Opposite changes of regulatory T cell blood content may differentially contribute to atherosclerosis or lymphoproliferative disorders

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Received: 11.12.2015. Accepted: 15.01.2016.

ABSTRACT

Background. Chronic autoimmune inflammation in arterial wall may lead to atherosclerosis progression.

Objective. The aim of this study was to investigate the association between Treg, Th17 and B1a cell blood frequencies as well as IgM autoantibodies to oxLDL and the abundance of carotid atherosclerosis.

Material and methods. 18 patients with increased IMT (intima-media thickness) and 65 patients with different severity of carotid atherosclerotic plaques were included. Treg, Th17 and B1a cell blood frequencies were assessed via direct immunofluorescence staining and flow cytometry, oxLDL as well as IgM autoantibodies to oxLDL were measured with commercial kits.

Results. We observed higher values of Treg in patients without carotid atherosclerosis. Patients with intact carotid arteries as compared to patients with mild atherosclerotic plaques had decreased Th1 levels. OxLDL IgM levels were higher in patients with intact carotid arteries. Patients who received statin treatment had higher levels of Treg. Immunophenotyping of B cells revealed two cases of monoclonal B-cell lymphocytosis and 1 case of B-CLL (B-cell chronic lymphocytic leukemia) in elderly patients with intact carotid arteries.

Conclusion. We hypothesize that certain parameters of cell immunity may hamper atherosclerosis while protecting from lymphoproliferative disorders.

KEY WORDS: atherosclerosis, regulatory T cells, T helpers 17, monoclonal B-cell lymphocytosis, B-cell chronic lymphocytic leukemia

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INTRODUCTION

The main function of the immune system is the elimination of pathogens and neoplastic cells. Certain disturbances in processes of antigen recognition and cell activation underlie the creation and progression of cancer and chronic inflammatory diseases. Atherosclerosis is accompanied by autoimmune inflammation in arterial wall, and different types of innate and adaptive immune cells were shown to be involved in initiation and progression of atherosclerotic plaques (AP). CD4+CD25+Foxp3+ regulatory T cells (Treg) possess anti-atherogenic activity, while IL-17 producing CD4+ T cells (Th17) are supposed to maintain inflammation in arterial wall. Previous data indicated that shift in Treg/Th17 cell balance in favor of proinflammatory Th17 was associated with coronary atherosclerosis progression and multivessel disease [1, 2]. CD19+CD5+CD27-B cells (B1a) have been reported to reduce atherosclerosis via secretion of natural IgM antibodies that participate in the elimination of pro-atherogenic lipoproteins [3]. Data concerning carotid atherosclerosis are less clear. Increased intima-media thickness (IMT) and/or AP in carotid arteries have been shown to predict the occurrence of both stroke and myocardial infarction independently of traditional cardiovascular risk factors [4].

OBJECTIVE

The aim of this study was to investigate the association between Treg, Th17 and B1a cell blood concentrations and the abundance of carotid atherosclerosis.

MATERIALS AND METHODS

Patients

The study was approved by the Institutional Ethics Committee in compliance with the Declaration of Helsinki. Written consent was obtained from each patient. From October 2014 to November 2014, 83 patients who faced the need for carotid sonography were enrolled in the study. The exclusion criteria were: acute coronary syndrome (ACS), stroke or interventions in previous 6 months, liver or renal failure, infectious/inflammatory disease, and current use of immunosuppressive drugs and vitamin D as factors that influence lymphocyte subpopulations levels independently.

Vascular ultrasound

IMT and AP in carotid arteries were analyzed using a high-resolution ultrasound system with linear array transducer 3–9 MHz (PHILIPS iU22 ultrasound system, Philips Inc., Eindhoven, the Netherlands) in compliance with *Mannheim carotid intima--media thickness and plaque consensus* (2011) [5] and consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force (2008) [6]. AP were assessed in distal parts of common carotid arteries, in bifurcation of common carotid arteries, in internal carotid arteries (ICA) at both sides in longitudinal (anterior oblique, side and posterior oblique) and transversal planes. Carotid plaques were defined as focal structures encroaching into the arterial lumen of at least 0.5 mm or 50% of surrounding IMT value, or demonstrating thickness greater than 1.5 mm as measured from the intima–lumen interface to the media-adventitia [5]. Based on these data, patients were categorized into 3 groups: group 1 – plaque-negative, IMT thickening above 1.1 mm or local plaques with mild stenosis of ICA less than 30% (n = 31); group 2 – diffuse plaques with moderate ICA stenosis less than 50% (n = 32); and group 3 – diffuse plaques with severe ICA stenosis 50% and above (n = 20). In group 1, 18 patients had increased IMT and 13 patients – mild carotid atherosclerosis (20–30%).

There were no significant differences in anamnestic, clinical (age, smoking status, body mass index, arterial hypertension), and biochemical characteristics (glucose, cholesterol, triglycerides) between the groups. 12 patients did not receive statin therapy before admission (predominantly in group 1, see table 1), others had been receiving statins (atorvastatin 20 mg daily or rosuvastatin 10 mg daily), acetylsalicylic acid 75 mg daily, β-blockers (bisoprolol 2.5–10 mg daily) and ACE inhibitors (enalapril 5–20 mg daily) for at least 1 month before the enrollment.

Lymphocyte immunophenotyping

Whole blood was collected in a vacutainer tubes with sodium citrate as a anticoagulant. Samples were processed within 2 h after being collected. For surface antigen staining the following antibodies and reagents were used: CD4-FITC, CD25-PE, CD-127-PC5, CD45-APC, CD19-FITC, CD5-PE, CD27-PC5, CD-23-APC, and lysing and fixing solutions (Beckman Coulter, Becton Dickinson Immunocytometry Systems). The intracellular antigen analysis was performed in mononuclear leukocytes. The cells were isolated by density gradient centrifugation (Histopaque®-1077, Sigma-Aldrich). For cytokine detection, mononuclear cells were additionally cultivated in the presence of 25 ng/ml PMA, 1 μg/ml ionomycin, and 10 μg/ml monensin for 4 h. Cell staining was performed with CD4-PC5, FoxP3-Alexa488, IFN--γ-PE, IL-17a-Alexa488 and appropriate isotypic controls, and with a FoxP3 Staining Buffer Set (all reagents from eBioscience) in accordance with the manufacturer's manual. Samples were analyzed with two-laser FACSCalibur flow cytometer equipped

TABLE 1.
Clinical data of patients.

	Group 1 (n = 31)	Group 2 (n = 32)	Group 3 (n = 20)	p
Males	30 (97%)	26 (81%)	17 (85%)	0.10
Age (years)	58 (50–63)	60 (53-66)	65 (59–68)	0.07
BMI (kg/m^2)	27 (25–31)	28 (26-29)	29 (27–31)	0.45
Smoking	6 (19%)	11 (34%)	8 (40%)	0.12
Hypertension	19 (61%)	25 (78%)	14 (70%)	0.18
Statin therapy	**21 (68%)**	**31 (97%)**	**19 (95%)**	**p1/2 = 0.007; p1/3 = 0.05**
TC (mmol/l)	**5.0 (4.4–6.0)**	**4.8 (4.2–5.6)**	**4.3 (3.8–5.1)**	**p1/3 = 0.01**
LDL (mmol/l)	2.1 (1.6–2.8)	1.8 (1.4–2.4)	1.5 (0.6–2.8)	0.76
HDL (mmol/l)	0.9 (0.8–1.0)	0.8 (0.6–0.9)	0.7 (0.6–1.3)	0.40
TG (mmol/l)	**2.4 (1.7–3.1)**	**3.6 (2.4–4.5)**	**3.3 (2.1–4.0)**	**p1/2 = 0.01**
Glucose (mmol/l)	5.5 (5.2–5.8)	5.3 (4.8–5.8)	5.2 (4.7–5.7)	0.27
CRP	1.7 (0.6–3.7)	1.5 (0.9–9.8)	1.2 (0.7–3.2)	0.67

BMI – body mass index, TC – total cholesterol, HDL – high density lipoproteins, LDL – low density lipoproteins, TG – triglycerides, CRP – C-reactive protein.

with CellQuest Pro software (Becton Dickinson Immunocytometry Systems). Lymphocytes were gated according to light scattering and CD45 expression. Treg were identified as CD4+CD25^{high} CD127^{low} cells and CD4+FoxP3+, Th1 as CD4+IFN-γ+, Th17 as CD4+IL-17a+, B1a cells as CD19+CD5+CD27-.

Lipid parameters

Values of total cholesterol (TC), cholesterol of high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TG) were tested enzymatically on the "Architect 8000" (Abbott) analyzer. Oxidized LDL (oxLDL) were measured with commercial kits "Mercodia" (Sweden).

Concentration of immunoglobulins

Concentration of immunoglobulins was measured by commercial kit "Vector-Best" (Russia). Autoantibodies (AAb) to oxLDL were determined by ELISA with plates coated by oxidized low-density lipoproteins (5 μg per well) as published for AAb against lipoprotein(a) [7]. LDL were isolated from human plasma by ultracentrifugation with gradient of density of NaBr [8] and then were oxidized with Cu^{2+} as described previously [9].

STATISTICS

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

RESULTS

The levels of circulating CD4+CD25^{high}CD127^{low} Treg were significantly lower in patients with severe ICA atherosclerosis (group 3) compared to patients with mild and moderate atherosclerosis (stenosis less than 50%, group 1 and 2). No differences in other T cells subpopulation content between groups were observed (tab. 2).

Spearman's test showed a moderate opposite correlation between CD4+CD25^{high}CD127^{low} Treg levels and the percentage of maximal carotid stenosis ($r = -0.44$; $p < 0.05$).

In group 1, patients with increased IMT as compared to patients with mild atherosclerotic plaques had decreased Th1 levels (7.7% [5.8–10.0] vs. 11% [8.0–12.8], % of lymphocytes; $p = 0.05$) and increased levels of CD4+CD25^{high}CD127^{low} T cells (6.1% [5.0–7.6] vs. 4.8% [3.9–5.8], % of CD4+ cells; $p < 0.05$). Total B cells and B1a cells concentrations were comparable in all the groups (tab. 2).

We found increased oxLDL IgM serum concentrations (fig. 1b, tab. 2) in group 1 and 2 than in group 3 while oxLDL concentrations were comparable in all the groups. No correlations between oxLDL and oxLDL IgM serum concentrations were observed, neither between B cells and B cell subsets and oxLDL IgM serum concentrations.

Patients who had been receiving statin therapy and with the lower TC levels (4.6 mmol/l [4.2–5.3] vs. 5.9 mmol/l [5.2–7.0]; $p = 0.0005$) had decreased oxLDL serum concentration (9.4 mU/l [7.6–11.9] vs. 12.5 mU/l [9.8–16.8]; $p < 0.05$)

FIGURE 1.
The frequencies of CD4+CD25^{high}CD127^{low} Treg in patients with carotid atherosclerosis (a) serum concentrations of oxLDL IgM in patients with carotid atherosclerosis (b).

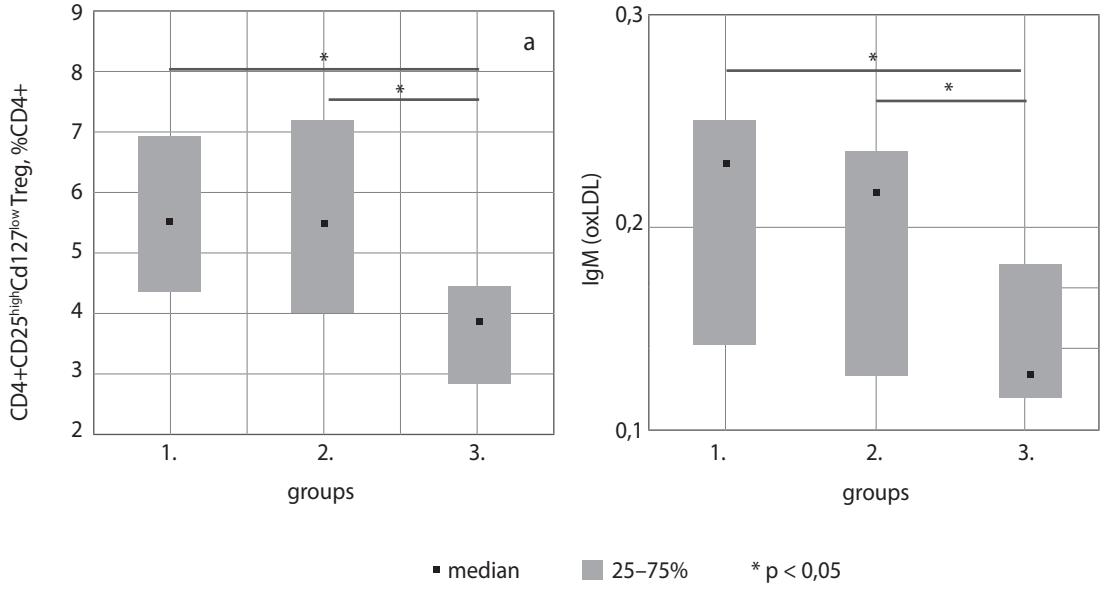


TABLE 2.
Comparison of the percentages and absolute values of circulating lymphocytes subpopulations and AAbs to oxLDL concentrations in blood between the groups of patients.

	Group 1 (n = 31)	Group 2 (n = 32)	Group 3 (n = 20)	p
Leukocytes (10^6/ml)	7.2 (6.0–8.0)	7.6 (7.2–8.0)	7.2 (6.4–8.2)	0.08
Lymphocytes (%)	27 (24–32)	26 (21–31)	28 (24–32)	0.61
Lymphocytes (10^6/ml)	1.9 (1.5–2.1)	2.1 (1.7–2.7)	2.3 (1.4–2.5)	0.23
CD4+ (%)	38 (33–45)	42 (34–44)	43 (34–48)	0.48
CD4+ (10^6/ml)	0.72 (0.61–0.81)	0.84 (0.68–1.05)	0.71 (0.59–1.12)	0.22
CD4+CD25^{high}CD127^{low} Treg (% CD4+)	**5.6 (4.4–6.9)**	**5.4 (4.0–7.2)**	**3.9 (2.8–4.2)**	**p1/3 = 0.001 p2/3 = 0.007**
CD4+CD25^{high}CD127^{low} Treg (10^3/ml)	**36 (28–52)**	**43 (32–68)**	**27 (19–48)**	**p1/3 = 0.01 p2/3 = 0.001**
CD4+FoxP3+ Treg (% CD4+)	7.5 (6.0–8.6)	7.0 (6.2–9.3)	6.5 (4.9–9.0)	0.73
CD4+FoxP3+ Treg (10^3/ml)	53 (38–80)	62 (51-76)	53 (34–84)	0.30
Th17 (% CD4+)	1.1 (0.8–1.6)	1.3 (0.7–1.8)	1.3 (0.7–1.9)	0.99
Th17 (10^3/ml)	8.1 (5.7–13)	10.7 (6.5–14.4)	8.4 (6.7–14.5)	0.56
CD4+CD25^{high}CD127^{low} Treg/Th17	5.7 (3.0–7.1)	5.9 (2.3–8.3)	4.2 (1.9–4.5)	0.28
CD4+FoxP3+ Treg/Th17	7.8 (3.7–10.8)	7.5 (3.3–11.0)	7.0 (3.7–8.7)	0.76
Th1 (% CD4+)	22 (18–31)	21 (15–28)	18 (16–26)	0.49
Th1 (10^3/ml)	181 (111–220)	174 (143–254)	188 (98–262)	0.83
CD19+ B total (% lymphocytes)	11 (7–20)	9 (7–12)	8 (7.5–8.8)	0.33
CD19+ B total (10^3/ml)	180 (133–464)	198 (170–303)	114 (113–130)	0.42
CD19+CD5+CD27- B1 (%) lymphocytes	18 (10–27)	20 (12–34)	26 (12–39)	0.43
CD19+CD5+CD27- B1 (10^3/ml)	29 (15–37)	43 (26–53)	44 (17–50)	0.33
oxLDL (U/ml)	10.0 (7.6–15.9)	10.2 (8.4–13.0)	10.0 (7.9–12.0)	0.91
IgM (oxLDL), o.u.	**0.22 (0.14–0.25)**	**0.20 (0.13–0.24)**	**0.14 (0.12–0.18)**	**p1/3 = 0.004 p2/3 = 0.04**

Treg – regulatory T-cells, Th1 – T-helpers 1, Th17 – T-helpers producing IL-17, oxLDL – oxidized low density lipoproteins.

as well as increased concentrations of CD4+FoxP3+ Treg cells (% of lymphocytes) (3.0% [2.5–4.0%] vs. 2.2% [1.9–2.7%]; $p < 0.01$). There were no differences in Th1, Th17, CD4+CD25^{high} CD127^{low} Treg and B cell subpopulations, and AAbs blood levels between patients on or off statin therapy.

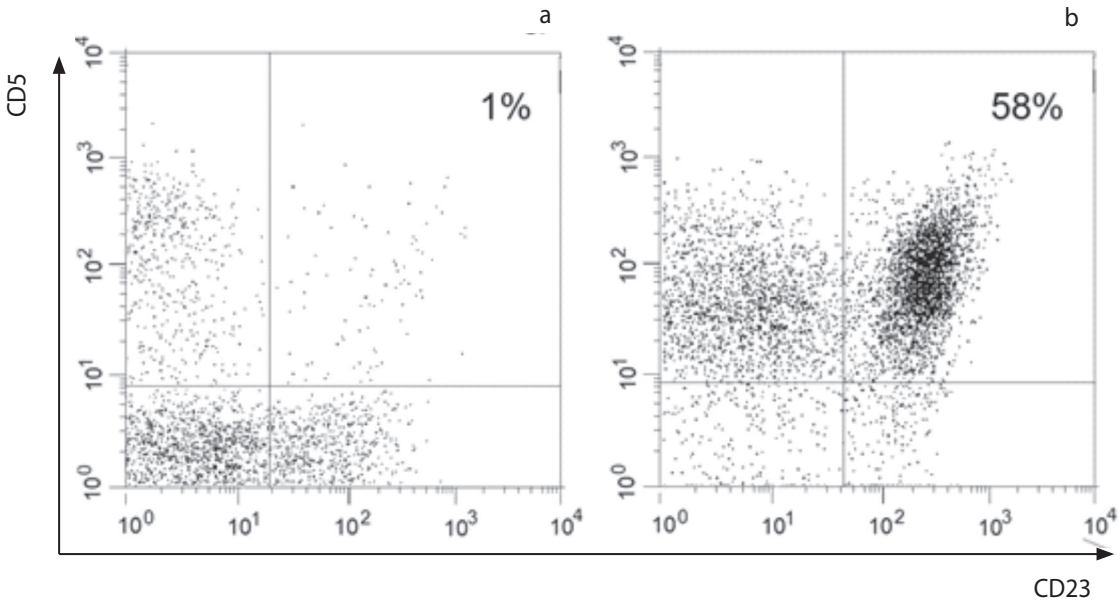
During the study, we initially diagnosed an atypical CD19+CD5+CD27+B cell clone expansion in 3 elderly patients with intact carotid arteries (group 1) (Fisher's exact two-tailed $p = 0.01$ vs. patients with carotid atherosclerosis). The frequencies of CD19+CD5+CD27+ cells were elevated (58–95% from B cells) in these 3 patients (fig. 2b).

We additionally stained B cells using antibodies to CD23-APC according to clinical recommendations for diagnostics of B-CLL (B-cell chronic lymphocytic leukemia) in these 3 patients. The occurrence of CD19+CD5+CD23+ cell clone of 5000 cells/μl, the presence of lymphadenopathy, organomegaly and cytopenia in 1 patient was indicative of B-CLL, while the presence of CD19+CD5+CD23+ cell clones of 200 and 500 cells/μl and the absence of symptoms were indicative of monoclonal B-cell lymphocytosis (MBCL) in the other 2 patients [10, 11].

Patient 1, 57-year-old male. Laboratory findings: leukocytes 7.0 × 10^9/l, lymphocytes 27%. Carotid ultrasound findings: no carotid and femoral atherosclerosis, IMT 1.1 mm. Neither splenomegaly nor lymphadenopathy was observed. Immunological findings: CD19+ B cells 14% of all lymphocytes, CD19+CD5+CD23+ cells – 70% of B cells. CD4+CD25^{high} CD127^{low} Treg – 3.2%, CD4+FoxP3+ Treg – 5.2% and Th17 – 0.75% of total CD4+ lymphocyte count, CD4+CD25^{high}CD127^{low} Treg/Th17 ratio – 4.3, CD4+FoxP3+ Treg/Th17 – 6.9.

Patient 2, 61-year-old male. Laboratory findings: leukocytes 4.9 × 10^9/l, lymphocytes 30%. Carotid ultrasound findings: no carotid and femoral atherosclerosis, IMT 0.73 mm. Neither splenomegaly nor lymphadenopathy was observed. Immunological findings: CD19+ B cells 36% of lymphocytes, CD19+CD5+CD23+ cells – 58% of B cells (fig. 2b). 7.8%, CD4+FoxP3+ Treg – 10.2% and Th17 – 0.9% of CD4+ lymphocytes, CD4+CD25^{high}CD127^{low} Treg/Th17 ratio – 8.7, CD4+FoxP3+ Treg/Th17 – 11.3.

FIGURE 2.
The examples of CD5 and CD23 expression by B cells: (a) the example shows a minor subpopulation of double-positive CD5+CD23+; (b) the expansion (clonal proliferation) of CD5+CD23+ cells. The dot plots for CD19+ lymphocytes are shown.



Patient 3, 82-year-old male. Laboratory findings: leukocytes 9.5 × 10^9/l, lymphocytes 66.8%, anemia (hemoglobin 120 g/l, erythrocytes 3.97 × 10^{12}/l), thrombocytopenia (platelets 99 × 10^9/l). Carotid ultrasound findings: no carotid and femoral atherosclerosis, IMT 1.2 mm. Computed tomography findings: splenomegaly, lymphadenopathy. Immunological findings: CD19+ B cells – 75% of lymphocytes, CD19+CD5+CD23+ cells – 95% of all B cells. CD4+CD25^{high}CD127^{low} Treg – 10%, CD4+FoxP3+ Treg – 19.4%, Th17 – 0.7% of CD4+ lymphocytes, CD4+CD25^{high}CD127^{low} Treg/Th17 ratio – 14.3, CD4+FoxP3+ Treg/Th17 – 27.7.

DISCUSSION

Treg are critical in maintaining immune homeostasis, preventing autoimmune diseases and hypersensitivity reactions and providing tolerance after transplantation. On the other hand, Treg are also known to suppress antitumor immunity [12]. In general population, the mean number of $CD4+CD25^{high}CD127^{low}$ Treg in healthy subjects is 1.65–5.75% of CD4+ T-lymphocytes and decreases with age [13].

A large body of experimental data indicated a protective role of Treg in atherosclerosis [14]. Most clinical studies were focused on coronary atherosclerosis and acute coronary syndromes and showed decreased Treg level in patients after myocardial infarction and unstable angina as well as with multivessel coronary artery disease [1, 2, 15].

Some studies revealed that the shift in Treg/Th17 cell balance in favor of Th17 cells was associated with carotid atherosclerosis [1, 16]. In our study, we confirmed these findings by observing lower $CD4+CD25^{high}CD127^{low}$ Treg blood levels in patients with significant ICA lesions. A negative correlation between $CD4+CD25^{high}CD127^{low}$ T cells and percentage of ICA stenosis was observed. This might reflect the anti-atherogenic mechanisms deficiency in patients with severe carotid atherosclerosis. The assessment of ICA atherosclerosis is more useful in individual stroke prediction than common carotid or bifurcational atherosclerosis [17]. Nevertheless, the prospective study by Wigren et al. did not reveal the association between Treg blood levels and ischemic stroke [14]. We speculate that T-cell disbalance participates in atherosclerosis progression but more studies need to be conducted to verify its role in plaque destabilization and atherothrombosis.

Statin treatment induces the accumulation of Treg in atherosclerotic plaque [18]. Statins increase the frequencies of circulating CD4+FoxP3+ Treg in healthy individuals [19] and in patients with ACS [15]. Here we demonstrated higher frequencies of CD4+FoxP3+ Treg and lower oxLDL blood levels in patients with carotid atherosclerosis who had been receiving statins. That could result from pleiotropic effects of statins and their ability to suppress plaque inflammation [20, 21]. This fact could also explain the comparable values of Treg between group 1 and 2 in our study as the patients from group 2 might have higher Treg levels due to statin treatment.

According to recent data, IgM to modified lipoproteins promote their clearance and play a protective role in atherogenesis. A strong association between decreased levels of natural IgM to oxLDL and atherosclerosis abundance was demonstrated [22]. Here we observed lower oxLDL IgM serum concentrations in patients with significant carotid lesions. At the same time, we did not observe either a correlation between B1a cells content and IgM to oxLDL level or oxLDL concentration and IgM to oxLDL level. B1a cell levels were also comparable between the groups. We suggest that anti-oxLDL IgM production depends on the amount and functional activity of specific B1a cells while the common pool of B1a cells could remain constant.

B-cell phenotyping allowed us to reveal an atypical CD19+CD5+CD27+CD23+ B cell clone expansion in 3 patients from group 1 (with intact carotid arteries) as compared to patients from groups 2 and 3 (with carotid atherosclerosis). Our findings may only be considered complimentary and did not have any statistical significance but we speculate that their presentation and discussion may be interesting from hypothesis--generating point of view.

In general, the population of CD19+CD5+CD27+ B cells is negligible and accounts for about 1% of all B cells. These were the values we observed also in other patients (fig. 2a). Atypical CD19+CD5+CD27+ cells may be indicative of B-CLL [23].

B-CLL and MBCL are characterized by asymptomatic monoclonal expansion of B lymphocytes in peripheral blood. The incidence of MBCL in subjects over 40 years old varies from 1% to 18%, which can be partially due to the availability and sensitivity of flow cytometric examination.

Treg frequencies are increased in patients with B-CLL and MBCL [24, 25]. The level of Th17 and IL-17 were shown to be decreased in patients with CLL indicating a distortion of the immunological balance to regulatory component in CLL pathogenesis [26]. We revealed increased Treg content and Treg/Th17 ratios in patients with lymphoproliferative disorder. In contrast, as was previously discussed, the shift of Treg/Th17 cell balance in favor of Th17 cells was associated with coronary and carotid atherosclerosis progression and destabilization [1, 2, 16]. According to Budczies et al., there is an inverse association between non-smoking-related cancers and atherosclerosis; lymphoproliferative disorders were associated with much smaller incidence of atherosclerosis ($OR = 0.41$; $p < 0.0001$) [27].

CONCLUSIONS

Taken together, these data may assume different immunological profiles of atherosclerosis and CLL with the predominance

of proinflammatory or regulatory components respectively. We hypothesized that high levels of Treg, especially in patients with B-CLL and MBCL, protect them from atherosclerosis. More research needs to be conducted to determine if these changes are primary or secondary ones, and including patients with different types of cancer will allow us to better understand the role of Treg in atherosclerosis protection.

Acknowledgements

The study was supported by the Ministry of Healthcare of the Russian Federation (RK 114052670029) and Russian Basic Research Foundation (14-04-00679).

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Authors' contributions:
All authors equally contributed to the clinical data collection, analysis of the data and to writing the manuscript.