Original Paper

Heme Oxygenase-1 Restores Impaired GARP⁺CD4⁺CD25⁺ Regulatory T Cells from Patients with Acute Coronary Syndrome by Upregulating LAP and GARP Expression on Activated T Lymphocytes

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Key Words

Heme oxygenase-1 • GARP⁺CD4⁺CD25⁺Tregs • LAP • Foxp3 • Acute coronary syndrome

Abstract

Background: Accumulating evidence shows that the pathological autoreactive immune response is responsible for plaque rupture and the subsequent onset of acute coronary syndrome (ACS). Naturally occurring CD4⁺CD25⁺regulatory T cells (nTregs) are indispensable in suppressing the pathological autoreactive immune response and maintaining immune homeostasis. However, the number and the suppressive function of glycoprotein-A repetitions predominant (GARP)⁺CD4⁺CD25⁺ activated nTregs were impaired in patients with ACS. Recent evidence suggests that heme oxygenase-1 (HO-1) can regulate the adaptive immune response by promoting the expression of Foxp3. We therefore hypothesized that HO-1 may enhance the function of GARP⁺CD4⁺CD25⁺Tregs in patients with ACS and thus regulate immune imbalance. **Methods:** T lymphocytes were isolated from healthy volunteers (control, n=30) and patients with stable angina (SA, n=40) or ACS (n=51). Half of these cells were treated with an HO-1 inducer (hemin) for 48 h, and the other half were incubated with complete RPMI-1640 medium. The frequencies of T-helper 1 (Th1), Th2, Th17 and latency-associated peptide (LAP)⁺CD4⁺ T cells and the expression of Foxp3 and GARP by CD4⁺CD25⁺T cells were then assessed by measuring flow cytometry after stimulation *in vitro*. The suppressive function of activated Tregs was measured by thymidine uptake. The levels of transforming growth factor-1 (TGF-β1) in the plasma were measured using enzyme-linked immunosorbent assay

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(ELISA). The expression levels of the genes encoding these proteins were analyzed by real-time polymerase chain reaction. **Results:** Patients with ACS exhibited an impaired number and suppressive function of GARP⁺ CD4⁺ CD25⁺Tregs and a mixed Th1/Th17-dominant T cell response when compared with the SA and control groups. The expression of LAP in T cells was also lower in patients with ACS compared to patients with SA and the control individuals. Treatment with an HO-1 inducer enhanced the biological activity of GARP⁺ CD4⁺ CD25⁺Tregs and resulted in increased expression of LAP and GARP by activated T cells. **Conclusions:** The reduced number and impaired suppressive function of GARP⁺ CD4⁺ CD25⁺Tregs result in excess effector T cell proliferation, leading to plaque instability and the onset of ACS. HO-1 can effectively restore impaired GARP⁺ CD4⁺ CD25⁺Tregs from patients with ACS by promoting LAP and GARP expression on activated T cells.

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Introduction

Although atherosclerosis (AS) is a complicated pathophysiological process that involves multifactorial disorders, an increasing number of studies have demonstrated that AS is a chronic immunoinflammatory [1, 2]. AS is the pathological basis of acute coronary syndrome (ACS), which is defined as the erosion or rupture of coronary atherosclerotic plaques with subsequent platelet aggregation and formation of an intracoronary thrombus [3].

One of the key causes of coronary atherosclerotic plaque destabilization is that a considerable number of activated T cells are recruited in peripheral circulation and atherosclerotic plaques of patients with ACS [4, 5], and these T cells exert proatherogenic and plaque-destabilizing effects [6, 7]. Numerous investigations have reported remarkably increased T helper-1(Th1) cells in the plaques and peripheral blood of patients with ACS [8-10]. This predominance of Th1 cells elicits a strong immunoinflammatory cascade via the secretion of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) that leads to plaque instability and rupture [11]. In addition, Th17 was shown to play a role in the promotion of plaque instability of ACS by producing Interleukin 17 (IL-17), IL-6, TNF- α , macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory chemokine-2 (MIP-2) and matrix metalloproteases [12, 13]. Therefore, the mixed Th1/Th17-dominant T cell response is largely responsible for the development of ACS because of the pivotal role of these cells in the progress of AS and in plaque destabilization.

Regulatory T cells (Tregs) are activated by antigens and then efficiently maintain peripheral immune homeostasis by inhibiting the hyperproliferation of Th1 and Th17 cells. However, clinical data have indicated that the frequency of activated nTregs is decreased and their functional properties are compromised in patients with ACS [14, 15]. The forkhead/winged helix (Foxp3) transcription factor is a master transcriptional regulator of nTregs cells and is thought to control both the development and function of this T cell subset [16]. In both humans and mice, defective Foxp3 signaling causes nTreg insufficiency and dysfunction leading to the hyperactivation of effector T cells and fatal autoimmune diseases [17, 18]. Therefore, Foxp3 was previously considered to be a specific biological molecular marker for activated nTregs. However, further studies showed that Foxp3 expression is also found in activated effector T cells (Tresp) in humans [19]. Thus, the use of Foxp3 as specific biomarker for activated human nTregs has been questioned. Recent studies have shown that glycoprotein A repetitions predominant (GARP) is specifically expressed on activated human nTregs and is not induced on non-nTregs after T cell receptor (TCR) [20]. Moreover, GARP⁺ CD4⁺ CD25^{hi} Foxp3⁺Tregs showed greater suppressive function than GARP⁻CD4⁺ CD25^{hi} Foxp3⁺Tregs [21]. Thus, GARP⁺ CD4⁺ CD25⁺ Tregs may be more suitable for distinguishing bona fide activated human nTregs with high suppressive capacity from Tresp and induced Tregs. TGF- β is a pleiotropic cytokine and can be secreted or expressed on the membrane of many cell types in a small latent form along with the latency-associated peptide (LAP) that non-covalently associates with mature TGF- β to inhibit its activity. Latent TGF- β must cleave the LAP to acquire biological activity. Sockis et al. confirmed that the membrane protein

GARP serves as a receptor for latent TGF- β on the surface of activated human Tregs, where releases active TGF- β by directly binding to the LAP of latent TGF- β [22].

Heme oxygenase (HO) is a rate-limiting enzyme in heme degradation that releases biliverdin, carbon monoxide, and free iron by catalyzing heme. The HO family consists of the three isoforms HO-1, HO-2, and HO-3. Among these isoforms, HO-1 is the most important heme-degrading enzyme and is induced by its own substrate, oxidative stress and in response to inflammatory cytokines [23]. Although many studies have indicated that HO-1 may play a role in adaptive immunity, the mechanism of HO-1 in this process remained controversial [24-26]. Recent research further demonstrated that HO-1 is involved in the regulation of adaptive immunity by up-regulating the expression of Foxp3 on CD4⁺CD25⁺Tregs in animal models [27]. However, no studies have examined whether HO-1 could enhance the number and the suppressor function of GARP⁺CD4⁺CD25⁺Tregs in patients with ACS. Thus, the current study sought to investigate the effect of HO-1 on the levels and immunosuppressive function of GARP⁺CD4⁺CD25⁺Tregs in different groups of patients and explore the potential mechanisms by which HO-1 regulates adaptive immunity.

Materials and Methods

Patients

After rigorous screening, a total of 121 patients from Wuhan Union Hospital were recruited to the study during the period from November 2012 to March 2014. The study included the following three patient groups: (1) Healthy volunteers (control; 21 men and 9 women, mean age 53 $\pm$ 6 years) with no angina and coronary risk factors were recruited. All of these healthy volunteers had normal electrocardiography and no evidence of atherosclerosis by coronary angiography; (2) Patients with stable angina (SA; 28 men and 12 women, mean age 57 $\pm$ 5 years), including those with typical effort angina lasting more than 3 months without changes in angina frequency or severity and coronary angiography showing coronary artery stenosis $\geq$ 75%; and (3) Patients with acute coronary syndrome (ACS) including 30 cases of acute myocardial infarction (AMI) and 21 cases of unstable angina (UA); 35 men and 16 women; mean age 56 $\pm$ 5 years). Unstable angina was defined as chest pain at rest with transient myocardial ischemia electrocardiographic changes (ST-segment depression and/or T-wave inversions) and the absence of serum biomarkers of myocardial necrosis. Acute myocardial infarction was confirmed by a significant increase in the levels of myocardial necrosis biomarkers (especially creatine kinase MB and troponin I), as well as severe, persistent, and typically substernal pain.

The following patients were excluded from our study: (1) those with a history of taking statins or anti-inflammatory drugs; (2) those with rheumatic heart disease, or valvular heart disease; (3) those with inflammatory disease, malignant disease, advanced liver disease, or renal failure; (4) those with thromboembolism, disseminated intravascular coagulation, or connective tissue disease; and (5) those with atrial fibrillation or a pacemaker.

All of the patients signed informed consent forms. Our research strictly conformed to the principles of the Declaration of Helsinki and was approved by the ethics committee of Tongji Medical College of Huazhong University of Science and Technology.

Blood samples

Blood samples were collected from all patients upon admission and within 24 h of the onset of symptoms. After centrifugation, peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient separation for the isolation of T lymphocytes. The plasma was aliquoted and stored at -80°C until further use.

Isolation of T cells and TCR stimulation

After washing twice in RPMI-1640 medium (Gibco), PBMCs were resuspended at a density of 3 $\times$ 10⁷ cells/ml in complete culture medium (RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 10% heat-inactivated fetal calf serum, Gibco). Cells were cultured in a volume of 1 ml at 37°C in 5% CO₂ in a nylon wool column for 2 h, and 6 ml of prewarmed medium was

passed through the column to obtain T cells. Flow cytometry was performed to detect the purity of the isolated T cells. The T cells were equally divided and half were incubated in complete culture medium for 48h and then stimulated with soluble anti-human CD3 (3 µg/ml, eBioscience) and anti-human CD28 (1 µg/ml, eBioscience) antibodies in a 24-well plate (1×10⁶/well) for 24 h at 37°C under 5% CO₂. The remaining T cells were incubated with 10 ng/ml hemin in a 24-well plate (1×10⁶/well) for 48 h and then stimulated with anti-human CD3 (3 µg/ml, eBioscience) and anti-human CD28 (1 µg/ml, eBioscience) antibodies for 24 h at 37°C under 5% CO₂.

Flow cytometric analysis

After 24 h of culture, the isolated T cells were harvested and placed in sterile tubes. The cells were then centrifuged at 420 × g for 6 min and aliquoted into two tubes to stain for Tregs and Th cells.

For Tregs analysis, cell surface staining was performed using anti-human CD4-FITC (eBioscience), anti-human CD25-APC (eBioscience), anti-human GARP-PE (eBioscience), and anti-human LAP-PECy7 (eBioscience) antibodies at 4°C for 30 min. After surface staining, the cells were washed in phosphate-buffered saline (PBS), fixed and permeabilized with fix/permeabilization reagent at 4°C for 30 min, washed twice in 1×permeabilization-buffer and incubated with an anti-human Foxp3-PECy5 (eBioscience) antibody at 4°C for 30 min.

For Th cell analysis, T cells were seeded at a density of 1×10⁶cells/well in RPMI-1640 medium with 10% heat-inactivated fetal calf serum (Gibco) and stimulated with phorbol myristate acetate (PMA, 50 ng/ml, Sigma), ionomycin (1 µM, Sigma) and monensin (500 ng/ml, eBioscience) for 4 h at 37°C under 5% CO₂. The cells were then incubated with anti-human CD4-FITC (eBioscience) antibody at 4°C for 30 min, washed twice in 1× permeabilization-buffer and stained with anti-human IFN-γ- PE, anti-human IL-17-PE or anti-human IL-4-PE (eBioscience) antibody at 4°C for 30 min.

All of the cells were analyzed using a FACScan cytometer equipped with CellQuest software (BD Bioscience Pharmingen).

Proliferation and functional suppression assays

After 24 h of stimulation with soluble anti-human CD3 and anti-human CD28 antibodies, PBMCs from control (n=10), SA (n=10) and ACS (n=11) patients were purified using a CD4+T cell positive isolation kit (Miltenyi Biotec, Bergisch Gladbach Germany) and FACS sorting using a FACS Aria (BD Biosciences) to obtain purified GARP+CD4+CD25+Tregs and GARP-CD4+CD25-Tresps. The purities of the GARP+ CD4+CD25+regulatory T cells and GARP-CD4+CD25-Tresps were determined by flow cytometry and were greater than 95%.

The following groups were used to measure the proliferation or suppression of Tresps and Tregs: (1) GARP-CD4+CD25-Tresps cultured alone (104/well); (2) GARP+CD4+CD25+Tregs cultured alone (104/well); and (3) Tregs and Tresps co- cultured at different Tregs/Tresps ratios (1:1, 1:2, 1:4 and 1:8). All of the cells were cultured in complete RPMI-1640 in a final volume of 200 µl in the presence of 10⁵ T cell-depleted and irradiated antigen-presenting cells per well. The cells were incubated with plate-bound anti-human CD3 (5 µg/ml, eBioscience) and soluble anti-human CD28 (2 µg/ml, eBioscience) antibodies at 37°C in 5% CO₂ for 72 h in 96-well plates. Next, 1 µl of [3H]-thymidine (eBiosciences) was added to each well for the final 16 h of culture, and proliferation was assessed using a scintillation counter (PerkinElmer). Suppression was expressed as a percentage of the positive control.

Quantitative real-time polymerase chain reaction (RT-PCR)

According to the manufacturer's instructions, total RNA was extracted with RNAiso Plus (Takara), and cDNA was synthesized using a RNA PCR Kit (Takara). Target genes were analyzed using SYBR Green Master Mix (Takara) with an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Each sample was analyzed at least in duplicate. The primer sequences used are listed in Table 1.

The relative mRNA levels were calculated using the comparative CT formula $2^{-\Delta\Delta CT}$ and normalized to the expression of the housekeeping gene GAPDH.

Treatment of T Cells with hemin

Freshly isolated T cells were treated with hemin at various concentrations for different time periods, and the expression of HO-1 mRNA was measured by RT-PCR. Half of the T cells isolated from each patient

were incubated with 10ng/ml hemin in a 24-well plate (1×10^6 /well) for 48 h and then stimulated with soluble anti-human CD3 (3 μ g/ml) and anti-human CD28 (1 μ g/ml) antibodies for 24 h at 37°C under 5% CO₂. After stimulation, the frequency of Th1, Th2, and Th17 cells and GARP⁺CD4⁺CD25⁺Tregs, the function of GARP⁺CD4⁺CD25⁺Tregs and the related gene expression were re-evaluated.

Cytokine detection of plasma TGF- β

The levels of TGF- β 1 in the plasma were measured using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience). All samples were assayed at least in triplicate.

Statistical analysis

Values were expressed as the mean $\pm$ standard deviation (SD). Significant differences between the three groups were determined using a one-way analysis of variance (ANOVA) with the Newman-Keuls post-test and the Dunnett adjustment test. Differences in T cells before and after treatment with HO-1 were compared by the Wilcoxon signed-rank test. Spearman's correlation was performed as a test of correlation between two continuous variables. P values < 0.05 were considered statistically significant.

Results

Basic clinical characteristics

The baseline characteristics of the control, SA and ACS groups are listed in Table 2. There were no significant differences in age, gender, risk factors, or medications between the groups.

The frequency of GARP⁺CD4⁺CD25⁺Tregs is reduced in activated T cells from patients with ACS

The T cells from all the subjects were analyzed after activation with anti-CD3 and anti-CD28 antibodies. The frequencies of CD4⁺CD25⁺T cells were detected by flow cytometry (Fig. 1A), and no significant differences were detected among the three groups (P=0.36). However, after measuring the expression of GARP in CD4⁺CD25⁺T

Table 1. RT-PCR primer sequences

Gene	Sequence(5'-3')
Foxp3 sense	CACTTGCAGACACCATTTGC
Foxp3 antisense	CTCTTCTTCCTTGAACCCCA
T-bet sense	GATGTTTGTGGACGTGGTCTTG
T-bet antisense	CTTTCCACACTGCACCCACTT
IFN- γ sense	GGTTCTCTTGGCTGTTACTGCC
IFN- γ antisense	TTGGACATTCAAGTCAGTTACC
GATA3 sense	GGACGCGGCGCAGTA C
GATA3 antisense	TGCCTTGACCGTCGATGTTA
IL-4 sense	GCCACCATGAGAAGGACACT
IL-4 antisense	ACTCTGGTTGGCTTCTCTCA
TGF- β 1 sense	CAGCAACAATTCTGGCGATAC
TGF- β 1 antisense	TGGGCGCTAAGGCGAAAG
ROR γ t sense	GCAATGGAAGTGGTGTGGTT
ROR γ t antisense	AGGATGCTTTGGCGATGAGTC
IL-17 sense	ACCAATCCCAAAAGGTCCTC
IL-17 antisense	GGGGACAGAGTTCATGTGGT
HO-1 sense	ATGACACCAAGGACCAGAGC
HO-1 antisense	GTGTAAGGACCCATCGGAGA
GARP sense	CGTCCCGAGACTCATCTAC
GARP antisense	GGTCAGGTGCTCAAGAAAGC
GAPDH sense	GAGTCAACGGATTTGGTCGT
GAPDH antisense	GACAAGCTTCCCGTTCTCAG

Table 2. Clinical characteristics of the study population.

Values are expressed as the mean $\pm$ SD or as a number. SA: Stable angina pectoris; ACS: acute coronary syndromes; TC: total cholesterol; TG: total triglycerides; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; CCB: calcium channel blocker; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker. ##P > 0.05 vs. Control or SA; # P > 0.05 vs. SA

Characteristics	Control (n=30)	SA (n=40)	ACS (n=51)
Age(years)	53 $\pm$ 6	57 $\pm$ 5	56 $\pm$ 5 ##
Gender(male/Female)	21/9	28/12	35/16 ##
Risk factors			
Hypertension, n(%)	-	25(63)	35(69) #
Diabetes mellitus, n(%)	-	9(23)	14(27) #
Smoking, n(%)	-	21(53)	31(61) #
TC,(mmol/L)	4.54 $\pm$ 0.36	4.65 $\pm$ 0.55	4.76 $\pm$ 0.49 ##
TG,(mmol/L)	1.73 $\pm$ 0.41	2.12 $\pm$ 0.36	2.01 $\pm$ 0.4 ##
LDL-C,(mmol/L)	2.25 $\pm$ 0.31	2.63 $\pm$ 0.27	2.77 $\pm$ 0.25 ##
HDL-C,(mmol/L)	1.21 $\pm$ 0.16	1.14 $\pm$ 0.22	1.12 $\pm$ 0.14 ##
Medications			
Aspirin (%)	-	19(47.5)	27(52.9) #
β -Blockers, n(%)	-	13(32.5)	20(39.2) #
CCB, n (%)	-	20(50)	28(54.9) #
ACEI/ARB, n (%)	-	13(32.5)	18(35.3) #
Nitrates, n (%)	-	21(52.5)	30(58.8) #

Fig. 1. The expression of GARP was reduced on activated T cells from patients with ACS. Activated T cells were stained with labeled antibodies from control (n=20), SA (n=30) and ACS (n=40) patients. A. Representative FSC/SSC dot plot showing the gated CD4⁺T cells by the isotype control and representative FACS plots showing the frequencies of CD4⁺CD25⁺T cells among the stimulated T cells from each group. B. CD4⁺CD25⁺T cells were gated by the isotype control, and the representative GARP expression on activated CD4⁺CD25⁺T cells from one patient in each group is shown. C. The frequencies of CD4⁺CD25⁺T cells and the expression of GARP on CD4⁺CD25⁺ subsets were analyzed in each group. D. Comparison of the mRNA levels of GARP in each group. Error bars, SD; ** P < 0.01 vs. control or SA; ns, P > 0.05 vs. control.

cells (Fig. 1B), we found that the expression of GARP was sharply reduced in patients with ACS (6.02±1.05%) compared with patients in the SA and control groups (9.09±1.23%,

Fig. 2. GARP⁺CD4⁺CD25⁺Tregs function was compromised in patients with ACS. GARP⁺CD4⁺CD25⁺Tregs and GARP⁺CD4⁺CD25⁺Tresps were isolated by magnetic bead and FACS sorting from control (n=10), SA (n=10), ACS (n=11) groups. A. The proliferative capacities of GARP⁺CD4⁺CD25⁺Tregs and Tresps were measured and compared among the three groups. B. The suppressive properties of GARP⁺CD4⁺CD25⁺Tregs from each group were assayed at different ratios and expressed as the mean ± SD. C. the plasma TGF-β1 concentrations were compared between the three groups. D. the INF-γ, IL-4 and IL-17 mRNA expression among T cells from ACS, SA and healthy group. Error bars, SD; ** P < 0.01 vs. control or SA; ns, P > 0.05 vs. control.

9.58±1.51%, respectively P<0.01; Fig.1B) after stimulation *in vitro*, However no significant differences were observed between the SA and control groups (P=0.39; Fig. 1C). The expression of the GARP gene was consistent with these results (P<0.01; Fig. 1D).

The function of GARP+CD4+CD25+Tregs is significantly impaired in patients with ACS

GARP⁺CD4⁺CD25⁺Tregs and GARP⁺CD4⁺CD25⁺Tresps were isolated using magnetic bead and FACS sorting from the control (n=10), SA (n=10), ACS (n=11) groups (purity >95%). There were no significant differences in the proliferation of GARP⁺CD4⁺CD25⁺Tresps (P=0.73) or GARP⁺CD4⁺CD25⁺Tregs (P=0.61) between the groups (Fig. 2A).

The suppressor function of GARP⁺CD4⁺CD25⁺Tregs from control (n=10), SA (n=10), ACS (n=11) groups was assessed by co-culturing with autologous Tresps at varying ratios of Tregs to Tresps (1:1, 1:2, 1:4, and 1:8) and expressed as the mean±SD. The suppressive properties of Tregs were reduced when increased numbers of Tresps were added to the culture (Fig. 2B). In addition, the suppressor function of Tregs from patients with ACS was significantly impaired in different ratios compared with Tregs isolated from the SA and control group (P<0.01; Fig. 2B).

Because TGF-β plays an important role in the suppressive properties of Tregs, plasma TGF-β1 concentrations were measured in each group. The TGF-β1 concentrations were as follows: 10.2±2.3 ng/ml for the ACS group; 15.7±2.6 ng/ml for the SA group; and 16.9±2.9 ng/

Fig. 3. The level of HO-1 mRNA in freshly isolated T cells from each group, and the expression of HO-1 mRNA after treatment with hemin at various concentrations and at different times *in vitro*. A. The expression of HO-1 mRNA in freshly isolated T cells from the control (n=10), SA (n=10), ACS (n=11) groups. B. The expression of HO-1 mRNA in T cells from 10 patients with ACS treated with various doses of hemin. C. The expression of HO-1 mRNA in T cells from 10 patients with ACS treated with 10 ng/ml hemin at different time points. Error bars, SD; * P < 0.05 vs. 48h; ** P < 0.01 vs. control or SA; ns, P > 0.05 vs. control.

ml for the control group. The concentrations of TGF- β 1 in ACS individuals were significantly lower than those in SA or control individuals ($P < 0.01$; Fig. 2C).

Meanwhile, the IFN- γ , IL-4 and IL-17 mRNA expression levels of stimulated T cells from each group were analyzed. we found that the expression of IFN- γ and IL-17 gene expression levels were marked increases in patients with ACS compared with patients in the SA and control groups (all $P < 0.01$; Fig. 2D). However no significant differences were observed between the SA and control groups. A similar result was not observed on the IL-4 mRNA expression ($P = 0.46$; Fig. 2D).

The level of HO-1 mRNA in freshly isolated T cells from each group and the expression of HO-1 mRNA of T cells from the ACS group following treatment with hemin

First, we detected the levels of HO-1 mRNA of freshly isolated T cells from the three groups, and found that the level of HO-1 mRNA was significantly lower in T cells from the ACS group than those from the SA and control groups ($P < 0.01$); however, there was no obvious difference between the SA group and control group ($P = 0.34$; Fig. 3A). Then, T cells from ACS individuals were treated with hemin, a HO-1 inducer, at various concentrations (0, 10, 30, 50, and 70 ng/ml) for different periods of time (0, 24, 48, and 72 h). As shown in Fig. 3B, 10 ng/ml hemin was sufficient to markedly induce the expression of HO-1 mRNA. We found that the maximal expression of HO-1 mRNA was induced by treating cells with 10 ng/ml hemin for 48 h ($P < 0.05$; Fig. 3C).

Fig. 4. HO-1 had a significant effect on GARP⁺CD4⁺CD25⁺Tregs, Th1 and Th17 cells in patients with ACS. T cells from all of the subjects were treated with 10 ng/ml of hemin for 48 h. A. GARP expression on CD4⁺CD25⁺T cells was measured and compared between the three groups after stimulation *in vitro*. B. An equivalent increase in the suppressor function of GARP⁺CD4⁺CD25⁺Tregs was observed in the control (n=10), SA (n=10) and ACS (n=11) groups. C. GARP gene expression was compared between the treated and untreated cells from each group. D. The TGF-β1 gene expression was compared between the treated and untreated cells from each group. E. The frequencies of Th1, Th17 and Th2 cells were measured and compared to samples that were not treated with hemin. Error bars, SD; ** P < 0.01 compared to the no hemin group; ns, P > 0.05 compared to the no hemin group.

Assessment of the effect of HO-1 on the expression of GARP and function of activated Tregs and on the frequency of Th1, Th17 and Th2 cells in each group

The T cells from all of the study subjects were treated with 10 ng/ml hemin for 48 h and subsequently activated by anti-CD3 and anti-CD28 antibodies. We then re-evaluated the effect of HO-1 on GARP expression and the function of the activated Tregs among the three groups. As shown in Fig. 4A, the expression of GARP on CD4⁺CD25⁺T cells was 9.83±1.28%

Fig. 5. The effect of HO-1 on the expression of certain genes. T cells from control (n=20), SA (n=30), and ACS (n=40) patients were co-cultured with 10 ng/ml of hemin for 48h and then stimulated with anti-CD3 and anti-CD28 antibodies. The relative mRNA expression levels of T-bet, IFN- γ , ROR γ t, IL-17, GATA3 and IL-4 were detected and compared with the cells that did not receive hemin in each group. Error bars, SD; ** P < 0.01 compared with the no hemin group; ns, P > 0.05 compared with the no hemin group.

in the control group, $9.32 \pm 1.16\%$ in the SA group, and $9.08 \pm 1.56\%$ in the ACS group after stimulation *in vitro*, and no significant differences were found among the three groups ($P=0.68$). The effect of HO-1 on the suppressive properties of activated Tregs was also measured in the three groups. Although incubation with hemin increased the ability of the Tregs to suppress the proliferation of Tregs in each group, the changes in the suppressor function of Tregs from patients with ACS were more significant than those from patients with SA or the control group ($P<0.01$; Fig. 4B). In addition, T cells from the ACS group were more sensitive to HO-1 in terms of GARP and TGF- $\beta 1$ mRNA induction compared with cells from the other two groups ($P<0.01$; Fig. 4C and D).

We then found that the frequencies of Th1 and Th17 cells were decreased to different degrees in the three groups. As shown in Fig. 4E, the frequency of Th1 and Th17 cells was significantly lower among T cells treated with hemin compared with untreated cells in the ACS group ($9.76 \pm 1.6\%$ vs. $20.3 \pm 2.53\%$, $P<0.01$; $1.45 \pm 0.86\%$ vs. $3.17 \pm 1.12\%$, $P<0.01$, respectively). A similar decrease was not observed in the SA group ($P=0.52$ and $P=0.4$, respectively) or the control group ($P=0.6$ and $P=0.5$, respectively). However, there were no significant differences in the frequencies of Th2 cells compared with cells left untreated in

Fig. 6. Decreased expression of LAP and Foxp3 in activated T cells from patients with ACS. T cells were isolated and stimulated from control (n=20), SA (n=30) and ACS (n=40) patients. A. CD4⁺T cells were gated by the isotype control. Representative FACS images show LAP expression on stimulated CD4⁺ T cells. B. Representative FSC/SSC dot plot showing the gated CD4⁺CD25⁺T cells by the isotype control and representative FACS results showing Foxp3 expression in CD4⁺CD25⁺T cells from a single patient in each group after stimulation *in vitro*. C. Comparison of LAP and Foxp3 expression among the three groups. D. The gene expression Foxp3 was measured in each group. Error bars, SD; ** P < 0.01 vs. control or SA; ns, P > 0.05 vs. control.

the control group, the SA group or the ACS group (P=0.71, P=0.58, and P=0.24, respectively; Fig. 4E).

Assessment of the effect of HO-1 on gene expression in Th1, Th17 and Th2 cells in each group

Compared with cells left untreated, the Th1-associated T-bet and IFN- γ , and Th17-associated ROR γ t and IL-17 gene expression levels were markedly lower in the ACS group than in the SA and control groups (all P < 0.01; Fig. 5A-D). However, no obvious differences

Fig. 7. HO-1 significantly promoted the expression of LAP and Foxp3 in activated T cells from patients with ACS. T cells isolated from control (n=20), SA (n=30) and ACS (n=40) patients were incubated with 10 ng/ml hemin for 48 h and subsequently stimulated with anti-CD3 and anti-CD28 antibodies. A. CD4⁺T cells were gated by the isotype control. Representative FACS plots showing the LAP expression by CD4⁺T cells in each group. B. Representative FSC/SSC dot plot showing the gated CD4⁺CD25⁺T cells by the isotype control and representative FACS results showing Foxp3 expression in CD4⁺CD25⁺T cells from each group. C. The expression of LAP and Foxp3 were analyzed and compared to samples that were not treated with hemin. D. The gene expression of Foxp3 was measured and compared to samples that were not treated with hemin. Error bars, SD; **P < 0.01 compared to the no hemin group; ns, P > 0.05 compared to the no hemin group.

between the treated and untreated cells were observed in the T-bet, IFN- γ , ROR γ t and IL-17 gene expression levels between the SA (P=0.31, P=0.38, P=0.7, and P=0.32, respectively) and control (P=0.47, P=0.54, P=0.73, and P=0.56, respectively) groups. In addition, no significant attenuation of Th2-related GATA3 or IL-4 gene expression was observed in the control

Fig. 8. GARP⁺CD4⁺CD25⁺Treg cells were closely correlated with Foxp3 expression in CD4⁺CD25⁺T cells and LAP⁺CD4⁺T cells. After 24 h of stimulation, the untreated T cells from control (n=20), SA (n=30) and ACS (n=40) groups were used to analyze the relationships among GARP, LAP and Foxp3 expression. A. GARP⁺CD4⁺CD25⁺Treg levels positively correlated with LAP⁺CD4⁺T cells (r=0.65, P<0.01). B. GARP⁺CD4⁺CD25⁺Treg levels positively correlated with Foxp3 expression in CD4⁺CD25⁺T cells (r=0.77, P<0.01).

(P=0.66 and P=0.71, respectively), SA (P=0.53 and P=0.64, respectively), or ACS groups (P=0.35 and P=0.56, respectively) (Fig. 5E and F).

Attenuated expression of LAP and Foxp3 in stimulated T cells from the ACS group

The expression of LAP and Foxp3 in stimulated T cells from each group were detected by performing flow cytometric analysis. The expression of LAP on activated T cells and Foxp3 in activated CD4⁺CD25⁺T cells were clearly lower in the ACS group (3.74±1.36% and, 44.65±7.42%, respectively) than in patients with SA (8.06±1.63% and 77.55±8.96%, respectively) or control subjects (8.18±1.77% and 79.32±5.61%, respectively) (P<0.01; Fig. 6 A-C). However, no significant differences were found between the SA and control groups for the expressions of LAP and Foxp3 (P=0.61 and P=0.76, respectively). Similar results were observed for the expression of the Foxp3 gene (P<0.01; Fig. 6D).

Assessment of the effect of HO-1 on the expression of LAP and Foxp3 in activated T cells from patients with ACS

Following incubation with hemin, HO-1 promoted the expression of LAP and Foxp3 in the ACS groups (Fig. 7A-C). We found that T cells from the ACS group were more sensitive to HO-1 in terms of Foxp3 and LAP induction compared with cells from the other two groups (P<0.01) after stimulation *in vitro*. In comparison, no clear differences were observed in the SA and control groups for the expression of LAP (P=0.33 and P=0.46, respectively) and Foxp3 (P=0.52 and P=0.57, respectively) compared with cells left untreated. In addition, we confirmed these results by measuring the gene expression of Foxp3 after treating the T cells with hemin (P<0.01; Fig. 7D).

Spearman correlation analysis of the expression of Foxp3 and LAP with GARP

After 24 h of stimulation with soluble anti-human CD3 and anti-human CD28 antibodies, The untreated T cells from the control (n=20), SA (n=30) and ACS (n=40) groups were used to evaluate the relationships among GARP, LAP and Foxp3 expression using Spearman's correlation analysis. As shown in Fig. 8A, LAP⁺CD4⁺T cells showed a positive correlation with the frequencies of GARP⁺CD4⁺CD25⁺T cells (r=0.65, P<0.01). In addition, there was a positive correlation between the expression of GARP and Foxp3 in CD4⁺CD25⁺T cells (r=0.77, P<0.01; Fig. 8B).

Discussion

The pathogenic immune response typically results from the excessive expansion of effector T cells and has been shown to be responsible for plaque rupture and the subsequent onset of ACS [5, 9]. Tregs are indispensable for suppressing the pathological autoreactive immune response and maintaining immune homeostasis. In some rodent models, Tregs were shown to be atheroprotective and to play a crucial role in promoting plaque stability following the induction or adoptive transfer of Tregs [28, 29]. Tregs must be first activated by antigens before developing suppressive functions [30, 31]. Therefore, promoting the function of activated Tregs may help prevent the development and destabilization of atherosclerotic plaques. GARP is identified as a biomarker specific for activated human nTregs and is not induced in non-nTregs after T cell receptor (TCR) [20]. GARP⁺ CD4⁺ CD25⁺ Tregs can be used to distinguish activated Tregs. In the current study, we confirmed that the frequencies and function of GARP⁺ CD4⁺ CD25⁺ Tregs were compromised in patients with ACS. These GARP⁺ CD4⁺ CD25⁺ Tregs could not effectively control the excess proliferation of effector T cells, thus leading to a severe immune response in patients with ACS. HO-1 has been shown to interfere with the innate immune response through the regulation of innate immune cells. In the current study, we demonstrated the effects of HO-1 on adaptive immunity. In particular, HO-1 dramatically promoted the generation of GARP⁺ CD4⁺ CD25⁺ Tregs and enhanced their suppressive properties to decrease the pathogenic immune response in patients with ACS. These effects of HO-1 are likely achieved through the upregulated expression of GARP and LAP on activated human T cells.

Caligiuri indicated that one important reason for the pathogenic autoaggressive immune response in ACS is a flawed regulatory lymphocyte network [32]. After stimulation *in vitro*, we detected the number of CD4⁺ CD25⁺ T cells but found no significant differences in the frequencies of CD4⁺ CD25⁺ T cells between patients with ACS compared with patients with SA or with the control individuals. Next, we measured GARP expression on CD4⁺ CD25⁺ T cells and found that the expression of GARP was significantly lower in T cells isolated from ACS subjects than in SA or control subjects. In addition, our previous research had demonstrated that expression of GARP in freshly isolated CD4⁺ T cells from ACS patients significantly decreased too [14]. GARP is important for the expression of Foxp3 and for maintaining the suppressive function of activated Tregs [33]. Accordingly, our research further corroborated that the frequencies of GARP⁺ CD4⁺ CD25⁺ Tregs and the suppressor function of activated Tregs from ACS individuals were critically impaired compared to SA or control individuals. This evidence further indicated that the attenuated number of GARP⁺ CD4⁺ CD25⁺ Tregs was responsible for the impaired suppressive function and the destructive immune response characterized by the hyperproliferation of Th1 and Th17 cells in patients with ACS. The pro-inflammatory cytokines that are secreted by Th1 and Th17 destabilized the fibrous cap by promoting the activation of macrophages, inhibiting the proliferation, collagen synthesis and contractile protein expression of smooth muscle cells, and inhibiting the expression of scavenger receptor by macrophages. Together, these factors result in plaque rupture [34-36]. Our study showed that there were marked increases in the frequencies of Th1 and Th17 cells and in the relative gene expression of IFN- γ and IL-17 in patients with ACS. However, no significant differences were found between the SA and control groups. Thus, promoting GARP expression on CD4⁺ CD25⁺ T cells may serve as a potential therapeutic strategy for ACS.

HO-1 possesses antioxidant, antiapoptotic, and anti-inflammatory properties via the degradation of heme, and accumulating evidence also shows that the immunoregulatory function of HO-1 is due to its regulation of the function of Tregs [37, 38]. Choi et al. demonstrated that Treg function was inhibited via the pharmacologic blockade of HO-1 [39]. In addition, Xia et al. showed that HO-1 could regulate adaptive immunity by promoting the expression of Foxp3 in CD4⁺ CD25⁺ T cells and increasing the suppressor function of Tregs [27]. Our study found that the level of HO-1 mRNA was significantly lower in T cells from patients with ACS than in T cells from the SA and control groups. However, overexpression of HO-1 following heme treatment enhanced the number of GARP⁺ CD4⁺ CD25⁺ Tregs and restored

the suppressive property of activated Tregs from patients with ACS after stimulation *in vitro*. The increased frequencies and enhanced suppressive activity of GARP⁺ CD4⁺ CD25⁺ Tregs could effectively inhibit the hyperproliferation of Th1 cells to maintain immune homeostasis by direct cell-cell contact and the expression of anti-inflammatory cytokine TGF- β 1. HO-1 has been shown to suppress Th17 cell-mediated immune response in some animal models [40, 41]. Now, we further confirmed that HO-1 inhibited the Th17-dominant response in patients with ACS. The phosphorylation of STAT3, a key signaling molecule required for Foxp3 or ROR γ t expression, plays an important role in the process of Treg and Th17 cell differentiation. Zhang et al. discovered that HO-1 increased the Treg population and decreased the Th17 population by inhibiting the protein level of p-STAT3 [40]. Hahn demonstrated that Treg/Th17 differentiation was regulated by the GARP/TGF- β 1 complex under different inflammatory conditions [42] and Wilson indicated that TGF- β may actually suppress the differentiation of human Th17 cells [43]. We did not observe any clear effect of HO-1 on the cells from SA subjects. This may be due to the relative immune homeostasis in patients with SA. Because no significant differences were found between the SA and control groups. These results imply that HO-1 can effectively restore the impairments in Tregs from patients with ACS.

Although HO-1 enhances GARP expression and the suppressive function of activated Tregs, the mechanisms responsible for these effects remain elusive. The study by Xia et al. indicated that HO-1 induced the expression of membrane-bound TGF- β 1 as an important regulatory mechanism of augmenting Tregs cell function in an OVA-induced mouse asthmatic model [27]. TGF- β is secreted from T cells as a latent form in which LAP is non-covalently associated with mature TGF- β [44]. The expression of membrane-bound TGF- β can be assessed by detecting the levels of LAP on T cells. Therefore, we detected the expression of LAP on activated CD4⁺ cells in each group. Although there was a much lower LAP expression in patients with ACS, the expression of LAP was surprisingly increased following treatment with hemin. This effect of hemin on LAP may be ascribed to GARP and TGF- β . Indeed, Tran et al. indicated that GARP and LAP colocalized on the surface of activated Tregs and knockdown of GARP prevented surface LAP expression on activated Tregs [20]. Oida and co-workers demonstrated that the TGF- β induced the surface expression of LAP on murine CD4⁺ T cells [45]. In our study, patients with ACS showed low levels of TGF- β 1, GARP⁺ CD4⁺ CD25⁺ Tregs and LAP⁺ CD4⁺ T cells. Simultaneously, several studies have also shown that overexpression of HO-1 could affect TGF- β secretion [46].

Despite increased expression of LAP on activated CD4⁺ T cells, the non-covalent associations of TGF- β with LAP block the interaction with its receptor; this interaction is necessary for the function of TGF- β . To become active, mature TGF- β must be released from LAP. Stockis et al. documented that the membrane protein GARP was a receptor for LAP on the surface of activated human Tregs and showed that GARP directly interacted with LAP to release active TGF- β [22]. Our data showed that there was a positive correlation between LAP and GARP. Similarly, Wang and colleagues confirmed that GARP regulated the bioavailability and activation of TGF- β by binding to LAP [47]. Active TGF- β is a pleiotropic cytokine that regulates Foxp3 expression and the function of Tregs by inducing Smad2/3 phosphorylation and subsequent binding to the Foxp3 promoter [48, 49]. Our study found that there were lower levels of LAP and GARP on stimulated T cells from patients with ACS compared to patients with SA and the control subjects. These findings may account for the low expression of Foxp3 and the impaired suppressive function of activated Tregs from patients with ACS. In contrast, after increasing the expression of GARP and LAP with hemin treatment, we found that the expression of Foxp3 and the suppressive function of activated Tregs were enhanced. Probst-Kepper demonstrated that there was a positive feedback loop between GARP and Foxp3 and that up-regulation of Foxp3 can promote the expression of GARP in activated Tregs [33]. Our data showed that GARP is positively correlated with Foxp3 in CD4⁺ CD25⁺ T cells. Although we demonstrated a potential mechanism *in vitro*, no reliable atherosclerosis models are available to confirm these findings *in vivo*. Therefore, a transgenic mouse model is required to further study this hypothesis.

In conclusion, we further demonstrate that the reduced GARP expression and suppressive function of activated Tregs result in a mixed Th1/Th17-dominant T cell response in patients with ACS. In addition, our study is the first to illustrate that the immunoregulatory effect of HO-1 in ACS individuals is mediated by the upregulation of LAP and GARP expression on activated T cells. These findings will help us to more effectively explore therapies for ACS.

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