosol (source of biliverdin reductase), and 200 μl of sample homogenate. The activity of HO-1 was measured by spectrophotometric determination of bilirubin production at OD of 464 nm. One unit of HO-1 enzymatic activity was equivalent to 1 nmol bilirubin production per hour.

Enzyme-Linked Immunosorbent Assay

A 96-well plate was coated with 100 μl of 0.01% OVA overnight and then blocked with 200 μl of 1% bovine serum albumin for 2 hours. After three washes with PBS containing 0.05% Tween-20, the plate was incubated with 100 μl of 1:100 diluted serum from experimental mice at 37°C for 2 hours. Then 100 μl of HRP goat anti-mouse IgE Ab in 1:2000 dilution (Serotec, Raleigh, NC) were added for 2 hours, followed by tetramethylbenzidine (Jinmei Biotech Company, Shenzheng, China) to determine IgE level.

Ninety-six-well plates were also coated with either anti-mouse IL-10 or TGF- β 1 antibody (eBioscience, San Diego, CA) overnight at 4°C. After three washes with PBS, the plates were incubated with 100 μ l of 1:20 diluted serum samples at 4°C for 2 hours. Then biotin-conjugated anti-mouse IL-10 or TGF- β 1 antibody was added accordingly at room temperature for 1 hour, followed by avidin-HRP to determine serum levels of IL-10 and TGF- β 1.

Flow Cytometric Analysis

Five million splenic CD4⁺CD25⁺ T cells were isolated from C57/B6.129 mice using a mouse CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). The cells were stained with a panel of fluorescently conjugated antibodies: anti-CD4-APC, anti-CD25-PE (BD Pharmingen, San Diego, CA), anti-foxp3-FITC (eBioscience), biotinylated anti-TGF- β 1 (R&D Systems, Minneapolis, MN), anti-CD25-FITC, and anti-IL-10-PE (eBioscience). Cell surface and intranuclear stainings were performed as described previously.²³ Appropriate isotype controls were used for each experiment. After the staining, the samples were washed and centrifuged at 1500 $\times$ g for 5 minutes. Then they were fixed in 1% paraformaldehyde and counted in a FACScan (Bio-Rad, Hercules, CA). Analysis was performed with CellQuest software (Bio-Rad). A total of 10,000 to 50,000 events were acquired.

Proliferation Assay

CD4⁺ cells were irradiated with 4000 cGy. One hundred thousand antigen-presenting cells were mixed with 2 $\times$ 10⁴ responder CD4⁺CD25⁺ T cells in the presence or absence of CD4⁺CD25⁺ Treg cells in a 96-well round plate at 37°C for 48 ~ 56 hours. The ratio of CD25⁺ to CD25⁺ cells is 1:1. The cells in each well were stimulated with 10 μ g/ml of concanavalin A (Con A) or OVA. Proliferation was assessed by pulsing the cells with 1 μ Ci/well (0.037 MBq) of ³H-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) for an additional 72 hours. Count per minute (cpm) was measured by a Wallac Betaplate counter (Perkin-Elmer Life Sciences, Wellesley, MA).

Statistical Analysis

Data are presented as the mean $\pm$ SD. Analysis of variance with Fisher's posthoc analysis was used to test for significant differences between comparisons within individual sample sets. *P* < 0.05 was considered to be a statistical significance.

Results

In Vitro Characterization of HO-1 and Foxp3 mRNA Transcription in Splenic T Lymphocytes

To study the temporal relationship between HO-1 and foxp3 expression, T lymphocytes were isolated from

Figure 1. *In vitro* characterization of HO-1 and foxp3 mRNA expression in splenocytes (*n* = 6). **A:** The expression of foxp3 mRNA in the splenocytes stimulated with various doses of hemin. Fifty ng/ml of hemin significantly increased foxp3 mRNA level from its baseline. **B:** The time course of HO-1 mRNA expression in the cells treated with 50 ng/ml of hemin. Hemin treatment significantly induced HO-1 expression in the splenocytes at 24 hours, and the induction reached a maximal level by 48 hours. **C:** The expression of foxp3 mRNA of splenocytes at different time points after stimulation with 50 ng/ml of hemin. Foxp3 transcription attained its peak at 48 hours. These data are representative of three separate experiments.

BALB/c mouse spleen. These cells were treated with 0, 10, 30, 50, 70, and 90 ng/ml of hemin, a HO-1 inducer, for 48 hours. As shown in Figure 1A, 50 ng/ml of hemin significantly increased foxp3 mRNA expression from its baseline. Next, we defined the time course of HO-1 and foxp3 expression by treating the cells with 50 ng/ml of hemin. Real-time PCR was used to determine the transcriptions of HO-1 and foxp3. Hemin treatment significantly induced HO-1 expression in the splenocytes at 24 hours, and the induction reached a maximal level by 48 hours (Figure 1B). Likewise, foxp3 transcription attained its peak at 48 hours and was coincident with the maximal expression of HO-1 (Figure 1C).

Transcription and Protein Production of HO-1 and Foxp3 in Splenic CD4⁺CD25⁺ Treg Cells

In light of the above finding, we went further to examine whether HO-1 specifically regulated foxp3 in

Figure 2. HO-1 and foxp3 mRNA expression and their protein production in splenic CD4⁺CD25⁺ Treg cells *in vitro* ($n = 4$). **A:** HO-1 expression was enhanced by pcDNA3HO-1 transfection and hemin treatment in CD4⁺CD25⁺ Treg cells ($^{*}P < 0.01$). The up-regulation of HO-1 was induced by SnPP ($^{*}P < 0.05$). **B:** pcDNA3HO-1 transfection and hemin treatment augmented foxp3 transcription ($^{*}P < 0.05$), and the up-regulation of foxp3 by hemin was specifically attenuated by SnPP. **C:** Both pcDNA3HO-1 and hemin markedly increased HO-1 and foxp3 production. SnPP inhibited hemin-induced foxp3 production. These data are representative of three separate experiments.

CD4⁺CD25⁺ Treg cells. CD4⁺CD25⁺ Treg cells isolated from BALB/c mouse spleen were transfected with 1 μ g/ml of pcDNA3 control vector or a HO-1 expressing vector, pcDNA3HO-1. In a separate experiment, the Treg cells were treated with 50 ng/ml of hemin, 50 ng/ml of SnPP, and the combination of hemin with SnPP, respectively, for 48 hours. SnPP has been shown to up-regulate HO-1 expression.²⁴ However, it is a potent inhibitor by suppressing the enzymatic activity of HO-1.²⁵ As demonstrated in Figure 2, HO-1 expression was enhanced by pcDNA3HO-1 transfection or hemin treatment in CD4⁺CD25⁺ Treg cells (Figure 2A). The induction of HO-1 also augmented foxp3 transcription. The up-regulation of foxp3 by hemin was specifically attenuated by SnPP (Figure 2B).

We then correlated HO-1 and foxp3 transcription with their protein production. Control CD4⁺CD25⁺ Treg cells and the Treg cells treated with pcDNA3, pcDNA3HO-1, hemin, or SnPP were collected for Western blot analysis. The baseline production of HO-1 protein in the control and pcDNA3 group was low. Both pcDNA3HO-1 and hemin markedly increased HO-1 production. Furthermore, the increase of HO-1 was associated with enhancement of foxp3 protein level. Conversely, SnPP inhibited hemin-induced foxp3 production (Figure 2C).

Production of IL-10 and TGF- β 1 in Splenic CD4⁺CD25⁺ Treg Cells

The above experiments showed that HO-1 up-regulated foxp3 in CD4⁺CD25⁺ lymphocytes; thus it would be interesting to see whether HO-1 also enhanced the biological function of Treg cells such as secretion of effector cytokines. After 48 hours of pcDNA3HO-1 transfection or hemin treatment, culture supernatants of CD4⁺CD25⁺

Figure 3. Production of IL-10 and TGF- β 1 in splenic CD4⁺CD25⁺ Treg cells treated with hemin *in vitro* and the serum level of IL-10 in mice treated with hemin ($n = 6$). **A:** pcDNA3HO-1 and hemin significantly increased IL-10 secretion by Treg cells ($^{*}P < 0.05$), and hemin-enhanced IL-10 production was inhibited by SnPP. **B:** pcDNA3HO-1 and hemin did not alter secreted TGF- β 1 production. **C:** Hemin significantly augmented the serum IL-10 level in the mice challenged with OVA ($^{*}P < 0.05$). Hemin-mediated IL-10 production was inhibited by SnPP. These data are representative of three separate experiments.

Treg cells were collected to measure the concentrations of IL-10 and TGF- β 1. As shown in Figure 3A, up-regulation of HO-1 by pcDNA3HO-1 and hemin significantly increased IL-10 secretion in Treg cells, and hemin-enhanced IL-10 production was inhibited by SnPP. However, there was no significant difference of TGF- β 1 secretion among the experimental groups (Figure 3B).

Detection of Serum OVA-Specific IgE

We then validated the above results in an *in vivo* setting to determine whether induction of HO-1 could alleviate immune response in OVA-mediated asthmatic model. BALB/c and C57/B6.129 mice challenged with OVA were treated with hemin, SnPP, and hemin combined with SnPP, respectively. The serum was collected to measure

Figure 4. Serum level of OVA-specific IgE in the mice treated with hemin ($n = 6$). **A:** OVA challenge significantly augmented OVA-specific IgE production in C57/B6.129 mice ($^*P < 0.01$). However, hemin treatment attenuated the IgE. The suppression of hemin was specifically blocked by SnPP ($^*P < 0.05$). **B:** OVA challenge significantly increased the serum level of OVA-specific IgE in the B6.129P2-IL10^{tm1Cgn}/J mice ($^*P < 0.01$). In contrast to C57/B6.129 mice, hemin did not significantly suppress the IgE production ($^*P < 0.05$), although the level of OVA-specific IgE in OVA plus hemin group was lower than that in OVA alone group. These data are representative of three separate experiments.

the level of OVA-specific IgE. OVA challenge significantly increased the IgE level in C57/B6.129 mice. However, OVA-specific IgE level was suppressed in the mice receiving hemin. This hemin-mediated inhibition was reversed by SnPP (Figure 4A).

We also examined the effect of hemin in B6.129P2-IL10^{tm1Cgn}/J mice with IL-10 deficiency. Compared to control group, the serum level of OVA-specific IgE was significantly higher in the B6.129P2-IL10^{tm1Cgn}/J mice challenged with OVA. In contrast to C57/B6.129 mice, hemin did not significantly suppress IgE production in B6.129P2-IL10^{tm1Cgn}/J mice, although the level of OVA-specific IgE was lower in OVA plus hemin group than in OVA alone group (Figure 4B).

Cellular Infiltration in the Airway

To assess further the protective effect of HO-1 on airway inflammation in BALB/c and C57/B6.129 mice, the numbers of total cells and eosinophils in bronchial alveolar lavage fluid were measured in the OVA-challenged group treated with and without the HO-1 inducer. OVA sensitization resulted in a marked increase of total cell and eosinophil numbers in bronchial alveolar lavage fluid. Hemin significantly attenuated the cell influx. However, this inhibition was specifically reversed by SnPP (Figure 5, A and B).

Figure 5. Cellular infiltration in bronchial alveolar lavage fluid from the C57/B6.129 mice treated with hemin ($n = 6$). **A:** OVA challenge significantly induced total cell infiltration in the lung ($^*P < 0.001$). This was alleviated by hemin compared to OVA and SnPP treatment ($^+P < 0.01$), whereas SnPP inhibited the effect of hemin. **B:** OVA challenge significantly induced eosinophil infiltration in the lung ($^*P < 0.001$). This was alleviated by hemin compared to OVA and SnPP treatment ($^+P < 0.01$), conversely SnPP inhibited the effect of hemin. These data are representative of three separate experiments.

Serum Level of IL-10 and TGF- β 1

To evaluate whether HO-1 altered the function of Treg cells, we examined the serum levels of IL-10 and TGF- β 1 in BALB/c mice. The IL-10 level was significantly higher in the mice treated with hemin than that in the control and other experimental groups. SnPP inhibited the effect of hemin on IL-10 production (Figure 3C). In addition, serum level of TGF- β 1 was not affected by hemin in both BALB/c and B6.129P2-IL10^{tm1Cgn}/J mice (data not shown).

Expression and Production of HO-1, Foxp3, IL-10, and Membrane-Bound TGF- β 1 in Splenic CD4⁺CD25⁺ Treg Cells

After OVA challenge, systemic CD4⁺CD25⁺ Treg cells were isolated from the spleens of BALB/c mice treated with and without HO-1 agonist. Real-time PCR was performed to determine HO-1, foxp3, IL-10, and TGF- β 1 expression. Among all experimental groups, hemin significantly up-regulated HO-1 transcription. Pretreatment

Figure 6. Expression of HO-1, foxp3, IL-10, and membrane-bound TGF- β 1 in splenic CD4⁺CD25⁺ Treg cells ($n = 6$). **A:** Hemin significantly up-regulated HO-1 transcription ($*P < 0.01$). Pretreatment of OVA, SnPP, or hemin plus SnPP also increased the expression of HO-1 mRNA compared to control group ($*P < 0.05$). **B:** Hemin treatment significantly enhanced foxp3 expression ($*P < 0.05$). **C:** The expression of IL-10 was enhanced after hemin treatment ($*P < 0.05$). **D:** Hemin resulted in more TGF- β 1 transcription compared to other groups ($*P < 0.05$).

with OVA, SnPP, or hemin plus SnPP also increased the expression of HO-1 mRNA in comparison with control group mice (Figure 6A). Similar to the *in vitro* study, the induction of HO-1 by hemin was coincident with the increase of foxp3 expression, and the up-regulation of foxp3 by hemin was specifically attenuated by SnPP (Figure 6B). Moreover, the expression of IL-10 was enhanced after hemin treatment (Figure 6C). TGF- β 1 transcription was also increased in hemin-treated mice as compared to other groups (Figure 6D).

Next, the production of foxp3, IL-10, and membrane-bound TGF- β 1 in splenic CD4⁺CD25⁺ Treg cells of C57/B6.129 mice was examined by flow cytometry. As summarized in Table 1, the percentage of foxp3, IL-10, and membrane-bound TGF- β 1 of CD4⁺CD25⁺ Treg cells were suppressed in OVA-treated groups compared to control mice. However, pretreatment of hemin increased

the population of the cells expressing foxp3, IL-10, and membrane-bound TGF- β 1 compared to the mice treated with OVA alone. Furthermore, hemin-induced expansion of active Treg lymphocytes was specifically inhibited by SnPP. These data suggest that HO-1 up-regulates foxp3 and anti-inflammatory cytokines in the cells that previously did not express them.

Expression and Production of HO-1 and Foxp3 in Lung Tissues

We examined whether hemin induced HO-1 and foxp3 in inflamed local tissues. Lung samples from each experimental group were collected for real-time PCR and immunohistochemistry analysis. OVA challenge alone did affect HO-1 level in the airway. However, hemin significantly enhanced the local expression of HO-1 (Figure 7A). Likewise, foxp3 expression in the lung was augmented by the HO-1 inducer (Figure 7B). In addition, HO-1 production in the lung was elevated after OVA, SnPP, and hemin treatment (Figure 7A). Repeated administration of hemin not only increased lung HO-1 expression but also enhanced its enzymatic activity. The HO-1 activity in hemin group was 2.5 times higher than that in control group. Although pretreatment with SnPP or hemin plus SnPP also increased HO-1 protein, HO-1 activity was suppressed in the SnPP groups compared to that in control mice. The results are in agreement with our previous report.²⁶ Lastly, immunohistochemistry demonstrated that the production of foxp3 was only increased in the hemin group (Figure 7B). As shown in Figure 7, C and D, hemin also induced HO-1 expression in the lungs of B6.129P2-*Il10*^{tm1Cgn}/J mice (Figure 7C). However, foxp3 transcription was not significantly altered after hemin treatment (Figure 7D). This data indicates that IL-10 is implicated in HO-1-mediated up-regulation of foxp3.

Analysis of CD4⁺CD25⁺ Treg Cell Suppressive Function

Finally, we studied the direct suppressive effect of Treg cells after HO-1 induction. CD4⁺CD25⁺ Treg cells were harvested from the spleens of OVA-challenged mice with or without HO-1 induction. Treg cells were cultured together with CD4⁺CD25⁻ T-effector cells and antigen-presenting cells. The mixed cell population was then

Table 1. Expansion of Active Treg Cells by HO-1

Groups	Foxp3		IL-10		TGF- β	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Control	4.27	±1.48	37.09	±1.52	64.67	±14.97
OVA	2.65*	±0.61	19.42	±12.28	19.70 [‡]	±6.80
OVA + hemin	6.21	±1.75	46.47 [†]	±9.94	52.36	±16.25
OVA + SnPP	1.94*	±0.87	24.61	±6.74	29.57 [‡]	±18.15
OVA + hemin + SnPP	3.02*	±0.97	28.54	±1.24	38.15	±11.78

The expression of foxp3, intracellular IL-10, and membrane-bound TGF- β was analyzed by flow cytometry.

* $P < 0.01$, compared with control and OVA + hemin groups.

[†] $P < 0.05$, compared with OVA and OVA + SnPP groups.

[‡] $P < 0.05$, compared with control group.

Figure 7. Expression of HO-1 and foxp3 in lung tissues ($n = 6$). **A:** OVA, hemin, and SnPP significantly increased the expression of HO-1 mRNA in the lungs of the BALB/c mice ($*P < 0.05$). HO-1 production in the lung tissue was higher after SnPP and hemin treatment. **B:** Hemin significantly up-regulated foxp3 transcription and protein expression ($*P < 0.05$). **C:** Hemin significantly induced HO-1 expression in B6.129P2-Il10^{tm1Cgn}/J mice ($*P < 0.01$). **D:** Hemin did not alter foxp3 mRNA level in B6.129P2-Il10^{tm1Cgn}/J mice. These data are representative of three separate experiments.

stimulated with polyclonal antigen concanavalin A (Con A) or OVA. As shown in Figure 8, hemin did not alter maximal T-cell proliferation in the absence of CD4⁺CD25⁺ Treg cells. Addition of Treg lymphocytes suppressed T-cell proliferation in all experimental groups. However in the presence of Treg cells, the proliferation of effector T cells was higher in the group treated with OVA alone (Figure 8A). Hemin enhanced the regulatory activity of CD4⁺CD25⁺ Treg cells in the mice sensitized with OVA as evidenced by the reduction of cell proliferation. This hemin-mediated suppression was averted by SnPP (Figure 8A). In contrast, hemin did not alter T-cell proliferation in B6.129P2-Il10^{tm1Cgn}/J mice (Figure 8B). This suggests that IL-10 is involved in HO-1-mediated CD4⁺CD25⁺ Treg cell activity.

Discussion

HO-1 is a rate-limiting enzyme of heme metabolism. Recent studies show that HO-1 is a stress-induced protein. It is protective for tissue injury through multiple mechanisms including anti-oxidation, anti-inflammation, and anti-apoptosis. HO-1 catalyzes heme to generate biliverdin, bilirubin, and CO. These enzymatic products are in part responsible for HO-1 action.²⁷ The anti-inflammatory effect of HO-1 is

well studied in respiratory diseases. In several airway inflammatory models, HO-1 is found to reduce local inflammatory cell infiltration,²⁸ suppress inflammatory cytokines such as tumor necrosis factor- α , IL-1 β , and MIP-1 β , and augment anti-inflammatory cytokine IL-10. In addition, HO-1 can stabilize mast cells, lessen IgE production, and inhibit adhesion molecules.²⁹ Therefore, HO-1 plays an important role in preventing airway inflammation.

Using both *in vitro* and *in vivo* models, our study has revealed a novel anti-inflammatory mechanism of HO-1. In addition to its effect on nonspecific immune response, HO-1 is implicated in regulating adaptive immune system. It up-regulates foxp3 expression, activates CD4⁺CD25⁺ Treg cells and augments anti-inflammatory cytokine production, thereby alleviating airway inflammation. Moreover, the anti-inflammatory action of HO-1 is associated with its enzymatic activity.

Hemin is a substrate and inducer of HO-1, whereas SnPP is an inhibitor of HO-1. These two compounds have been widely used to study HO-1 function. Our previous study showed that the animals sensitized by OVA had a higher level of serum OVA-specific IgE, increased airway eosinophilia and airway hyperreactivity as compared to control mice. Thus, this animal model displayed several features of asthma. HO-1 markedly suppressed airway inflammation, including a decreased eosinophil infiltra-

Figure 8. Analysis of CD4⁺CD25⁺ Treg cell-suppressive function stimulated with Con A and OVA ($n = 4$). **A:** The mixed cell population was stimulated with Con A. OVA challenge increased the proliferation effect of T cells ($*P < 0.01$). However, Treg cells from hemin-treated mice significantly suppressed the effect of T-cell proliferation. This hemin-mediated suppression was averted by SnPP ($*P < 0.01$). **B:** The mixed cell population was stimulated with OVA. Treg cells from hemin-treated mice also suppressed the effect of T-cell proliferation ($*P < 0.05$). **C:** Hemin-treated Treg cells from B6.129P2-*It10^{m1Cgn}/J* mice were unable to suppress effector T-cell proliferation ($P > 0.05$). These data are representative of three separate experiments.

tion around the airway and attenuated airway hyperreactivity.²⁶ According to our previous study, we chose optimal doses of hemin and SnPP in this study. In addition, we used both C57/B6.129 and BALB/c mice to validate the result in different rodent strains.²⁶ Here, we have showed that hemin effectively induced HO-1 expression *in vitro* and *in vivo*. The induction of HO-1 coincided with foxp3 expression. Moreover, hemin treatment significantly attenuated OVA-mediated airway inflammation and IgE production in C57/B6.129 mice. The action of hemin is HO-1-specific because its anti-inflammatory effect was prevented by SnPP. These data suggest that the anti-inflammatory activity is directly related to HO-1. These results are in agreement with our previous finding and other reports.^{8,26,30}

In this study, we found that the HO-1 protein level was also up-regulated by SnPP administration; however, HO-1 activity was significantly inhibited. This finding is consistent with the work of Sardana and Kappas.²⁴ Furthermore, HO-1 effectively induced foxp3 expression. This finding indicates that the anti-inflammatory effect of HO-1 is correlated with its enzymatic activity.

It is well documented that Treg cells are essential for regulating effector immune cells, balancing Th1/Th2/

Th17 response. They play a pivotal role in controlling autoimmune diseases and maintaining immune tolerance.³¹ CD4⁺CD25⁺ cells are a well-characterized Treg population.¹¹ These CD4⁺ lymphocytes express a high level of CD25 on cell surface and possess potent immunosuppressive functions. Transcription factor foxp3 is not only highly expressed in Treg cells but also correlates with their activation.^{15,17,19} Thus, foxp3 is widely regarded as a marker of active Treg cells. CD4⁺CD25⁺ effector T lymphocytes seldom express foxp3.^{32,33} Therefore, we studied foxp3 expression to reflect the function of Treg cells. CD4⁺CD25⁺ Treg cells exert their regulatory effect on effector immune cells by both direct contact and indirect suppression. The direct contact requires the interaction of co-stimulatory molecules such as CTLA-4 between cells.^{34–36} The indirect suppression is mediated by anti-inflammatory cytokines including IL-10 and TGF- β , another well-known anti-inflammatory cytokine.³⁷ A recent study demonstrated that periphery CD4⁺CD25⁺ Treg cells are lower in asthmatic patients than that in control individuals.³⁸

In this study, we have found that hemin directly up-regulated HO-1 in CD4⁺CD25⁺ Treg cells in a cell culture system. Consequently, foxp3 expression and Treg activity were enhanced. However, it is unclear how the induction of HO-1 directly results in the up-regulation of foxp3. Some studies have shown interplay between HO-1, TGF- β , and IL-10. The overexpression of HO-1 can increase the secretion of IL-10. IL-10 and TGF- β also promote the conversion of CD4⁺CD25⁺ Treg cells to CD4⁺CD25⁺ Treg cells.^{39,40} It is well known that IL-10 is a key effector cytokine of CD4⁺CD25⁺ Treg cells.^{41,42} Therefore, we speculate that HO-1 induces IL-10, which further promotes foxp3 expression leading to activation of CD4⁺CD25⁺ Treg cells. To support this notion, we found that hemin failed to enhance foxp3 expression in IL-10-deficient mice although it up-regulated HO-1. The result suggests that HO-1 induces foxp3 expression, and activates CD4⁺CD25⁺ Treg cells via an IL-10-dependent mechanism during its anti-inflammatory action.

IL-10 is an anti-inflammatory cytokine produced by Treg cells. It controls inflammation and protects tissues and organs from inflammatory injury. Takamiya and co-workers⁴³ reported that HO-1 enhances the production of IL-10 and inhibits the release of Th2 cytokines, thereby suppressing IgE and eosinophilia. Th2-dominant airway hypersensitivity is further exaggerated by anti-IL-10 monoclonal antibody.⁴⁴ Several groups have reported that lack of HO-1 augments T-cell activation, leading to a proinflammatory state.^{45,46} Inoue and his colleagues show that transgenic expression of HO-1 cDNA increases the production of IL-10.⁴⁷ These animals are more resistant to endotoxin-pulmonary injury. In addition, it is reported that the secretion of IL-10 is significantly increased after induction of HO-1.⁴⁸ Moreover, inhibition of HO-1 markedly reduces the level of TGF- β .⁴⁹ These data suggest that induction of anti-inflammatory cytokines from Treg cells is a part of the mechanism by which HO-1 exerts its immunoregulation.

It is increasingly clear that TGF- β plays a pivotal role in regulation of T-cell subset differentiation. Recent studies

show that TGF- β in conjunction with IL-6 promotes the development of pathological Th17 cells, whereas TGF- β alone induces the development of foxp3 T cells with regulatory function.^{50–52} Ray and colleagues²³ demonstrated that foxp3 Treg cells can induce immune tolerance through a mechanism of direct cell-cell contact and that membrane-bound TGF- β is essential for this type of regulation. However, several animal and human studies have been unable to confirm the role of TGF- β in the regulation of foxp3 expression in CD4⁺CD25⁺ Treg cell. For this reason, we measured IL-10 and TGF- β 1 mRNA expression and protein production *in vivo* and *in vitro* after HO-1 induction. Interestingly, up-regulation of HO-1 by hemin or pcDNA3HO-1 specifically stimulated IL-10 but not TGF- β 1 secretion in this particular experimental setting. However, the transcription of IL-10 and TGF- β 1 was increased in the CD4⁺CD25⁺ Treg cell of BALB/c mice. Likewise, the transcription of IL-10 and TGF- β 1 was enhanced in C57/B6.129 mice. Although secreted TGF- β 1 was not altered in culture supernatants and sera, membrane-bound TGF- β 1 in CD4⁺CD25⁺ Treg cell was elevated after HO-1 induction. A recent study has found that overexpression of membrane-bound TGF- β 1 in foxp3⁺ Treg cells mitigates allergic airway inflammation through a Notch1-mediated mechanism.⁵³ It suggests that HO-1 induces the expression of membrane-bound TGF- β 1 as an important regulatory mechanism of augmenting CD4⁺CD25⁺ Treg cell function. Thus, our data indicate that IL-10 and membrane-bound TGF- β 1 play an important role in HO-1-mediated immunoprotection. Our previous study characterized the expression profile of Th1 cytokine (IFN- γ) and Th2 cytokine (IL-4) in an OVA-induced airway hypersensitivity model. OVA challenge suppressed IFN- γ and increased IL-4. The alteration of Th1/Th2 response was well correlated with the increase of airway resistance. Moreover, hemin significantly reduces IL-4 and airway resistance in the lung and increases serum IL-10 level.²⁶ Thus, it is feasible to postulate that up-regulation of IL-10 by HO-1 is involved in balancing Th1/Th2 reaction as evidenced by the protective effect of hemin. To further define the role of IL-10 in HO-1-mediated immunoregulation, we studied the protective effect of hemin in IL-10-deficient animals. Compared to wild-type mice with the same genetic background, hemin was unable to lessen the IgE production and airway inflammation elicited by OVA in B6.129P2-*Il10^{tm1Cgn}*/J mice. This is further supported by the fact that IL-10-deficient Treg cells failed to suppress T-cell proliferation. Meanwhile, we consider that IL-10 may have multiple roles in HO-1-mediated anti-inflammatory process, including up-regulation of foxp3 and direct suppression of inflammatory responses. Here we have showed that induction of HO-1 by hemin mitigated airway inflammation in OVA-induced asthmatic model. The immunoregulatory effect of HO-1 is in part mediated by IL-10.

In summary, our study has demonstrated that HO-1 can directly up-regulate foxp3 in CD4⁺CD25⁺ Treg cells. The activation of Treg cells contributes to the anti-inflammatory effect of HO-1, and this immunoregulation is also mediated by IL-10 and membrane-bound TGF- β 1.

Acknowledgment

We thank Sheri M. Skinner (Assistant Professor, Baylor College of Medicine, Houston, TX) for critical review of the manuscript.

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