Age-Associated Characteristics of CD4+ T-Cell Composition in Patients with Atherosclerosis

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Abstract: Background. We aimed to analyze the contents of the main CD4+ T-cell subsets in patients with atherosclerosis (AS) depending on age. Methods. Male patients with coronary and/or carotid AS, who are non-smokers, and who are receiving statins were divided into three age groups (I—<55 y.o. (n = 23), II—55–64 y.o. (n = 42), III—≥65 y.o. (n = 46)). Leukocyte phenotyping was performed by direct immunofluorescence and flow cytometry. For intracellular cytokine detection, blood mononuclear cells were pre-activated with phorbol 12-myristate 13-acetate and ionomycin in the presence of an intracellular vesicle transport blocker monensin. Results. The groups did not differ in traditional CVD risk factors and AS severity. The content of CD4+ T-cells was lower in group III and II than in group I. The content of CD4+CD25high Treg was lower in group III than in groups I and II. No differences in the quantities of the primed CD39+CD45RA+ and CD27high Treg, CD4+INFγ+ Th1, CD4+IL17+ Th17, and CD4+IL17+INFγ+ Th1/17 were observed. There were negative correlations between the values of CD4+ T-cells, CD4+CD45RA+ T-cells, CD4+CD25high Treg, CD4+CD25highCD45RA+ Treg, and age. Conclusion. In patients with AS, the age-related depletion of naive CD4+ T-cells also extends to the regulatory compartment. This phenomenon should be considered when studying the impact of the immune cells on the progression of AS.

Keywords: atherosclerosis; aging; CD4+ T-cells; Treg

1. Introduction

CD4+ T-lymphocytes play a major role in the regulation of adaptive immune response [1,2]. Naive CD45RA+ cells leave the thymus to mature in the secondary lymphoid organs into effector and memory cells [3]. Depending on the microenvironment, the activation of naive T-cells leads to their differentiation into effector cells, which are predominantly comprised of T-helpers (Th) type 1 producing INFγ, TNF; Th2 producing IL-4, -5, -10, -13, etc; and Th17 producing IL-17 and -22 [2]. The main contribution of regulatory T-cells (Tregs) to immune homeostasis is to prevent or limit effector T-cell activation. Tregs are characterized by the intracellular expression of transcription factor Foxp3, high membrane exposure of CD25, and the ability to produce anti-inflammatory cytokines TGFβ and IL-10. Naturally occurring Tregs develop primarily in the thymus, while inducible Tregs can also differentiate in the periphery during an active immune response [4].

Atherosclerosis (AS) is a chronic inflammatory disorder resulting from the accumulation of oxidized lipoproteins and other pro-atherogenic substances in the arterial intima [5]. It is hypothesized that a wide range of T-lymphocyte subtypes with different functions are involved in atherogenesis. Th1 contribute to maintenance of the inflammatory process and the acceleration of atherosclerosis [6,7]. Tregs were shown to possess anti-inflammatory and anti-atherogenic effects [8–11]. Data on the role of Th17 in the development of atherosclerosis are contradictory [12–15]; however, the majority of studies showed a pro-inflammatory activity of this cell type [16–20]. Th1/17 is a small subset of Th17 capable of producing...
both IL-17 and INF\(\gamma\). Increased blood frequencies of Th1/17 are associated with several autoimmune/inflammatory diseases, including atherosclerosis [14,21,22]. Age-related changes in the immune system are an important factor promoting the emergence and maintenance of chronic inflammatory diseases. Despite the data showing a decrease in the number of T-lymphocytes with age due to thymic involution, the composition of minor T-cell subpopulations including Tregs needs to be better understood. Several studies demonstrated an increase in Treg blood content and suppressive capacity in older people [23–25], while others did not observe any age-related changes in the Treg population [26]. Data on the state of effector subpopulations are sparse, with one recent paper showing some increases in the Th1 and Th17 blood contents in older healthy donors [27].

In the present study, we compared the blood content of CD4\(^+\) lymphocyte subpopulations, including the main effector and regulatory subsets, in patients with atherosclerosis of coronary and carotid arteries in different age groups.

2. Materials and Methods

The study was approved by the Institutional Ethics Committee. Written consent was obtained from each patient. A total of 120 non-smoking male patients with stable coronary artery disease who were scheduled for a coronary angiography were enrolled. Nine patients were excluded because coronary angiography was not performed. The severities of coronary and carotid AS were assessed using a coronary angiography and a vascular ultrasound, respectively. The exclusion criteria included acute coronary syndrome or interventions in the previous 6 months, history of stroke, neoplasms, liver or renal failure, infectious/inflammatory disease, decompensated diabetes mellitus, current use of immunosuppressive drugs, and smoking over the past 3 years. All patients had been receiving standard therapy with beta-blockers, acetylsalicylic acid/clopidogrel, ACE inhibitors/sartans, and statins.

Coronary angiography was performed via a trans-radial approach using a standard technique. The severity of coronary AS was determined by the degree of stenosis of the main coronary artery and assessed by one experienced independent observer. Coronary AS was defined as mild if no lesions beyond mild luminal irregularities were observed and if the stenosis was <40% of the lumen narrowing; median if the stenosis was >50%, or previous stenting of one or two coronary arteries; and severe if the stenosis was >50%, or previous stenting of 3 or more coronary arteries.

Duplex scanning of the carotid arteries was performed using a high-resolution ultrasound system with a linear array transducer 3–9 MHz. Atherosclerotic plaques were assessed in the distal parts of the common carotid artery (CCA), in the CCA bifurcation, and in the internal carotid artery (ICA) bilaterally in the longitudinal (anterior, lateral, and posterior planes) and transversal views. The severity of the carotid artery stenosis was determined using the ECST criteria (baseline stenosis site artery diameter/stenosis site artery diameter × 100%) [28].

2.1. Lymphocyte Immunophenotyping

Whole blood was collected in a sodium citrate anticoagulated vacutainer tube. The samples were processed within 2 h after being collected. For surface antigen staining, the following antibodies and reagents were used: CD3-FITC, CD3-PerCP, CD4-FITC, CD25-PE, CD127-PC5, CD39-FITC, CD127-PC5, CD45-APC, CD45RA-APC, CD278-APC, and lysing and fixing solutions (Beckman Coulter, Becton Dickinson Immunocytometry Systems, eBioscience, San Diego, CA, USA). The intracellular antigens analysis was performed in mononuclear leukocytes. The cells were isolated using density gradient centrifugation (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA). For cytokine detection, mononuclear cells were additionally cultivated in the presence of 25 ng/mL PMA, 1 \(\mu\)g/mL ionomycin, and 10 \(\mu\)g/mL monensin for 4 h. Cell staining was performed with CD4-PC5, FoxP3-Alexa488, INF\(\gamma\)-PE, IL17a-Alexa488, and relevant isotypic controls and with a FoxP3 Staining Buffer
Set (all reagents from eBioscience, San Diego, CA, USA) per the manufacturer’s manual. The samples were analyzed with a two-laser FACS Caliber flow cytometer equipped with CellQuest Pro software (Becton Dickinson Immunocytometry Systems). Lymphocytes were identified by light scattering parameters and CD45 expression pattern. Tregs were identified as CD4^+CD25^{high} (including CD45RA^+ naïve and CD45^- memory subsets) and CD4^+Foxp3^+, Th1 was identified as CD4^+INFγ^+, Th17 was identified as CD4^+IL17a^+ cells, and Th1/17 was identified as CD4^+IL17a^+INFγ^+ (Figure 1).

![Flow cytometry plots](image-url)

**Figure 1. Cont.**
Figure 1. Flow cytometric analysis (gating strategies) of CD4+ T-cells subpopulations.

2.2. Statistics

The data are presented as a median (25–75th percentile). Kruskal–Wallis ANOVA and Mann–Whitney U tests were used in multiple or paired comparisons, respectively. Chi-square or Fisher’s exact two-tailed test was used in multiple or paired comparisons of binary features, respectively. Spearman’s test was used for correlation analysis. The differences were considered statistically significant at \( p < 0.05 \).

3. Results

A total of 111 patients (median age 63 (55; 69)) were categorized into three groups according to age (I—<55 y.o. \((n = 23)\), II—55–64 y.o. \((n = 42)\), III—≥65 y.o. \((n = 46)\)). The groups were comparable in the traditional cardiovascular disease risk factors, coronary and carotid AS severity (Table 1), and medical treatment.

In patients over 65 years of age (group III), the absolute values of CD4+ T-cells and CD4+CD45RA+ T-cells, CD4+CD25high, and CD4+CD25highCD45RA+ Tregs were lower compared with those in groups I and II. The number of CD4+CD25highCD39+CD45RA− and CD4+CD25highCD278high Tregs did not change (Table 1). The absolute content of CD4+Foxp3+ Treg was lower in groups II and III vs. group I, but the differences did not achieve statistical significance.

The number of circulating Th17 and Th1 did not change depending on age (Table 2). Negative correlations were observed between absolute values of CD4+ T-cells \((r = -0.28)\), CD4+CD25high Treg \((r = -0.27)\), CD4+CD25highCD45RA+ Treg \((r = -0.24)\), CD4+CD45RA+ T-cells \((r = -0.36)\), CD4+CD45RA+/CD4+CD45RA− T-cells ratio \((r = -0.24)\), and age \((p < 0.05)\).
Table 1. Clinical characteristics of patients with atherosclerosis in different age groups.

| Parameter                                    | I (<55, n = 23) | II (55–64, n = 42) | III (≥65, n = 46) | p    |
|----------------------------------------------|-----------------|--------------------|-------------------|------|
| BMI, kg/m²                                    | 29.0 (28.0; 33.0) | 28.0 (25.0; 30.0) | 27.0 (26.0; 29.0) | 0.07 |
| Arterial hypertension, n (%)                 | 20 (87%)        | 35 (83%)           | 41 (89%)          | 0.87 |
| Diabetes mellitus, n (%)                     | 2 (8%)          | 1 (2%)             | 6 (13%)           | 0.18 |
| Severe coronary infarction, n (%)            | 12 (52%)        | 25 (59%)           | 24 (52%)          | 0.75 |

Coronary AS severity

| Coronary AS severity                                                                 | I (<55, n = 23) | II (55–64, n = 42) | III (≥65, n = 46) | p    |
|------------------------------------------------------------------------------------|-----------------|--------------------|-------------------|------|
| mild, n (%)                                                                        | 2 (8%)          | 0 (0%)             | 4 (8%)            | 0.41 |
| moderate, n (%)                                                                    | 12 (52%)        | 26 (62%)           | 23 (50%)          | 0.28 |
| severe, n (%)                                                                      | 10 (43%)        | 12 (28%)           | 22 (48%)          |      |
| CCA/CCA bifurcation stenosis, %                                                    | 37.5 (30.0; 45.0) | 30.0 (30.0; 40.0) | 40.0 (30.0; 45.0) | 0.21 |
| ICA stenosis, %                                                                    | 40.0 (35.0; 50.0) | 37.5 (30.0; 50.0) | 40.0 (25.0; 60.0) | 0.62 |
| Severe coronary AS + carotid artery stenosis > 50%, n (%)                          | 2 (8%)          | 0 (0%)             | 4 (8%)            | 0.41 |

The data are presented as a median (25%; 75%) or n (%).

Table 2. Immunological parameters of patients with atherosclerosis in different age groups.

| Parameter                                    | I (<55, n = 23) | II (55–64, n = 42) | III (≥65, n = 46) | p    |
|----------------------------------------------|-----------------|--------------------|-------------------|------|
| Lymphocytes, mln/mL                         | 2.1 (1.6; 2.6)  | 1.9 (1.5; 2.2)     | 1.8 (1.4; 2.2)    | 0.22 |
| CD4+ T-cells (10^3/mL)                      | 903.0 (585.6; 1113.8) | 745.4 (502.2; 924.0) | 646.3 (516.0; 806.4) | 0.03 |
| CD4^CD45RA^+ T-cells (10^3/mL)              | 365.0 (262.3; 478.2) | 422.3 (241.1; 553.0) | 197.2 (173.5; 297.5) |      |
| CD4^CD45RA^+CD4^CD45RA^- T-cells            | 0.23 (0.15; 0.33) | 0.23 (0.10; 0.30)  | 0.16 (0.09; 0.24) |      |
| CD4^CD25highCD127low Treg (10^3/mL)         | 35.0 (28.7; 54.4) | 31.0 (21.1; 43.6)  | 24.2 (18.4; 35.2) |      |
| CD4^CD25highCD39^CD45RA^- Treg (% CD25high Treg) | 53.5 (31.4; 64.3) | 56.0 (21.5; 64.5)  | 57.4 (45.1; 68.0) | 0.69 |
| CD4^CD45RA^+CD4^CD45RA^- T-cell             | 8.5 (6.1; 14.3)  | 6.6 (3.5; 12.7)    | 5.7 (2.2; 9.7)    | 0.11 |
| CD4^CD25highCD4RA^+ Treg (10^3/mL)          | 74.0 (53.5; 87.2) | 58.0 (35.0; 73.6)  | 54.0 (38.4; 73.3) | 0.19 |
| Th17 (10^3/mL)                              | 13.4 (8.1; 14.7) | 9.6 (4.8; 15.8)    | 11.3 (5.8; 16.6)  | 0.66 |
| Th1 (10^3/mL)                               | 212.0 (158.0; 258.7) | 142.3 (55.6; 225.0) | 143.5 (98.4; 239.7) | 0.19 |
| Th17/Th1 (10^3/mL)                          | 2.0 (1.4; 4.2)   | 2.0 (1.1; 3.7)     | 1.4 (0.6; 3.1)    | 0.52 |

The data are presented as a median (25%; 75%) or n (%).

4. Discussion

Age-related changes affect both innate and adaptive immunity. During aging, the number and repertoire of T-cells markedly are reduced due to thymus involution accompanied with the accumulation of memory cells and imbalance between CD4+ and CD8+ T-cells, with an increasing prevalence of CD8+ T-cells obtained from the older subjects, which was not mediated by changes in CTLA-4 expression or apoptosis.
Several studies have shown an increase in the frequencies of circulating Tregs during aging. Gregg et al. [23] observed an age-associated increase in the relative and absolute values of CD4\(^+\)CD25\(^{\text{high}}\) Treg in healthy volunteers, and no differences were found in the expression of surface markers of T-cell activation (CD69, HLA-DR, CD71, and CTLA-4) or in the suppressive capacity of Treg; however, the number of CD45RO\(^+\) cells was higher in older people. The frequencies of CD4\(^+\)Foxp3\(^+\) Treg in the blood of older subjects without confirmed disease was significantly higher than that in their younger counterparts; the former demonstrated a preserved or slightly increased expression of CTLA-4 and GITR, associated with the suppressive function of Treg, and a comparable expression of chemokine receptors CCR5 and CCR7 that mediate migration and homing [25].

The contradictory data on the composition of T-cell subsets in different age groups may be associated with heterogeneity of patients by gender and concomitant diseases. The frequencies of circulating CD4\(^+\) T-cells are higher in women [37], and there are gender-associated differences in cytokine production (INF\(_{\gamma}\) and IL-17) [38] and in autoimmune disease predominance and progression [39].

We studied age-related changes in the composition of CD4\(^+\) lymphocytes in a homogeneous group of non-smoking male patients with confirmed atherosclerotic disease of the coronary and carotid arteries. The frequencies of circulating CD4\(^+\) T-cells and CD4\(^+\)CD25\(^{\text{high}}\) Treg decreased with age in patients with atherosclerosis. The insufficient decrease in CD4\(^+\)Foxp3\(^+\) Treg in contrast with the decline in CD4\(^+\)CD25\(^{\text{high}}\) Treg was probably due to the partial compensation via induction of Treg in the periphery. These age-related alterations could be explained with a decrease in thymic production of naive lymphocytes, which leads to insufficient replenishment of T-cells with naive CD45RA\(^+\) lymphocytes. This is evidenced by the negative correlations between age and the content of CD45RA\(^+\) populations, including Tregs. Our data are consistent with the previously published comparison of the phenotype and functional characteristics of Treg in older and young, apparently healthy donors, with the former demonstrating a decrease in the level of naive CD45RA\(^+\) Treg and an increase in the level of cytokine-producing CD45RA\(^-\) Treg [40]. The relative content of Treg and CD45RA\(^+\)CD45RO\(^-\) naïve Treg is lower in patients with acute coronary syndrome compared with patients with stable angina or non-coronary chest pain syndrome; the content of CD45RA\(^-\) CD45RO\(^+\) Treg was comparable between groups [41]. We assume that Treg deficiency, which develops due to a decrease in the number of naive cells, may have a pathogenetic relationship with the initiation and destabilization of atherosclerosis. Further research is required to confirm this hypothesis.

5. Conclusions

In patients with AS, the age-related depletion of naive CD4\(^+\) T-cells also extends to the regulatory compartment. This phenomenon should be considered when studying the impact of the immune cells on the progression of AS.

Author Contributions: Conceptualization A.Y.F., A.V.P. and T.I.A.; methodology A.Y.F. and T.I.A.; validation A.Y.F., A.V.P. and T.I.A.; investigation A.Y.F. and T.I.A.; resources T.I.A. and A.V.P.; writing—original draft preparation, A.Y.F.; writing—review and editing, T.I.A. and A.V.P.; supervision T.I.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. The study was approved by the Russian Ministry of Health, project No. AAAA-A19-119022290045-1.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of National Medical Research Center of Cardiology of Ministry of Healthcare of Russian Federation (protocol No. 249 from 30 September 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request.
Conflicts of Interest: The authors declare no conflict of interest.

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