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CD45RO+ memory T-cells produce IL-17 in patients with atherosclerosis

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Abstract

Several CD4+ T helper (Th) cell subsets are shown to play a role in atherosclerotic lesion formation and progression. We investigated the frequencies of IL-17 and IFN-γ producing CD4+ T-cell subsets in the peripheral blood mononuclear cells (PBMCs) of 10 patients with atherosclerosis and 6 individuals with normal/insignificant coronary artery disease. Th1 and Th17 memory and effector T-cells were enumerated by flowcytometry and correlated with the clinical data and lipid profiles of the subjects. We found the *ex-vivo* (P=0.0001) and *in-vitro* production of IL-17 (P=0.001) but not IFN-γ by CD4+ memory T-cells of patients. CD45RO+ memory cells were the major producers of IL-17 and the CD4+CD45RO+PD-1- T-cells of the patients produced higher levels of IFN-γ than controls (P=0.02). Positive correlations between the frequency of CD4+CD45RO+IL-17+IFN-γ- T-cells and serum LDL-C (P=0.007), triglyceride (P=0.02), and systolic (P=0.001) and diastolic (P=0.009) blood pressures (BP) were found. The frequency of CD4+CD45RO+IL-17-IFN-γ- T-cells, which was higher in controls than patients, showed negative correlations with the serum LDL-C (P=0.01) and triglyceride (P=0.02) levels and systolic (P=0.003) and diastolic (P=0.01) BPs. The ex-vivo Th17 deviation of memory T-cells in atherosclerosis and high PD-1 expression are associated with the correlates of atherogenesis such as LDL, TG, and BP.

Key words: Th17, PD-1, memory T-cell, LDL, Atherosclerosis.

Introduction

Atherosclerosis is an inflammatory disease that is characterized by increasing levels of low-density lipoprotein cholesterol (LDL-C) in the blood. Different leukocyte populations are reported in the adventitia of normal arteries, which their frequency and distribution changes during formation, and progression of atherosclerotic lesions (1).

The initial step for formation of lesion is thought to be the endothelial dysfunction induced by oxidative and/or microbial factors (2). Endothelium then recruits different populations of leucocytes, including T-cells, to the injured site and attempts to repair itself. Circumstances that hamper endothelial repair result in the permeabilization of endothelial cells and migration of lymphocytes and monocytes into the intima, which further on, result in the formation of foam cells, migration of smooth muscle cells, and formation of the fatty streak and fibrous cap (3). At this stage, the macrophages and monocytes involved in the reaction start to die and cause the development of a necrotic core that is covered by fibrous cap. Atheromatous plaques continue to expand along with the wall of the affected artery (4).

It is now well accepted that local and systemic adaptive immune responses are also major determinants of atherogenesis, and several CD4+T helper (Th) cell subsets have been shown to play a role in the progression of the atherosclerotic lesion. While interferon (IFN)-γ secreting Th1 cells promote atherogenesis (5), Foxp3+ regulatory T-cells (Treg) decrease adaptive and innate immune responses and inhibit lesion formation (6). The role of other polarized T-cell subsets is still not well understood. The role of Th2 cells in atherogenesis remains to be conclusively defined, as this subset was proposed to limit atherogenesis, in part through IL-13 secretion (6), whereas other Th2 cytokines, in particular IL-4, might be pro-atherogenic (5, 7). Similarly, IL-17-secreting Th17 cells have been proposed to both promote and limit atherogenesis (7). However, recent reports consolidate the atherogenic role of Th17 cells in both humans and animal models (8, 9). Although each T-cell subset may exhibit a distinctive role in the atherogenesis, the activation/inhibition signals provoked by co-stimulatory/co-inhibitory molecules are of utmost importance in determining the functional fate of T-cells (10). Previous studies on the LDLR-/- /PD-L1-/- and LDLR-/-/PD-1-/- mice suggested a role for PD-1/PD-L1 pathway in the downregulation of atherogenic T-cell responses (11, 12). While studies on human subjects are scarce, downregulation of PD-1 on T-cells observed in patients with coronary artery disease (CAD) is shown to enhance CD4+ T-cell pro-inflammatory cytokine production (13).

In this study, we investigated the frequencies of IL-17 and IFN-γ producing CD4+ memory T-cell subsets as well as PD-1 expression on CD4+ memory T-cells in patients with carotid artery atherosclerosis and in control individuals. We also investigated the association of memory T-cell subsets with the correlates of atherogenesis and atheroprotection. Our results indicated a Th17 deviation of T-cell subsets in patients both before and after *in-vitro* stimulation. This increase was associated with the correlates of atherogenesis in our studied subjects.

Materials and methods

Subjects

After informed consent, 20 ml heparinized blood was obtained from each of the 10 non-diabetic, non-smoker patients (6 men and 4 women aged 50-60 yrs,

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

		Mean ± SD					
Number	**Sex**	**Age (years)**	**HDL (mg/dl)**	**LDL (mg/dl)**	**TG (mg/dl)**	**Systolic BP (mmHg)**	**Diastolic BP (mmHg)**
1	M	38	67	89	239	120	80
2	M	42	40	137	120	125	85
3	M	45	35	55	140	120	80
4	F	51	63	103	124	132	78
5	F	41	57	97	113	120	80
6	F	50	51	120	97	122	86
		44.5±5.16	52.17±12.69	100.16±28.04	138.83±51.04	123.16±4.75	81.5±3.20

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

		Mean ± SD					
Number	**Sex**	**Age (years)**	**HDL (mg/dl)**	**LDL (mg/dl)**	**TG (mg/dl)**	**Systolic BP (mmHg)**	**Diastolic BP (mmHg)**
1	M	60	50	146	216	138	87
2	M	60	70	169	215	148	94
3	M	57	52	185	167	152	85
4	M	60	50	174	220	148	88
5	M	57	38	183	210	140	90
6	M	60	40	145	250	138	82
7	F	53	40	145	165	125	90
8	F	50	33	175	280	165	95
9	F	57	34	187	220	142	95
10	F	55	40	178	183	135	88
		56.9±3.41	44.7±11.11	168.7±16.99	212.6±35.42	143.1±10.87	89.4±4.33

mean=56.9 ± 3.41 yrs) who were diagnosed with coronary artery disease confirmed by carotid angiography and had hypertension [systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg] and dyslipidemia according to the established guidelines (14-16). Control group consisted of 7 non-diabetic, non-smoker, normotensive, normolipemic individuals with normal/insignificant coronary artery disease confirmed by carotid angiography (4 men, 3 women aged 50-60 yrs, mean= 44.5 ± 5.16 yrs) from each of whom 20 ml blood was also obtained. The levels of LDL, TG and HDL and systolic and diastolic blood pressures (SBP and DBP) were measured for the patients and controls at the time of sampling (table 1 and 2). None of the patients and controls was on the Statins therapy.

Peripheral Blood Mononuclear Cells (PBMCs) Isolation

PBMCs were isolated by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Europe, GmbH, Germany) and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in fetal bovine serum (FBS Biosera, UK).

Flowcytometric analysis of Th17 and Th1 subsets

For enumeration of Th17 and Th1 cells, PBMCs (5×10^5 cells) were washed and stimulated with plate coated purified anti-CD3 (BD Pharmingen, 62.5 µg/ml) and soluble purified anti-CD28 (BD Pharmingen, 0.5 mg/ml) or phorbol myristate acetate (PMA, 50 ng/ml, Sigma) plus ionomycin (ION, 250 ng/ml, Sigma) at 37°C and 5% CO2. Golgi-stop was added after 1 hour and the cells were incubated for another 15 hours at 37°C and 5% CO2. Then the cells were washed and stained using conjugated antibodies: anti-CD45RO-FITC (BD Pharmingen), anti-CD4- PerCP (BD Pharmingen), anti-IFN-γ-APC (BD Pharmingen), and anti-IL-17-PE (BD Pharmingen) and were incubated in 4°C for 30 minutes. The cells were subsequently washed and resuspended in PBS containing 10% FBS. 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 cytokine expression. Flowcytometry analysis was carried out by flowjo software (version 7.6.2).

Flowcytometric analysis of PD-1 expression

For the analysis of PD-1 expression on memory T-cell subsets, PBMCs (5×10^5 cells) were washed and stimulated with plate-coated purified anti-CD3 (BD Pharming, 62.5 µg/ml) antibody and soluble purified anti-CD28 (BD Pharmingen, 0.5 mg/ml) at 37°C and 5% CO2. After 1 hour Golgi-stop was added and cells were incubated for another 15 hours at 37°C and 5% CO2. Then the cells were washed and stained for surface markers using conjugated antibodies: anti-CD45RO-FITC (BD Pharmingen), anti-CD4-PerCP (BD Pharmingen), and anti-PD-1-PE (BD Pharmingen) in 4°C for 30 minutes. The cells were subsequently washed and resuspended in PBS containing 10% FBS. We acquired 1×10^5 cells for each sample by FACScalibur flowcytometer. Live lymphocytes were gated by forward and side scatter and analyzed for surface marker expression. Flowcytometric analysis was done by flowjo software (version 7.6.2, Ashland, OR).

Lipid measurements

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

Statistical analysis

The Statistical analyses were performed using SPSS software (version 15, Chicago, IL) and GraphPad prism

(version 5, La Jolla, CA). Mann-Whitney U test was used for non-parametric comparison of the medians. P-values less than 0.05 were considered significant.

Results

Frequencies of T-cell subpopulations in patients with atherosclerosis and control subjects

We investigated the frequency of CD4+ T-cells by four-color flowcytometry subsequent to stimulation with PMA/ION and anti-CD3/anti-CD28 antibodies. Our results indicated that despite a trend of decreased original frequency of the non-stimulated CD4+CD45RO+ (memory) T-cells in patients, the difference between patients and controls was not statistically significant (16.89 ± 5.55% vs. 24.6 ± 5.80%, P=0.06). In the patient group, the frequency of PMA/ION stimulated CD4+CD45RO+ T-cells was not different from that of non-stimulated condition (P=0.5); however, in the control group the frequency of PMA/ION stimulated CD4+CD45RO+ T-cells was markedly decreased in comparison with non-stimulated condition (P=0.002). As a result, the frequency of CD4+CD45RO+ T-cells after stimulation with PMA/ION was greater in patients than controls (15.85 ± 4.91% vs. 10.47 ± 2.43%, P=0.007), while stimulation with anti-CD3/anti-CD28 antibodies only slightly increased the frequency of this T-cell subset in the controls (23.45 ± 4.91%) and patients (17.35 ± 5.04%, P= 0.04; figure 1).

Compared to the non-stimulated condition, PMA/ION decreased the frequencies of CD4+CD45RO- (effector) T-cells in both patients (24.63 ± 8.39% vs. 8.24 ± 5.18%, P<0.0001) and controls (22.98 ± 6.89% vs. 3.71 ± 2.37%, P=0.001). In the patient group, the frequency of CD4+CD45RO- T-cells after stimulation with PMA/ION remained higher than controls (P=0.01); but no evident difference were observed between controls and patients in non-stimulated condition (P=0.4). The stimulation by anti-CD3/anti-CD28 antibodies decreased this population in controls non-significantly (P=0.3) (figure 1).

The frequency of PMA/ION stimulated CD4-CD45RO+ T-cells in the controls was higher than the patients (28.48 ± 5.83% vs. 17.45 ± 5.30%, P=0.004). Compared to the non-stimulated condition (10.56 ± 3.08% and 11.81 ± 4.36%), the frequency of this population increased after stimulation with PMA/ION in both controls (P=0.001) and patients (P=0.01).

Frequencies of IL-17 and IFN-γ producing CD4+CD45RO+ memory T-cells

As shown in figure 2, a visible population of CD4+CD45RO+ memory T-cells in the patients group (but not controls) produced IL-17 even before stimulation (7.60 ± 4.71% vs. 1.71 ± 0.94%, P=0.0001). The frequency of IL-17 producing CD4+CD45RO+ memory T-cells was higher in patients compared with controls both after stimulation with anti-CD3/anti-CD28 antibodies (8.79 ± 6.75% vs. 0.95 ± 0.82%, P=0.0001) and PMA/ION (19.21 ± 4.08% vs. 3.52 ± 3.89%, P=0.001).

After stimulation with anti-CD3/anti-CD28 antibodies, the frequency of the CD4+CD45RO+IL-17+IFN-γ+ T-cell subset increased in patients but not controls (0.65 ± 0.54% vs. 0.06 ± 0.05%, P=0.001). The frequency of the CD4+CD45RO+IL-17+IFN-γ+ T-cell subset increased in both patients and controls after stimulation with PMA/ION (8.62 ± 6.6% vs. 4.00 ± 2.36%) but was only significant in the patients group (0.33 ± 0.24% vs. 8.62 ± 6.6% P=0.01).

There was no IFN-γ production in the absence of IL-17 in the non-stimulated condition in patients and controls. The frequency of CD4+CD45RO+IL-17-IFN-γ+ T-cells in non-stimulated condition (0.71 ± 0.51% vs. 0.52 ± 0.51%, P=0.1) and after stimulation with anti-CD3/anti-CD28 antibodies showed no significant difference between patients and controls (12.17 ± 6.06% vs. 6.57 ± 5.80%, P=0.2). Stimulation with PMA/ION induced higher IFN-γ production in the control group (42 ± 24.29% vs. 21.78 ± 14.24%, P=0.0001) and stimulation with anti-CD3/anti-CD28 antibodies induced IFN-γ production in the patients group at a much lower level (figure 2).

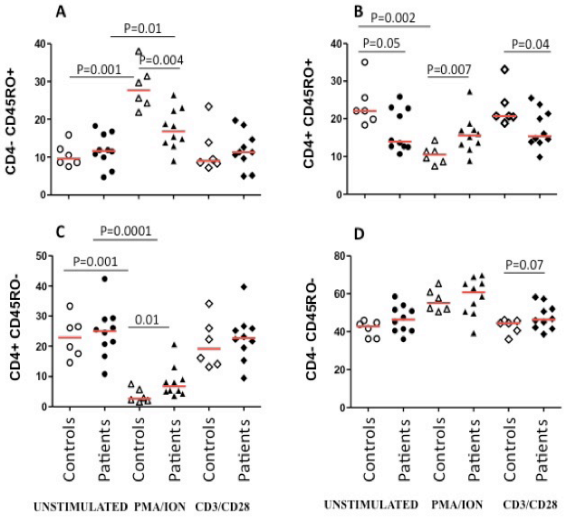

Figure 1. T-cell subpopulations **(A)** CD4-CD45RO+, **(B)** CD4+CD45RO+, **(C)** CD4+CD45RO-, and **(D)** CD4-CD45RO- of patients with atherosclerosis and controls in non-stimulated condition and after stimulation with PMA/ION and anti-CD3/anti-CD28 antibodies.

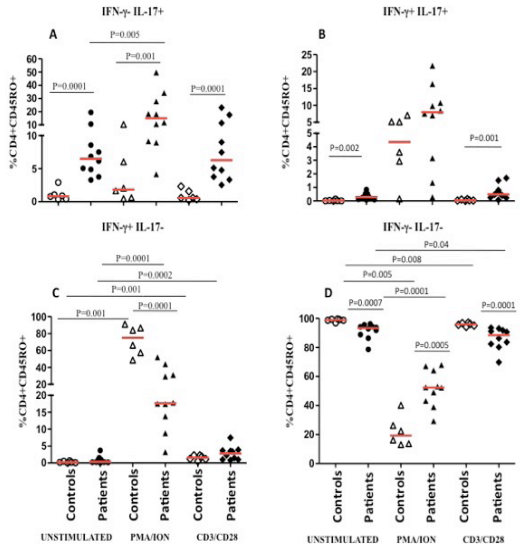

Figure 2. The frequencies of cytokine producing CD4+CD45RO+ T-cells **(A)** IFN-γ-IL-17+, **(B)** IFN-γ+IL-17+, **(C)** IFN-γ-+IL-17-, and **(D)** IFN-γ-IL-17- of patients with atherosclerosis and controls in non-stimulated condition and after stimulation with PMA/ION and anti-CD3/anti-CD28 antibodies.

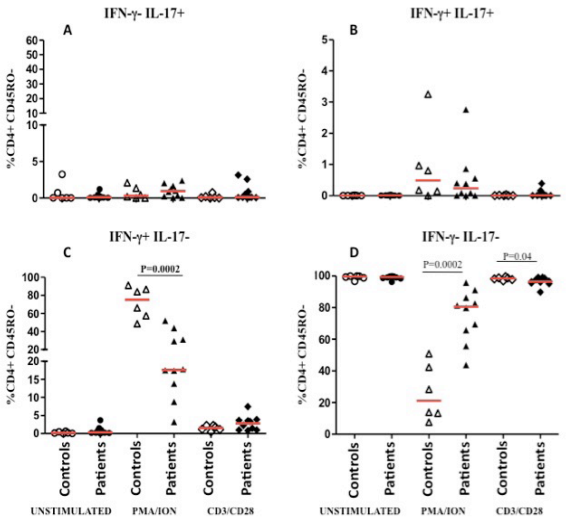

Figure 3. The frequencies of cytokine producing CD4+CD45RO- T-cells **(A)** IFN-γ-IL-17+, **(B)** IFN-γ+IL-17+, **(C)** IFN-γ-+IL-17-, and **(D)** IFN-γ-IL-17- in controls and patients after stimulation with PMA/ION and anti-CD3/anti-CD28 antibodies.

Frequencies of IL-17 and IFN-γ producing CD4+CD45RO- effector T-cells

The frequencies of IL-17 producing CD4+CD45RO- effector T-cells and IL-17/IFN-γ producing CD4+CD45RO- T-cells were not different between controls and patients. There was no IFN-γ production in the absence of IL-17 in the non-stimulated condition in patients and controls. After stimulation with PMA/ION, the frequency of CD4+CD45RO-IFN-γ+ T-cell subset increased in controls, compared with patients (63.36 ± 28.60% vs. 23.42 ± 15.38%, P= 0.0002). The frequency of CD4+CD45RO- T-cells that did not produce IL-17 and IFN-γ increased in patients after PMA/ION stimulation (34.63 ± 27.7% vs. 75.09 ± 16.33%, P=0.0002; figure 3). The representative dot plots of IL-17 and IFN-γ producing CD4+CD45RO+ memory and CD4+CD45RO- effector T-cells are shown in figure 4.

The Mean Fluorescent Intensity (MFI) of IL-17 in different T-cell subsets

Both stimulation conditions increased the MFI of IL-17 in patients and controls. In the CD4+CD45RO+ T-cell subset the MFI of IL-17 showed no significant difference between controls and patients in non-stimulated condition (P=0.1) and after stimulation with PMA/ION (P=0.1); however, after TCR stimulation the IL-17 MFI increased in patients compared with controls (P=0.04). In neither of the non-stimulated or stimulated conditions, the IL-17 MFI of CD4+CD45RO- T-cell subset was significantly different between controls and patients (P=0.2, P=0.2 and P=0.1, respectively; figure 5).

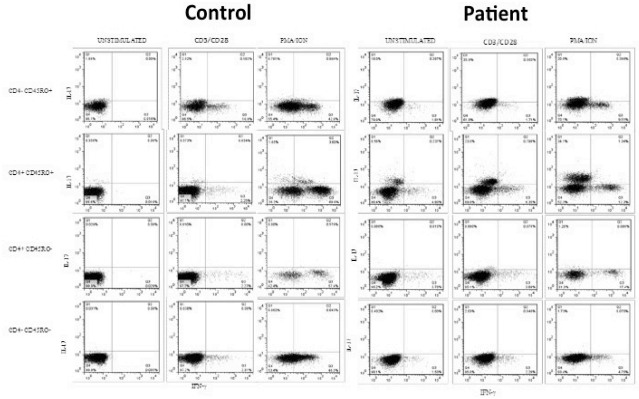

Figure 4. Representative dot plots illustrating production of IL-17 and IFN-γ in the CD4+ T-cell and CD4- T-cell subpopulations in a healthy individual and a patient with atherosclerosis in non-stimulated and stimulated conditions.

The Mean Fluorescent Intensity (MFI) of IFN-γ in different T-cell subsets

Both stimulation conditions increased the MFI of IFN-γ in patients and controls. For the CD4+CD45RO+ T-cell subset, the MFI of IFN-γ in non-stimulated condition and after stimulation with anti-CD3/anti-CD28 antibodies (P=0.06 and P=0.08, respectively) showed no significant difference between controls and patients. However, stimulation with PMA/ION induced greater expression (MFI) of IFN-γ in controls than patients (P=0.01). Moreover, after stimulation with PMA/ION, the IFN-γ MFI increased in both control and patient groups as compared with the non-stimulated condition (P=0.0003, P=0.0004, Figure 6). IFN-γ MFI in CD4+CD45RO- T-cells, showed no significant difference between controls and patients in non-stimulated condition (P=0.05) and after either of the stimulation conditions (P=0.1 and P=0.08; figure 5).

PD-1 expression on the CD4+ and CD4- T-cell subpopulations

The frequencies of PD-1 expressing T-cell subsets were also analyzed in the non-stimulated condition and after stimulation with anti-CD3/CD28 antibodies. The gating strategy and representative histograms are shown in figures 6a and 6b. The highest percentage of PD-1 expression in patients was observed on the CD4+CD45RO+ memory T-cells (figure 6b). The stimulation did not affect the PD-1 expression levels; however, a high percentage of CD4+CD45RO+ memory T-cells in all patients expressed PD-1 (figure 6c). In contrast, the majority of CD4+CD45RO- effector T-cells in patients did not express PD-1 as compared to the controls (figure 6c).

Correlation of Triglyceride (TG) levels with the frequencies of CD4+ and CD4- T-cell subpopulations

Serum triglyceride levels had negative correlation

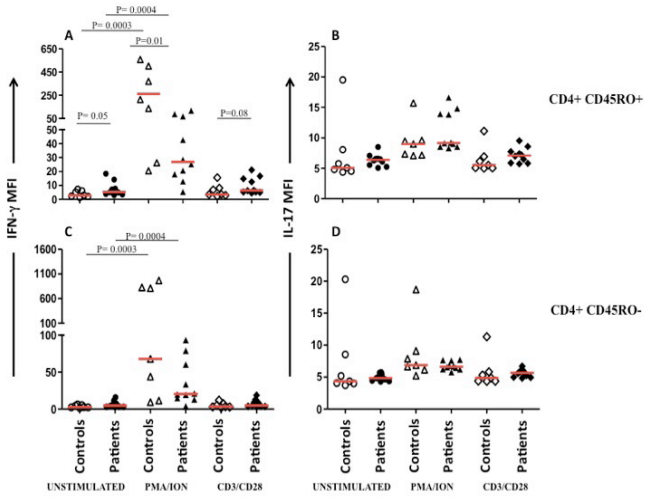

Figure 5. The MFI of IFN-γ and IL-17 cytokines in CD4+CD45RO+ (**A** and **B**), and CD4+CD45RO- (**C** and **D**) T-cells of patients and controls in non-stimulated and stimulated conditions.

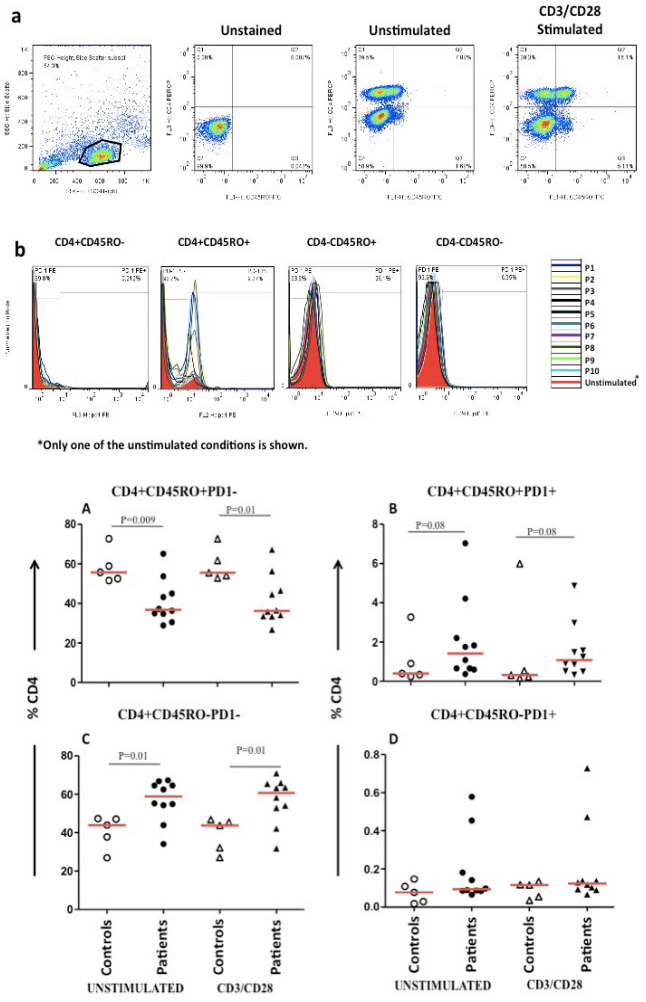

Figure 6. (A) Representative dot plots showing the gating strategy and percentage of memory and effector T-cells; **(B)** Representative histograms of PD-1 expression in different T-cell subpopulations of patients; **(C)** Comparison of the frequencies of memory and effector CD4+ T- cells based on the PD-1 expression between patients and controls.

with the frequencies of CD4+CD45RO+IL- 17-IFN-γ- (P=0.02), CD4-CD45RO+IL-17-IFN-γ- (P=0.02), and CD4+CD45RO+PD-1- T-cells (P=0.01). TG also had positive correlations with the frequencies of CD4+CD45RO+IL-17+IFN-γ- (P=0.02), CD4-CD45RO+IL-17+IFN-γ- (P=0.02) and CD4+CD45RO-PD-1- (P=0.06) T-cells. Some of these correlations are shown in figure 7a.

Correlation of HDL Cholesterol (HDL-C) levels with the frequencies of CD4+ and CD4- T- cell subpopulations

Serum HDL-C level showed a positive correlation with the frequency of CD4+CD45RO+PD-1- memory T-cells (P=0.01). Increased HDL-C levels in controls were associated with an increase in this population (figure 7a). HDL-C had an insignificant negative correlation with the frequency of CD4+CD45RO-PD-1- T-cells in patients (P=0.09).

Correlation of LDL Cholesterol (LDL-C) levels with the frequencies of CD4+ and CD4- T- cell subpopulations

LDL-C levels in patients' sera had positive correlations with the frequencies of CD4+CD45RO+IL-17+IFN-γ- (P=0.007) and CD4-CD45RO+IL-17+IFN-γ- (P=0.01) memory T-cells (figure 7b). Increase in the LDL-C levels in patients was concomitant with the enhanced frequencies of these subpopulations. LDL-C had negative correlations with the frequencies of CD4+CD45RO+IL-17-IFN-γ- (P=0.01), CD4-CD45RO+IL-17-IFN-γ- (P=0.01), CD4-CD45RO-IL-17-IFN-γ- (P=0.05), and CD4+CD45RO+PD-1- T-cells (P=0.05). Similarly, we observed that the lower the LDL-C levels in controls, the higher the frequencies of these subpopulations.

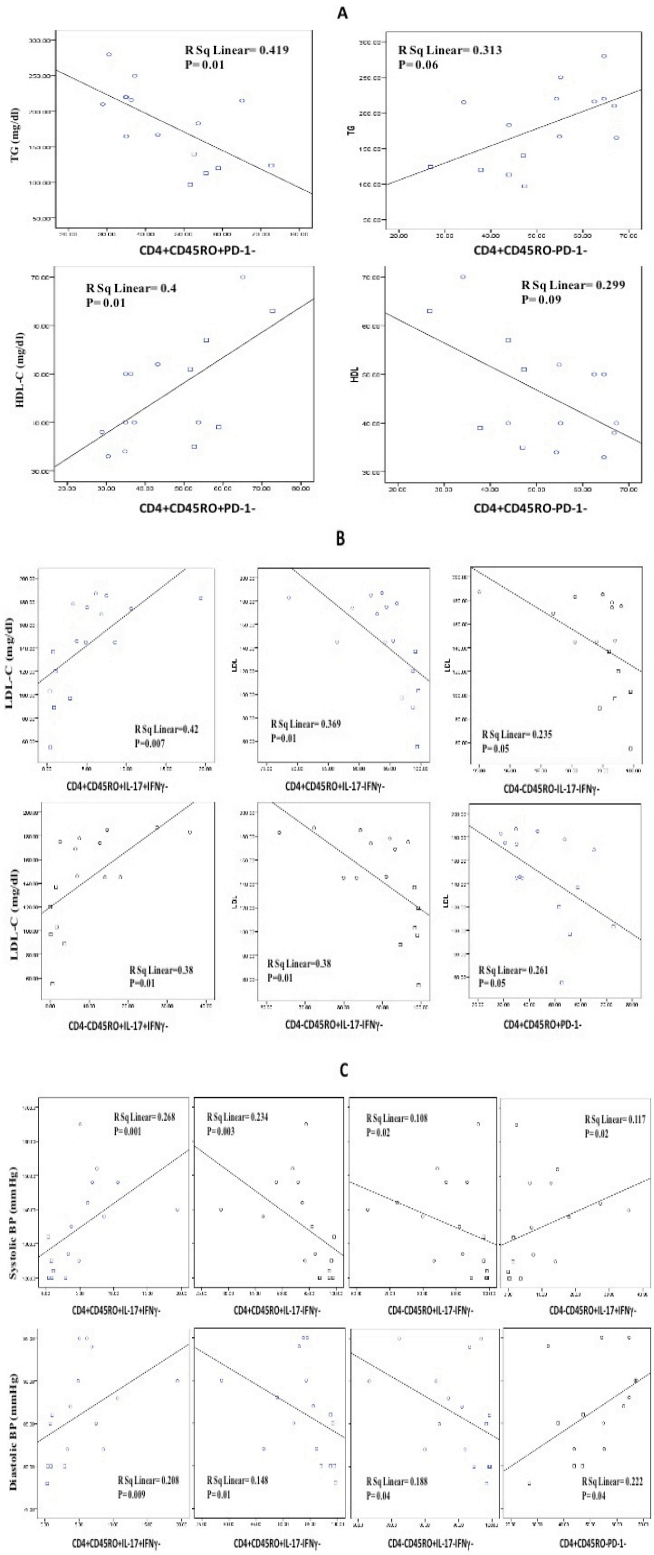

Figure 7. (A) Correlation of Triglyceride and HDL levels with PD-1- effector and memory CD4+ T-cells; **(B)** Correlation of serum LDL levels with different T-cell subpopulations; **(C)** Correlation of Systolic and Diastolic BP levels with different T-cell subpopulations.

Correlation of systolic blood pressure with the frequencies of CD4+ and CD4- T-cell subpopulations

Systolic blood pressure had positive correlations with the frequencies of CD4+CD45RO+IL-17+IFN-γ- (P=0.001) and CD4-CD45RO+IL-17+IFN-γ- (P=0.02) memory T-cells and negative correlation with the frequency of CD4+CD45RO+IL-17-IFN-γ- (P=0.003) and CD4-CD45RO+IL-17-IFN-γ- memory T-cells (P=0.02) (figure 7c).

Correlation of diastolic blood pressure with the frequencies of CD4+ and CD4- T-cell subpopulations

Diastolic blood pressure had positive correlations with the frequencies of CD4+CD45RO+IL-17+IFN-γ- (P=0.009) and CD4+CD45RO-PD-1- T-cells (P=0.04). It showed negative correlations with the frequencies of CD4+CD45RO+IL-17-IFN-γ- (P=0.01) and CD4-CD45RO+IL-17- IFN-γ- (P=0.04) (figure 7c).

Discussion

One of the main findings of this study was the *ex-vivo* and *in-vitro* production of IL-17 but not IFN-γ by CD4+ memory and effector T-cells of patients with atherosclerosis confirmed by carotid angiography. In addition, while the percentage of IFN-γ producing cells was not significantly different between patients and controls, the percentage of CD4+ T-cells producing both IL-17 and IFN-γ was increased in patients. The lower IFN-γ MFI and the higher IL-17 MFI observed in patients compared to controls are also suggestive of a Th1 to Th17 shift in CD4+ T-cells of patients with atherosclerosis. Previous studies in mouse and human models point to the role of both Th1 and Th17 cells in the progression of atherosclerosis. However, there is not much information with regard to the T-cell subsets that are responsible for production of these cytokines in terms of memory or effector phenotype in humans. A recent study in LDb mice (Ldlr-/-/Apobec1-/-) showed the accumulation of Th17 cells in the lymphoid organs of these nonobese hyperlipidemic mice (8). The Th17 cells in these mice were reported to be of effector- memory phenotype and expressed high levels of CD44 and the mRNA of Rorc as well as IL-21, IL-22 and IL-23r. The secreted IL-17 level in the sera of the LDb mice was also shown to be significantly increased compared to the wild type mice (8). In another study, splenic T-cells of ApoE-/- mice that were fed on a western-type diet showed a time-dependent Th17 deviation upon stimulation *in-vitro* (9). Progressive accumulation of T-cells in all vascular layers of the human aortic wall is beautifully shown in a recent study (17). The authors represent data showing that CD45RO+ memory T-cells are present in high numbers in the intima of vulnerable plaques and progressive lesions. Plaque healing; however, results in the reduction of these cells in the area (18). Our results indicated that among the CD4+ T-cells, CD45RO+ memory cells were the major producers of IL-17. While in the CD4- (CD8+) subset, the effector T-cells (CD45RO-) were responsible for producing IL-17. We also observed an *ex-vivo* decrease in the frequency of CD4+CD45RO+ T-cells in patients compared to controls. The susceptibility of memory-like Tregs to apoptosis has already been suggested (19). A high percentage of CD4+CD45RO+ memory T-cells in all patients expressed PD-1, while the percentage of CD4+CD45RO-PD-1+ effector T-cells was not different between patients and controls. Previous studies have shown that over 80% of Treg cells express CD45RO molecule (20). Therefore, it is possible that a decrease in the population of Treg cells (which express PD-1 molecule) in an atherogenic condition provides the opportunity for Th17 cells to prevail. We did not specifically investigate the Tregs in the current study but a new study has shown frequency and functional defects in the naïve-like and memory-like Treg cells in patients with coronary artery disease (21).

We also found a positive correlation between the frequency of CD4+CD45RO+IL-17+IFN-γ- T-cells with the serum LDL, triglyceride levels, and systolic and diastolic blood pressures in the studied subjects. On the other hand, the frequency of CD4+CD45RO+IL-17-IFN-γ- T-cells, which was higher in controls than patients, showed a negative correlation with the serum LDL and triglyceride levels as well as systolic and diastolic blood pressure. These findings are in agreement with the findings of a previous study that also showed the effect of ox-LDL on Th17 deviation (8). Interestingly, the frequency of CD4+CD45RO+PD-1- T-cells showed a positive correlation with HDL levels and a negative correlation with the serum LDL and triglyceride levels in patients. These cells in the peripheral blood of patients produced significantly higher levels of IFN-γ than that of controls. Currently, we do not know the function of this subset in the atheroprotection or atherogenesis but the observation that these cells produce IFN-γ is noteworthy and needs further investigation. In Conclusion, our study showed that there is an *ex-vivo* Th17 deviation and high PD-1 expression of CD4+CD45RO+ memory T-cells in the atherosclerosis, which is associated with the correlates of atherogenesis such as LDL, TG, and BP. Moreover, we showed a decrease of PD-1 expression on Th1 cells in our patients. Both conditions correlated with the presence of atherosclerosis.

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