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Original Paper

Impairment of Circulating $CD4^+CD25^+GARP^+$ Regulatory T Cells in Patients with Acute Coronary Syndrome

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Key Words
Atherosclerosis • Regulatory T cells • GARP • Immune system • Acute coronary syndrome

Abstract
Background: Atherosclerosis (AS) is an inflammatory and immune disease. Regulatory T cells (Tregs) suppress the activation of T cells and have been shown to play a protective role during the pathogenesis of AS. However, specific markers for Tregs are lacking. Recently, glycoprotein A repetitions predominant (GARP) was discovered as a specific marker of activated Tregs, and we therefore utilized GARP as a specific surface marker for Tregs in the current study. ***Methods:*** To assess whether $GARP^+$ Tregs are downregulated in patients with acute coronary syndrome (ACS), we examined $CD4^+CD25^+GARP^+$ T cell frequencies as well as their associated cytokines and suppressive function. Additionally, we compared GARP expression to that of FOXP3, which may be more sensitive as a marker of activated Tregs in patients with ACS. ***Results:*** Patients with ACS demonstrated a significant decrease in circulating $CD4^+CD25^+GARP^+$ Tregs. Moreover, the suppressive function of Tregs and levels of related cytokines were also impaired in ACS patients compared to those with stable angina (SA) or normal coronary artery (NCA). Additionally, after TCR stimulation, peripheral blood mononuclear cells (PBMCs) from patients with ACS exhibited a decrease in $CD4^+CD25^+GARP^+$ Tregs. ***Conclusions:*** These finding indicate that circulating $CD4^+CD25^+GARP^+$ Tregs are impaired in patients withACS. Thus, targeting GARP may promote the protective function of Tregs in ACS.

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Introduction

Atherosclerosis is an inflammatory disease involving immunological dissonance [1-4]. Activated T cells, particularly Th1 and Th17 cells, have been associated with plaque stabilization [5-7], through the release of inflammatory cytokines that promote plaque rupture and induce acute coronary syndrome (ACS), including unstable angina (UA) and acute myocardial infarction (AMI) [8, 9].

Regulatory T cells (Tregs) maintain immunological tolerance and have been shown to play pivotal roles in protection against the progression of atherosclerosis [10, 11]. In particular, multiple groups have reported that natural Tregs are inhibitors of atherosclerosis [12-14], and these $CD4^+CD25^+$ natural Tregs were also found to be downregulated in patients with ACS [15].

The transcription factor Foxp3 is fundamental for the function and development of Tregs [16]. However, recent observations have questioned FOXP3 as a bona fide marker of human Tregs and have provided evidence that additional markers are required to identify cells with a regulatory phenotype [17-20]. Many additional molecules, such as CD25, glucocorticoid-induced TNFR related protein (GITR), and cytotoxic T lymphocyte antigen 4 (CTLA-4), have been proposed as markers for Tregs, although these molecules are also expressed during T cell activation or differentiation [21]. Recently, glycoprotein-A repetitions predominant (GARP or LRRC32), a transmembrane protein containing leucine-rich repeats, was identified as specific marker of activated human Tregs [22-24].

Several studies have shown that GARP controls FOXP3 expression through a positive feedback loop that is involved in maintenance of the phenotype and function of activated Tregs [20, 25]. In this study, we examined the frequencies and function of $CD4^+CD25^+GARP^+$ Tregs in different groups of patients. Using GARP as a novel marker of circulating activated human Tregs, our results are the first to show that these cells are impaired in ACS patients.

Materials and Methods

Patients

We recruited 154 patients between September 2011 and September 2012 at the Union Hospital, Wuhan, Hubei province, China. These patients were classified into 3 groups: (1) Stable angina pectoris (SA) (24 men and 20 women, mean age 57±6 years); (2) Acute coronary syndrome (ACS) (39 men and 28 women, mean age 58±8 years); and (3) Normal coronary artery (NCA) (25 men and 18 women, mean age 58±7 years). All NCA patients demonstrated normal coronary arteries, as determined by angiography, and had no clinical or electrocardiographic signs of CAD.

Inclusion criteria

The inclusion criteria for SA consisted of typical exertion chest discomfort that was associated with down-sloping or horizontal ST-segment depression >1 mm in an exercise test.

The inclusion criteria for ACS included chest pain at rest with definite ischemic electrocardiographic changes (ST-segment changes and/or T-wave inversions). The patients were diagnosed with myocardial infarction according to a rise and/or fall in cardiac biomarker values (preferably troponin) at least one value above the 99th percentile of the upper reference limit and at least one of the following criteria: symptoms of ischemia; new or presumably new significant ST-T changes or new LBBB; the development of pathological Q waves in the ECG; imaging evidence of a new loss of viable myocardium or new regional wall motion abnormality; or the identification of an intracoronary thrombus by angiography.

Patients recruited to the control group included those undergoing coronary angiography for indications other than CAD symptoms, who had no electrocardiographic signs of CAD, and those with normal coronary arteries on angiography.

Exclusion criteria

Patients with rheumatic heart disease, valvular heart disease, receiving treatment with anti-inflammatory drugs, connective tissue disease, advanced liver disease, renal failure, or malignancies were excluded from the study.

Ethical considerations

Patients and controls provided written informed consent. Additionally, this investigation conformed to the principles outlined in the Declaration of Helsinki and was approved by the ethics committee of the Tongji Medical College of Huazhong University of Science and Technology.

Blood samples

Peripheral blood samples were obtained from patients by sterile venipuncture using a 21-gauge needle at 7AM on the day of admission. The samples were collected into collection tubes containing 68 USP units of lithium heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and then used for cell culture, flow cytometric analysis, and real time-polymerase chain reaction (PCR). After centrifugation, the serum was collected, aliquoted, and frozen at -80°C for determination of cytokines levels.

PBMC culture and short-term TCR stimulations

PBMCs were washed twice in serum-free RPMI-1640 medium (ATCC modification A1049101 Gibco) and then resuspended at a density of 2×10^6 cells/ml in RPMI-1640 medium, which was supplemented with 10% heat-inactivated fetal calf serum (Gibco), 600 μg/ml glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. The cell suspension was seeded in 24-well culture plates. For GARP induction, cells were stimulated with soluble anti-CD3 and anti-CD28 antibodies (2 μg/ml each) (eBioscience) for 24 hours. The incubator was set to 37°C with a 5% CO_2 environment. After 24 hours of culture, the contents of each well were transferred to 5-ml sterile tubes. The cells were then centrifuged at 1,500×g for 5 minutes.

Flow cytometric analysis

PBMCs were aliquoted into tubes and washed twice in phosphate- buffered saline (PBS). The cells were then stained with the relevant antibodies (Abs) in PBS, containing 2% FCS and 0.1% sodium azide at 4°C for 30 min. These samples were then washed twice and analyzed by flow cytometry using a FACS Calibur (BD Biosciences). The following Abs were used for surface staining: fluorescein isothiocyanate (FITC) anti-human CD4 (eBioscience), PerCP/Cy5.5 anti-human CD25 (eBioscience), and phycoerythrin (PE) anti-human GARP (Alexis Biochemicals).

For FOXP3 intracellular staining, cells were first stained with surface markers, as described above. Then, the cells were washed and resuspended in 1×fixation/permeabilization buffer according to the manufacturer's instructions, followed by two washes in 1× permeabilization buffer. The cells were then stained with APC anti-human Foxp3 Ab (eBioscience), followed by two washes and subsequent analysis by flow cytometry using a FACS Calibur (BD Immunocytometry Systems). All data were analyzed using the FlowJo7.6.1 software program (Treestar Inc).

Proliferation and suppression assays

$CD4^+$ T cells were purified from PBMCs using a $CD4^+$ T cell positive isolation Kit II (Miltenyi Biotec, Gladbach, Germany). These purified $CD4^+$ T cells were then stained with anti-human CD25-PerCP/Cy5.5 and anti-human GARP-PE for 30 min at 4°C. After surface staining, responder T cells (Tresps; $CD4^+CD25^{int/low}GARP^-$T cells) and $CD4^+CD25^{high}GARP^+$ Tregs were obtained by FACS sorting using a FACS Aria (BD Biosciences). The purity of $CD4^+CD25^{int/low}GARP^-$T cells was >95%, and the purity of $CD4^+CD25^{high}GARP^+$ Tregs was >93%. We next divided the Tregs and Tresps into three groups: 1) $CD4^+CD25^{high}GARP^+$ Tregs (1×10^4 cells/well) cultured alone. After three days of culture, we collected the supernatants of all three groups to detect the anti-inflammatory cytokines IL-10 and TGF-β1. 2) $CD4^+CD25^{int/low}GARP^-$ T cells (1×10^4 cells/well) cultured alone; and 3) $CD4^+CD25^{high}GARP^+$ Tregs and $CD4^+CD25^{int/low}GARP^-$ T cells co-cultured at different ratios (Tregs/Tresps ratios: 1:1, 1:2, 1:4 and 1:8). Specifically, the T cells were incubated in complete RPMI-1640, as abovementioned, and cultured at 37°C with plate-bound anti-CD3 (5 μg/ml, eBioscience) and soluble anti-CD28 (2 μg/ml, eBioscience) in 5% CO_2 for 72 h in U-bottom 96-well plates.

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Table 1. Real-Time PCR Primer Sequences

Molecule	Sequence (5'- 3')
GARP sense	CACCAAGACAAAGTGCCCTG
GARP antisense	CGAAGTGCTGTGTAGAAGCC
FOXP3 sense	CCCGGATGTGAGAAGGTCTT
FOXP3 antisense	CTTGTCGGATGATGCCACAG
GAPDH sense	CCAGAACATCATCCCTGCCT
GAPDH antisense	CCTGCTTCACCACCTTCTTG

Table 2. Clinical data of patients in the NCA, SA and ACS groups. All values are expressed as the mean ± S.D. or the number or percentage of enrolled patients. NCA: normal coronary artery; SA: stable angina; ACS: acute coronary syndrome; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker

Characteristics	NCA n=43	SA n=44	ACS n=67	p
Age(years)	58±7	57±6	58±8	p=0.747
Sex(male/female)	25/18	24/20	39/28	p=0.919
Risk factors				
Hypertension, n (%)	17 (39.5%)	20 (45.5%)	38 (56.7%)	p=0.151
Diabetes, n (%)	13 (30.2%)	18 (40.9%)	32 (47.8%)	p=0.189
Hyperlipidaemia, n (%)	16 (37.2%)	22 (50.0 %)	39 (58.2%)	p=0.070
Tobacco, n (%)	16 (37.2%)	19 (43.2%)	29 (43.3%)	p=0.676
Obesity, n (%)	12 (27.9%)	15 (34.1%)	32 (47.8%)	p=0.072
Medications				
Aspirin, n (%)	32 (74.4%)	35 (79.5%)	57 (85.1%)	p=0.273
ACEI/ARB, n (%)	15 (34.9%)	23 (52.3%)	42 (62.7%)	p=0.011
Beta-blockers, n (%)	14 (32.6%)	21 (47.7%)	35 (52.2%)	p=0.083
Calcium blockers, n (%)	11 (25.6%)	12 (27.3%)	25 (37.3%)	p=0.032
Nitrates, n (%)	3 (7.0%)	17 (38.6%)	40 (59.7%)	p<0.001
Statins, n (%)	16 (37.2%)	32 (72.7%)	55 (82.1%)	p<0.001

All cells were cultured in a final volume of 200 µl. [3H]-thymidine (1 µl, Amersham Biosciences) was added to each well 16 h prior to harvest, and the incorporation of [3H]-thymidine was assayed by scintillation counting (PerkinElmer).

Real-time Polymerase Chain Reaction

Total RNA was extracted using RNAiso Plus (code: D9108A;Takara) from 2×10^6 PBMCs and reverse transcribed to cDNA using an RNA PCR Kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. The expression of target genes was quantified using SYBR Green Master Mix (Takara, Japan) with an ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA). All reactions were performed in at least duplicate for each sample. Primer pairs were designed using the Primer 3.0 software and were synthesized by Invitrogen in Shanghai (Table 1).

The relative mRNA expression level was calculated using the comparative CT formula $2^{-\Delta\Delta CT}$. The results were normalized to GAPDH.

Cytokine Assays

The levels of biologically active form of TGF-β1 and IL-10 in patient plasma and $CD4^+CD25^{high}GARP^+$ Tregs culture supernatant were measured using an enzyme-linked immunosorbent assay (ELISA) (eBioscience) according to the manufacturer's instructions. The minimal detectable concentrations were 8.6 pg/ml for TGF-β 1 and 2 pg/ml for IL-10. The intra-assay and inter-assay variation coefficients for all ELISAs were <10%. All samples were measured in duplicate.

Statistical Analysis

All variance data were expressed as the mean ± standard deviation (SD) and analyzed by ANOVA. When significance was found, the Newman–Keuls test was performed for post-hoc analysis to detect differences among groups. For ranked data, Pearson's chi square test or Fisher's exact test was performed for comparisons between groups. Spearman's correlation analysis was used to detect any correlation between the variables. GraphPad Prism 6.0 and SPSS 17.0 software were used for data analysis. Invariably, two-tailed p values <0.05 were considered statistically significant.

Results

Basic Clinical Characteristics

The basic clinical characteristics of the patients are summarized in Table 2. There were no significant differences in age, gender, hyperlipidemia, diabetes, hypertension, or smoking

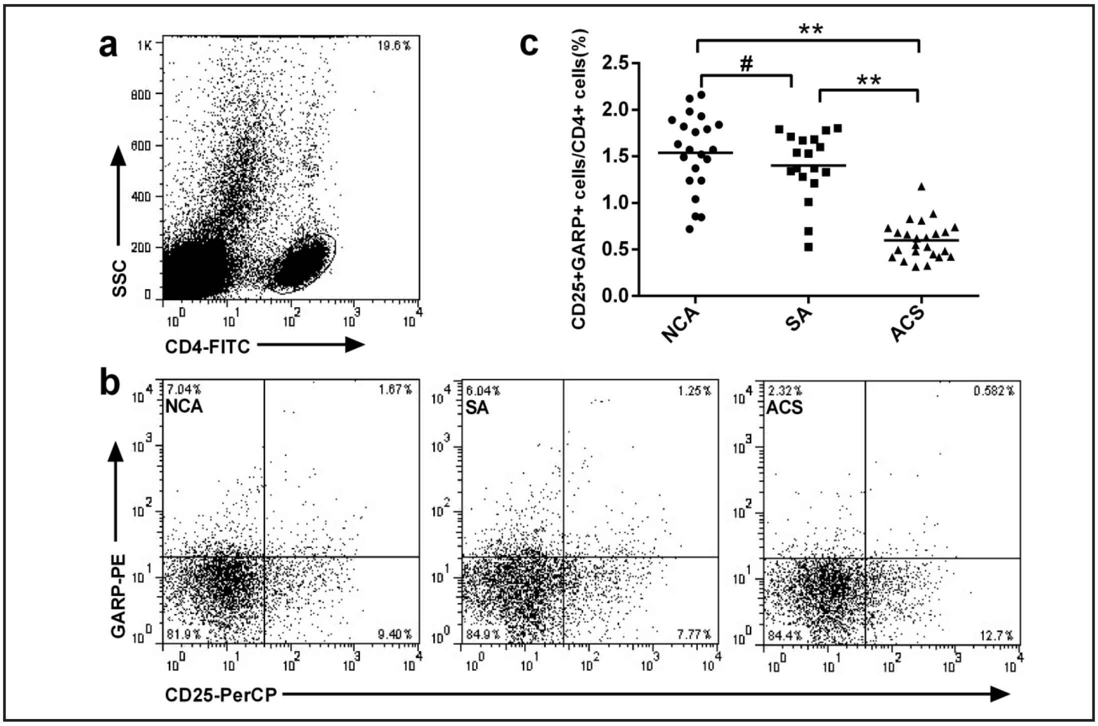


Fig. 1. The circulating CD4⁺CD25⁺GARP⁺ Treg frequency is decreased in patients with ACS. a. Representative FSC/SSC dot plot shows the gated CD4⁺ T cells. b. Representative FACS analyses of single patients from each group indicate the frequencies of CD4⁺CD25⁺GARP⁺ Tregs. c. Comparison of the results from all 3 groups (NCA: n=22; SA: n=18, ACS: n=24). *p<0.05 vs. SA; **p<0.01 vs. NCA; #p>0.05 vs. NCA.

between the three groups. Other pre-medications are also listed in Table 2. In particular, we compared the CD4⁺CD25⁺GARP⁺ Treg levels between patients with and without pre-medication with angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, stains, nitrates, and calcium blockers, and these factors did not influence the level of CD4⁺CD25⁺GARP⁺ Tregs (data not shown).

The circulating CD4+CD25+GARP+ Tregs frequencies are decreased in ACS patients

We measured the frequency of circulating CD4⁺CD25⁺GARP⁺ Tregs using flow cytometry (Fig. 1a-b). As shown in Fig. 1c, we found that the frequencies of CD4⁺CD25⁺GARP⁺ Tregs were significantly lower in the ACS group (0.60±0.20%) compared to the NCA (1.54±0.41%) and SA (1.42±0.39%) groups ($p<0.01$), whereas there was no distinct difference between the SA and NCA groups.

The frequencies of stimulated CD4+CD25+GARP+ Tregs and CD4+CD25+GARP+FOXP3+ Tregs are decreased in ACS patients

We next measured the frequency of activated Tregs after 24 hours of TCR stimulation. For all of the groups examined, the frequency of CD4⁺CD25⁺ T cells was significantly higher than that observed during rest conditions (Fig. 2a-b); however, there was no significant difference among the three groups ($p=0.84$) (Fig. 2d). To investigate the sensitivity of different molecules to mark activated Tregs in patients with ACS, we stained for FOXP3 and GARP in CD4⁺CD25⁺Tregs after the TCR stimulation (Fig. 2c). Interestingly, upon analyzing the frequency of CD4⁺CD25⁺FOXP3⁺ Tregs, there was no significant difference among the three groups ($p=0.46$) (Fig. 2e). However, the frequency of activated CD4⁺CD25⁺GARP⁺ Tregs (GARP⁺/CD4⁺CD25⁺ T cells) was decreased in the ACS group (5.99±1.55%) compared to the NCA (9.63±2.13%) and SA (8.94±1.49%) groups ($p<0.01$), whereas there was no significant difference between the NCA and SA groups ($p=0.52$). As shown in Fig. 2g, we also found

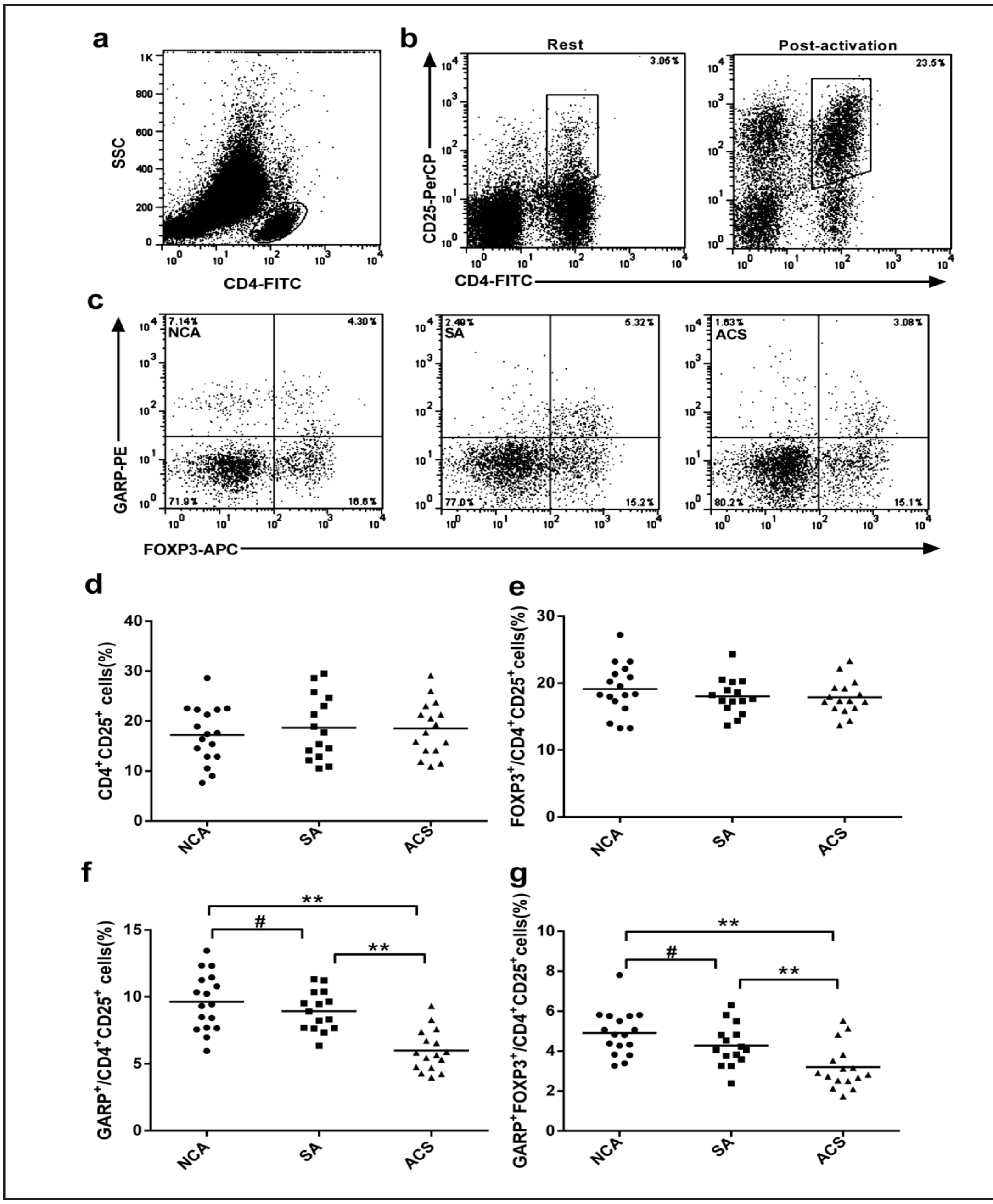


Fig. 2. The frequencies of stimulated $CD4^+CD25^+GARP^+$ Tregs and $CD4^+CD25^+Foxp3^+GARP^+$ Tregs are decreased in ACS patients. PBMCs were freshly isolated (NCA: n=17; SA: n=15; ACS: n=16) and stimulated with CD3/CD28 antibodies for 24 hours. The cells were then stained with four types of antibodies and analyzed using flow cytometry (BD). a. Representative FSC/SSC dot plot shows the gated $CD4^+$ T cells. b. The post-stimulation $CD4^+CD25^+$ T cells were expanded. c. Representative FACS analyses of single patients from each group show the frequencies of $GARP^+$ T cells (numbers in the upper quadrants), $FOXP3^+$ T cells (numbers in the right quadrants), and $GARP^+FOXP3^+$ T cells (numbers in the upper right quadrants) gated on expanded $CD4^+CD25^+$ T cells. d. Comparison of stimulated $CD4^+CD25^+/CD4^+$T cell frequencies among the three groups. e. The percentages of $FOXP3^+/CD4^+CD25^+$ T cells, based on FACS analyses, were comparable among the NCA, SA, and ACS groups. f. The percentages of $GARP^+/CD4^+CD25^+$ T cells, based on FACS analyses, were comparable among the NCA, SA, and ACS groups. g. The percentages of $GARP^+FOXP3^+/CD4^+CD25^+$T cells, based on FACS analyses, were comparable among the NCA, SA, and ACS groups. *$p<0.05$ vs. SA; **$p<0.01$ vs. NCA; #$p>0.05$ vs. NCA.

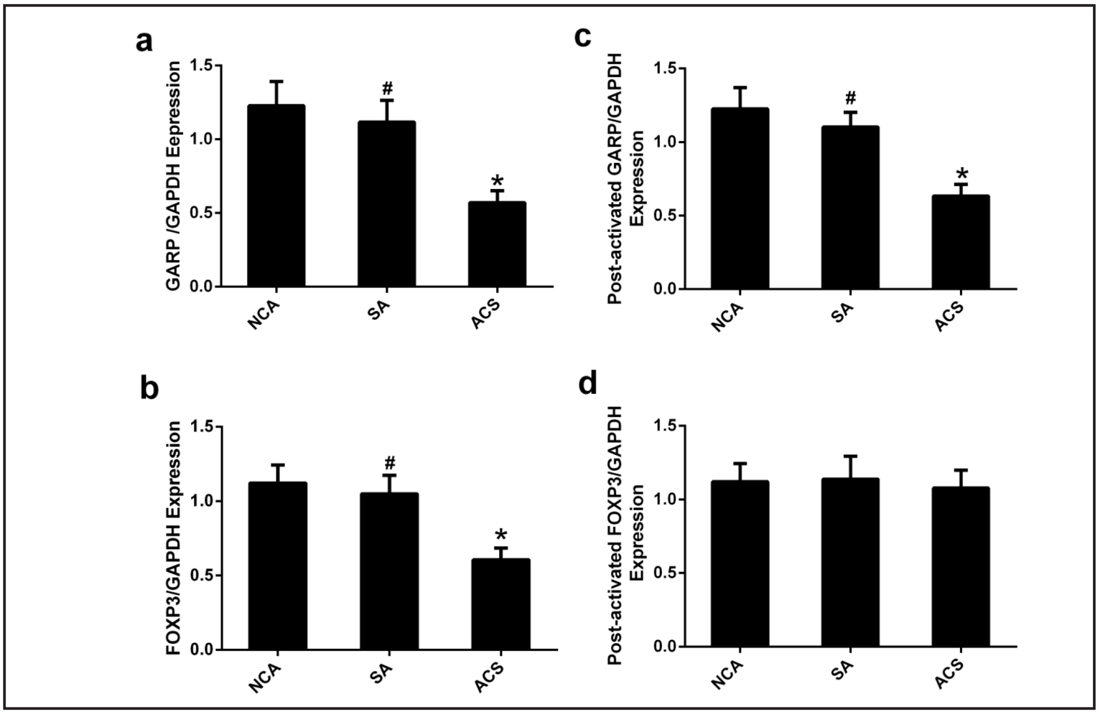


Fig. 3. Relative mRNA expression of GARP and FOXP3 in unstimulated and stimulated PBMCs of patients (NCA: n=22; SA: n=21; ACS: n=22). a and b show the expression of unstimulated PMBCs. c and d show the expression of the simulated PBMCs. *p<0.05 vs. NCA and SA. #p>0.05 vs. NCA.

that the frequency of $CD4^+CD25^+FOXP3^+GARP^+$ Tregs ($FOXP3^+GARP^+/CD4^+CD25^+$ T cells) was markedly lower in the ACS group (3.20±1.11%) compared to the NCA (4.91±1.23%) and SA (4.28±1.04%) groups ($p<0.01$), whereas there was no significant difference between NCA and SA groups ($p=0.53$).

Gene expression levels of GARP and Foxp3 in unstimulated and stimulated PBMCs from patients with ACS

We next measured the levels of GARP and FOXP3 mRNA in unstimulated and stimulated PBMCs by RT-PCR. In freshly isolated PBMCs, the expression of GARP was significantly decreased in the ACS group (0.57±0.29) compared to the SA (1.11±0.56) and NCA (1.22±0.62) groups ($p<0.05$) (Fig. 3a). Similar to the findings of previous studies, the expression of FOXP3 was decreased in the ACS group (0.61±0.37) compared to the NCA (1.12±0.56) and SA (1.05±0.57) groups ($p<0.05$) (Fig. 3b). There was no significant difference in FOXP3 or GARP expression in cells obtained from NCA and SA patients (Fig. 3a and 3b). In stimulated PBMCs, the expression of GARP was decreased in ACS patients (0.63±0.36) compared to NCA (1.26±0.65) and SA (1.10±0.45) patients ($p<0.05$) (Fig. 3c); however, there was no difference in the expression of FOXP3 among the three groups ($p=0.95$) (Fig. 3d).

The function of CD4+CD25highGARP+ Tregs is compromised in patients with ACS

We next isolated $CD4^+$ T cells from PBMCs, and the purity of the $CD4^+$ T cells was >95%, as determined by FACS (Fig. 4a). We then isolated $CD4^+CD25^{high}GARP^+$ Tregs and $CD4^+CD25^{int/low}GARP^-$ Tresps by FACS sorting using a FACS Aria (Fig. 4b). Following TCR stimulation, $CD4^+CD25^{int/low}GARP^-$ Tresps from all three groups exhibited similar rates of proliferation, whereas the proliferation of $CD4^+CD25^{high}GARP^+$ Tregs was minimal, which suggested that these cells were anergic to TCR stimulation (Fig. 4c). Then, the ability of $CD4^+CD25^{high}GARP^+$ Tregs to inhibit the proliferation of $CD4^+CD25^{int/low}GARP^-$ Tresps was determined using a [3H]-thymidine incorporation assay in co-cultures of Tregs and Tresps in different ratios (1:1, 1:2, 1:4, and 1:8). This result demonstrated that $CD4^+CD25^{high}GARP^+$ Tregs from ACS

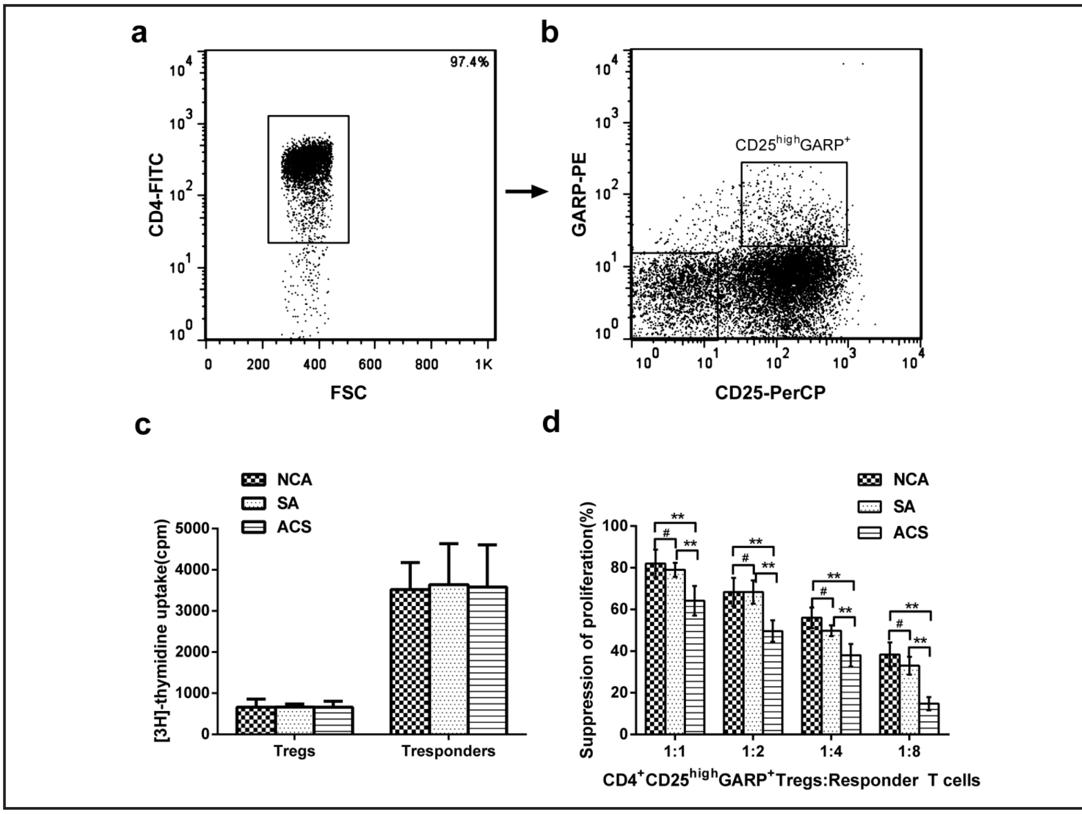


Fig. 4. $CD4^+CD25^{high}GARP^+$Tregs from patients with ACS had an impaired capacity to suppress responder T cell proliferation. a. The identification of the purity of the $CD4^+$ T cells. b. Induction and sorting of $GARP^+$ T cells. $CD4^+$T cells were stimulated through TCR for 48 hours and stained for GARP and CD25. c. Purified $CD4^+CD25^{high}GARP^+$Tregs and responder T cells ($CD4^+CD25^{int/low}GARP^-$Tresps) were assayed comparatively for their proliferative capacity between the three groups. d. Reduced suppressive function of $CD4^+CD25^{high}GARP^+$ Tregs from patients with ACS, as indicated by suppression assay. **P<0.01 vs. NCA; *p<0.05 vs. SA; #p>0.05 vs. NCA.

patients exhibited a reduced capacity to suppress the proliferation of Tresps at all ratios compared to cells from the NCA and SA groups (p<0.01) (Fig. 4d). We also assessed the TGF-β1 and IL-10 levels in the supernatants of cultured $CD4^+CD25^{high}GARP^+$ Tregs. The result indicated that TGF-β1 levels were reduced in the ACS group (9.96±1.77 ng/ml) compared with SA (17.36±3.80 ng/ml) and NCA (18.6±5.91 ng/ml) groups (p<0.01) (Fig. 5a), although there were no differences in IL-10 levels among the three groups (p=0.85) (Fig. 5b).

Serum levels of TGF-β1 are decreased in patients with ACS

As shown in Fig. 5c, the TGF-β1 levels were decreased in patients with ACS (11.93±3.56 pg/ml) compared to the SA (16.20±4.65 pg/ml) and NCA (17.11±5.02 pg/ml) patients (p<0.01). In contrast, there was no difference in IL-10 levels among the three groups (NCA, 27.44±7.40 pg/ml l; SA, 28.94±6.50 pg/ml and ACS; 28.02±7.24 pg/ml) (p=0.79) (Fig. 5d). In addition, as shown in Fig. 6, the level of TGF-β1 showed a positive correlation with the frequency of circulating $CD4^+CD25^+GARP^+$ Tregs (r=0.592, p<0.01) in the three groups.

Discussion

Atherosclerosis is an inflammatory and immune-mediated disease [2, 4]. Regulatory T cells represent a unique type of T cell that can suppress inflammatory responses,

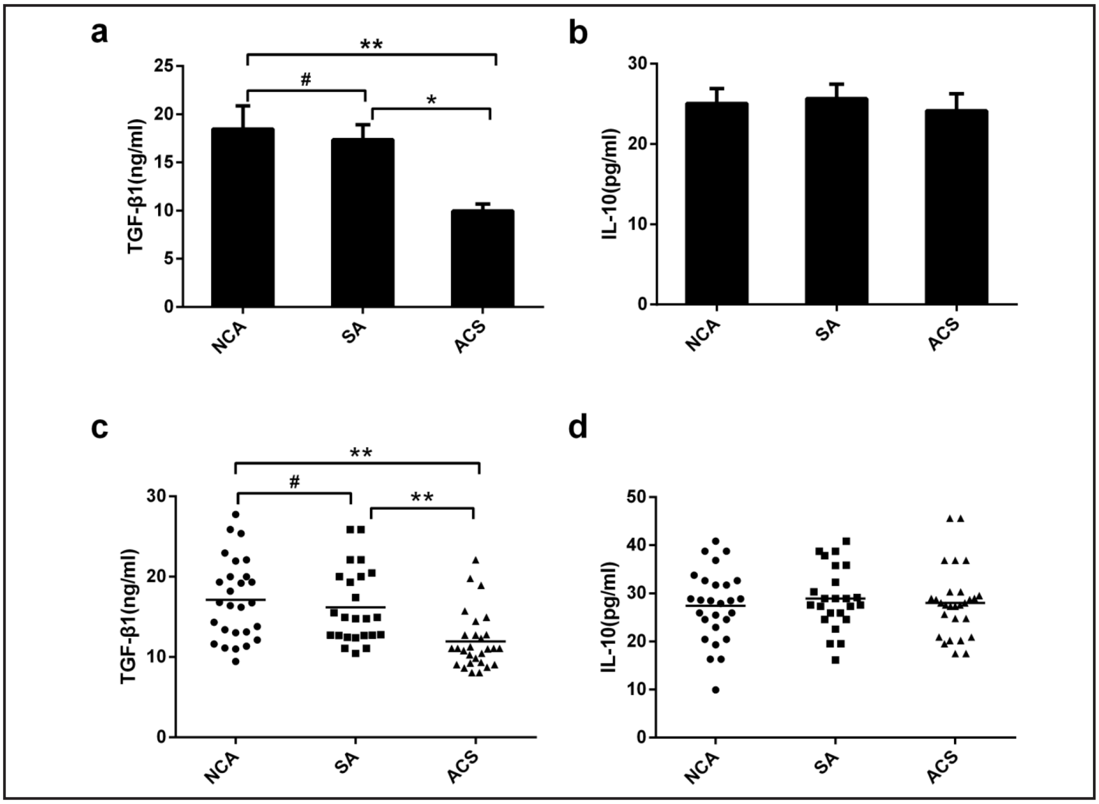
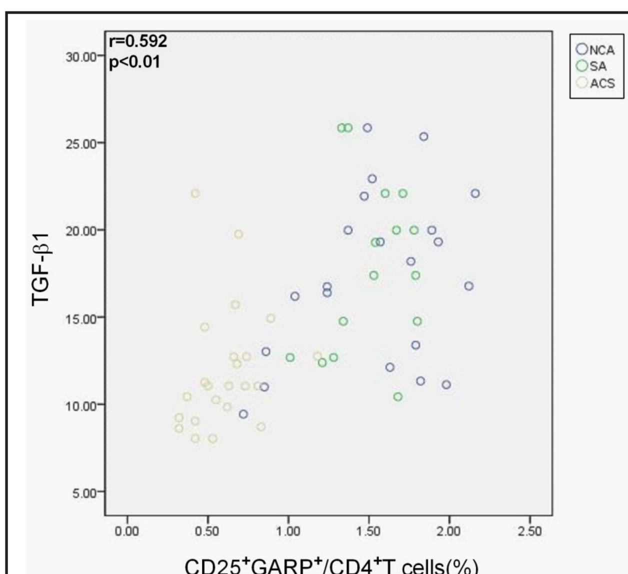


Fig. 5. TGF-β1 and IL-10 secretion in supernatants of the cultured CD4+CD25highGARP+ Tregs and human plasma (NCA: n=27; SA: n=24; ACS: n=28) among the three groups. a. levels of TGF-β1 in the supernatants of the cultured CD4+CD25highGARP+ Tregs. b. levels of IL-10 in the supernatants of the cultured CD4+CD25highGARP+ Tregs. c. The levels of TGF-β1 were decreased in the ACS group serum compared with that in SA and NCA groups. d. The levels of IL-10 had no significant difference among three groups. **P<0.01 vs. NCA; * p<0.05 vs. SA or NCA; # p>0.05 vs. NCA.

Fig. 6. Spearman correlation of human circulating CD4+CD25+GARP+ Tregs and TGF-β1 of serum. TGF-β1 concentrations positively correlate with a proportion of CD4+CD25+GARP+ /CD4+ T cells (r=0.592, p<0.01).



counterbalance plaque formation, and play a pivotal role in inhibiting atherosclerosis initiation and progression. Previous studies have demonstrated that CD4+CD25+FOXP3+ Tregs are downregulated in patients with coronary artery disease (CAD) [8, 9, 15, 26].

However, few studies have focused on identifying specific markers of activated Tregs in patients with CAD. Recently, GARP was identified as a novel marker of activated Tregs [23, 25, 27]. Therefore, in the current study, we utilized GARP as an activated Treg marker and hypothesized that the frequency and function of $GARP^+$ Tregs may be impaired in patients with ACS.

Using freshly isolated $CD4^+CD25^+GARP^+$ Tregs from patients with ACS and SA, we compared the frequency of $CD4^+CD25^+GARP^+$ Tregs to that observed in the control groups (NCA). As expected, the frequency of $CD4^+CD25^+GARP^+$ Tregs was significantly lower in patients with ACS compared to the SA and NCA groups, which indicated that the activation of Tregs was impaired in vivo. Previous studies have shown that siRNA-mediated downregulation of GARP in Tregs could substantially impair FOXP3 expression and their suppressive function [28]. We also found that the gene expression levels of FOXP3 and GARP among patient PBMCs were reduced compared to the control group. Thus, our results support previous findings indicating that circulating $CD4^+CD25^+FOXP3^+$ Tregs are reduced in patients with ACS [8, 9, 15, 26].

Wang et al. reported that GARP is more reliable than FOXP3 in characterizing activated Tregs in chronic inflammatory diseases, such as HIV [29]. Thus we sought to investigate which of these two markers would be a more sensitive marker of Treg activation during ACS. We isolated PBMCs, and after 24 hours of TCR stimulation, we detected the frequencies of $CD4^+CD25^+FOXP^+$ Tregs, $CD4^+CD25^+GARP^+$ Tregs and $CD4^+CD25^+GARP^+FOXP^+$ Tregs from different patients. As predicted, the frequency of $CD4^+CD25^+GARP^+FOXP^+$ Tregs in the ACS group was much lower than that observed in the NCA and SA groups. The frequency of $CD4^+CD25^+GARP^+$ Tregs was also lower in the ACS groups compared to the NCA and SA groups. Interestingly, however, there was no difference in the frequency of $CD4^+CD25^+FOXP3^+$ Tregs after TCR stimulation among the three groups. Thus, in ACS patients, GARP seems to be a more sensitive marker of Treg activation than FOXP3.

To assess the function of $GARP^+$ Tregs in patients among the three groups, we co-cultured $CD4^+CD25^{high}GARP^+$ Tregs and $CD4^+CD25^{int/low}GARP^-$ Tresps at different ratios, and the results showed that $CD4^+CD25^{high}GARP^+$ Tregs from the ACS group displayed a reduced capacity to suppress the proliferation of Tresps relative to that observed for the SA and NCA groups. Thus, our results suggested that natural Tregs as well as activated Tregs were impaired in patients with ACS.

GARP is a cell surface receptor for latency-associated peptide (LAP), which is a linker pro-peptide for the activated form of TGF-β [23]. Our group also found that the percentages of $CD4^+LAP^+GARP^+$ T cells were reduced in ACS patients compared to controls (unpublished data). Wang et al. previously demonstrated that the expression of the LAP-TGF-β complex, when bound to GARP, could induce the expression of FOXP3 through TGF-β receptors signaling [29]. Moreover, previously work has demonstrated an imbalance between Tregs and Th17 cells during the progression of atherosclerosis [8], and recent reports have indicated that GARP/LAP complexes can adjust the balance between Tregs and Th17 cells [29]. Therefore, we hypothesize that impaired $CD4^+GARP^+LAP^+$ T cells from ACS patients may lead to this imbalance in the Treg/Th17 ratio, which may then intensify plaque ruptures.

$CD4^+CD25^+GARP^+$ T cells represent activated Tregs with suppressive activity, as these cells secrete both TGF-β1 and IL-10 in vitro. In addition, GARP can regulate the bioavailability and activation of TGF-β1 [30]. Our findings showed that the serum TGF-β1 levels of ACS patients were lower than those observed in NCA and SA patients. Moreover, this difference was positively correlated with the frequency of $CD4^+CD25^+GARP^+$ Tregs, although there was no significant difference in IL-10 levels. These data are similar to those reported in previous studies [26, 31] and may be explained by the finding that LAP (a precursor of TGF-β1) forms complexes with GARP; in particular, these complexes are downregulated in ACS [our unpublished data] and may therefore lead to a reduction in the level of TGF-β1.

Interestingly, Hahn et al. demonstrated that soluble GARP under inflammatory conditions (together with IL-6 and IL-23) could promote Th17 differentiation; however, GARP also cooperates with TGF-β1 to induce Treg differentiation. Moreover, GARP, which is considered a safeguard of the regulatory phenotype, is part of a positive feedback loop that involves FOXP3 and can adjust Treg and Th17 cell differentiation under distinct inflammatory conditions [32]. Therefore, GARP is more than a maker of activated Tregs; as a double-edged sword, this molecule likely function to adjust the imbalance of Treg and Th17 cells during atherosclerosis. Although animal research has examined TGF-β1/GARP complexes, which regulate Treg and Th17 cell differentiation [33, 34], few studies have employed disease models, particularly atherosclerosis models. Therefore, future research should more carefully address this potential mechanism.

In conclusion, our study is the first to use GARP as a surface marker of Tregs in patients with ACS, and our results demonstrate that the frequency of circulating $CD4^+CD25^+GARP^+$ Tregs is decreased and that their suppressive functions are impaired in patients with ACS. These findings may provide new targets for atherosclerosis therapy, such as the use of soluble GARP as an anti-inflammatory immunosuppressive drug [32], as well as methods aimed at correcting the imbalanced immune response to stabilize plaques.

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