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Senescent CD153+ T Lymphocytes Increase in the Peripheral Blood of Patients with Thromboangiitis Obliterans

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ABSTRACT

Background: Buerger's disease, also known as Thromboangiitis Obliterans (TAO), is a progressive, inflammatory vascular disease with unknown etiology.

Objective: To address the degree of T cell immunosenescence in this inflammatory disease, the frequency of senescent T cells expressing CD57 and/or CD153 (CD30L) in patients with TAO.

Methods: In this study, nine male cigarette smoker patients with TAO, nine male healthy cigarette smokers, and nine male healthy non-smoker blood donors were enrolled. PBMCs were extracted from the blood of all participants and stored in liquid nitrogen before use. The percentages of senescent T cells were detected by flow cytometry. The results were analyzed using non-parametric statistical tests.

Results: The frequencies of senescent $CD3^+CD4^+CD57^+CD153^+$ and $CD3^+CD4^+CD57^-CD153^+$ T cells significantly increased in patients compared with the non-smoker controls (p=0.01 and p=0.04, respectively). The frequency of senescent $CD3^+CD4^-CD57^-CD153+$ T cells was higher in patients compared with the smoker controls (p=0.02). In patients with TAO, $CD57^+CD153^-$ cells were more frequent in $CD3^{hi}CD4^-$ and $CD3^{hi}CD4^+$ T cells compared with the $CD3^{lo}CD4^-$ and $CD3^{lo}CD4^+$ T cells (p=0.008 and p=0.0002, respectively). Conversely, the frequency of $CD57^-CD153^+$ T cells was significantly higher in $CD3^{lo}CD4^-$ T cells compared with the $CD3^{hi}CD4^-$ T cells (p=0.004). The percentage of $CD3^+CD4^+CD57^+CD153^-$ T cells correlated negatively with smoking level in smoker controls (p=0.02, Spearman r=-0.80).

Conclusion: Elevated frequencies of senescent $CD4^+CD57^+CD153^+$ and $CD4^+CD57^-CD153^+$ T cells in patients compared with non-smoker and smoker controls suggest the contribution of immunosenescence in TAO.

Keywords: CD57, CD153/CD30L, Cigarette Smoking, Immunosenescence, Thromboangiitis Obliterans

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INTRODUCTION

Buerger's disease, also known as Thromboangiitis Obliterans (TAO), is a common illness in the Middle East and Asia, including Iran (1-3). TAO is a progressive, inflammatory vascular disease with unknown etiology that mostly affects men with an age range of 20 to 50 years (4, 5). One of the suggested causative factors for inflammation in TAO is tobacco use and smoking cessation is the only effective way to prevent its progression (6, 7). Since inflammation is at the core of disease progression/flare-ups, it makes sense to investigate the role of the immune system in the development and progression of TAO (8). Also, the autoimmune responses directed against vascular endothelium and tobacco antigens are suggested to play a role in the disease (4, 9). In this regard, circulating auto-antibodies against collagens, cardiolipin, and elastins, as well as the increase in pro-inflammatory cytokines levels are reported in patients with TAO (5, 10, 11). Other immunopathologic criteria include the deposition of immune complexes in the blood vessels of TAO patients and the infiltration of inflammatory cells along the internal elastic lamina (5, 11). Oxidative stress and free radicals produced during chronic inflammation can cause lipid peroxidation leading to endothelial damage and platelet dysfunction and thereby increasing the thrombotic events (8, 12-14). Uncontrolled inflammation and oxidative stress induce a variety of cytokines and chemokines which in turn participate in the chronic inflammatory damage to the cells (15, 16). One of the complications of oxidative stress and chronic inflammatory cytokine release (such as IL-6 and IL-8) is the damage to DNA and telomeres, increased p53, and mitochondrial dysfunction which contribute to replicative senescence (17-20). Cellular senescence, an irreversible cell cycle arrest, is triggered by various stimuli/pathways, among which cytokines are shared (21, 22). Cytokines not only induce senescence but are also produced by senescent immune and non-immune cells (23). Senescent cells are metabolically active and secrete complex senescence-associated secretory phenotype (SASP) including various pro-inflammatory factors, chemokines, cytokines, proteases, and growth factors (24). The secreted SASPs are capable of further feedback and generating more mitochondrial ROS and DNA damages and further senescence induction cycles (25).

Cellular senescence also occurs in the immune cells, an event which is part of the broader phenomenon known as immunosenescence (26). Immunosenescence has been the subject of thorough investigation and is linked to several inflammatory diseases of the cardiovascular system including atherosclerosis (27, 28). Also, age-related diseases including cancer and myocardial infarction are suggested to be associated with immunosenescence (29-31). However, the premature aging of the immune system in young adults with chronic inflammatory diseases is a matter of concern (32, 33). Senescent immune cells display changes in their phenotype which helps their characterization in the whole lymphocyte population. Decrease in expression levels of CD28, CD27, and CCR7 and an increase in expression levels of CD57, CD153, and PD-1 on T cells are known as markers of T cell immunosenescence (34, 35). CD153 (CD30L) is a 40 KDa member of the TNF protein family with the conserved N-terminal cytoplasmic domain across the species and is expressed on activated T cells and B cells (36, 37). CD153 regulates proliferation and cytokine production in T cells via reverse signaling (38). Several studies showed that $CD4^{+}PD\text{-}1^{+}CD153^{+}$ T cells represent a T cell population with the feature of cellular senescence (34, 39) and using CD153 vaccination successfully reduced the number of senescent T cells in mice (40). Moreover, the association of the CD153 molecule with CD3 stabilizes TCR/CD3 complex on the T cell surface and leads to the production of a specific set of cytokines along with the proliferation of SA-T cells (41). CD57 is a 110 KDa glycoprotein and its

expression on senescent T cells is well known (35). Senescent $CD4^+CD57^+$ T cells show more inflammatory phenotypes and are associated with worse outcomes in patients with heart failure (28). We previously reported that in patients with atherosclerosis, the frequency of senescent $CD4^+CD153^+CD57^+$ cTfh cells increases (42), however, the studies on TAO and immunosenescence are scarce (43, 44).

Due to the central role of T lymphocytes in the immune responses, T cell immunosenescence is one of the critical points in the progression of chronic inflammatory diseases and may be an important therapeutic target. Therefore, in the current study of patients with TAO, we assessed the frequency of senescent $CD4^+$ and $CD4^-$ T cells that expressed CD57 and/or CD153 and compared those with the healthy smokers and the non-smoker control groups.

MATERIALS AND METHODS

Subjects

After informed consent, a total of nine non-atherosclerotic, non-diabetic, smoker male patients with TAO (aged 55.33±2.86 years) comprised the patients group.

The diagnosis was made by collaborating specialists based on the following inclusion criteria: 1. ischemic limb disease, 2. onset of symptoms before 50 years of age, 3. infrapopliteal arterial occlusive disease, 4. clinical symptoms of distal venous insufficiency, 5. ischemic involvement of the nerves with pain, 6. absence of atherosclerosis in CT angiography, 7. absence of emboli in CT angiography, 8. lack of calcification in arterial walls, 9. smoking, 10. arterial wall thickness, 11. high level of collateral vessels under the knee, 12. corkscrew collateral appearance on angiography. Also, the exclusion criteria were as follows: 1. autoimmune diseases, 2. other vasculitis, 3. diabetes, 4. hypertension, 5. kidney diseases, 6. acute infection, 7. lack of consent.

In addition, nine healthy smoker males (aged 51.22±4.18 years) and nine healthy non-smoker males (aged 43.22±2.48 years) blood donors were included in the two control groups. The blood donors entered the study after informed consent. A collaborating physician evaluated them for cardiovascular and pulmonary diseases, hypertension, hyperthyroidism, history of stroke, diabetes, cancer, and autoimmune diseases. They were also assessed for acute (common cold, influenza, lung infection) and chronic infectious diseases (urinary tract infection, brucellosis, tuberculosis, and typhoid). Also, those who had a recent surgery and dentistry procedure and those who were on any medications such as antibiotics, aspirin, and propranolol, or had recently received any vaccine or blood products were excluded from the study. Individuals with a history of travel to endemic malaria areas in the country, tattooing, acupuncture, and risky behaviors were also excluded from the study criteria. All included controls tested negative for infectious diseases such as hepatitis C, hepatitis B, HIV, and syphilis.

For patients and smoker controls, the smoking level was measured as pack-year (PY) based on the formula below:

Number of packs*number of years of smoking

Every pack was defined as 20 cigarettes. The values and the mean PY of smoking are shown in Table 1.

Collection of Blood and Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Six ml blood sample of patients with TAO as well as six ml blood sample of smoker and non-smoker healthy controls were collected in heparin containing tubes. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Europe, GmbH, Germany). Isolated PBMCs were suspended by adding fresh and cold freezing media (1 ml freezing media to each 3×10^6 cells) containing 90% FBS (Shellmax, Iran) and 10% DMSO (Sigma, USA), and the cells were stored in liquid nitrogen until thawing.

Table 1. Level of smoking (PY) in patients with TAO and healthy smoker controls.

Patients	Age (year)	Packs (No.)	Smoking (Years)	Pack-Year (PY)	Healthy smoker controls	Age (year)	Packs (No.)	Smoking (Years)	Pack-Year (PY)	*p*-value
Case 1	55	1	15	15	Cont. 1	47	1/4	30	7.50	
Case 2	49	2	10	20	Cont. 2	54	2	36	72	
Case 3	58	2	13	26	Cont. 3	53	1/4	15	3.75	
Case 4	52	2	10	20	Cont. 4	52	6/20	5	1.5	
Case 5	57	2	10	20	Cont. 5	42	1	16	16	
Case 6*	57	2	13	26	Cont. 6	54	1/2	30	15	
Case 7*	55	2	10	20	Cont. 7	57	7/20	30	10.5	
Case 8	58	1	10	10	Cont. 8	52	3/20	35	5.25	
Case 9*	57	3	10	30	Cont. 9	50	NK[#]	NK	NK	
				Mean±SD 19.66±6.03					Mean±SD 16.43±23.03	0.06

*Cases who underwent amputation; #NK=Not known

Flow-cytometry Analysis

Cryotubes containing frozen PBMCs were quickly thawed in a 37 °C water bath after removing from liquid nitrogen, and cells were re-suspended in pre-warmed RPMI 1640 (Shellmax, Iran) to wash. After washing, the Trypan blue dye exclusion test was used to determine the number of viable cells and the viability of all samples was higher than 95%. Then, cells were incubated with conjugated antibodies at 4 °C for 20 min. for staining. The antibodies used included mouse anti-human CD4-PerCP (BioLegend, San Diego, CA, USA), mouse anti-human CD3-FITC (BioLegend, San Diego, CA, USA), mouse anti-human CD57-PE (BioLegend, San Diego, CA, USA), and mouse anti-human CD153-APC (R&D, USA). Unstained, single stained and FMO tubes were used for the definition of flow cytometry gates. The cells were enumerated and the samples were analyzed using FACS Calibur and the analysis of results was performed using FlowJo software (v7.6.2). Median fluorescence intensity (MFI) was used to analyze the expression levels of CD57 and CD153 in T cells. We then normalized the MFI data by using the following equation: Normalized MFI=MFI of the test sample - MFI of unstained sample in the same experiment.

ESR and CRP Blood Levels

ESR and CRP levels were extracted from patients' laboratory results at the time of admission. ESR was measured in Westergren-Katz tubes after 1 h. CRP test was done by latex-enhanced nephelometry.

Statistical Analyses

All statistical analyses were performed using Graphpad Prism version 9 software. The Kruskal-Wallis test was used for the comparison of variables between the patients, healthy smokers and non-smoker controls. The Mann-Whitney test was used for the comparison of variables between $CD3^{lo}CD4^{-}$ and $CD3^{hi}CD4^{-}$ cells in the patients and also to compare the smoking level (PY) of the patients and smoker controls. The Spearman's (Rho) rank test was used to evaluate the potential correlation between the frequency of senescent $CD4^{+}$and $CD4^{-}$T cells and inflammatory factors (ESR and CRP) in the patients and the correlation between the frequency of senescent T cells and smoking level (PY) in the patients and smoker controls. P-values less than 0.05 were considered statistically significant. The following symbols were applied to indicate statistically significant findings: *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Analysis of Demographical Criteria of the Patients and the Controls

All the patients and controls were male and there was no statistically significant difference in the age between the patients and the healthy smoker controls (p=0.28), however, non-smoker controls were younger compared to the smoker controls and patients (p=0.03 and p<0.001, respectively). Also, there was a non-significant higher level of smoking in the patients with TAO compared with the smoker controls (supplementary Fig. 1).

Frequency of CD57 and CD153 Expressing $CD3^+CD4^+$and $CD3^+CD4^-$ T cells

The frequencies of CD57 and CD153 expressing T cells were analyzed in all the study groups. The representative dot plots of patients, healthy non-smokers, and smoker groups are shown in Fig. 1 (A-C, respectively). We observed that the percentages of $CD3^+CD4^+CD57^+CD153^+$ and $CD3^+CD4^+CD57^-CD153^+$ T cells significantly increased in patients with TAO compared to healthy non-smoker individuals (p=0.01 and p=0.04, Figs. 1D and 1E, respectively). Moreover, while the difference between the frequencies of $CD3^+CD4^-CD57^+CD153^+$ T cells in patients, healthy smokers, and non-smoker individuals did not reach a significant level, the frequencies of $CD3^+CD4^-CD57^-CD153^+$ T cells significantly increased in patients in comparison with the healthy smoker individuals (p=0.02 Figs. 1F and 1G).

Indeed, our data showed that the mean frequency of $CD3^+CD4^+CD57^-CD153^+$ T cells (7.57±9.04) was 19 times higher than the mean frequency of $CD3^+CD4^+CD57^+CD153^+$ (0.39±1.11) T cells in patients. The ratios of mean frequency of $CD3^+CD4^+CD57^-CD153^+$ T cells to $CD3^+CD4^+CD57^+CD153^+$ T cells in the healthy smokers and non-smoker individuals were 25 (2.50/0.10) and 40 (2.40/0.060), respectively.

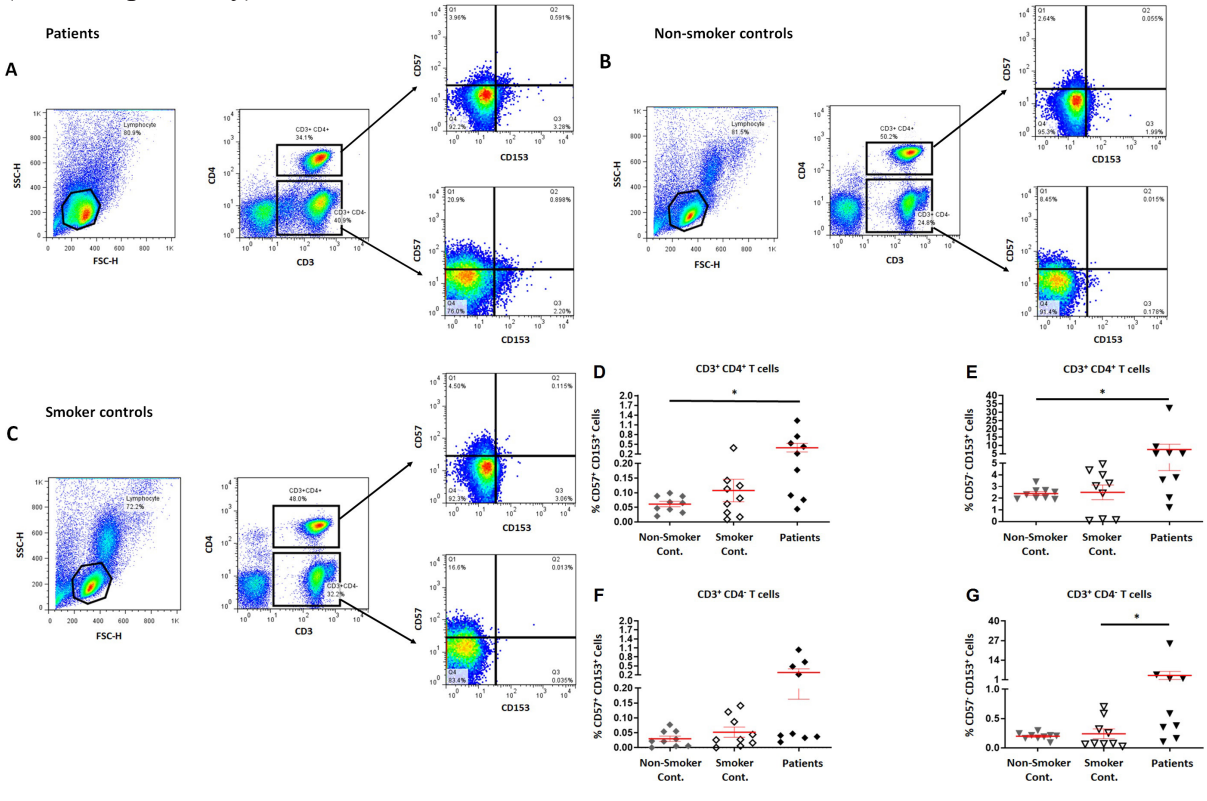

Fig. 1. The frequencies of CD57 and CD153 expressing T cells. Representative flow-cytometry dot plots demonstrating CD153 and CD57 expressing T cells in (A) patients, (B) healthy non-smokers, and (C) healthy smokers. The mean±SD of percentages of (D) $CD3^+CD4^+CD57^+CD153^+$ T cells, (E) $CD3^+CD4^+CD57^-CD153^+$ T cells (F) $CD3^+CD4^-CD57^+CD153^+$ T cells, (G) $CD3^+CD4^-CD57^-CD153^+$ T cells are shown. Data analyses were performed using the non-parametric Kruskal-Wallis test; *p<0.05.

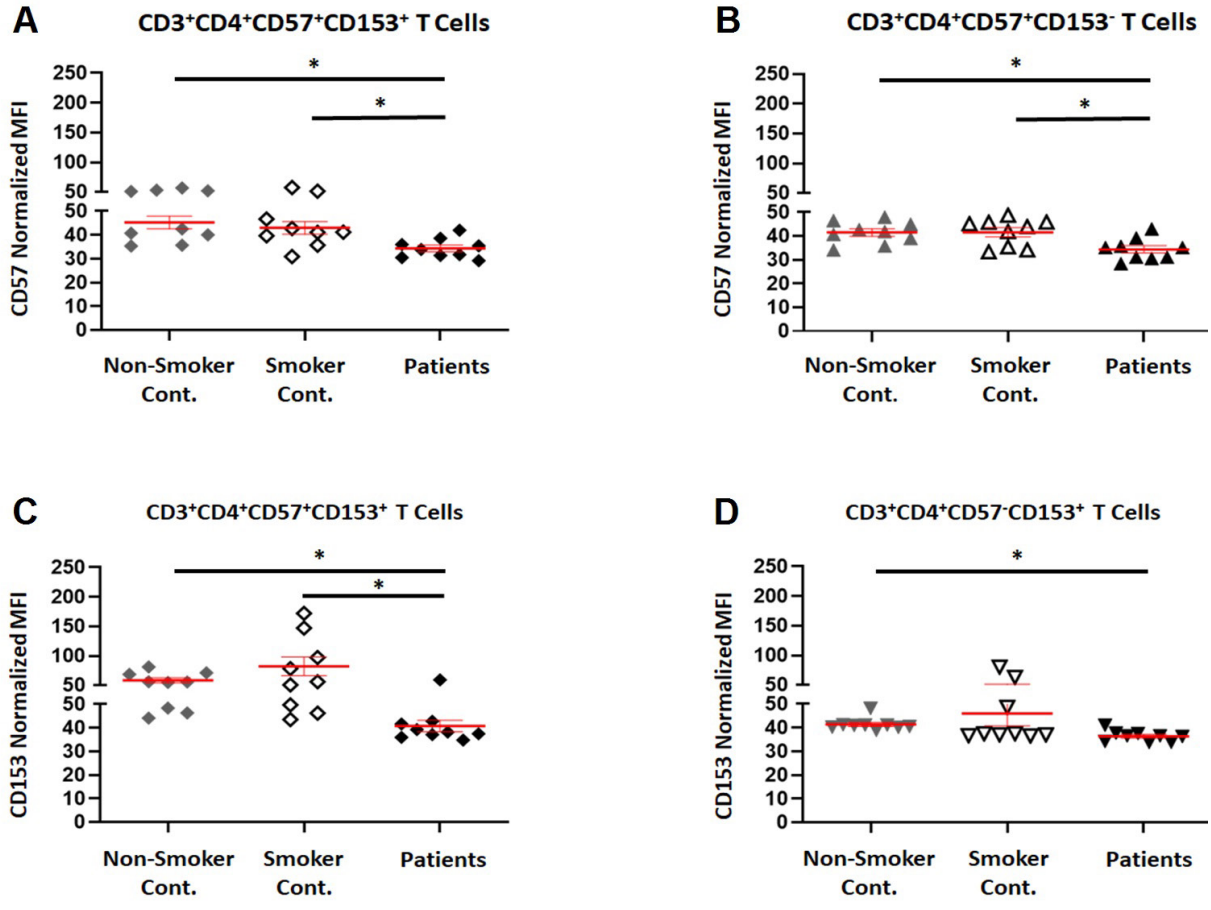

Fig. 2. Decreased expression of CD57 and CD153 on $CD4^+$ T cells of patients with TAO compared to both control groups. (A) The mean±SD of CD57 nMFI in $CD3^+CD4^+CD57^+CD153^+$ T cells, (B) $CD3^+CD4^+CD57^+CD153^-$ T cells, and (C) The mean±SD of CD153 nMFI in $CD3^+CD4^+CD57^+CD153^+$ T cells, and (D) $CD3^+CD4^+CD57^-CD153^+$ T cells are shown. Data are shown as mean±SD and analyzed by the non-parametric Kruskal-Wallis test; *p<0.05.

There was a significant negative correlation between the smoking level and frequency of $CD3^+CD4^+CD57^+CD153^-$ T cell subset in the smoker controls (p=0.02, Supplementary Figs. 2 and 3).

The Normalized Median Fluorescent Intensity (nMFI) of CD57 and CD153 in $CD3^+CD4^+$ T cells.

Our data showed that the nMFI of CD57 on both $CD57^+CD153^+$ (p=0.03 and p=0.04, respectively, Fig. 2A.) and $CD57^+CD153^-$ T cells (p=0.04 and p=0.01, respectively, Fig. 2B) significantly decreased in patients with TAO compared to healthy smoker and non-smoker individuals. The nMFI of CD153 also significantly decreased on $CD57^+CD153^+$ T cells in patients in comparison with the healthy smoker and non-smoker individuals (p=0.002 and p=0.01, respectively, Fig. 2C). Whereas the nMFI of CD153 decreased significantly on $CD57^-CD153^+$ in patients only in comparison with the healthy non-smoker control group (p=0.04, Fig. 2D).

The Frequency of $CD3^{lo}CD4^-$ and $CD3^{hi}CD4^-$ T cells Expressing CD57 and CD153 in Patients with TAO.

As shown in Fig. 3A and Supplementary Fig. 4, a decrease in the expression of CD3 was obvious in $CD4^-$ T cells of four TAO patients. Grouping of $CD4^-$ T cells of patients to $CD3^{lo}CD4^-$ and $CD3^{hi}CD4^-$ cells and assessing the expression of CD57 and CD153 on them were also performed. According to our results, despite the significant decrease of $CD57^+CD153^-$ cells in $CD3^{lo}CD4^-$ T cells compared to

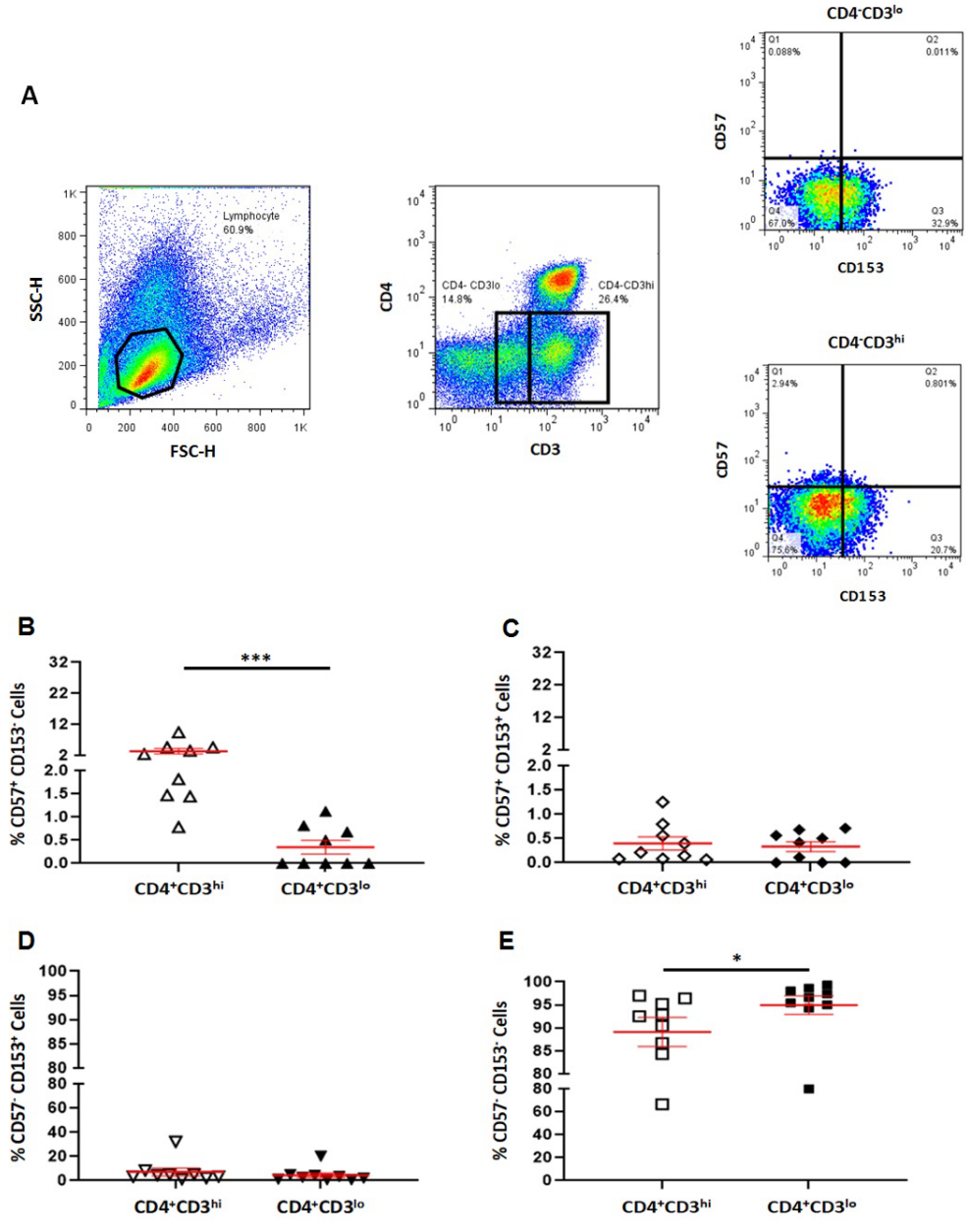

Fig. 3. The frequencies of $CD3^{lo}CD4^{-}$ and $CD3^{hi}CD4^{-}$ T cells expressing CD57 and CD153 in TAO patients. (A) Representative flow-cytometry dot plots showing CD153 and CD57 expressing $CD4^{+}$ T cells in patients, (B) The mean±SD of percentage of $CD3^{+}CD4^{-}CD57^{+}CD153^{-}$ T cells, (C) $CD3^{+}CD4^{-}CD57^{+}CD153^{+}$ T cells, (D) $CD3^{+}CD4^{-}CD57^{-}CD153^{+}$ T cells, (E) $CD3^{+}CD4^{-}CD57^{-}CD153^{-}$ T cells. Data analyses were performed using the non-parametric Mann-Whitney test; *p<0.05, **p<0.01 and ***p<0.001.

$CD3^{hi}CD4^{-}$ T cells (p=0.008, Fig. 3B), the frequency of both $CD57^{-}CD153^{+}$ and $CD57^{-}CD153^{-}$cells significantly increased in $CD3^{lo}CD4^{-}$ T cells group compared to $CD3^{hi}CD4^{-}$ T cells (p=0.004, Fig. 3D, and p=0.03, Fig. 3E, respectively).

The Frequency of $CD3^{lo}CD4^{+}$ and $CD3^{hi}CD4^{+}$ T cells Expressing CD57 and CD153 in TAO Patients

Similar gating on $CD3^{hi}$ and $CD3^{lo}CD4^{+}$ T cells was performed to assess the expression of CD57 and CD153 on cells. There was no significant downregulation of CD3 on $CD4^{+}$ T cells in patients and the frequency of $CD3^{lo}CD4^{+}$ T cells was generally low. There was, however, a significant decrease of $CD57^{+}CD153^{-}$ cells in the $CD3^{lo}CD4^{+}$ T cells group compared to $CD3^{hi}CD4^{+}$ T cells (p=0.0002, Fig. 4B).

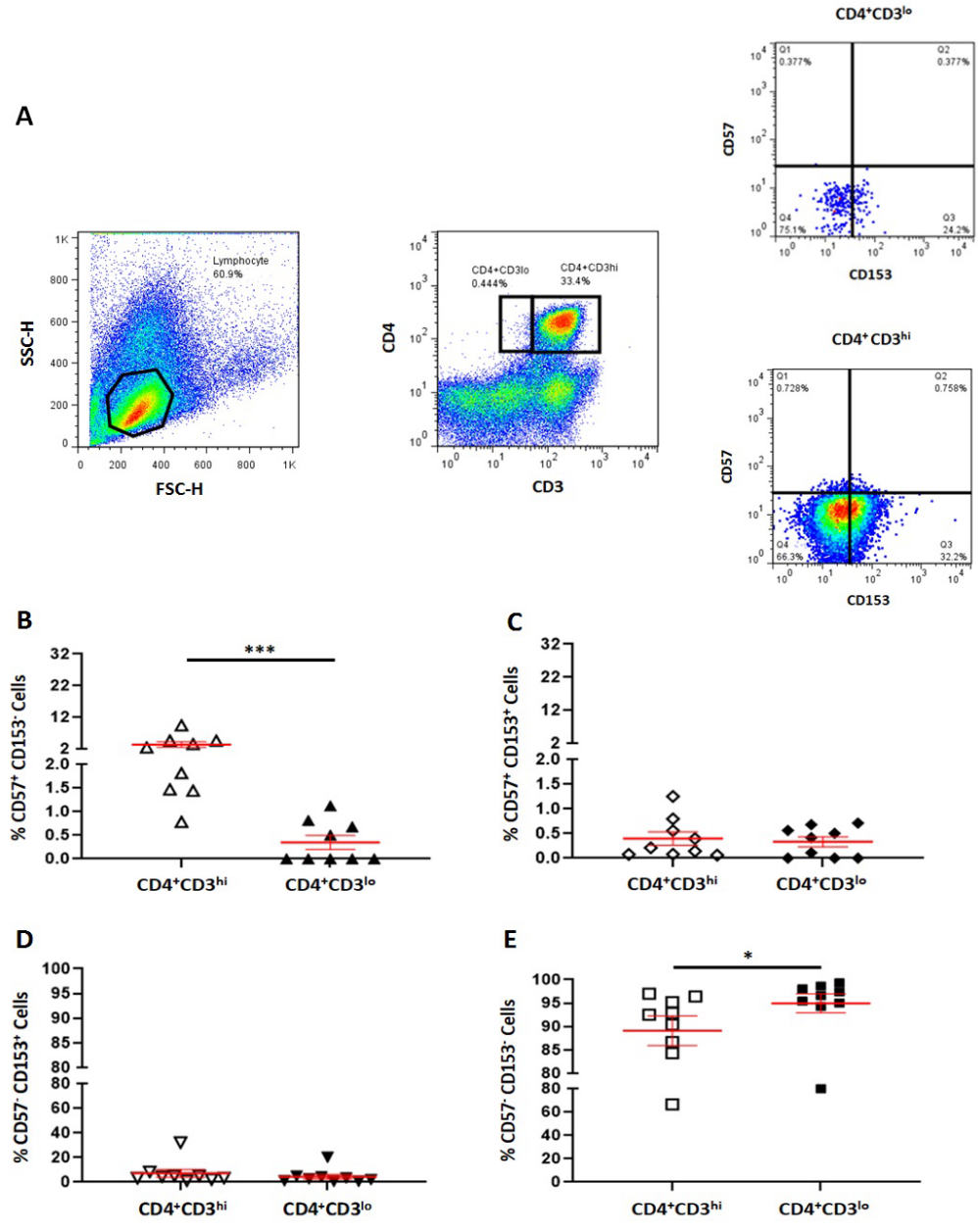

Fig. 4. The frequencies of $CD3^{lo}CD4^{+}$ and $CD3^{hi}CD4^{+}$ T cells expressing CD57 and CD153 in TAO patients. (A) Representative flow-cytometry dot plots displaying CD153 and CD57 expressing $CD4^{+}$T cells in patients, (B) The mean±SD of percentage of $CD3^{+}CD4^{+}CD57^{+}CD153^{-}$ T cells, (C) $CD3^{+}CD4^{+}CD57^{+}CD153^{+}$ T cells, (D) $CD3^{+}CD4^{+}CD57^{-}CD153^{+}$ T cells, (E) $CD3^{+}CD4^{+}CD57^{-}CD153^{-}$ T cells. Data analyses were done using the non-parametric Mann-Whitney test; *p<0.05 and ***p<0.001.

The Normalized Median Fluorescent Intensity (nMFI) of CD57 and CD153 in $CD3^{lo}CD4^{-}$, $CD3^{hi}CD4^{-}$, $CD3^{lo}CD4^{+}$, and $CD3^{hi}CD4^{+}$ T cells.

Our data showed that the nMFI of CD57 significantly decreased in $CD3^{lo}$ T cells of both $CD4^{-}$ and $CD4^{+}$ T cell groups compared with the $CD3^{hi}$ T cells (p=0.0003, Fig. 5A and p=0.0002, Fig. 5C). Also the nMFI of CD57 significantly decreased in $CD3^{lo}CD4^{+}$ T cells compared to $CD3^{hi}CD4^{+}$ T cells (p=0.03, Fig. 5D). While, the nMFI of CD153 in $CD3^{lo}CD4^{-}$ T cells significantly increased in comparison with the $CD3^{hi}CD4^{-}$ T cells (p=0.03, Fig. 5B).

The Correlation between the Frequency of Senescent $CD4^{+}$and $CD4^{-}$ T cells with ESR and CRP in TAO Patients

The mean ESR and CRP levels in patients with TAO were 37.16±4.53 mm/h

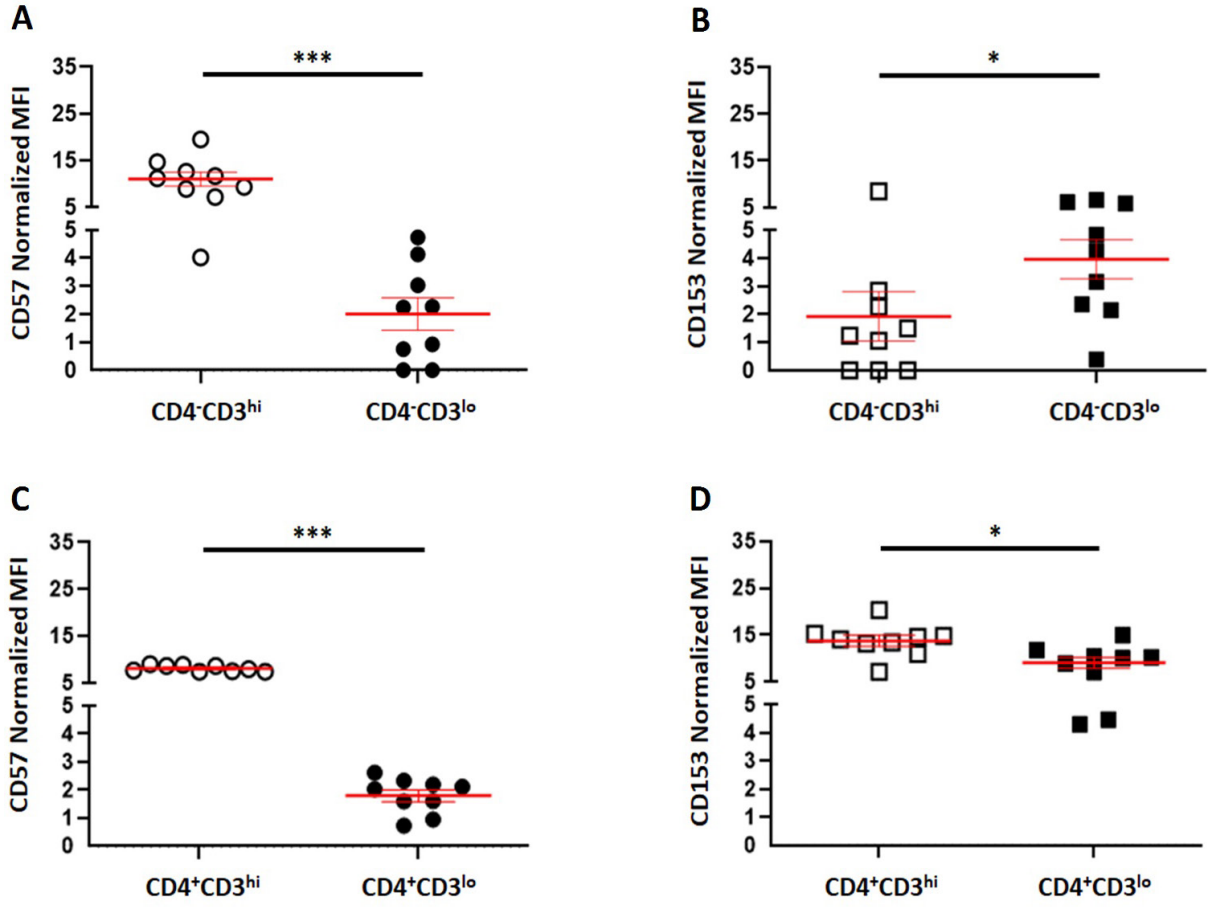

Fig. 5. The nMFI of CD57 and CD153 in $CD3^{lo}CD4^-$, $CD3^{hi}CD4^-$, $CD3^{lo}CD4^+$ and $CD3^{hi}CD4^+$ T cells in patients with TAO. (A) The mean±SD of nMFI of CD57 in $CD4^-$ T cells, (B) CD153 in $CD4^-$ T cells, (C) CD57 in $CD4^+$ T cells, and (D) CD153 in $CD4^+$ T cells are shown. Data analyses were done using the non-parametric Mann-Whitney test; *p<0.05 and ***p<0.001.

and 3.96±0.25 mg/L, respectively. Our data showed that there was no significant correlation between the frequencies of senescent $CD4^-$ and $CD4^+$ T cells and ESR and CRP inflammatory factors in patients with TAO (data not shown).

DISCUSSION

In the current study, we found that the frequency of $CD3^+CD4^+CD57^+CD153^+$ and $CD3^+CD4^+CD57^-CD153^+$ T cells increased in TAO patients compared to healthy non-smoker individuals, and this increase was only partly related to the smoking status as the healthy smokers showed just a slight increase in these cell populations. This observation was stronger in the $CD3^+CD4^-CD57^-CD153^+$ T cells which showed higher frequency in patients with TAO than in the healthy smokers. We also observed that nMFI of CD57 and CD153 showed a similar levels of expression on T cells of smoker and non-smoker controls which were different from patients with TAO. Having said so, the frequency of senescent T cells showed an increasing trend from healthy non-smokers to healthy smokers to TAO patients. This supports the role of tobacco smoking as only one of the factors involved in the pathogenesis of TAO. As seen in Table 1, the patients with TAO had a marginally higher level of smoking (measured in PY) compared to the smoker controls of the same age and gender. Therefore, the level of exposure to cigarette smoke and its toxic ingredients may affect the level of immunosenescence, as well. These findings are in accordance with previous studies that showed cigarette smoking upregulates the

genes that are associated with senescence, for example, KLRG1 and B3GAT1 (coding for CD57) in multiple cells of the immune system such as $CD8^+$ T cells, $CD4^+$ T cells, and NK cells and lead to premature aging and smoking-mediated diseases (45). Moreover, smoking increases the activity of senescence-associated-β-galactosidase (SA-β-Gal) and the production of inflammatory cytokines in $CCR6^+$ Th17 cells (46). It is also known that repeated exposure to antigens and stimulation of T cells lead to telomere shortening and replicative senescence (47). Although tobacco antigens are among the candidate antigens that may induce replicative senescence in T cells of smokers, they do not show similar effects in patients with TAO and smokers of the same age and gender without TAO. This notion is in line with the observed association of MHC class I and class II alleles as well as other genes of the immune system with TAO (48-50). The immune system is in constant encounter with pathogenic microorganisms and is also involved in detoxifying the toxic materials and stress-inducing components (51, 52). These encounters may induce or accelerate inflammation in genetically susceptible individuals. In this regard, it is suggested that pathogens involved in periodontal diseases or even intracellular *Rickettsia* infection may have a role in the development of TAO (53, 54). It is shown that in addition to autoantibodies, *Rickettsia rickettsii* specific antibodies are found in a high percentage of patients with TAO (9, 55, 56). It should, however, be noted that the level of smoking between our healthy controls and the patients with TAO was not similar (supplementary Figs. 2 and 3) which again confirms the importance of smoking levels in TAO disease. Currently, we do not know if the higher smoking level contributes to the susceptibility of the endothelium to select pathogens or even if there is a cooperation between pathogenic organisms (such as *Rickettsia)* and toxin-induced endothelial lesions in the development or progression of TAO. The analysis of the correlation between the smoking level and senescent T cell subsets showed a different trend in smoker controls and patients (Supplementary Fig. 2) which is worth studying in bigger cohorts. It should be noted that both innate and adaptive immunity frequently remove senescent cells through a mechanism called the senescence surveillance (57). The chemo-attractant activity of SASPs and the expression of MHC class I polypeptide-related sequence A (MICA) and UL16 binding protein 2 (ULBP2) on the cellular membrane in response to DNA damages and oncogenes induce the elimination of senescent cells (58). Clearance of senescent cells is required to combat susceptibility to age-related diseases (59). Therefore, it is probable that the observed decrease in the frequency of senescent $CD57^+$ T cells in healthy smokers in response to higher smoking levels may be a defensive mechanism to protect the body from the deleterious effects of senescent cell accumulation. While the lack of sufficient clearance of senescent cells in some smokers could be considered a factor that makes them prone to Buerger's disease.

Our study indicates that patients with TAO experience an increase in T cells with different immunosenescence phenotypes. CD153 is an activation marker also associated with the TCR-induced secretion of osteopontin and cytokines in senescence-associated T cells and macrophages (60, 61). Interestingly, CD153 co-stimulation in senescence-associated T cells seems to be refractory to the inhibition of the PD-1 signaling pathway which makes it a potent stimulating signal (39). A recent mice study showed that CD153 engages with TCR/CD3 on SA-T cells and enhances TCR signaling and activation (41). Also, the lack of CD153 or its receptor (CD30) in mice decreased the frequency of senescent lymphocytes (41). Despite the low number of cases of TAO in our study and lack of in-vitro stimulation, four out of nine patients showed a clear downregulation of CD3 on their T cells, a sign of the activation status of T cells. Interestingly, the frequency of $CD57^+$

T cells was lower in $CD3^{lo}$ T cells compared with the $CD3^{hi}$ T cells in both $CD4^+$ and $CD4^-$ populations. This was despite the similar or higher frequency of the $CD153^+$ T cells in the $CD3^{lo}$ population compared with the $CD3^{hi}$ T cells in these populations. Comparison of the normalized MFI of CD57 between $CD3^{lo}$ and $CD3^{hi}$ T cells showed that in both $CD4^+$ and $CD4^-$ T cells, the expression of CD57 was lower in $CD3^{lo}$ compared with the $CD3^{hi}$ population. The findings regarding CD153 were somewhat different between $CD4^+$ and $CD4^-$ T cells. While $CD4^-CD3^{lo}$ T cells had higher frequency and expression of CD153, the $CD4^+CD3^{lo}$ T cells did not show such a clear-cut difference. This observation, although in a small group of patients, is important because previous studies have suggested a difference in the role of the CD153 pathway between $CD4^+$ and $CD8^+$ T cells (62). In general, we conclude that the activation rate in T cells of TAO patients is high. Also, from these data one might hypothesize that the $CD3^{lo}$ T cells are at the earlier stages of senescence compared with the $CD3^{hi}$ T cells. This hypothesis, however; needs further investigation of the kinetics of CD153 and CD57 expression on senescent T cells. In this regard, our previous study showed that treatment with a senolytic drug would shift cTfh cells away from $CD57^+CD153^+$ to either of single positive populations (42).

Additionally, we observed no correlation between ESR and CRP levels (as inflammatory factors) with the frequency of senescent $CD4^+$ T cells in TAO patients. This finding confirms the results of previous studies have shown that ESR and CRP levels do not increase in patients with TAO (4, 63). These markers are indicators of acute inflammation followed by infection, tissue necrosis, etc. and they return to their normal levels after a few days. They are expected to be unchanged in chronic inflammatory disorders such as TAO (64, 65).

Limitations of our study are the low number of cases, the younger age of healthy controls, and the lack of PD-1 in our phenotyping cocktail. That being said, we applied the analysis of covariance (ANCOVA) to remove the effect of age difference as a confounding factor between groups. None of the results changed after removing the effect of confounding factors. Regarding the number of cases, it should be considered that TAO is generally not very frequent and the sampling was performed during the COVID-19 pandemic which also hampered recruiting healthy non-smokers of higher age. Despite all the limitations, our study has included a smoker control group who were in the same age range as patients with TAO, and from our point of view including this group decreases the confounding effect of smoking and age in our study.

CONCLUSION

In conclusion, due to the increased frequency of $CD4^+CD57^+CD153^+$, $CD4^+CD57^-CD153^+$, and $CD4^-CD57^-CD153^+$ T ($CD3^+$) cells in patients with TAO, we suggest that senescence in T cells is a player in this disease. This is even more important considering that TAO mostly affects young and middle-aged individuals in whom we generally do not expect senescence to be an issue. More in-depth study of the cause, extent and kinetics of immunosenescence in patients with TAO may pave the way to finding new therapeutic solutions for this disease. In this regard, Senolytic drugs may be helpful in the clearance or rejuvenating of senescent cells and may be helpful in preventing disease progression (66). It is also worth investigating whether $CD57^-CD153^+$ T cells are at earlier stages of activation before senescence than $CD57^+CD153^+$ cells which may be activated senescent T cells.

AUTHORS' CONTRIBUTION

NM, performed the experiments, acquired the data, participated in data analysis and prepared the draft. LK, HGJ, and AH

participated in the diagnosis of patients and the examination of the controls provided the samples, and participated in the interpretation of data and the approval of the draft. NKH participated in data analysis and preparation of the draft. MD, conceptualized and designed the study, supervised the study, and corrected the draft.

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CONFLICT OF INTEREST

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