$CD4^+Foxp3^+$ Treg and its $ICOS^+$ Subsets in Patients with Myocardial Infarction

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ABSTRACT

Background: Atherosclerosis is a multifactorial disorder with chronic inflammatory conditions in which immune cells play a significant role in its pathogenic process. Regulatory T cells (Treg), as a part of immune system, are involved in controlling autoimmune and inflammatory diseases. Quantitative and/or functional alteration of Tregs has been shown to play an atheroprotective role and may also promote plaque stabilization. **Objective:** To assess if inducible costimulatory molecule (ICOS) expression on one subtype of Treg cells with high suppressive potential correlates with the pathogenesis of atherosclerosis. **Methods:** Patients with myocardial infarction (MI) and/or stable angina (SA), diagnosed as atherosclerosis by angiography, and a group of individuals with normal coronary angiography (NCA) were recruited for the present study. Peripheral blood mononuclear cells (PBMCs) were prepared and the expression of ICOS, Foxp3 and CD4 molecules was tested by flowcytometry. **Results:** The percentage of $CD4^+Foxp3^+$ Treg cells was reduced in MI group compared to NCA and SA groups ($p<0.005$). Evaluation of the two Treg subsets according to ICOS expression showed a decreased $ICOS^+/ICOS^-$ Treg ratio in MI and SA groups compared to NCA individuals ($p=0.002$ and $p=0.048$, respectively). **Conclusion:** The present data indicate that Tregs and its $ICOS^+$ subsets are decreased in patients with MI or SA, suggesting a potential role for Treg in atherosclerosis progression or onset of acute coronary syndrome.

Keywords: Acute Coronary Syndrome, Atherosclerosis, ICOS, Myocardial Infarction, Regulatory T Cells, Stable Angina

INTRODUCTION

Atherosclerosis is a multifactorial disorder in which, immune mechanisms play important roles in its pathogenesis. Evidences indicate that innate and adaptive immunities are involved in atheroma progression and its stability (1-3). The enhanced T helper-1 (Th1)

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response in the atherosclerosis indicates a dysregulation of the immune response during the course of the disease. It has been stated that an imbalance in T-helper subsets could play an important role in plaque rupture and the onset of ACS (including unstable angina (UA) and myocardial infarction (MI)) (4,5). Regulatory T cells (Tregs), one of the Tcell subsets characterized by CD4 and FOXP3 expression, regulate immunological responsiveness by their suppressive effects on effector Th cells (6). Alterations of Treg cell subsets or their function have been found in local and peripheral atherosclerotic lesions of patients with myocardial infarction (7-9). Moreover, it was revealed that Th17/Treg imbalance exists in patients with ACS and also during atherogenesis in $APOE^{-/-}$ mice (10-12). Reports suggest that adoptive transferring of Treg cells are able to attenuate atherosclerosis in animal models (13,14). Additionally, adoptive Treg transfer could improve MI induced cardiac remodeling through inhibition of inflammatory cytokine production.(15,16). Overall, the mentioned studies have proposed an atheroprotective role for Treg cells.

Furthermore, $CD4^+$ T cell responses to antigen are modulated by co-stimulatory signals. Inducible co-stimulatory molecules (ICOS) have an important role in modulating T-cell activation and Th2 differentiation (17,18). It has been shown that ICOS play a protective role in regulation of atherosclerosis through its effect on regulatory T-cell responses (19, 20). $FOXP3^+$ Treg cells have been divided in to two subsets according to ICOS expression (21). $ICOS^{high}$ Treg subsets are more suppressive than $ICOS^{low}$ or $ICOS^-$ Treg, probably due to a higher expression of inhibitory cytokines i.e. IL-10 and TGF-β (21).

Our study was aimed at assessing the frequency of circulating $CD4^+Foxp3^+$ Treg and its $ICOS^+$ subset in patients with myocardial infarction (MI) as compared with those with stable angina (SA) and angiographically normal coronary subjects.

MATERIALS AND METHODS

Patients. Three groups of subjects were recruited as following; group 1, MI patients who were admitted to the University hospital due to myocardial infarction (n=28); group 2, patients with stable angina approved for atherosclerosis by angiography (n=27); and group 3, Angiographically Normal Coronary subjects (NCA) as a control group (n=25). Informed consent was obtained from each individual who participated in this study.

Myocardial infarction was diagnosed by the elevation of troponin I (>0.4 ng/ml), CK-MB (>5ng/ml) or definite ST-segment elevations (>2 mm) in at least two consecutive leads. Patients with stable angina were from the outpatient clinic and they had a similar extent of coronary suffering as that found in MI patients on recent angiography. Control subjects were selected on the basis of a recent angiography showing NCA.

Exclusion criteria were as follows; age below 18 or above 80 years, renal failure, known history of cancer or chronic-immune-mediated disorders, or current use of immunosuppressive agents. Patients with MI and stable angina had a similar extent of coronary atherosclerosis. No statical difference was found among the three groups regarding to the age and sex.

Blood Samples. The time interval between MI symptom onset and blood sampling was less than 24h in all cases. The samples were collected in EDTA containing tubes. Peripheral blood mononuclear cells (PBMCs) were separated over Ficoll-Hypaque (Nyco

med Oslo, Norway) gradient and analyzed by flowcytometry (Becton-Dickinson, USA).

Cell Culture. Since ICOS (CD278) expression is low on T cells, 24h cell culture resulted in an optimum of ICOS surface expression (22). PBMCs were suspended at a density of 2×10^6 cells/ml in complete culture medium (RPMI 1640, Biosera supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM glutamine and 10% heat-inactivated fetal calf serum, Gibco). The cell suspension was transferred to every well of 24-well plates. The incubator was set at 37°C with a 5% CO2 atmosphere. After 24h of culturing, the contents of the well were transferred to 5-ml sterile tubes. The cells were then centrifuged at 1500 rpm for 5 min. To analyze the Treg cells, PBMCs were aliquoted into tubes for further staining.

Surface and Intracellular Staining for Flow-Cytometry Analysis. Cells were aliquoted into tubes and washed once in phosphate-buffered saline (PBS). For Treg analysis, the cells were incubated for at least 30 minutes with PerCP anti-human CD4 (RPA-T4, eBioscience, Cat.No.45-0049-42) and phyco-erythrin (PE) anti-human ICOS (ISA-3, eBioscience, Cat.No.12-9948-73) in the dark at 4°C.

After washing twice with PBS, the cells were permeabilized by fixation and permeabilization buffer according to the manufacturer's instructions (eBioscience, Cat. No. 00-5123, Cat. No. 00-8333) for further intracellular staining by FITC anti-human Foxp3 (PCH101, eBioscience, Cat. No. 11-4776-42). Mouse isotype control antibody (eBioscience) was used to confirm the specificity of primary antibody binding and to exclude the non-specific Fc receptor binding to cells or other cellular proteins in the flow cytometry experiments. Isolated PBMCs were stained with anti-CD4–PerCP, anti-Foxp3-FITC and anti-ICOS–PE. The cells were gated on lymphocytes by their forward- and side-scatter features. The percentage of cells expressing CD4, Foxp3 and ICOS among gated lymphocytes was determined by three-color flow cytometry. Stained cells were analyzed by flowcytometer using a FACScan cytometer equipped with CellQuest software (BD).

Statistical Analysis. ANOVA test was applied to analyze differences among the three groups. Values are expressed as mean ± SD in the text. SPSS software was used for the analysis of the data. A probable value of $p<0.05$ was considered to be statistically significant.

RESULTS

As summarized in Table1, there were no significant differences in age, gender, and number of diseased vessels among the 3 groups ($p>0.05$). Among the risk factors analyzed, diabetes showed a significant considerable difference between the groups ($p=0.039$). Regarding to the type of medication in MI and SA groups, a significant difference was obvious for the nitrate and ASA consumption ($p=0.002$ and $p=0.02$, respectively).

Although there were differences in the WBC population of the MI, SA and the NCA groups in this study , no statistically significant difference was obtained for the percentage of $CD4^+$ T cells or the number of lymphocytes ($p<0.05$).

The percentage of $CD4^+Foxp3^+$Treg cells decreased in the MI and SA groups compared to the NCA group (1.93 ± 1.07%, and 2.9 ± 1.18% vs. 4.09 ± 1.16 %., $p<0.001$, $p=0.001$, respectively). Moreover, there was a statistically significant difference between

the MI and the SA groups (p=0.005) (Table 2).

Table 1. Demographic and clinical characteristics of the CAD patients.

Characteristics	MI (n=28)	SA (n=27)	NCA (n=25)	P-value
Age (years)	61.14 ± 9.75	59.07 ± 9.71	55.28 ± 11.34	0.118
Sex (male/female)	17/11	19.8	13/12	0.396
Number of diseased Vessels	1.8±0.9	1.7±0.5	-	0.883
Risk factors				
Smoking %(n)	25 (7)	14.8 (4)	12 (3)	0.43
Hypertension %(n)	46.42 (13)	29.62 (8)	40 (10)	0.436
Diabetes mellitus %(n)	46.42 (13)	44.44 (12)	16 (4)	0.039*
Hyperlipidaemia %(n)	53.57 (15)	37.03 (10)	28 (7)	0.154
Medications				
Aspirin %(n)	46.42 (13)	66.6 (18)	28 (7)	0.02*
Statins %(n)	42.8 (12)	55.5 (15)	28 (7)	0.133
Calcium blockers %(n)	7.14 (2)	7.4 (2)	0	0.348
Beta-blockers %(n)	50 (14)	55.5 (15)	44 (11)	0.7
ACEI %(n)	50 (14)	55.5(15)	44 (11)	0.865
Nitrates %(n)	42.8 (12)	14.8 (4)	4 (1)	0.002

The values are expressed as mean ± SD or the number of cases. MI= myocardial infarction; SA= stable angina; NCA; Normal coronary angiography, ACEI= angiotensin-converting enzyme inhibitor; *= p<0.05.

In the study of the two $CD4^+Foxp3^+$ Treg subsets, according to ICOS expression, both $ICOS^+$ and $ICOS^-$ subsets were decreased in atherosclerosis patients. However, Treg reduction mostly occurred in $ICOS^+$ subset (Table 2).

Compared to NCA population, the ratio of $ICOS^+/ICOS^-$ Treg is lower in MI and SA groups (1.06 ± 0.6, and 1.24 ± 0.46 vs. 1.63 ± 0.65, p=0.002, and p=0.048; respectively). No significant difference was found in ICOS ratio between SA and MI groups (p=0.47) (Table 2).

Table 2. Cell subsets and molecule expression in CAD patients.

Assessment	MI (n=28)	SA (n=27)	NCA (n=25)	P-value
White Blood Cells	6412 ± 1542¥	6609 ± 2303¥	8423 ± 3001¥	NCA and SA: 0.957 NCA and MI: 0.016 SA and MI : 0.022
Lymphocyte (% of WBC)	2592 ± 1346¥ (35.6 ± 7.71)	2482 ± 661¥ (32.34 ± 11.95)	2259 ± 651¥ (31.44 ± 10.07)	NCA and SA: 0.523 NCA and MI: 0.361 SA and MI : 0.946
$CD4^+$ T cells (%)	44.9 ± 7.8	49.8 ± 11	49.3 ± 8.5	NCA and SA:0.979 NCA and MI:0.202 SA and MI:0.128
$CD4^+Foxp3^+$ T cells (%)	1.93 ± 1.07* #	2.9 ± 1.18*	4.09 ± 1.16	NCA and SA: 0.001 NCA and MI< 0.001 SA and MI : 0.005
$CD4^+Foxp3^+$ $ICOS^+$ T cells (%)	0.92 ± 0.62*#	1.58 ± 0.62*	2.47 ± 0.74	NCA and SA< 0.001 NCA and MI<0.001 SA and MI : 0.001
$CD4^+Foxp3^+$ $ICOS^-$ T cells£ (%)	1 ± 0.59*	1.37 ± 0.63	1.61 ± 0.64	NCA and SA:0.337 NCA and MI:0.002 SA and MI : 0.086
T cell Ratio€	1.06%±0.60*	1.24%±0.46*	1.63%±0.65	NCA and SA =0.048 NCA and MI=0.002 SA and MI=0.475

Values are expressed as mean ± SD. MI= Myocardial infarction; SA= stable angina; NCA= Normal coronary angiography; ¥= cell/ml; *p< 0.05 vs. control; # p<0.05 vs.SA; £= Total Treg – ICOS+Treg; €=CD4+Foxp3+ICOS+/ CD4+Foxp3+$ICOS^-$

DISCUSSION

It has been illustrated that both innate and adaptive immune mechanisms play important roles in atherosclerosis pathogenesis (1-3). Studies show that immune regulatory cells have atheroprotective role. For instance, In experimental mouse model, adoptive transferring of natural or antigen specific induced Treg cells can ameliorate atherosclerosis (13,14,23-25). Moreover, numerical and functional changes of Treg subsets have been observed in atherosclerosis (7,8,12,13).

We found that the frequency of $CD4^+Foxp3^+$Treg cells are significantly lower in patients with coronary heart disease (MI and SA) compared to the NCA.

Interestingly, our data showed a marked decrease in $CD4^+Foxp3^+$ Treg in MI and SA patients. Mor et al. showed that peripheral Tregs were significantly reduced in patients with ACS indicating a potential role for Tregs in atheroma progression (7). Other studies showed that the decreased Treg cells were associated with atherosclerosis development, progression and unstability (8,12,26). Tcell activation and inflammatory cytokines (IFN-γ) promote plaque disruption and subsequently thrombus and ACS onset (2,27). Conversly, TGF-β induces plaque stability (28). Overall, we assume that a decrease in Treg may account for an uncontrolled T cell activity leading to a plaque rupture and MI.

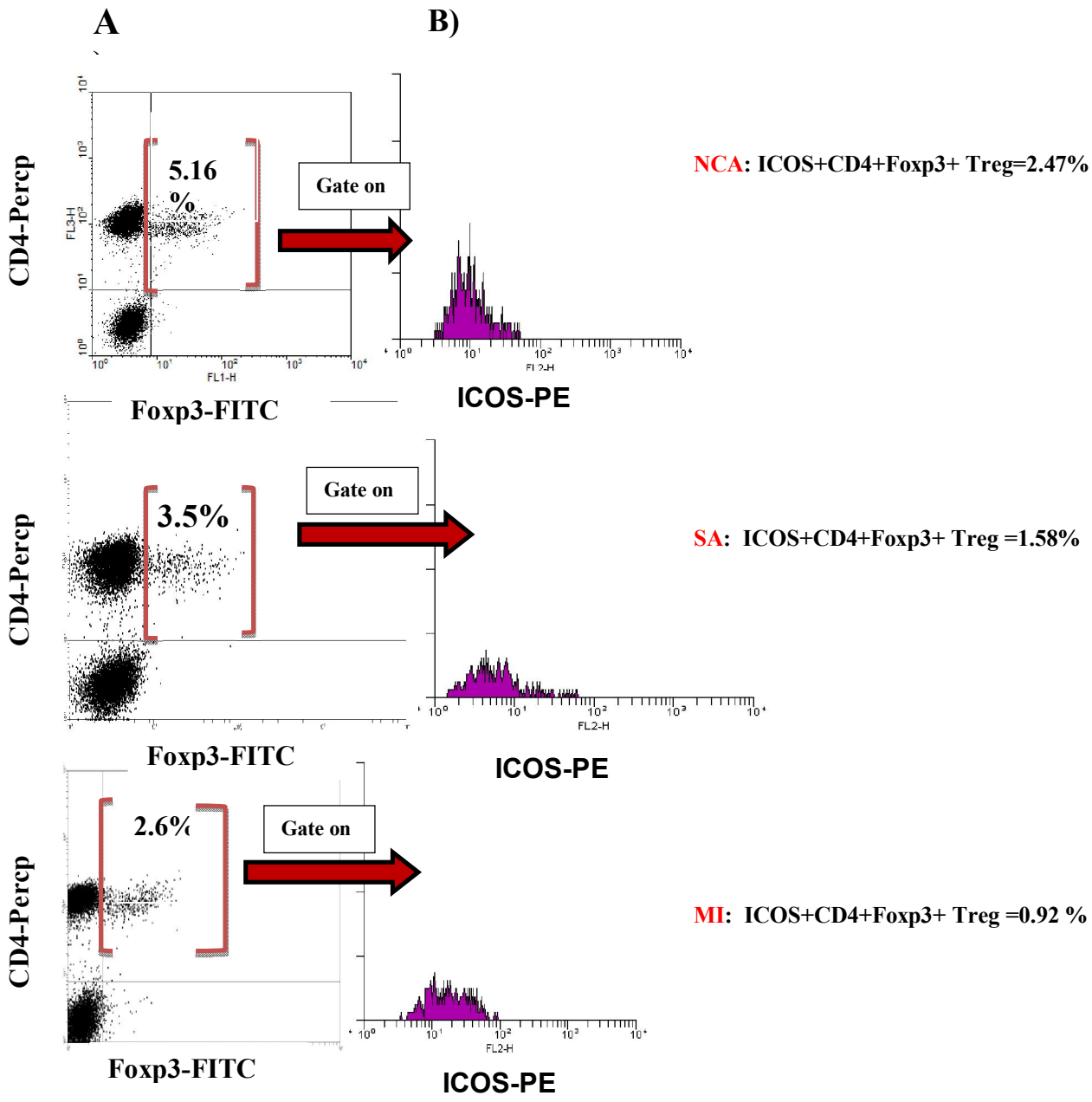

Figure 1. PBMCs from patients with MI, SA, and control subjects were stained with labeled antibodies as described in methods and materials. A) Plots in intern box represent $CD4^+FOXP3^+$ T cells population. $CD4^+FOXP3^+$ T cells were gated on flow cytometer. B) Representative ICOS expression plot and histogram for $CD4^+FOXP3^+$ T subsets from each group was shown. MI: acute myocardial infarction; SA: stable angina; the percentage of positive cells was shown in each box.

Our data together with others support the suggestion that Treg cells have a protective role in atherosclerosis progression and a stabilizing effect on plaque rupture (7,8,12). Recently, according to the differential expression of a costimulatory receptor (ICOS), two subsets of naturally occurring $FOXP3^+$ Treg cells were identified in thymus, ton-

sils,lymph nodes and the periphery (21). The suppressive function of $ICOS^{high}$ Treg subset was more efficient compared to $ICOS^{low}$ or $ICOS^{-}$ Treg, probably due to a higher expression of certain inhibitory cytokines (IL-10, TGF-β) (21). ICOS/ICOSL interactions control the responses of already activated T cells (29). ICOS is particularly effective in enhancing IL-10 production (30). Furthermore, experiments showed that ICOS plays an atheroprotective role, interestingly through its effect on Treg cell responses (19,20). Additionally it has been found that ICOS expression on Treg cells in malignancy could be associated with its high suppressive capacity (22).

We evaluated the alteration of $ICOS^{+}$/$ICOS^{-}$ subsets of Treg cells in patients with coronary heart disease. Our data showed that the majority of Treg population is $ICOS^{+}$ in coronary heart disease (MI and SA) patients. $ICOS^{+}$Treg/$ICOS^{-}$Treg ratio was significantly reduced in MI and SA groups compared to the control. So we may hypothesize that $ICOS^{+}$Treg/ $ICOS^{-}$Treg ratio is skewed toward the $ICOS^{-}$ population which was assumed in previous studies as a population with decreased suppressive function (21,22). Observed results indicate that the lower expression of ICOS on Treg cells in atherosclerosis seems to support Afek et al. (19) and Gotsman et al. (20) suggestions regarding the protective role of ICOS in atherosclerosis by its influence on Treg cells (19,20). It could be concluded that modulation of the immune responses by Treg induction or enhancement of Treg function might be a strategy for inhibiting atherosclerosis progression and ACS occurrence. Moreover, enhancement of Treg responses, through manipulation of the ICOS pathway, might have a therapeutic potential for atherosclerotic disease.

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