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*Original Article*

# Methylation of the FOXP3 upstream enhancer as a clinical indicator of defective regulatory T cells in patients with acute coronary syndrome

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**Abstract:** Atherosclerosis is an immune-mediated inflammatory process, which acts as the main cause of acute coronary syndrome (ACS). Regulatory CD4+CD25+FOXP3+T cells (Tregs) are thought to play a major role in inhibiting the formation and progression of atherosclerosis. However, the exact role played by Tregs in the pathogenesis of ACS is yet remained unclear. FOXP3 is a key regulator of Treg formation and function. Demethylation at the CpG-rich island of FOXP3 upstream enhancers can alter FOXP3 expression, and may affect Treg function during the development of ACS. This study investigated the immunosuppressive function and methylation status of a FOXP3 upstream enhancer in Tregs in ACS patients. Notably, Tregs from ACS patients exhibited a significantly lower immunosuppressive effect on Teffs. Furthermore, the methylation status of the FOXP3 upstream enhancer was significantly increased in ACS patients. Consistent with these observations, Tregs originated from ACS patients manifested significantly lower levels of FOXP3 mRNA. The immunosuppressive effect of Tregs on Teffs was compromised in ACS patients. Together, our data suggest that examination of the methylation status of the FOXP3 upstream enhancer might be a novel approach to diagnose ACS and to differentiate ACS subtypes.

**Keywords:** Acute coronary syndrome, regulatory T cells, effector T cells, forkhead box P3, DNA methylation, immunosuppressive function

## Introduction

Atherosclerosis is the pathologic basis of coronary artery disease (CAD) [1]. Acute coronary syndrome (ACS), the most serious manifestation of CAD [2], is a multifactorial disease in which the immune mechanism plays a crucial role [3]. The majority of T cells found in atherosclerotic lesions are activated effector and/or memory $CD4^+$ T cells [4]. These effector T cells (Teffs) are associated with plaque destabilization and exert multiple pro-inflammatory effects by releasing effector cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor β (TNF-β) [5, 6], which contribute to the recruitment of T cells, plaque rupture, and the onset of ACS [7].

Regulatory CD4+CD25+FOXP3+T cells (Tregs) are specialized cells which suppress immune function [8, 9]. By restraining excessive immune responses and promoting self-tolerance, Tregs play an important role in protecting against the development and progression of atherosclerosis [10]. Thus, defective Tregs are thought to promote the progression of atherosclerosis, and even the onset of ACS. Recently, some investigators have suggested that a decrease in Tregs might contribute to the development and progression of ACS [11-13]. However, other studies have reported elevated Treg numbers in ACS patients [8, 14-16]. Beyond assessing their numbers, few studies have evaluated the function of Tregs in ACS, and some have reported conflicting results regarding the immunosuppressive effect of Tregs on Teffs [17-19].

The transcription factor forkhead box protein 3 (FOXP3) is specifically expressed in Tregs and indispensable for their immunosuppressive func-

**Table 1.** Demographic and clinical data for the ACS patients and control subjects

| | Control | ACS | P |
|---|---|---|---|
| No. of participants | 52 | 60 | |
| Age (years) | 58.62 ± 12.07 | 60.28 ± 11.11 | 0.211 |
| Risk factors, % | | | |
| Hypertension | 36.5 | 50 | 0.149 |
| Hypercholesteremia | 34.6 | 46.8 | 0.189 |
| Diabetes | 30.8 | 41.9 | 0.218 |
| Smoking | 36.5 | 48.4 | 0.203 |
| Family history | 42.3 | 46.8 | 0.633 |

tion [20-22]. FOXP3 is a key regulator of Treg formation and function, and its expression can be regulated by several epigenetically liable enhancers and promoters [23]. Additionally, in murine systems, a non-intronic upstream enhancer of FOXP3 contains an evolutionarily conserved CpG-rich island that remains non-methylated in Tregs [24]. Demethylation of the CpG-rich island not only alters FOXP3 expression, but also affects the immunosuppressive function of Tregs [25]. Furthermore, other study discovered a novel differentially methylated region (DMR) within a CpG-rich island positioned adjacent to the murine FOXP3 upstream enhancer homologue, which was demonstrated to have enhancer activity that was subject to methylation-induced silencing in vitro [24]. Additionally, an epigenome-wide analysis identified several differentially methylated positions associated with the rheumatoid arthritis (RA) phenotype in monocytes [26]. However, no similar studies have been conducted with ACS Treg subsets. We thus investigated the methylation status, in which, we tested the immunosuppressive activity of Tregs on Teffs, and examined whether a FOXP3 upstream enhancer was epigenetically modified in patients with ACS. Our results suggest that methylation of the FOXP3 upstream enhancer is significantly increased in ACS patients.

## Materials and methods

### *Study population*

Peripheral blood was collected from 60 subjects admitted to the Cardiovascular Department of Tongji Hospital with a diagnosis of ACS and 52 healthy controls based on criteria in our previous studies [10]. All of the 60 enrolled ACS patients had angiographically confirmed coronary artery disease (≥ 50% stenosis), defined as typical chest pain at rest occurring < 48 hours from hospital admission or ECG changes suggesting myocardial ischemia with or without an increase in serum markers of myocardial damage. The exclusion criteria in the study included: (1) patients with an infectious or inflammatory disease; (2) patients with an autoimmune or hematological disease; (3) patients with liver or kidney dysfunction. This study was approved by the Medical Ethical Committee of Tongji Hospital (Wuhan, China). Written informed consent was obtained from each participant. The clinical features of the enrolled patients and healthy control subjects are shown in **Table 1**.

### *Isolation and identification of Tregs*

Tregs were enriched using a human Tregs Isolation Kit (Miltenyi Biotec; Bergisch Gladback, Germany) and a Mini-MACS separation system (Miltenyi Biotec, Germany). The isolated Tregs and Teffs were stained with either antibodies against anti-human CD4-FITC (RPA-T4) and CD25-APC (4E3) or CD127-PE (eBioscience; San Diego, CA, USA). As FOXP3 is a transcription factor which localizes to the nucleus, it cannot be used as a surface marker of Tregs. Interestingly, CD127 expression is inversely with FOXP3 expression and Treg suppressor activity. Thus, the best way at present to identify human Tregs appears to be co-expression of high levels of CD4 and CD25 as well as low levels of CD127 [27]. The purity of the isolated PBMCs was determined to be above 90% and the identities of the cell surface markers were analyzed by flow cytometry (FCM; BD Bioscience; Franklin Lakes, NJ, USA).

### *Detection of the treg suppression function by personal and crossover co-culture studies*

To compare the suppression function of Tregs obtained from normal subjects and ACS patients, personal Tregs obtained from ACS patients or control subjects were co-cultured with personal Teffs at different ratios (Tregs:Teffs = 0:8, 1:8, 1:4, 1:2, and 1:1). To further investigate differences in the suppression function of Tregs from the two groups of subjects, we

performed crossover co-culture studies at different ratios using Teffs obtained from control subjects with Tregs from ACS patients or control subjects, and Teffs obtained from ACS patients with Tregs from ACS patients or control subjects. The Tregs were titrated with Teffs at the following ratios (Tregs:Teffs = 1:8, 1:4, 1:2, 1:1), with the number of Teffs being held constant at $1.0 \times 10^5$ cells/mL. After 5 days of culture, the proliferation index (PI) of CFSE-labeled Teffs was measured by flow cytometry (BD Bioscience, USA). The Treg suppression ratio was calculated using the following formula: [(1 - (PI of Tregs:Teffs - x:8)/(PI of Tregs:Teffs - 0:8) × 100%].

*Proliferation/function assay of CFSE-labeled Teffs*

Purified CFSE-labeled Teffs ($1 \times 10^5$ cells/well) from ACS patients and normal control subjects were cultured in plates which contained medium with monoclonal antibodies (eBioscience) against CD3 (2 ug/mL)/CD28 (1 ug/mL), medium with CD3/CD28 plus an allo-stimulated antigen, and medium with CD3/CD28 plus an auto-stimulated antigen, for 72 hrs at 37°C with 5% $CO_2$. After culture, the proliferation index of the Teffs was measured by flow cytometry.

*Methylation level of the FOXP3 upstream enhancer*

Genomic DNA used in the DNA methylation assay was isolated from subsets of Tregs and Teffs obtained from ACS patients and healthy control subjects using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 (TaKaRa; Dalian, China). Bisulfile conversion of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen; Hilden, Germany). Real-time PCR primers were designed using Methyl Primer Express software. The degree of DNA methylation was assayed at each CpG dinucleotide locus in the relevant FOXP3 upstream enhancer regions, and expressed as the mean percent methylation.

*Real-time quantitative PCR (RT-PCR) of FOXP3 mRNA*

Total RNA was extracted from Tregs obtained from ACS patients and control subjects using TRI Reagent (Takara Biotechnology; Shiga, Japan), and reverse transcribed to cDNA using reverse transcriptase (Invitrogen; Karlsruhe, Germany). Real-time RT-PCR reactions were carried out in SYBR Green PCR Master Mix (TOYOBO; Osaka, Japan), and quantitative analysis of FOXP3 mRNA was performed using the StepOne Quantitative real-time PCR System (Applied Biosystems; Foster City, CA, USA). The data were normalized to the expression level of GAPDH. PCR reactions were 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 15 s. The primers used for FOXP3 were: sense 5'-TGACCAAGGCTTCATCTGTG-3' and antisense 5'-GAGGAACTCTGGGAATGTGC-3'. The β-actin primers were sense 5'-AGCGAGCATCCCCCAAAGTT-3' and antisense 5'-GGGCACGAAGGCTCATCATT-3'.

*Cytokine measurements*

The levels of cytokines IL-10, IFN-γ, and TGF-β1 in plasma and cultured cells were detected using enzyme-linked immunosorbent assays (ELISAs) (NeoBioscience Technology; Beijing, China). The minimal detectable concentrations (pg/mL) were 15 (IL-10), 1 (IFN-γ), and 1 (TGF-β1), respectively. Non-detectable cytokine concentrations were assigned to a value of 0.01 for inclusion into the analysis.

*Statistical analysis*

All data were analyzed using the SPSS 17.0 and GraphPad Prism 5.0 software. Differences between groups were analyzed using the non-parametric Mann-Whitney U test, and relationships between numeric variables were analyzed using the Pearson's correlation index. Error bars represent standard deviations from the mean. *P*-values < 0.05 were considered statistically significant.

## Results

*Patient demographics*

Samples of Tregs and Teffs were obtained from 60 patients with ACS and 52 healthy control subjects. There were no significant differences between the two groups in terms of age and the percentage of risk factors of ACS as shown in **Table 1**. Since FOXP3 is located on chromosome Xp 11.23, which is subjected to X-inactivation in females, only male subjects were included in the study (**Table 1**).

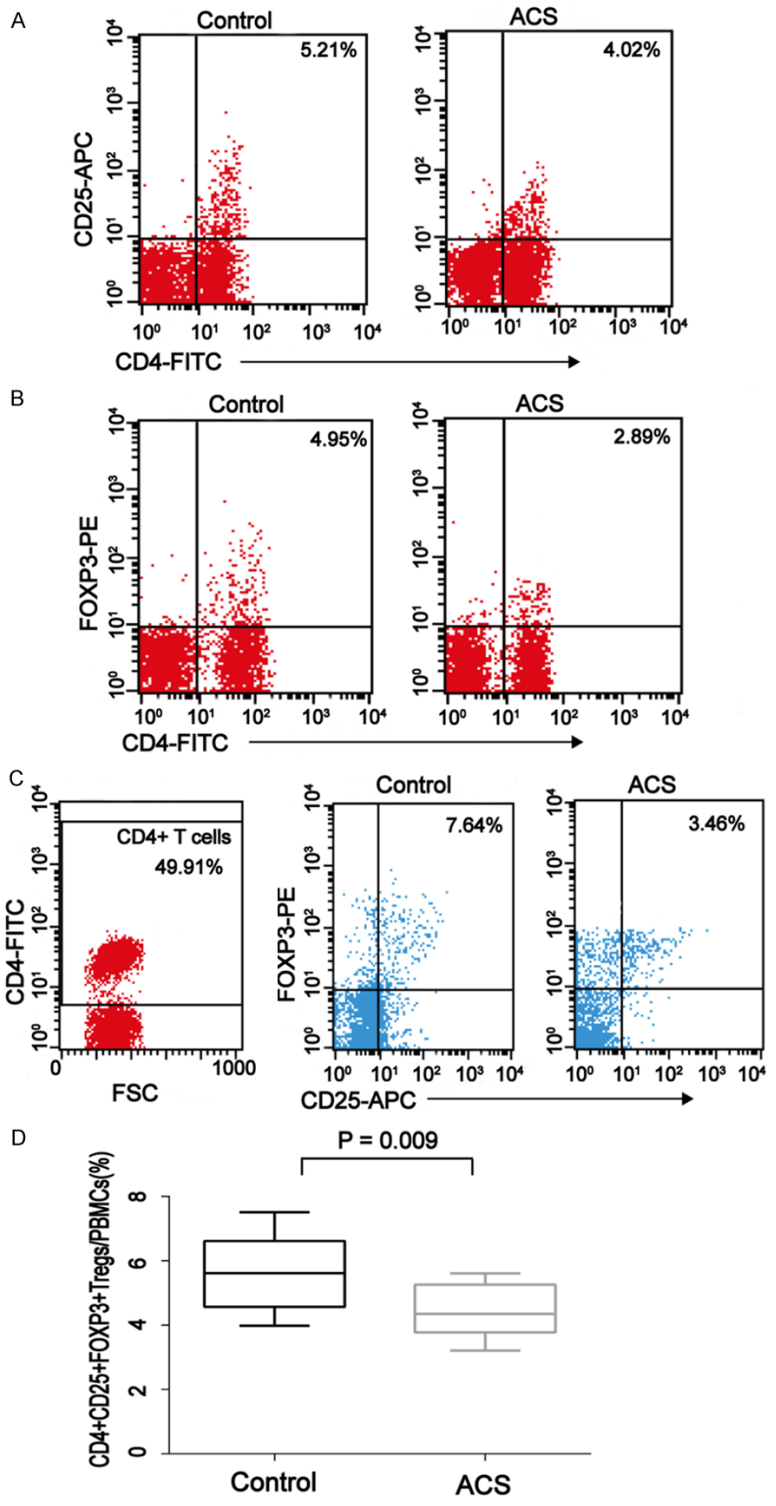


**Figure 1.** Flow cytometric analysis of frequency of Tregs obtained from the controls and ACS patients. (A) The frequency of CD4+CD25+ cells in mononuclear cells in ACS patients and control groups, respectively. (B) The frequency of CD4+FOXP3+ cells in mononuclear cells in the two control groups, respectively. (C) The frequency of CD4+CD25+FOXP3+ Tregs in mononuclear cells in the two groups, respectively. (D) The percentage of CD4+CD25+FOXP3+ Tregs in PBMCs in the two groups, corresponding data in (C). Data are presented as the mean ± SD. P = 0.009.

*The percentage of Tregs was decreased in ACS patients*

Some studies have suggested that ACS patients have reduced numbers of Tregs, while other studies have reported the opposite results. Due to the importance of Tregs for ACS, we sought to re-tackle this question. Our experimental results showed that the frequencies of Tregs were decreased in the ACS patients compared to the control subjects (P = 0.009) (**Figure 1**). However, up to now, few study investigators showed that the suppression function of Tregs to Teffs. Therefore, we questioned the function of Tregs in ACS patients.

*Tregs from ACS patients manifested decreased suppression function*

As the two groups of subjects in our study had similar frequencies of FOXP3$^+$ Tregs, we examined the ability of allo-specific Tregs to suppress Teffs by conducting *in vitro* co-culture studies. To detect the ratio of Tregs required for suppression, personal Tregs obtained from ACS patients and control subjects were co-cultured with personal Teffs at different ratios (Tregs:Teffs = 0:8, 1:8, 1:4, 1:2, and 1:1). We found that Tregs obtained from ACS patients had a lower suppressive ability when compared with Tregs obtained from control subjects. When using the same proportion of Tregs to Teffs, Tregs from ACS patients exhibited a lower suppressive ability at all of the ratios tested. Furthermore, the prolifera-

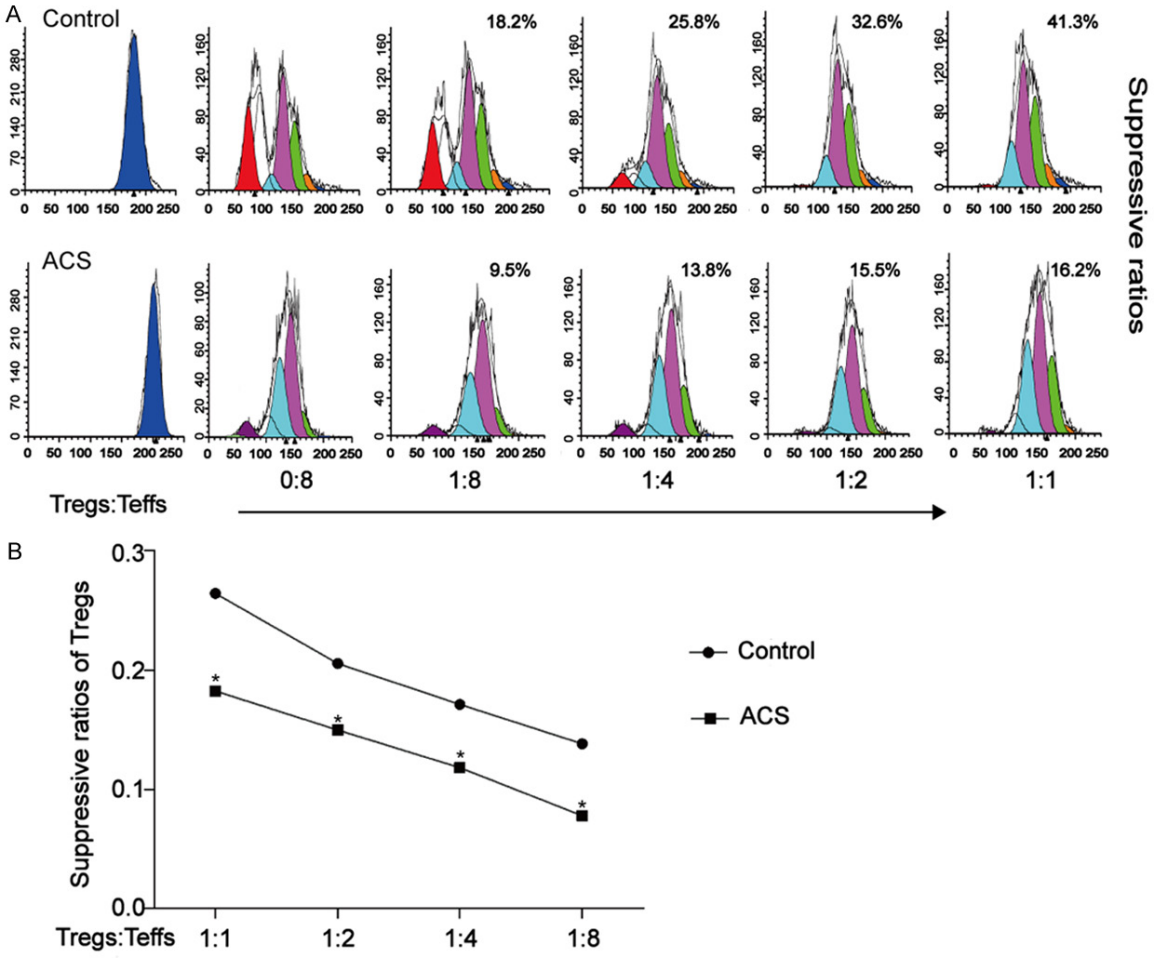


**Figure 2.** The immunosuppressive effect of Tregs on Teffs in the two groups. (Personal co-culture) A: CFSE-labeled Teffs were co-cultured with respective Tregs at different ratios in ACS and control groups, and the proliferation of Teffs was demonstrated by flow cytometry results in the FITC channel. The Treg suppression ratio was calculated using the following formula: [(1 - (PI of Tregs:Teffs - x:8)/(PI of Tregs:Teffs - 0:8) × 100%]. The specific percentage data represented the ratio of suppression function of Tregs in the two groups. B: The Tregs were stimulated with soluble anti-CD3/anti-CD28 antibodies for 7 days; after which, the proliferation index of the CFSE-labeled Teffs was determined. The Treg suppression ratio was also calculated using the same formula. Data represent results from at least 8 different subjects in each group. *P < 0.01.

tion of allo-specific Teffs obtained from ACS patients was reduced in a dose-dependent manner based on the ratio of Tregs to Teffs (P < 0.01) (**Figure 2**).

*The capacity for proliferation of Teffs was similar between ACS patients and control subjects*

We next examined the proliferative capacity of Teffs isolated from ACS patients and control subjects. Teffs cultured in 1640 medium (10% fetal bovine serum) showed a good proliferative capacity when stimulated with soluble anti-CD3/CD28 antibody or by means of allo-stimulation. When Teffs were cultured without Tregs (100,000:0), their proliferation index ranged from 5.6 to 33.8. Teffs obtained from the two groups of subjects showed no significant difference in their proliferation index in all groups (P > 0.05) (**Figure 3**).

*Tregs from ACS patients exhibited a lower ability to suppress Teffs at different ratios*

To further investigate the differences for the suppression function of Tregs between the two groups of subjects, we performed crossover co-culture studies at different ratios (1:8, 1:4, 1:2, 1:1) using Teffs obtained from control subjects with Tregs from ACS patients or control subjects, and Teffs from ACS patients with Tregs from ACS patients or control subjects.

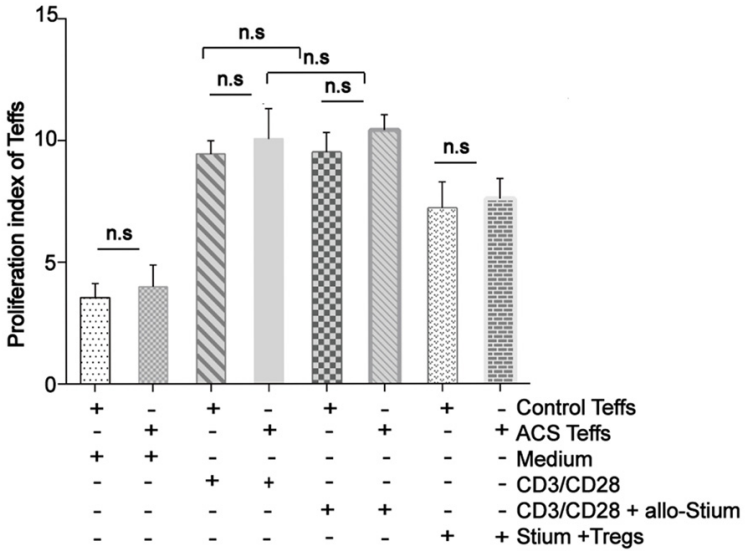


**Figure 3.** Analyses of proliferation indexes for Teffs obtained from ACS patients and control subjects. Teffs obtained from ACS patients and control subjects showed no significant difference in their proliferation index when cultured in medium, medium with CD3/CD28, medium with CD3/CD28 plus allo-stimulated antigen, and medium with CD3/CD28 plus auto-stimulated antigen, respectively. Error bars indicate SD; n.s, P > 0.05 compared to the controls.

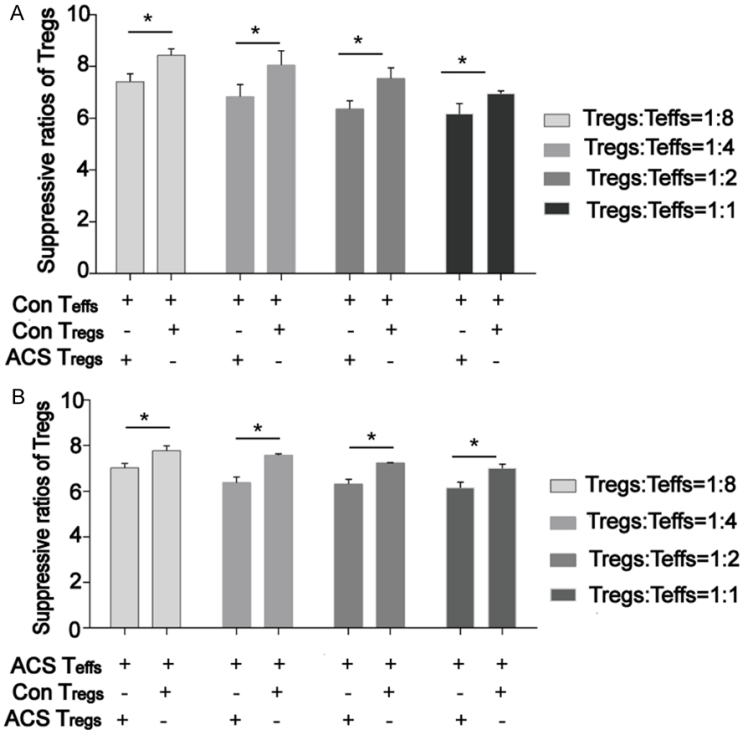


**Figure 4.** Measurement of immunosuppressive effect of Tregs on Teffs in the two groups. (Crossover co-culture) A: Crossover experiment using co-cultured control Teffs with Tregs from ACS patients or control subjects at variable titration ratios to detect the Treg suppression function in the two groups of subjects. B: Crossover experiments using ACS Teffs with Tregs from ACS patients or control subjects at the same ratios. Tregs from ACS patients had reduced suppressive effects when compared with control Tregs. *P < 0.05.

Tregs derived from ACS patients demonstrated a lower suppressive ability when compared with Tregs from control subjects (**Figure 4A**). Of note, this finding was consistent with results obtained once we cross-over co-cultured Teffs from ACS patients with Tregs from ACS patients or controls (**Figure 4B**). Collectively, these data suggest that Tregs in ACS patients have an impaired ability to suppress Teffs, and this impairment may contribute to the development and progression of ACS.

*Methylation status of the FOXP3 upstream enhancer*

To investigate a potential cause for the impaired suppression function of Tregs, we examined the methylation status of the FOXP3 upstream enhancer, which plays an important role in regulating Treg function. To avoid possible artifacts due to random X chromosome inactivation in females, the cells were isolated only from male ACS patients and age-matched control subjects. Pyrosequencing analysis was used to detect the methylation status of the 8 CpG-rich islands at the -5835 to -5794 upstream enhancer region of the FOXP3 gene (**Figure 5A-C**). The results identified a specific FOXP3 upstream CpG motif in the FOXP3 enhancer. The methylation levels of the FOXP3 upstream enhancer in Tregs from ACS patients were significantly higher than those in Tregs from control subjects (P < 0.05) (**Figure 5D**).

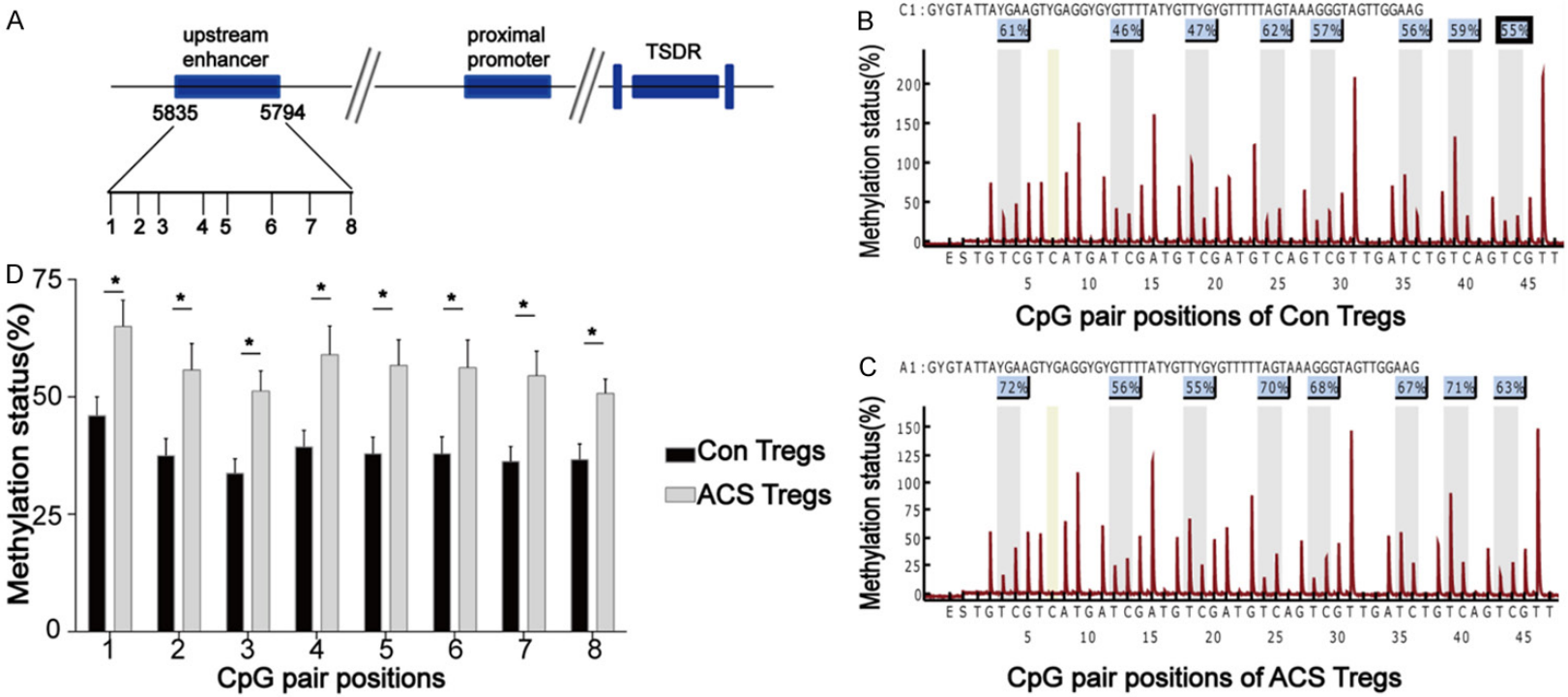


**Figure 5.** Determination of methylation status in the FOXP3 upstream enhancer of Tregs and Teffs. (A) Schematic representation of the upstream enhancer regions in the FOXP3 gene locus. Pyrosequencing data for the 8 CpG pair positions in the FOXP3 upstream enhancer. The relative level of methylation at each position is displayed in a small colored box just above each CpG motif of Tregs obtained from the controls (B) and Tregs obtained from the ACS patients (C). (D) Comparison of the methylation status of the FOXP3 upstream enhancer in Tregs obtained from ACS patients and control subjects, corresponding data in (B and C). Data are presented as the mean ± SD. *P < 0.05.

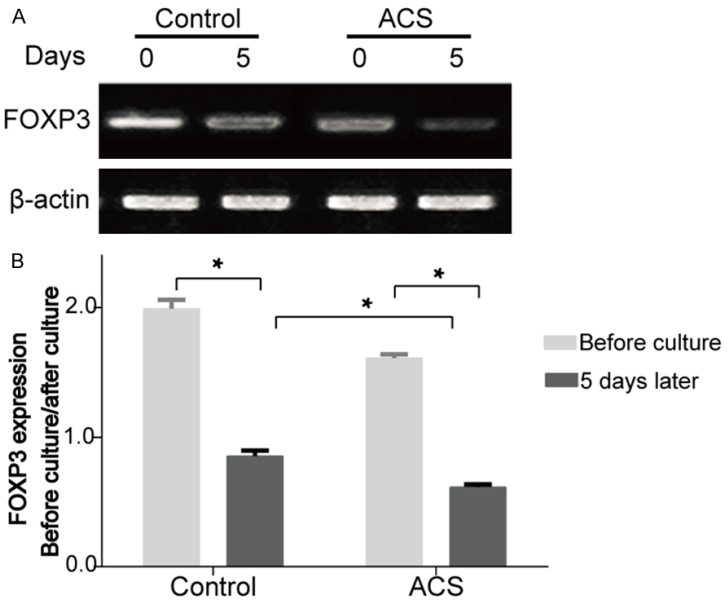


**Figure 6.** Treg expression of FOXP3 mRNA on day 0 and after 5 days of culture. (A) Expression of FOXP3 mRNA was decreased after 5 days of culture when compared with expression on day 0, in both ACS patients and control subjects. ACS patients exhibited lower levels of FOXP3 mRNA expression when compared with expression in control subjects. (B) The relative fold of (A). *P < 0.05.

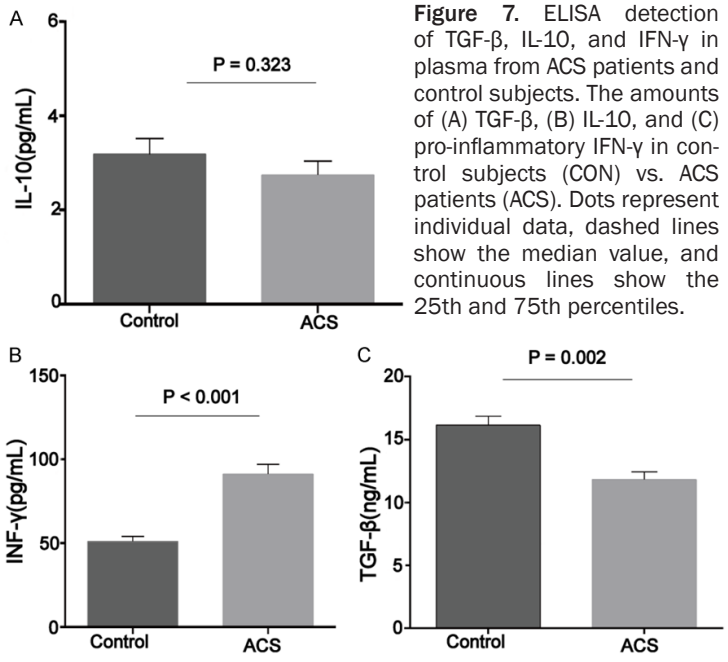


**Figure 7.** ELISA detection of TGF-β, IL-10, and IFN-γ in plasma from ACS patients and control subjects. The amounts of (A) TGF-β, (B) IL-10, and (C) pro-inflammatory IFN-γ in control subjects (CON) vs. ACS patients (ACS). Dots represent individual data, dashed lines show the median value, and continuous lines show the 25th and 75th percentiles.

*ACS patients were characterized by lower FOXP3 mRNA*

RT-PCR was next employed to examine the expression levels of FOXP3 mRNA in freshly isolated Tregs, as well as Tregs which had been cultured for 5 days. The levels of FOXP3 mRNA expression in non-cultured Tregs obtained from ACS patients were significantly lower than the levels in Tregs obtained from control subjects ($P < 0.05$). After 5 days of culture, the levels of FOXP3 mRNA expression in Tregs from both ACS patients and control subjects were significantly lower when compared with those expression levels of freshly isolated cells ($P < 0.05$). Moreover, the mean level of FOXP3 mRNA expression in ACS patients was significantly lower than that in control subjects ($P < 0.05$) (**Figure 6**).

*Serum levels of TGF-β1, INF-γ, and IL-10 in the plasma of ACS patients*

ACS patients had significantly lower plasma levels of TGF-β1, but significantly higher plasma levels of the pro-inflammatory cytokine IFN-γ when compared with those levels in control subjects ($P = 0.002$ and $P < 0.001$, respectively) (**Figure 7B** and **7C**). However, the two groups of subjects had similar levels of IL-10 ($P = 0.323$) (**Figure 7A**). In agreement with previous findings, the Tregs from ACS patients demonstrated a reduced anti-inflammatory function, but an enhanced pro-inflammatory function.

## Discussion

Tregs are now recognized to play an important role in regulating T-cell-mediated immune responses by suppressing Teffs proliferation during the initiation and progression of atherosclerosis [28]. Recently, several groups have reported lower Treg frequencies in ACS patients when compared with Treg frequencies in healthy age-matched control subjects [29]. In contrast, another study found no decrease in Tregs counts in ACS patients [15]; thus the role of Tregs

in ACS has remained controversial. The ACS patients in our study had lower percentages of Tregs compared to the control subjects. And we suggest that in ACS patients, the number of Tregs might not be the sole factor affecting their immunosuppressive function of Treg.

While defective Treg functions have been associated with autoimmune diseases such as rheumatoid arthritis, T1DM, and autoimmune thyroid disease [30, 31], only limited data has been available concerning the immunosuppressive effects of Tregs on Teffs in patients with ACS. Therefore, we examined the suppressive effects of Tregs on Teffs in ACS patients and control subjects by co-culturing Tregs with CFSE-labeled Teffs at different ratios. Our results showed that the immunosuppressive effect of Tregs on Teffs was significantly reduced in ACS patients. Additionally, we found no significant difference in the proliferative capabilities of Teffs obtained from the ACS patients and control subjects. This result further confirmed that the impaired suppression function of Tregs in ACS patients is due to the Tregs themselves, rather than an enhanced proliferative capability of Teffs. This conclusion is also supported by data obtained in previous studies [32]. Thus, we speculate that the reduced immunosuppressive effect of Tregs on Teffs in ACS patients may be a crucial factor contributing to the onset and progression of ACS. Also, the number of Tregs was not the sole factor affecting their total immunosuppressive capacity, which might explain why the percentage of Tregs was not decreased in some ACS patients.

The development and function of Tregs is governed by FOXP3, a winged-helix family transcription factor, which acts as a major controller of gene expression in Tregs [33]. Several regulatory regions within the FOXP3 locus, e.g., the upstream enhancer, proximal promoter, and Treg-specific demethylated region (TSDR), are known to participate in controlling gene expression, and can be epigenetically regulated [24]. Increasing evidence suggests that the upstream enhancer of FOXP3 contains an evolutionarily conserved CpG-rich island that is not methylated in Tregs, but is methylated in naive or activated conventional T (Tconv) cells as well as TGF-β1-induced Tregs in rodent species [34]. Hypomethylation of cis-elements at the FOXP3 locus permits FOXP3 binding, as well as the binding or other transcription factors that constitutively activate FOXP3 [25]. Nevertheless, until now, the methylation status of the upstream FOXP3 enhancer has not been richly investigated in ACS patients. Our results showed that the FOXP3 upstream enhancer was hypomethylated in Tregs at the same CpG-rich islands in both ACS patients and control subjects. Thus, we believe that the methylation status of the FOXP3 upstream enhancer might play a vital role in maintaining the immunosuppressive function of Tregs. However, the degree of methylation of the FOXP3 upstream enhancer in ACS patients was significantly greater than that in control subjects. Therefore, we suggest that the methylation status of the FOXP3 upstream enhancer might serve as a marker to discriminate the functional status of Tregs in ACS patients.

Methylation of the CpG-rich islands is associated with gene silencing. Demethylation at the CpG motif in the FOXP3 upstream enhancer has been associated with expression of FOXP3 [25]. Kennedy et al [10] reported that the methylation status of the upstream enhancer was crucial for achieving the enhancer activity needed for expression of FOXP3 mRNA. In the current study, we found significantly reduced levels of FOXP3 mRNA expression in the ACS group, which was consistent with our results concerning the methylation status of the FOXP3 upstream enhancer. Our analysis revealed that FOXP3 mRNA expression was inversely correlated with the methylation status of the FOXP3 upstream enhancer, and also with lower levels of TGF-β and higher levels of IFN-γ in ACS patients. The analysis also showed that methylation levels of the upstream FOXP3 enhancer were negatively correlated with Treg function in both groups. Therefore, we believe that the methylation status of the FOXP3 upstream enhancer determines FOXP3 expression, and might further affect the suppression function of Tregs in ACS patients.

Our study has some limitations that should be mentioned: (1) the study sample size was relatively small, and this limited the statistical power of our group analyses, and (2) the factors that increased the degrees of methylation or demethylation in the specific FOXP3 gene region relevant to the function of Tregs in ACS were not investigated. Further studies are needed to determine the mechanism underlying the Treg dysfunction observed in cases of ACS.

## Conclusions

In summary, our results suggest that the Tregs of ACS patients have a lower immune suppressive function. Because methylation of the FOXP3 upstream enhancer was increased in the Tregs of ACS patients, the methylation status of that enhancer might serve as a surrogate biomarker for Treg function in patients with ACS and other diseases. Moreover, epigenetic regulation of the FOXP3 upstream enhancer may represent a novel target for therapeutic intervention in cases of ACS.

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## Disclosure of conflict of interest

None.

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