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Human Mesenchymal Stem Cells Elevate $CD4^+CD25^+CD127^{low/-}$ Regulatory T Cells of Asthmatic Patients via Heme Oxygenase-1

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ABSTRACT

Up-regulation of $CD4^+CD25^+CD127^{low/-}$ regulatory T cells (Tregs) is a new target in the treatment of asthma. Human bone marrow mesenchymal stem cells can up-regulate $CD4^+CD25^+CD127^{low/-}$ regulatory T cells *in vitro*, meanwhile, heme oxygenase-1 (HO-1) plays an important role in the development and maintenance of $CD4^+CD25^+$ regulatory T cells. However the mechanism has not yet been adequately understood. Hence, we wondered what effect of Heme Oxygenase-1 made on regulation of $CD4^+CD25^+CD127^{low/-}$ regulatory T cells mediated by mesenchymal stem cells.

Peripheral blood mononuclear cells isolated from asthmatic patients and healthy controls were co-cultured with human bone marrow mesenchymal stem cells which were pretreated with Hemin (the revulsive of Heme Oxygenase-1), Protoporphyrin IX zinc (the inhibitor of Heme Oxygenase-1) and saline.

The expression of Heme Oxygenase-1 in MSCs was enhanced by Hemin and inhibited by Protoporphyrin zinc *in vitro*. Overexpression of Heme Oxygenase-1 elevated the proportion of $CD4^+CD25^+CD127^{low/-}$ regulatory T cells in $CD4^+$ T cells, meanwhile, inhibition of Heme Oxygenase-1 decreased the proportion of $CD4^+CD25^+CD127^{low/-}$ regulatory T cells in $CD4^+$ T cells as compared with mesenchymal stem cells alone.

Taken together, these data demonstrated that Heme Oxygenase-1 contributed to the up-regulation of $CD4^+CD25^+CD127^{low/-}$ regulatory T cells mediated by mesenchymal stem cells in asthma.

Keywords: Asthma; Heme oxygenase-1; Mesenchymal stromal cells; T-Lymphocytes, Regulatory

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INTRODUCTION

Allergic asthma is a disease characterized by chronic inflammation of the airways, airway hyperresponsiveness, airway wall remodeling and respiratory dysfunction. Classically, imbalance of Th1, Th2, Th17 and regulatory T cells has been considered integral to the pathogenesis of asthma.[1] $CD4^+CD25^+$ regulatory T cells (Tregs) are a distinct population of $CD4^+$ T cells that possess a variety of immunomodulatory effects in allergic diseases.[2,3] $CD4^+CD25^+$ Tregs in asthma play a crucial role in the suppression of the immune responses induced by Th2 cells.[2,4-6] Studies have shown that the regulatory T cells, which possess the ability of immunosuppressive activities, express high levels of CD4, CD25 and are low or negative for CD127.[7,8] Thus, up-regulating $CD4^+CD25^+CD127^{low/-}$ Tregs is a new target in the treatment of asthma.

Bone marrow mesenchymal stem cells (MSCs), which are multipotent cells, can be induced to differentiate under appropriate conditions into several lineages, such as bone, cartilage, adipocytes, muscle, tendon and ligament.[9] Moreover, MSCs are able to modulate T cell responses and induce immunosuppressive effects,[10,11] making it possible to be a potential therapeutic agent for various immunological disorders.[12,13] It is reported that bone marrow MSCs can increase the number of regulatory T cells and inhibit the proliferation and activation of T lymphocytes *in vitro*.[14] In our previous studies, MSCs were capable of up-regulating the proportion of regulatory T cells in peripheral blood and attenuating the airway inflammation of asthmatic mice. Based on these results, we hypothesized that MSCs could be used as a new therapeutic method in the treatment of asthma.

Heme oxygenases (HOs) are the rate-limiting intracellular enzymes that degrade heme to biliverdin, carbon monoxide and free divalent iron. The inducible form of HOs, heme oxygenase-1 (HO-1), has been considered as an anti-inflammatory and immunosuppressive molecule.[15] It is demonstrated that forkhead box P3 transcription factor (Foxp3), which is required for the development and maintenance of $CD4^+CD25^+$ Tregs, can be enhanced by HO-1.[16] Moreover, HO-1 is able to enhance the immunomodulatory effects of regulatory T cells by increasing the secretion of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β).[17] Additionally, adult human MSCs express HO-1,[18] suggesting a potential role for HO-1 in the immunosuppressive effects of MSCs. Zinc protoporphyrin IX (ZnPP) is a member of metalloporphyrins in which the heme iron is replaced by zinc, which becomes a competitive inhibitor of heme oxygenase (HO). In this study, we investigated the regulation of HO-1 by Hemin and ZnPP and the role of HO-1 contributed to the up-regulation of $CD4^+CD25^+CD127^{low/-}$ Tregs mediated by MSCs in asthma.

MATERIALS AND METHODS

Ethics Statement

Ten cases of asthmatic patients with acute episode and 10 age-, sex- and race-matched cases of healthy controls were enrolled in the study after signing an institutional review board-approved informed consent form. Those with confirmed diagnosis of asthma having acute episode according to GINA's diagnostic criteria were included in case group. The exclusion criterion was patients on immunomodulatory or immunosuppressive therapy at the time of blood sample collection. The treatment-free period was at least 3 months for glucocorticoid, cyclophosphamide, azathioprine and other immunosuppressive medications.

MSC Culture

Human bone marrow MSCs were obtained from Cyagen Biosciences (Guangzhou, China). Cell purity was confirmed by flow cytometry (>95%). The phenotypic properties were determined on the basis of CD29, CD44, CD73, CD105 and CD166 expression and the absence of CD11a, CD34 and CD45. MSCs cultured 1×10^5 cells per well were pretreated with 0, 15, 30, 45, 60 μmol/L Hemin (the revulsive of HO-1, Sigma, USA) and 0, 5, 10, 15, 20 μmol/L Protoporphyrin zinc (ZnPP, the inhibitor of HO-1, Sigma, USA), respectively for 24 hours before RNA extraction.

Mononuclear Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 cases of patients with acute episode of asthma and 10 cases of healthy controls using Ficoll (GE Healthcare, UK) density gradient centrifugation.

MSC Co-Culture with Pbmcs

MSCs were seeded on six-well flat-bottomed plates by 1×10^5 MSCs per well. Then, MSCs were pretreated with 30μmol/L Hemin, 10μmol/L ZnPP and saline respectively for 24 hours. Then, 25 μg/mL Mitomycin C from streptomyces caespitosus (MMC, Sigma, USA) was added to the cells for 30 minutes, inhibiting cell proliferation. After replacing the medium, MSCs were washed by Phosphate Buffered Saline (PBS) for 3 times to remove the Hemin and ZnPP. Then, 1×10^6 PBMCs were added to be incubated with the MSCs. Lectin from phaseolus vulgaris (PHA, Sigma, USA) were used for 72 hours to promote the proliferation of lymphocytes before flow cytometry analysis. In this study, group A, MSCs+Hemin+PBMCs+PHA, group B, MSCs+ZnPP+PBMCs+PHA and group C, MSCs+Saline+PBMCs+PHA were experimental groups. Group D, PBMCs+PHA, was control group.

Realtime RT-PCR Analysis

HO-1 gene expression of MSCs was determined by means of Realtime PCR analysis. Total RNA was isolated using RNeasy kit (Invitrogen, USA), and cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (TaKaRa Biotechnology, Dalian, China). The primers for HO-1 were purchased from TaKaRa (Dalian, China) and gene expression was measured by realtime PCR using SYBR Premix Ex Taq (TaKaRa Biotechnology, Dalian, China). The primer was as follows: HO-1, (forward) 5'-CAC GCA TAT ACC CGC TAC CT-3' and (reverse) 5'-AAG GCG GTC TTA GCC TCT TC-3'; Results were expressed for each sample as relative gene expression normalized for the GAPDH mRNA expression.

Western Blot Analysis

The concentration of total protein extracted from MSCs with different concentrations of hemin or ZnPP was determined with a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, USA). Equal amounts of protein were separated by 10% SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Bedford, USA) using a mini trans-blot. Rat anti-human HO-1 (Cell Signaling Technology, Boston, USA), was used to detect the expression of homologous proteins. β-actin (Santa Cruz Biotechnology, CA, USA) was used as an internal control. Electrochemiluminescence was performed with a Chemilmager 5500 imaging system (San Leandro, CA, USA), according to the manufacturer's instructions.

Flow Cytometry Assay

The liquid supernatant of group A, B, C and D was collected to examine the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells by flow cytometry on a FACS Calibur system (EPICS ALTRA, Beckman Coulter, Fullerton, CA, USA). CD4, CD25 and CD127 were identified in the different groups using appropriate fluorescent conjugated antibodies of FITC anti-human CD4, PE anti-human CD25 and APC anti-human CD127 (eBioscience, USA). The data were collected and processed using the FlowJo FACS analysis software (Tree Star, Ashland, OR, USA).

Statistical Analysis

Data were presented as mean±standard deviation. Statistical analysis of data was conducted using SPSS13.0 software. Two-independent samples *t* test was used to show the difference between the patients and healthy controls. The means were then compared using a one-way ANOVA with LSD among groups. Values of $p<0.05$ were considered significant.

RESULTS

Pattern of HO-1 Expression in MSCs Preincubated with Different Concentrations of Hemin

To explore the effect of hemin on expression of HO-1 in MSCs, Realtime RT-PCR and western blot were performed. MSCs were preincubated with 0, 15, 30, 45, 60 μmol/L Hemin for 24 hours. The expression of HO-1 in MSCs could be increased by Hemin *in vitro*. Hemin-mediated induction of HO-1 was in a dose-dependent fashion. With the higher the concentration of Hemin added to MSCs, the expression of HO-1 mRNA and protein were higher ($p=0.014$) (Figure 1).

Pattern of HO-1 Expression in MSCs Preincubated with Different Concentrations of Znpp

To study the effect of ZnPP on expression of HO-1 in MSCs, MSCs were pretreated with 0, 5, 10, 15, 20 μmol/L ZnPP for 24 hours. The expression of HO-1 in MSCs could be inhibited by ZnPP *in vitro*. With the increasing concentration of ZnPP added to MSCs, the expression of HO-1 mRNA and protein were decreased ($p=0.021$) (Figure 2).

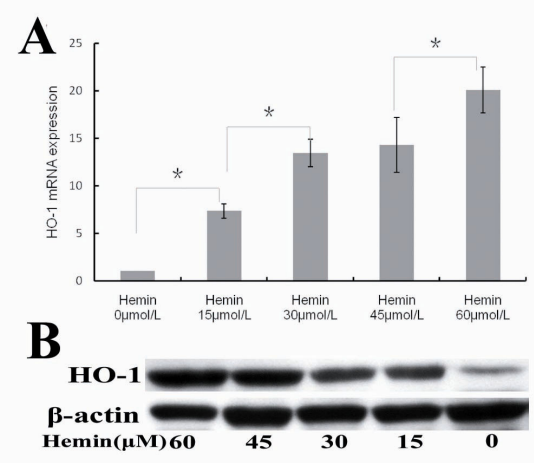

Figure 1. The expression of HO-1 mRNA and protein in MSCs preincubated with different concentrations of Hemin. Pretreated with different concentrations of Hemin for 24 hours, HO-1 expression was assessed in MSCs by Realtime RT-PCR and Western blot. (A) The expression of HO-1 mRNA in MSCs preincubated with different concentrations of Hemin. (B) Western blot analysis of HO-1 protein expression in MSCs preincubated with different concentrations of Hemin. Hemin-mediated induction of HO-1 was in a dose-dependent fashion. With the higher the concentration of Hemin added to MSCs, the expression of HO-1 mRNA and protein were higher. *p<0.05.

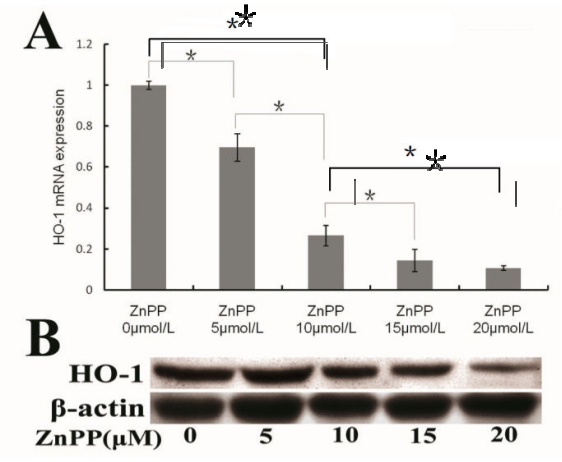

Figure 2. The expression of HO-1 mRNA and protein in MSCs preincubated with different concentrations of ZnPP. Pretreated with different concentrations of ZnPP for 24 hours, HO-1 expression was assessed in MSCs by Realtime RT-PCR and Western blot. (A) The expression of HO-1 mRNA in MSCs preincubated with different concentrations of ZnPP. (B) Western blot analysis of HO-1 protein expression in MSCs preincubated with different concentrations of ZnPP. With the increasing concentration of ZnPP added to MSCs, the expression of HO-1 mRNA and protein were getting lower. *p<0.05.

HO-1 Contributes to the MSC-Mediated Induction of $CD4^+CD25^+CD127^{low/-}$ Tregs

To study the effect of HO-1 in the regulation of regulatory T cells mediated by MSCs, PBMCs isolated from asthmatic patients and healthy controls were co-cultured with human bone marrow MSCs which were preincubated with Hemin, ZnPP and saline.

In patients with acute episode of asthma, the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells was 74.23±1.49% in group A (MSCs+Hemin+PBMCs+PHA), 47.84±4.58% in group B (MSCs+ZnPP+PBMCs+PHA), 61.08±3.58% in group C (MSCs+Saline+PBMCs+PHA) and 6.10±0.85% in group D (PBMCs+PHA). In healthy controls, the proportion was 77.78±1.75% in group A, 53.57±4.50% in group B, 67.05±2.57% in group C and 9.12±1.87% in group D (p<0.001) (Figures 3 and 4).

Both in asthmatic patients and healthy controls, the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells could be up-regulated by MSCs. In case of MSCs pretreated with hemin, the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs was higher than MSCs alone. Thus, after induction of HO-1, MSCs could lead to the higher proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs. Induction of HO-1 in MSCs markedly increased their ability to induce $CD4^+CD25^+CD127^{low/-}$ Tregs. Whereas, MSCs pretreated with ZnPP had lower proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs compared to MSCs alone. Inhibition of HO-1 in MSCs markedly reduced their ability to induce $CD4^+CD25^+CD127^{low/-}$ Tregs.

Increased Expression of $CD4^+CD25^+CD127^{low/-}$ Tregs after Treatment of PBMCs with MSCs (MSCs+ Hemin/MSCs+ ZnPP)

To explore the effect of $CD4^+CD25^+CD127^{low/-}$ Tregs in asthmatic patients and healthy controls with treatment of MSCs (MSCs+Hemin/ MSCs+ZnPP), we analyzed the data on increased expression of $CD4^+CD25^+CD127^{low/-}$ Tregs after treatment of PBMCs with MSCs (MSCs+Hemin/MSCs+ZnPP). Although the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells of asthmatic patients is less than healthy controls (p<0.001) (figure5D), but after the treatment of PBMCs with MSCs (MSCs+Hemin/MSCs+ZnPP), the increased

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expression of $CD4^+CD25^+CD127^{low/-}$ Tregs in asthmatic patients was almost equal to healthy controls.($p>0.05$) (Fig. 5A-5C). It means that, when asthma patients were compared with healthy controls, MSCs (MSCs+Hemin/MSCs+ZnPP) could increase $CD4^+CD25^+CD127^{low/-}$ Tregs at the same increment.

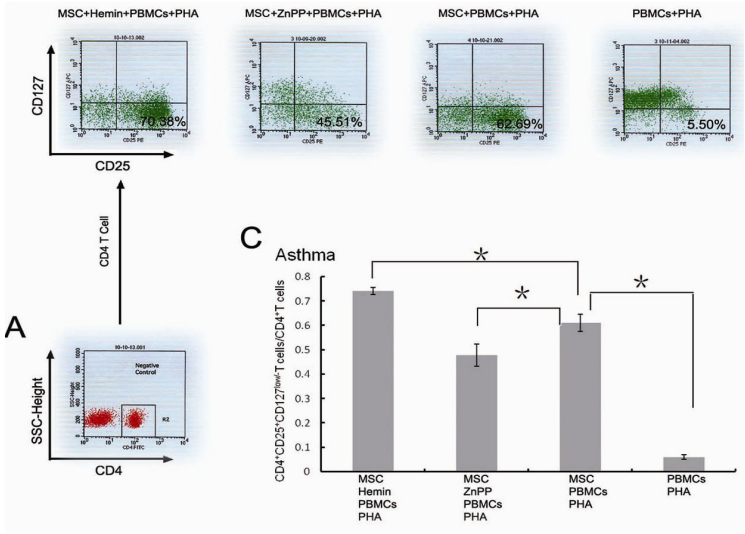

Figure 3. The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells in 4 different groups of asthmatic patients. PBMCs isolated from 10 asthmatic patients were co-cultured with MSCs pretreated with Hemin, ZnPP and saline. The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells were assessed by flow cytometry. (A) Expression of $CD4^+$T Cells. (B) Representatives of $CD4^+CD25^+CD127^{low/-}$ Tregs in 4 groups. (C) The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells in four groups; Induction of HO-1 could increase the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs, while inhibition of HO-1 could decrease the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs as compared with MSCs alone. *$p<0.001$.

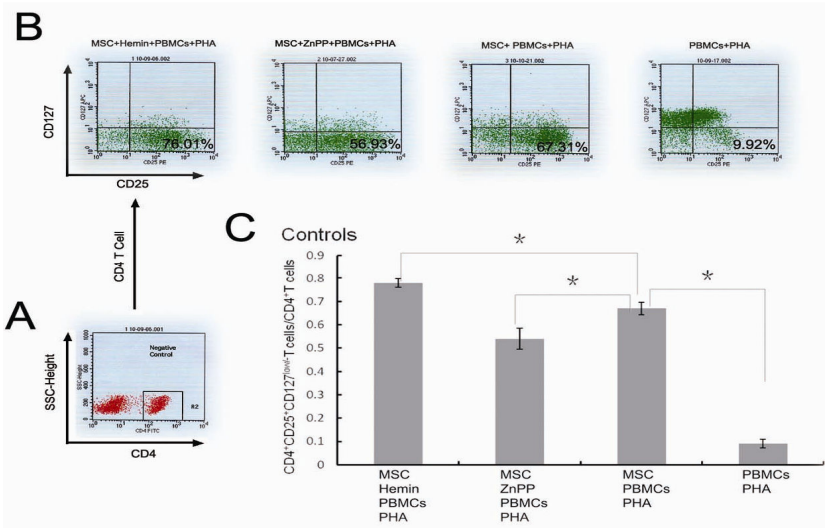

Figure 4. The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells in 4 groups of healthy controls. PBMCs isolated from 10 healthy controls were co-cultured with MSCs pretreated with Hemin, ZnPP and saline. The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells were assessed by flow cytometry. (A) Expression of $CD4^+$ T Cells. (B) Representatives of $CD4^+CD25^+CD127^{low/-}$ Tregs in 4 groups. (C) The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells in four groups; Induction of HO-1 could increase the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs, while inhibition of HO-1 could decrease the proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs as compared with MSCs alone. *$p<0.001$.

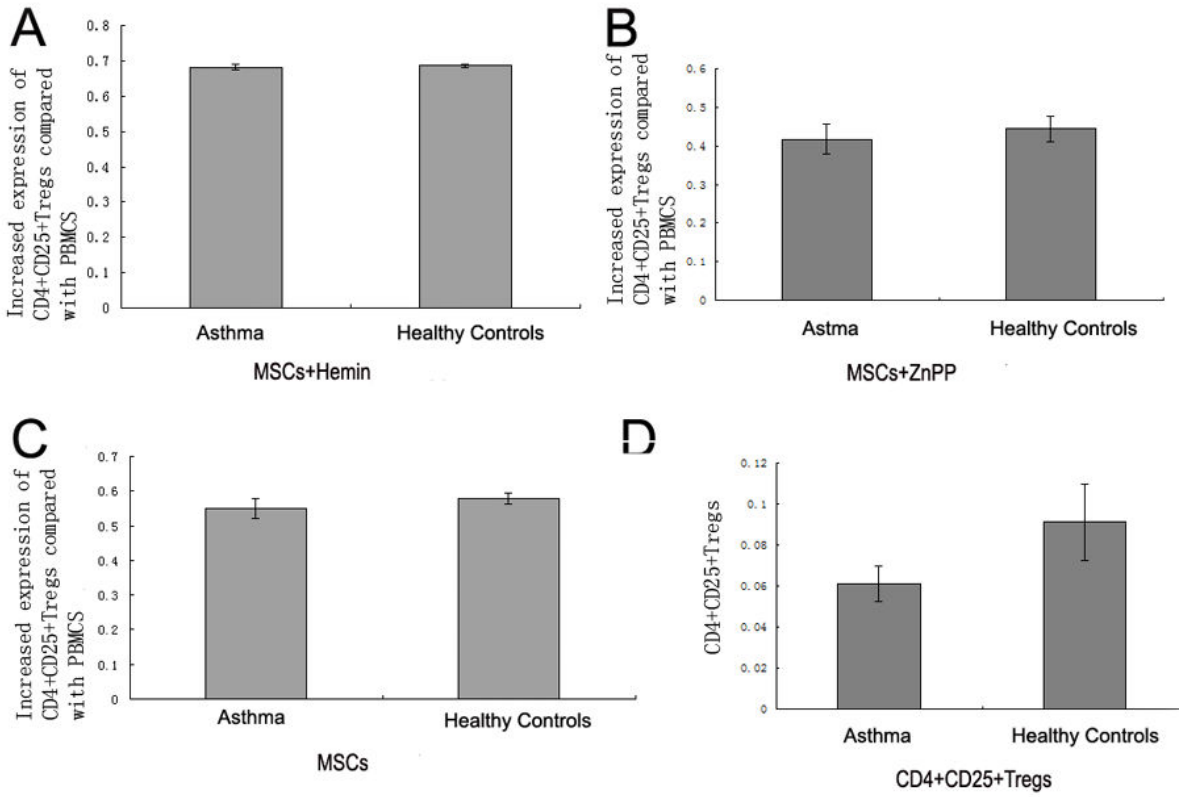

Figure 5. The increased expression of $CD4^+CD25^+CD127^{low/-}$ Tregs after the treatment of PBMCs with MSCs, MSCs+ Hemin and MSCs+ ZnPP. (A) The increased expression of $CD4^+CD25^+CD127^{low/-}$ Tregs after the treatment of PBMCs with MSCs+Hemin, there was no significant difference in asthmatic patients compared with healthy controls. (p=0.104) (B) The increased expression of $CD4^+CD25^+CD127^{low/-}$ Tregs after the treatment of PBMCs with MSCs+ZnPP, there was no significant difference in asthmatic patients compared with healthy controls. (p=0.11) (C) The increased expression of $CD4^+CD25^+CD127^{low/-}$ Tregs after the treatment of PBMCs with MSCs, there was no significant difference in asthmatic patients compared with healthy controls. (p=0.09) (D) The proportion of $CD4^+CD25^+CD127^{low/-}$ Tregs in $CD4^+$ T cells of asthmatic patients was lower compared to healthy controls (p<0.001).

DISCUSSION

It has been suggested that MSCs possess remarkable immunosuppressive properties by modulating the proliferation and activities of the major immune cell populations (T cells, B cells, natural killer cells, dendritic cells, etc.) and inducing regulatory T cells both *in vivo* and *in vitro*.[10-12,19,20] The previous study shows that MSCs can up-regulate $CD4^+CD25^+$ Tregs and actively prevent the induction of allergen driven pathology of asthmatic mice.[21-27] Unfortunately, mechanism of MSCs in up-regulating $CD4^+CD25^+$ Tregs is still unknown.

This study demonstrated that MSCs can up-regulate $CD4^+CD25^+CD127^{low/-}$ Tregs through a HO-1-dependent mechanism. Chabannes et al have shown HO-1 plays an important role in immunosuppressive effects in human bone marrow MSCs,[18] and Dimitrios Mougiakakos et al have demonstrated that HO-1 is involved in the MSC-mediated induction of $IL\text{-}10^+$ T regulatory (Tr1) cells and $TGF\text{-}\beta^+$ T helper (Th3) cells.[28] Our study identified that the expression of HO-1 in MSCs could be induced by Hemin and inhibited by ZnPP *in vitro*. In our following experiments, induction of HO-1 in MSCs markedly increased their ability to induce $CD4^+CD25^+CD127^{low/-}$ Tregs, whereas, inhibition of HO-1 in MSCs markedly reduced their ability to induce $CD4^+CD25^+CD127^{low/-}$ Tregs. Thus, we demonstrated that HO-1 contributed to the up-

regulation of $CD4^+CD25^+CD127^{low/-}$ Tregs mediated by MSCs in asthma.

HO-1, which has been viewed as a cytoprotective protein, can ameliorate the effects of inflammatory cellular damage, suggesting important functions in both innate and adaptive immune responses.[29,30] In an animal model of asthma, intra-abdominal injection of HO-1 reduced airway inflammation and decreased IgE production in serum.[31,32] One study showed that up-regulation of HO-1 before ovalbumin challenge in sensitized mice results in decreased neutrophils and eosinophils influx to the airways.[30] Xia et al have demonstrated that induction of HO-1 can augment IL-10 and TGF-β production and $CD4^+CD25^+Foxp3^+$ Tregs function, thereby leading to decrease of ovalbumin-specific IgE level and eosinophil infiltration in bronchial alveolar lavage fluid.[16] HO-1 also exerted its protective effect on asthma through a mechanism mediated by $CD4^+CD25^+Foxp3^+$ Tregs, which was in accordance with our results.[17,33]

Human Treg cells were first characterized as $CD4^+CD25^+$ T cells in 2001 by several groups based on the finding in 1995 that mouse Treg cells constitutively express CD25. Similarly, in 2003, FOXP3 was described as a master control gene for mouse Treg cell development and function, and subsequent studies have confirmed FOXP3 as a specific marker for human Treg cells. With regard to the new marker, Liu and colleagues demonstrated that CD127 expression is down-modulated on Treg cells, inversely correlating with the expression of Treg marker FOXP3. The study of Liu W. et al. argued that FoxP3 interacted with the CD127 promoter and might have contributed to reduced expression of CD127 in Treg cells. Hence, $CD127^{low/-}$ was as a new marker for $CD4^+CD25^+FOXP3^+$Treg cells from the year of 2006.[34]

Allergic inflammation has been characterized as a Th2-cell-predominant disease. Therefore, efforts to alter the balance of Th1 and Th2 cells in asthma have been aggressively pursued, either by promoting Th1-cell responses or inhibiting Th2-cell cytokines.[35] More recently, our understanding of the immune response was significantly altered by reports on the $CD4^+CD25^+$ regulatory T cell lineage, whose ability is to suppress the immune responses induced by Th2 cells.[2,4,5] It has been demonstrated that there are reduction of regulatory T cells in many immunological diseases,[2] including allergic asthma, but their important function and mechanisms in immunoregulation are far from being illustrated. $CD4^+CD25^+$ Tregs could modulate allergic airway inflammation by dampening Th2-cytokine responses. In the previous study, ovalbumin-challenged mice showed decreased number of $CD4^+CD25^+$ Tregs in the spleen. Studies have shown that the regulatory T cells, which have immunosuppressive activities, express high level of CD4, CD25 and low or negative CD127.[7,8] Our data and a study by Dao Nguyen et al reported a decrease in $CD4^+CD25^+CD127^{low/-}$ Tregs in the activated PBMCs derived from asthmatic patients.[36] MSCs could up-regulate $CD4^+CD25^+CD127^{low/-}$ Tregs, thus providing a new therapeutic modality of MSCs in asthmatic patients.

ACKNOWLEDGEMENTS

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