Resting and Activated Natural Tregs Decrease in the Peripheral Blood of Patients with Atherosclerosis

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ABSTRACT

Background: Atherosclerosis is a chronic inflammatory disease affecting large and medium arteries. CD4+ T cells are known to play a role in the progression of the disease. CD4+CD25+Foxp3+ natural Treg (nTreg) cells seem to have a protective role in the disease and their reduction in acute coronary syndrome is recently shown. **Objective:** To investigate the frequency of nTreg subsets in the peripheral blood of patients with atherosclerosis. **Methods:** Confirmation of atherosclerosis was done by angiography and 15 ml heparinized blood was obtained from each of the 13 non-diabetic patients and 13 non-diabetic, non-smoker individuals with normal/insignificant coronary artery disease which was also confirmed by angiography. Lipid profiles of the patients and controls were measured at the time of sampling. Mononuclear cells were used for both RNA extraction and immunophenotyping by real-time PCR and flowcytometry techniques, respectively. **Results:** In natural Treg subsets, the frequency of CD4+CD45RO-CD25+Foxp3^{lo} T-cells (resting nTregs) was greater in controls than patients (p=0.02). The frequency of CD4+CD45RO+CD25^{hi}Foxp3^{hi} T-cells (activated nTregs) was significantly higher in controls compared with patients (p=0.02). However, the frequency of CD4+CD25+CD45RO+Foxp3- T-cells (effector/memory) increased in patients compared with controls (p=0.01). Both the MFI and gene expression of Foxp3 were higher in control group than in patients (p=0.015 and p=0.017, respectively). Moreover, the TGF-β gene expression showed a decrease in the peripheral blood mononuclear cells of patients compared with controls (p=0.03). **Conclusion:** Decrease in both subsets of resting and activated nTregs along with a decrease in the expression of Foxp3 and TGF-β genes in patients with atherosclerosis suggests phenotypic changes in these subsets, which may as well be correlated with a more inflammatory profile in their lymphocytes.

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Keywords: Atherosclerosis, Natural Tregs, CD45RO, FOXP3

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INTRODUCTION

Regulatory T cells (Treg), as a specific subpopulation of T cells, comprise 5–10% of all peripheral CD4+ T cells, and are critical in the regulation of immune response, homeostasis and tolerance (1). Tregs are divided to natural Tregs and inducible Treg subsets (2). Natural CD4+CD25+ Tregs are produced in the thymus and play a critical role in suppression of self-reactive immune responses and maintenance of self-tolerance (3). Natural CD4+CD25+Foxp3+ Tregs can suppress different types of immune cells including CD4+ and CD8+ T cells, B cells, natural killer (NK) cells, NKT cells, monocytes, and dendritic cells (DCs). A main function of natural CD4+CD25+ Tregs is suppression of activation of naïve T cells, but they can also inhibit activated effector T cells and memory CD4+ and CD8+ cells (4). The other main subset of Tregs, iTregs, develop from naive T cells in the periphery during an active immune response. iTregs can be further divided to Tr1 cells that produce IL-10, and a TGFβ-secreting subset also termed Th3 cells, as well as CD4+CD25-Foxp3+ cells (5,6). Three populations of Foxp3 expressing nTreg cells are known as CD25+CD45RA+Foxp3^{lo}Ki67- resting Treg cells (rTregs), CD25^{hi}CD45RA-Foxp3^{hi} ki67+CTLA-4+CD95+GITR+ (glucocorticoid-induced TNF-receptor-related protein) activated Treg cells (aTregs), and CD25+CD45RA-Foxp3^{lo} non-Treg cells (7,8). The former populations both have suppressive functions and the latter is non-suppressive and cytokine secreting in vitro (8,9). In this regard, the suppressive populations inhibit proliferation, whereas, CD4+CD25+CD45RA+Foxp3^{lo} cells secrete proinflammatory cytokines such as IL-17, IL-2, and IFN-γ and have minimal inhibitory function. Treg subpopulations vary in proportions and function as reported in different autoimmune disorders (8,9).

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries, in which endothelial dysfunction and vascular inflammation is followed by the accumulation of modified lipid, inflammatory cells and cell debris in ‘plaques’ within the vascular wall (10). Although most cells in fatty streaks of the plaque are macrophage-derived foam cells, T cells and macrophages are found in all stages of the disease (11). T cells constitute about 10% of all cells in human plaques of which 70% are CD4+ T cells (12). Different subsets of inflammatory, effector and regulatory T cells, are found in the plaques. Two recent reports have shown the decreased number of Tregs in human atherosclerosis (13,14). The role of natural Tregs (nTregs) in experimental atherosclerosis was initially demonstrated in a study where depletion of peripheral Tregs by anti-CD25 monoclonal antibodies increased the size of atherosclerotic lesion and vulnerability in apolipoprotein E deficient (ApoE−/−) mice (15). Human atherosclerotic lesions contain only limited number of Tregs (1%–5% of all T cells) when compared with other chronically inflamed tissues (Tregs ≤25%). Patients with coronary artery disease (CAD) have reduced frequency of peripheral Treg cells (16), which is directly associated with carotid atherosclerotic plaque vulnerability and inversely correlated with infiltrative mature dendritic cells (17,18). Treg cells, by reducing blood cholesterol levels, prevent the accumulation of inflammatory cells and secretion of pro-inflammatory cytokines, increase conversion of M1 to M2 macrophages (19), increase secretion of anti-inflammatory cytokines such as IL-35, IL-10 and TGF-β and inhibit B cell activation, and thereby protect against atherosclerosis (20). Despite the important role of Tregs in inflammatory diseases, detailed phenotypic and functional studies on these cells in atherosclerosis are scarce. In this study, we investigated the frequencies of Treg subsets in patients with atherosclerosis and

compared it with control individuals. Our results indicated that the frequencies of resting and activated nTregs were greater in controls than patients. However, the frequency of effctor/memory T-cells was higher in patients compared with controls.

MATERIALS AND METHODS

Subjects. After detection and confirmation of atherosclerosis by peripheral angiography, 15 ml heparinized blood was obtained from each of the 13 non-diabetic patients (6 men and 7 women aged 39-77 yrs) who were diagnosed with coronary artery disease. Control group consisted of 13 non-diabetic, non-smoker individuals with normal/insignificant coronary artery disease confirmed by peripheral angiography (5 men and 8 women aged 47-72 yrs). The levels of low-density lipoproteins (LDL), Triglycerides (TG), and Cholesterol (Chol) were measured for the patients and controls at the time of sampling. Body Mass index (BMI) of patients were also calculated based on the demographical information asked in a questionnaire (Tables 1 and 2). Peripheral blood mononuclear cells (PBMCs) were separated from blood by density gradient centrifugation on Ficoll. Part of the PBMCs (3×10^6) were used for RNA extraction and real-time PCR experiments for detection of gene expression and the rest (3×10^6) were used for Immunophenotyping of cells by flowcytometry.

Table 1. The demographical, lipid profile and BMI of the control individuals at the time of entry to the study.

Number	Sex	Age (years)	TG (mg/dl)	CHOL (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	BMI
1	F	55	70	140	52	74	26.67
2	M	72	110	160	49	89	25.39
3	F	52	64	153	58	82.2	29.59
4	M	55	71	132	52	65.8	27.34
5	F	53	130	200	71	73	23.44
6	M	47	140	190	65	68.6	23.12
7	M	55	468	271	117	60.4	25.63
8	M	49	85	125	56	52	21.80
9	F	64	64	148	80	55.2	26.67
10	F	60	72	162	69	78.6	31.11
11	F	57	64	143	31	99.2	23.47
12	F	52	68	145	67	34	20.57
13	F	60	132	142	68	38	25.81
Mean ± SD		56 ± 6.6	62 ± 6.3	153 ± 22	60 ± 13	67 ± 19	25.4 ± 2.97

Peripheral Blood Mononuclear Cells (PBMCs) Isolation. PBMCs were isolated by density gradient centrifugation (Ficoll-Paque PLUS, Inno-train, Germany) and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in fetal bovine serum (FBS Biosera, UK).

Table 2. The demographical, lipid profile and BMI of the patients at the time of entry to the study.

Number	Sex	Age (years)	TG (mg/dl)	CHOL (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	BMI
1	F	64	94	114	51	44.2	20.45
2	M	61	88	90	34	38.4	23.44
3	F	49	76	108	42	50.8	18.94
4	M	76	56	118	58	48.8	19.48
5	F	70	57	163	75	76.6	35.67
6	M	65	125	164	65	74	25.71
7	F	64	120	190	80	86	21.48
8	M	68	105	170	90	59	19.03
9	M	39	154	198	97	70.2	25.26
10	M	74	82	152	70	65.6	22.20
11	F	42	166	235	134	67.8	18.26
12	F	77	122	229	138	66.6	34.17
13	F	65	153	185	80	74.4	27.77
Mean ± SD		62 ± 12	107 ± 36	162 ± 45	78 ± 31	63 ± 14	24 ± 5.6

Flowcytometric Analysis of Treg Subsets. For enumeration of Treg cells, PBMCs (5×10^5 cells) were washed and stained using conjugated antibodies: anti-CD45RO-FITC (BD Pharmingen), anti-CD4-PerCP (BD Pharmingen), anti-CD25-APC (BD Pharmingen), and anti-Foxp3-PE (BD Pharmingen) and were incubated in 4°C for 30 minutes. The cells were subsequently washed and re-suspended in PBS containing 10% FBS. For intracellular staining of Foxp3 molecules, the cells were fixed and permeabilized by Foxp3 buffer set (BD, USA) before adding the conjugated Foxp3 antibody. For each sample, 1×10^5 cells were acquired by FACScalibur™ flowcytometer. Live lymphocytes were gated on forward and side scatter and further analyzed for surface marker and further cytokine expression analysis by real-time PCR (see next section). Flowcytometry analysis was carried out by flowjo software (version 7.6.2). For analyzing mean fluorescent intensity (MFI), geometric mean was used. Representative dot-plots and gating strategies for immunophenotyping analyses are shown in Figures 1, 2 and 3.

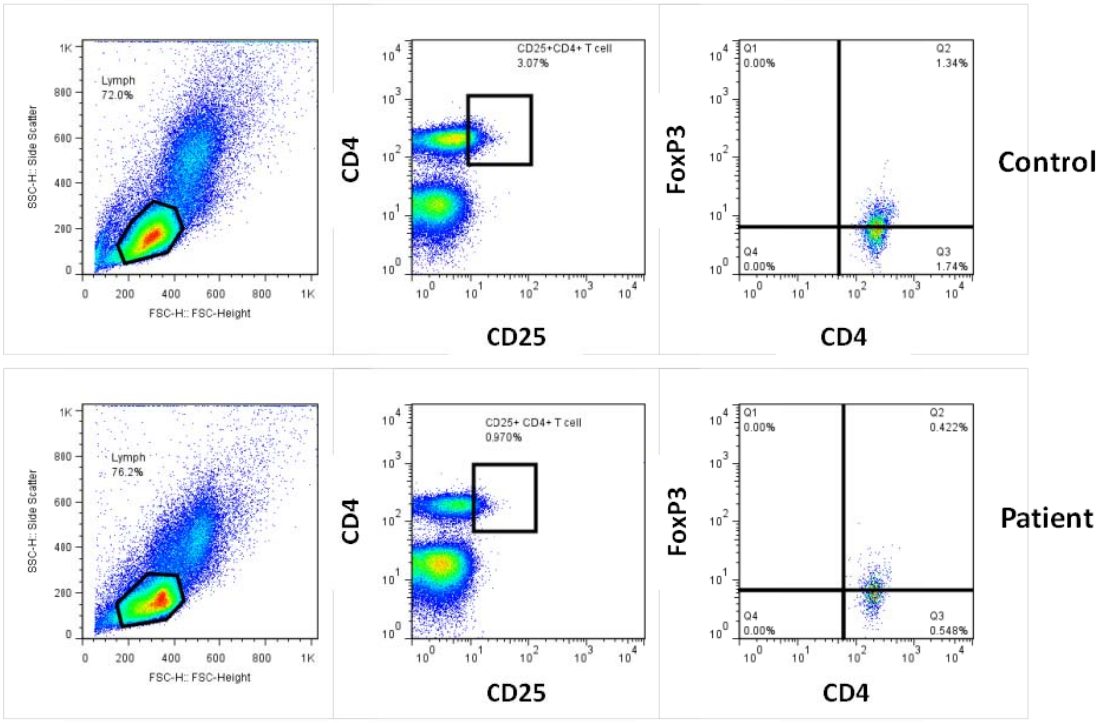

Figure 1. Representative dot plots showing the gating strategy and percentage of Tregs. The gating was performed on lymphocytes and then CD4+CD25+ T cells were gated. On this population, cells with expression of Foxp3 were differentiated from those without Foxp3 expression.

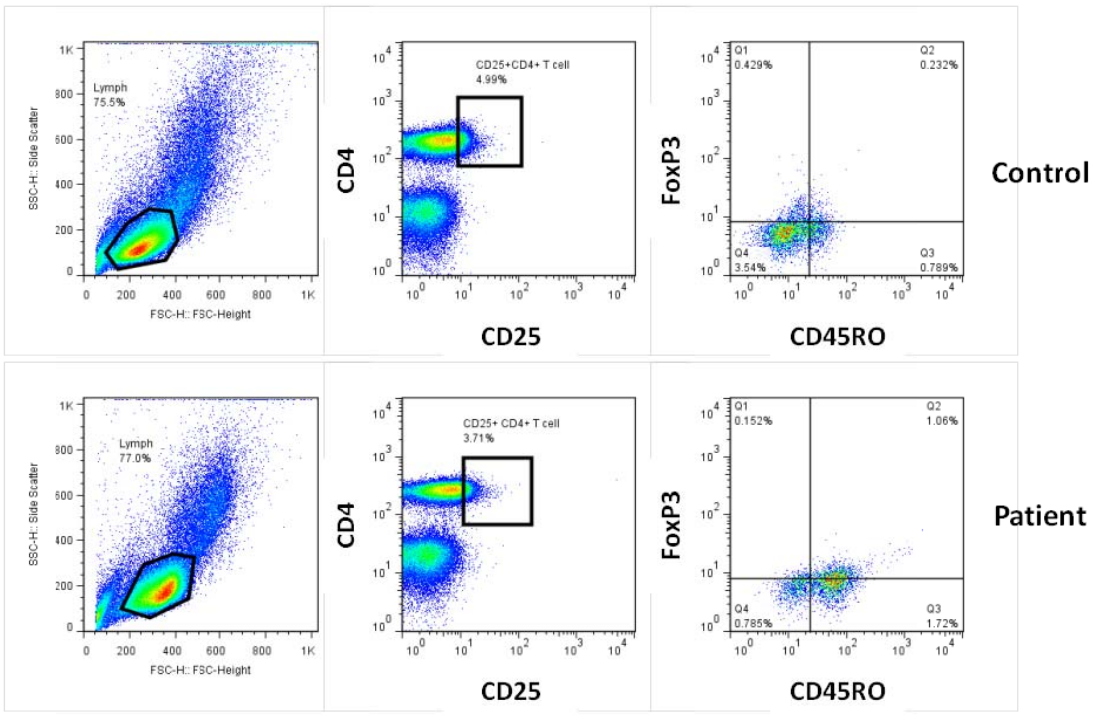

Figure 2. Representative dot plots showing the gating strategy and percentage of nTreg subsets. The gating was performed on lymphocytes and then CD4+CD25+ T cells were gated. On this population the subsets were defined based o the expression of Foxp3 and CD45RO.

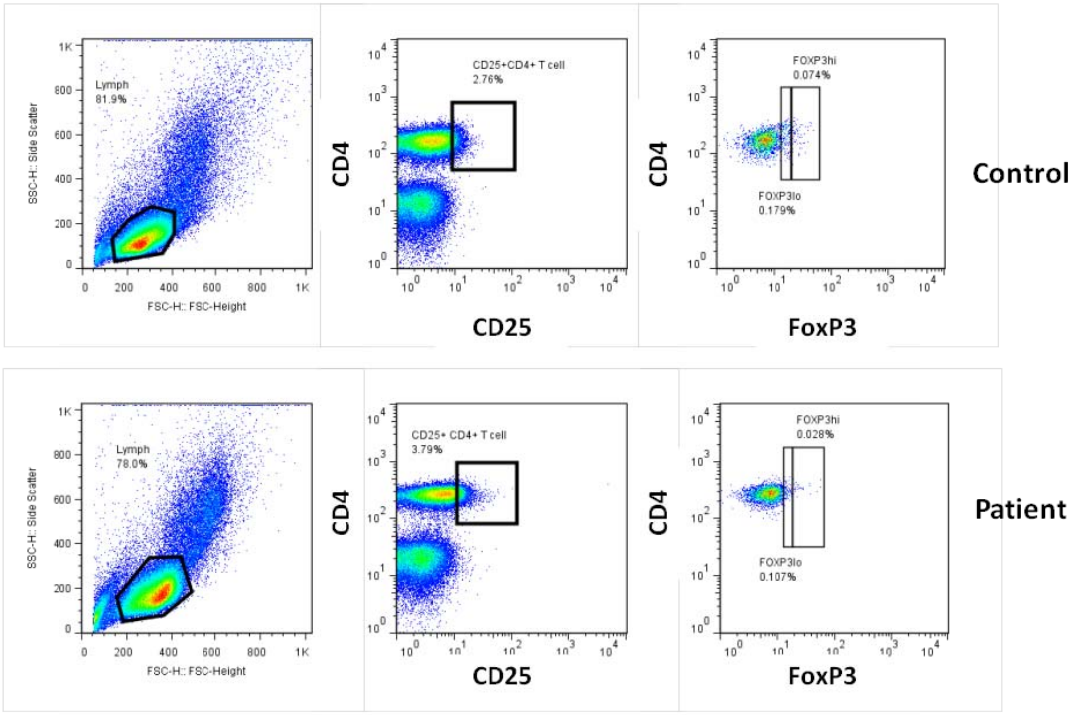

Figure 3. Representative dot plots showing the gating strategy and percentage of Tregs and effector T-cells. The gating was performed on lymphocytes and then CD4+CD25+ T cells were gated. On this population and among the foxp3+ cells (based on a more conservative gating than figure 1) cells with Hi expression of Foxp3 were differentiated from those with Low Foxp3 expression.

Real-Time qPCR. Total RNA was extracted from 1 to 5×10^5 cells using Trizol reagent. The total RNA preparation was reverse transcribed using Superscript II reverse transcriptase, oligo (dT) and random hexamer primer (Thermo Fisher, USA) in a final volume of 20 µl. For real-time quantitative PCR (RT-qPCR), the reaction mixture contained 2 µL cDNA, 10 µL SYBER GREEN premix TAQ2 (Takara, Korea), and 0.8 µl forward and reverse primers in a final volume of 20 µl. qPCR was performed on a ABI-7500 system (USA) under the following conditions: stage 1, 95°C for 30 seconds; and stage 2, 95°C for 5 seconds, 60°C for 34 seconds. Amplification (50 cycles) was carried out and relative expression of each gene was determined by normalization to 18s. The primer sequences were as follows: Foxp3 sense 5′-CATGATCAGCCTCACACCAC-3′ and antisense 5′-CCACTTGCAGACACCATTTG-3′; TGF-β sense 5′-TGGTTGAGCCGTGGAGGGGA-3′ and antisense 5′-CTCGGCGGCCGGTAGTGAAC-3′; 18s sense 5′-CGAACGTCTGCCCTATCAACTT-3′ and antisense 5′-ACCCGTGGTCACCATGGTA-3′. All primers were supplied by metabion (Germany).

Lipid Measurements. Trigelyceride, total Cholesterol, HDL- and LDL-Cholesterol were measured enzymatically in the sera by the diagnostic laboratory of Dena Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.

Statistical Analysis. The Statistical analyses were performed using SPSS software (version 22, Chicago, IL) and GraphPad prism (version 6, La Jolla, CA). Mann-Whitney U test was used for non-parametric comparison of the medians. Spearman's rho rank test was used to test the correlations. P values less than 0.05 were considered significant.

RESULTS

Frequencies of Foxp3+ lymphocytes and Foxp3 MFI in controls and patients. Our results indicated that the frequencies of CD4+Foxp3+ and CD4-Foxp3+ lymphocytes were greater in controls than patients but only in CD4+ population reached a significant level (p=0.0089), (Figure 4). In the CD4+ and CD4- populations in total lymphocytes, Foxp3 MFI increased in controls compared with patients (p=0.01, p=0.02, p=0.03, respectively) (Figure 5).

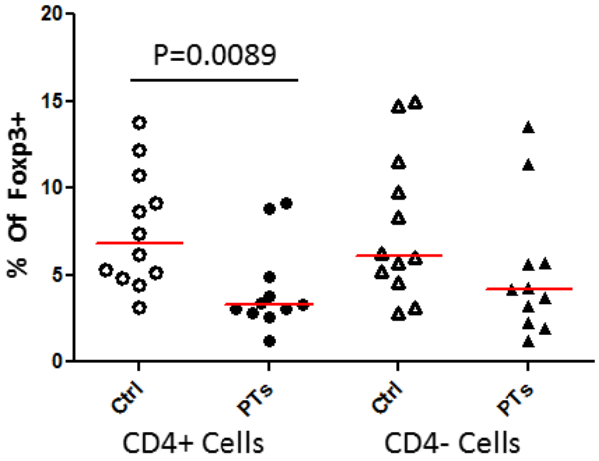

Figure 4. Frequency of Foxp3+ lymphocytes in CD4+ and CD4- population of patients and controls. Percent total is shown.

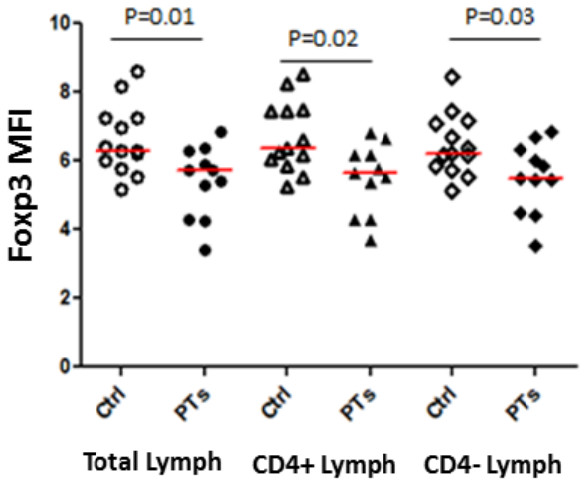

Figure 5. Foxp3 MFI in total lymphocytes and CD4+ and CD4- lymphocytes of patients and controls. Geometric mean is calculated.

Natural Treg subpopulations in the peripheral bloods of patients and controls. The frequency of CD4+CD25+CD45RO+Foxp3- T-cells (effctor/memory T-cell) increased in patients compared with controls (p=0.01, Figure 6). In natural Treg subsets, the frequency of CD45RO-Foxp3^{+} T-cells (resting nTregs) was greater in controls than patients (p=0.02, Figure 6).

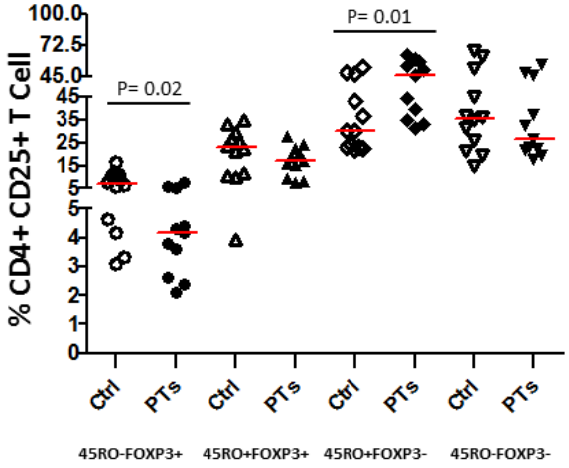

Figure 6. Frequency of T cell subpopulations based on the expression of CD4, CD25, CD45RO and Foxp3 in the peripheral blood of patient and controls. Percent of parent population (CD4+CD25+) is shown.

Table 3. Frequency of activated Tregs and activated/memory cells based on the expression of CD25 and Foxp3 in CD4+CD45RO+ T-cells of patients and controls (see also Figure 7).

Subsets	Control (n=13) Mean ± SD (%)#	Patients (n=11) Mean ± SD (%)
CD4+CD25+CD45RO+	1.98 ± 2.14	1.64 ± 1.12
CD4+CD45RO+CD25+Foxp3^{hi}	0.21 ± 0.2	0.10 ± 0.09
CD4+CD45RO+CD25+Foxp3^{lo}	0.50 ± 0.62	0.27 ± 0.26
CD4+CD45RO+CD25^{hi}Foxp3^{hi}	0.06 ± 0.06	0.01 ± 0.01
CD4+CD45RO+CD25^{lo}Foxp3^{lo}	0.38 ± 0.46	0.22 ± 0.21

Percent total (of 100,000 cells) is shown.

However, further analysis of nTreg subsets showed that the frequency of CD45RO+CD25^{hi}Foxp3^{hi} T-cells (activated nTregs) increased in controls compared with patients (p=0.02, Figure 7).

Foxp3^{lo}CD25^{lo}CD45RO+ and Foxp3^{hi}CD25^{hi}CD45RO+ in the Peripheral Bloods of Patients and Controls. Our results indicated that the frequency of CD4+CD45RO+CD25+Foxp3^{hi} cells was higher in controls compared with patients but the difference between patients and controls was not statistically significant (Table 3).

Further investigation showed that the frequency of CD4+CD45RO+CD25^{hi}Foxp3^{hi} (effector Tregs) was higher in controls compared with patients (P=0.02) while the frequency of CD4+CD45RO+CD25^{lo}Foxp3^{lo}(effector/memory T-cells) was lower in controls compared with patients (Figure 7).

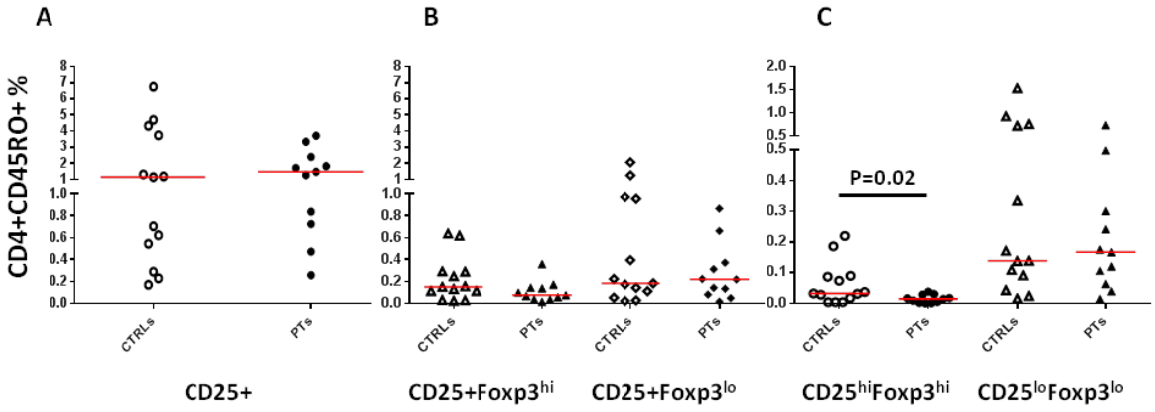

Figure 7. Frequency of activated Tregs and activated/memory cells based on the expression of CD25 and Foxp3 in CD4+CD45RO+ T-cells of patients and controls. (A) Comparison of CD4+CD45RO+CD25+ T cells in controls and patients as a measure of activated T cells (which express CD25 upon stimulation) and activated Tregs. (B) Foxp3 is taken into consideration to differentiate activated T cells and activated Tregs. (C) The differential expression levels of CD25 and foxp3 on activate/ memory T cells (CD25^{lo}foxp3^{lo}) and Tregs (CD25^{hi}foxp3^{hi}). All the percentages are percent total.

Foxp3 MFI in CD4+CD25+ and CD4+CD25+Foxp3+ population. In CD4+CD25+ and CD4+CD25+Foxp3+ populations the MFI of Foxp3 was higher in controls compared with patients (Figure 8).

mRNA Expression of Foxp3 and TGF-β in Total Lymphocytes of Patients and Controls. Data of this experiments revealed that in control group, Foxp3 was expressed in a greater level than in patients with atherosclerosis (p=0.017). Also, TGF-β gene expression was lower in patients compared with controls (p=0.03, Figure 9).

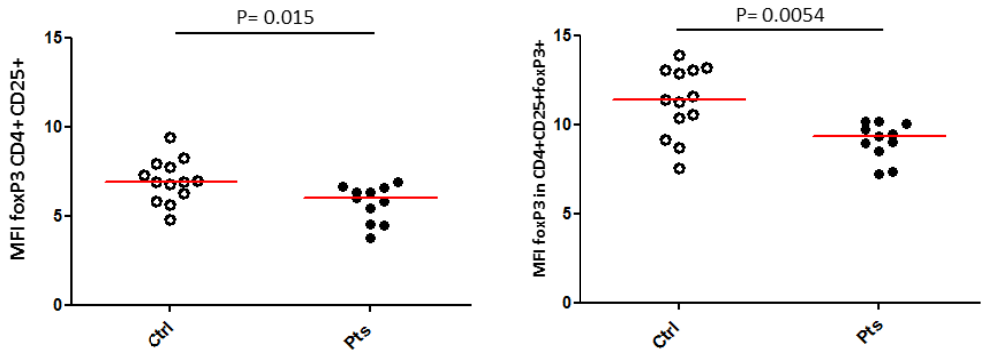

Figure 8. Foxp3 MFI in CD4+CD25+ and CD4-CD25+Foxp3+ subpopulations in the peripheral blood of patient and controls. Since part of CD4+CD25+ T cells were Foxp3-, the MFI of Foxp3+ cells were also analyzed separately.

Correlation of Bood Pressure with the Frequencies of CD4+ T-Cell Subpopulations in Patients with Atherosclerosis. The frequencies of CD4+CD25+CD45RO- Foxp3+ (nTreg) cells were higher in patients with normal blood pressure (<13/9) compared with patients with high blood pressure (>13/9). The frequencies of CD4+CD25+CD45RO+Foxp3+ (aTreg) cells were higher in patients with normal blood pressure (<13/9) whereas the frequencies of CD4+CD25+CD45RO+Foxp3- (effector or memory T-cell) in patients with high blood pressure were more than patients with normal blood pressure. However, none of these differences reached the significant level (Figure 10).

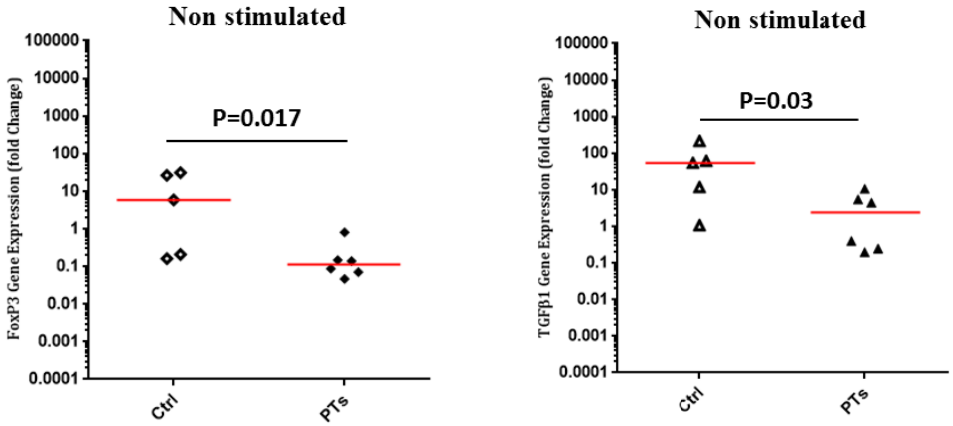

Figure 9. mRNA expression of Foxp3 and TGF-β genes in the total lymphocytes of patients and controls. No stimulation was done before extraction of RNA.

Correlation of Triglycerides with the Frequencies of CD4+ T- Cell Subpopulations in Patients with Atherosclerosis. Our results showed that the frequencies of CD4+CD25+CD45RO-Foxp3+ (nTreg) were higher in patients with normal levels of

triglycerides (<140 mg/dl) compared with patients with increased levels of triglycerides (>140 mg/dl). However, none of these differences reached the significant level (Figure 10).

Correlation of Cholesterol Levels with the Frequencies of CD4+ T-Cell Subpopulations in Patients with Atherosclerosis. Our result showed that the frequencies of CD4+CD25+CD45RO-Foxp3+ (nTreg) were higher in patients with normal levels of cholesterol (<200 mg/dl) compared with patients with elevated levels of Cholesterol (>200 mg/dl). But the frequencies of CD4+CD25+CD45RO+Foxp3 and CD4+CD25+CD45RO+Foxp3- in patients with high levels of Cholesterol were more than patients with normal levels of Cholesterol. However, none of these differences reached the significant level (Figure 10).

Correlation of LDL-Cholesterol with the Frequencies of CD4+ T-Cell Subpopulations in Patients with Atherosclerosis. The frequencies of CD4+CD25+CD45RO-Foxp3+ (nTreg) cells were higher in patients with normal levels of LDL-Cholesterol (<110 mg/dl) compared with patients with elevated levels of LDL-Cholesterol (>110 mg/dl). But the frequencies of CD4+CD25+CD45RO+Foxp3 and CD4+CD25+CD45RO+Foxp3- in patients with high levels of LDL-Cholesterol were more than patients with normal levels of LDL-Cholesterol. However, none of these differences reached the significant level (Figure 10).

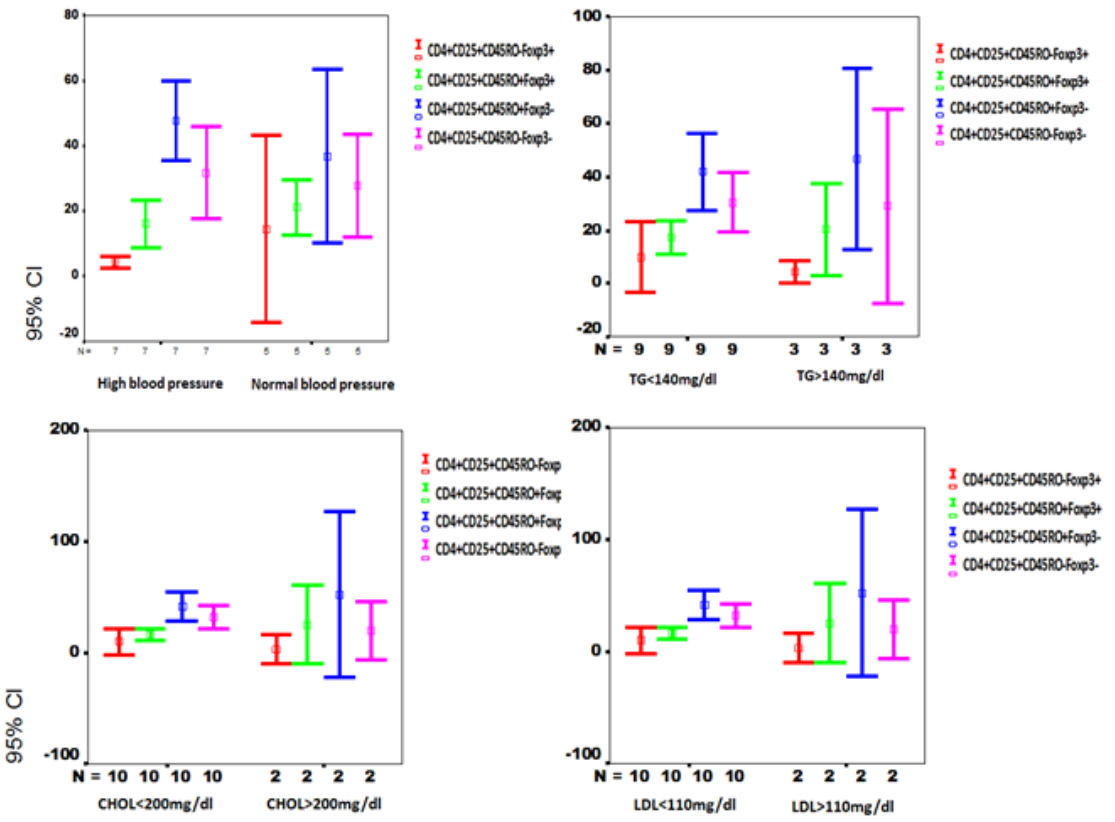

Figure 10. Correlation of triglycerides, Cholesterol, LDL and blood pressure with the frequencies of CD4+ T-cell subpopulations in patients with atherosclerosis.

DISCUSSION

Our results showed that in patients with atherosclerosis, the frequencies of both aTregs and nTregs were decreased and the frequency of effector/memory T-cells was increased. Previous studies have only investigated the number of total nTregs and showed that the frequency of Foxp3+ Treg cells decreased in all stages of human atherosclerotic lesions, as measured in surgical or biopsy samples (21). In our study, too, the frequencies of CD4+ Foxp3+ and CD4- Foxp3+ lymphocytes were greater in controls than patients. Similarly, the frequency of Foxp3+ Treg cells was reduced in the peripheral circulation of patients with carotid artery plaques (22). A decrease in the number of Treg cells has been associated with carotid atherosclerotic plaque vulnerability and is inversely correlated with infiltrative mature dendritic cells (17,18). We studied the frequency of resting and activated Tregs in order to investigate the possible population shift from resting to activated Tregs or vice versa in atherosclerosis. A recent study showed that the immune cell types involved in regulatory mechanisms might be over stimulated in the early pre-clinical phase of atherosclerosis (23). Moreover, a correlation exists between CD4+CD25^{hi}FoxP3+ cell frequency and circulating lipids (23). Another study showed that CD45RA−Foxp3^{hi} but not CD45RA+Foxp3^{lo} suppressive Tregs increased in the peripheral circulation of patients with head and neck squamous cell carcinoma and correlated with tumor progression (24). In our study a decrease in the CD4+CD45RO+CD25+ Foxp3^{hi} subset in atherosclerosis was observed but did not reach the significant level, however, when we considered CD25^{hi} and CD25^{lo} population into account, we found the significantly higher activated/effector Tregs (CD4+CD45RO+CD25^{hi}Foxp3^{hi}) subset in controls compared with patients. This was accompanied by a higher frequency of effector/memory T-cells (CD4+CD45RO+CD25^{lo}Foxp3^{lo}) in the peripheral blood of patients. Expression of Foxp3 molecule at the mRNA and protein level was also higher in PBMCs of controls compared with patients. Similarly TGF-β gene expression was decreased in patients.

In conclusion, our results suggest a decrease in the total nTreg (resting and activated) subsets in patients with atherosclerosis which shows that the resting population is not turning to activated nTreg upon chronic stimulation in atherosclerosis. Although the frequencies of nTregs were somewhat decreased in patients with elevated levels of triglycerides, cholesterol, LDL cholesterol and blood pressure, the differences between patients and controls were not statistically significant and we suggest a larger sample size would be needed to confirm our results. Previous studies indicated that hyper-lipidemia and high blood pressure were two main risk factors of atherosclerosis (25). Moreover, statin therapy can reduce lipid levels and increase Tregs function and frequency (26). Therefore, functional analysis of Tregs and its correlation with lipids profile and hypertension would also be meaningful.

ACKNOWLEDGEMENTS

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