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IL-33/IL-33R in various types of carotid artery atherosclerotic lesions

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ABSTRACT

Objective: Inflammation plays a crucial role in the progression of atherosclerotic plaques. The aim of the study was to investigate serum levels and expression of Interleukin-33 (IL-33) and ST2 receptor in atherosclerotic plaques and to analyze correlation with the type of the carotid plaques in patients with carotid disease.

Methods: This study included 191 consecutive patients submitted for carotid endarterectomy (CEA). Preoperative serum levels of IL-33 and soluble ST2 (sST2) were measured. Atherosclerotic plaques obtained during surgery were initially histologically classified and immunohistochemical analyzes of IL-33, IL-33R, CD68 and alpha-SMA expression was performed. Ultrasound assessment of the level of carotid stenosis in each patient was performed prior to carotid surgery. Demographic and clinical data such as gender, age, smoking status, blood pressure, glycaemia, hemoglobin and creatinine levels, and comorbidities were collected and the comparisons between variables were statistically evaluated.

Results: Serum levels of IL-33 (35.86 ± 7.93 pg/ml vs.12.29 ± 1.8 pg/ml, $p < 0.05$) and sST2 (183 ± 8.03 pg/ml vs. 122.31 ± 15.89 pg/ml, $p < 0.05$) were significantly higher in the group of CEA patients vs. healthy subjects. We demonstrated abundant tissue expression of IL-33 and ST2 in atherosclerotic carotid artery lesions. The levels of IL-33 and IL-33R expression were significantly higher in vulnerable plaques and significantly correlated with the degree of inflammatory cells infiltration in these plaques ($R = 0.579$, $p = 0.049$). Immunohistochemical analysis also revealed that cells responsible for IL-33 expression are not only mononuclear cells confined to inflammatory atherosclerotic lesions, but also smooth muscle cells which gained phenotypic characteristics of foam cells and were loaded with lipid droplets.

Conclusion: The obtained results confirm the importance of IL-33/ST2 axis in the process of atherosclerosis, and indicate its ambiguous function in immune response, whether as proinflammatory cytokine in advanced atherosclerotic lesions, or as profibrotic, in early lesions.

1. Introduction

Cardiovascular diseases remain leading cause of morbidity and mortality for decades, despite considerable efforts aiming to complete understanding of the process of atherosclerosis and consequences developing during the evolution of this process [1].

Atherosclerosis is a chronic inflammatory disease of large arterial blood vessels, materialized through atherosclerotic plaque formation [2]. Atherosclerotic plaque causes physical obstruction of blood flow due to blood vessel lumen stenosis, or may rupture, causing thrombosis and local infarction of tissues depending on blood supply by affected vessel.

Carotid artery disease presents one of most common and important form of atherosclerosis due to its high prevalence, and because brain tissue is very sensitive to blood flow reduction and may sustain serious irreversible damage even in short periods of blood flow deprivation. Additionally, surgical procedure, carotid endarterectomy, remains golden standard in treatment, and is one of few prophylactic surgical

Abbreviations: EAE, experimental autoimmune encephalomyelitis; hs-CRP, high-sensitivity C-reactive protein; IL-, interleukin; SMC, smooth muscle cells; DAMP, damage-associated molecular pattern molecules; MDCT, multidetector computed tomography arteriography; TIA, transient ischemic attack; AFX, amaurosis fugax; IFN-γ, interferon-gamma; CEA, carotid endarterectomy

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procedures currently. This fact is proven in numerous multicentre trials, and despite all periprocedural risks maintains advantage against endovascular procedures [3].

Despite growing evidence that inflammation plays essential role in the development of carotid artery disease, the precise molecular and cellular mechanisms underlying carotid plaque formation remain incompletely understood [4].

Studies have shown that levels of circulating inflammatory cytokines, high-sensitivity C-reactive protein (hs-CRP), interleukin (IL)-6, interleukin (IL)-1, tumour necrosis factor-α (TNF-α) or interleukin (IL)-18 have substantial predictive value for future cardiovascular events [5–7], carotid plaque instability and inicate increase of intima-media thickness of carotid arteries [8–10]. In contrast, elevated serum levels of anti-inflammatory cytokine interleukin (IL)-10 and Th2 cytokine interleukin (IL)-5 are associated with positive prognosis and desirable outcome of treatment in patients with acute coronary syndrome [11,12].

Interleukin (IL)-33 is a member of the IL-1 family of cytokines expressed constitutively by endothelial, epithelial, smooth muscle cells and cells of immune system [13]. When released from the cell, during cell necrosis or cell injury, it expresses its paracrine pro-inflammatory action through binding to IL-33 receptor (ST2, IL-33R) [14]. Apoptosis of the cell, on the other hand, provides no such response due to inactivation of IL-33 by caspases, activated during this controlled process.

Full-length, biologically active, IL-33 acts ambiguously [15,16], pro- and anti-inflammatory, by enhancing activity of mast cells, Th2 cells, regulatory T cells (Treg) and innate lymphoid cells. Due to its constitutive mode of expression, in contrast to majority of cytokines, IL-33 might function as an alarmin, danger signal, belonging to the larger family of damage-associated molecular pattern (DAMP) molecules [17].

Numerous studies in humans have shown a role of IL-33 in autoimmune, inflammatory, allergic, fibrotic and malignant disorders [18–24]. In mice, rendered deficient for the ApoE protein fed with high-fat diet, treatment with rIL-33 reduces aortic atherosclerotic plaque development and increases serum levels of atheroprotective type 2 cytokines [14]. Recent paper, however, suggests that deficiency of the endogenously produced IL-33 and its receptor ST2 does not impact the development of atherosclerosis in ApoE-deficient mice [25].

On the other hand, the role of IL-33 in human atherosclerosis was object of investigation in limited number of papers with controversial results. Interleukin (IL)-33 and IL-33 R (ST2) are both expressed in human atherosclerotic plaques [26]. Clinical data revealed that IL-33 serum levels were associated with mortality in patients with ST-elevation myocardial infarction (STEMI) [27]. Increased levels of circulating IL-33 after coronary stent implantation were associated with coronary in-stent restenosis [28]. Clinical studies have shown association between serum IL-33 levels and thrombotic complications after plaque rupture [29] and with progression of carotid atherosclerosis in patients with rheumatoid arthritis [30]. Interleukin-33 in human carotid plaques enhances prothrombotic activity of endothelial cells [31]. These differences between results obtained from animal models of atherosclerosis and from human studies may be explained with recent papers which put in light species specific differences in patterns of expression and immunomodulation of IL-33 [32].

2. Materials and Methods

2.1. Patients

Total of one hundred ninety one consecutive patients with atherosclerotic carotid disease admitted to Institute for cardiovascular disease “Dedinje”, Belgrade, Serbia for surgical treatment were enrolled in this study. The study was performed in accordance with the Declaration of Helsinki, with the approval of local ethics committee on human research (School of Medicine, University of Belgrade, Serbia) and informed consent was obtained from each study participant. Colour Doppler scan was performed on each patient for the evaluation of the degree of carotid stenosis, according to North American Symptomatic Carotid Endarterectomy Trial criteria [33]. Multidetector computed tomography arteriography (MDCT) was additionally performed to grade the severity of carotid artery stenosis. The patients selected for carotid endarterectomy (CEA) were either clinically symptomatic or asymptomatic. Symptomatic patients had in their previous medical history some form of neurological incident, transient ischemic attack

Table 1
Demographic and clinical characteristics of patients undergoing endarterectomy.

Symptoms	With symptoms	Without symptoms	P[b]
	n = 99 (49.7%)	n = 100 (50.2 %)	
Age	66.1 ± 7.6	67.0 ± 8.3	0.42
Gender			
Male	52 (47.7)	57 (52.3)	0.57
Female	47 (52.2)	43 (47.8)	
Diabetes mellitus			
Yes	33 (47.8)	36 (52.2)	0.762
No	66 (51.6)	64 (48.4)	
Smoking			
Yes	73 (49.3)	75 (50.7)	0.743
No	26 (51.0)	25 (49.0)	
Hypertension			
Yes	95 (50.3)	94 (49.7)	1
No	4 (40.0)	6 (60.0)	
Cholesterol			
mmol/L, (SD)	5.02 ± 0.11	4.87 ± 0.12	0.374
Triglycerides			
mmol/L, (SD)	1.52 ± 0.52	1.58 ± 0.08	0.554
Creatinine			
mmol/L, (SD)	87.94 ± 4.11	84.37 ± 2.70	0.465
PAD			
Present	13 (50)	13 (50)	1
Absent	86 (49.7)	87 (50.3)	
CAD			
Present	38 (44.7)	47 (55.3)	0.252
Absent	61 (53.5)	53 (46.5)	
Degree of carotid stenosis			
< 70%	7 (76.2)	14 (23.8)	
≥70% and < 90%	54 (90.4)	50 (9.6)	0.281
>90%	38 (90.5)	36 (9.5)	
Intraplaque inflammatory infiltration			
Present	4 (57.1)	3 (42.9)	0.721
Absent	95 (49.5)	97 (50.5)	
Intraplaque calcification			
Present	38 (52.0)	35 (47.9)	0.661
Absent	61 (48.4)	65 (51.6)	
Intraplaque hemorrhage			
Present	12 (94.1)	5 (5.9)	0.081
Absent	87 (88.5)	95 (11.5)	
Rupture of plaque			
Present	26 (46.4)	30 (53.6)	0.637
Absent	73 (51.0)	70 (48.9)	
AHA classification of carotid plaques			
IV	6 (46.1)	7 (53.8)	
Va	11 (55.0)	9 (45.0)	
Vb	18 (60.0)	12 (40.0)	0.321
Vc	29 (43.3)	38 (55.7)	
VIa	22 (43.1)	29 (56.9)	
VIb	11 (68.7)	5 (31.2)	

PAD, peripheral artery disease; CAD, coronary artery disease.
[a]Values are expressed as mean ± SE or absolute numbers with calculated percentage.
[b] Statistically significant (Chi-Square, Independent-Samples T, Mann-Whitney U and Kruskal-Wallis H tests; P < 0.05).

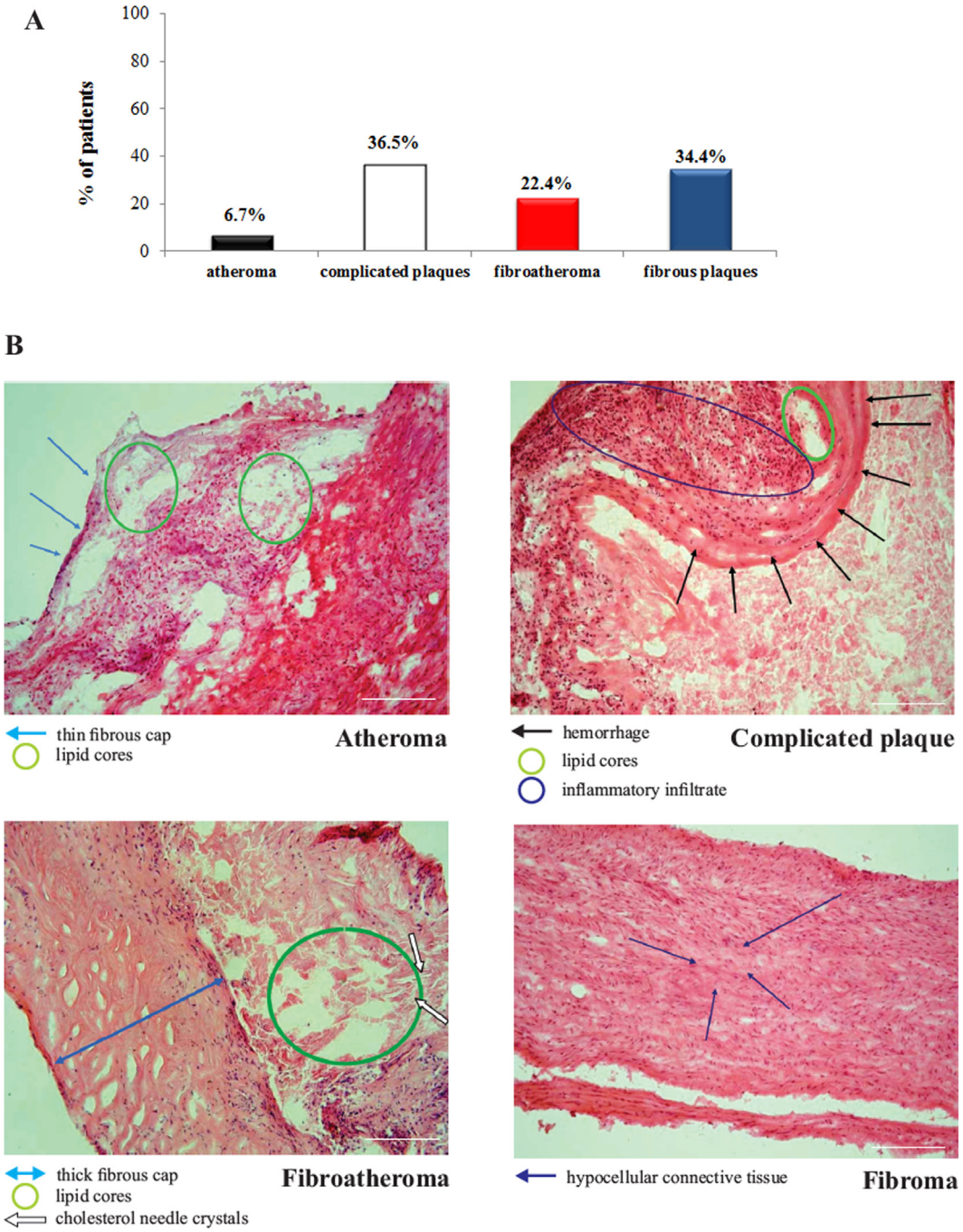

Fig. 1. Carotid plaque types based on histopathological analyses. (A) Distribution of CEA patients based on the histopathological analyses of carotid plaques (n = 191) (B) Representative photomicrographs of H&E stained atheroma with a thin fibrous cap, large lipid core, increased plaque inflammation and positive vascular remodeling (left panel, arrows, n = 13), fibroatheroma with large lipid core and cholesterol needle crystals, covered with thick fibrous cap (right panel, arrows, n = 43), fibrous plaque with dominance of hypocellular connective tissue (bottom panel, arrows, n = 66), complicated plaque with in-plaque hemorrhage, inflammatory infiltrate and lipid cores (bottom panel, arrows, n = 69). Magnification 200X, scale bar 250 μm.

(TIA), amaurosis fugax (AFX) or stroke [34].

All patients were selected through screening programs, performed by neurologists. For the purpose of this research, blood samples from patients and atherosclerotic plaques obtained during surgical procedure were collected. Furthermore, blood samples from 30 healthy volunteers matched for age and sex were also collected.

2.2. Histopathologic and immunohistochemical analysis of carotid atherosclerotic plaques

Atherosclerotic plaques (n = 191) surgically removed from patients' carotid arteries were histopathologically and immunohistochemically analyzed. Tissue samples were immediately frozen or embedded in paraffin, cut into sections 5 μm thick, stained using a hematoxylin-eosin and examined [35]. The grade of atherosclerosis in plaques was established according to the AHA classification of the American Heart Association Committee on Vascular Lesions of the Council of Atherosclerosis [36].

The evaluation of plaque stability was performed according to the criteria described previously by Moreno [37], Shindo [38], and Mehta and Shah [39]. Stable plaques had low lipid content, thick fibrous cap, low level of inflammation and absence of plaque complications. Briefly,

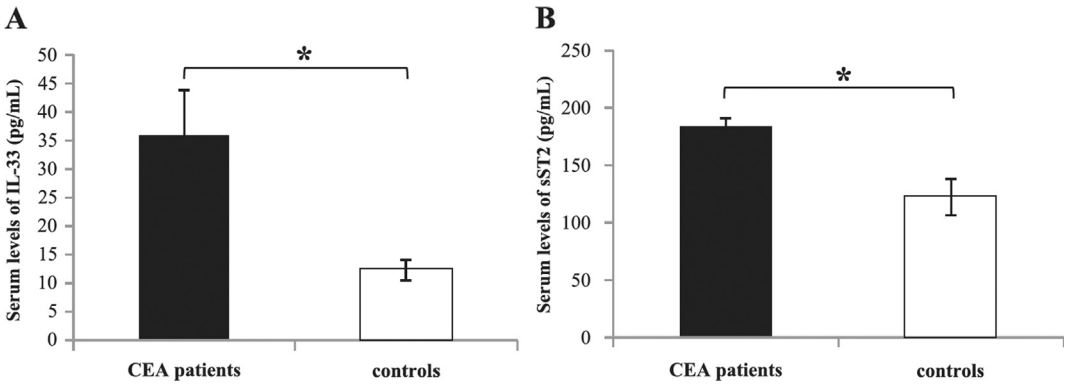

Fig. 2. Serum levels of IL-33 and sST2 in patients undergoing carotid endarterectomy. (A) IL-33 serum levels in patients with carotid disease and healthy controls (35.86 ± 7.93 pg/ml vs. 12.29 ± 1.8 pg/ml) (B) sST2serum levels in patients with carotid disease and controls (183 ± 8.03 vs. 122.31 ± 15.89 pg/ml), *P < 0.05; results are expressed as mean ± SE.

Table 2
Serum levels of IL-33 and sST2 in patients with or without diabetes and hypertension.

CEA patients	IL-33	P	sST2	P
with vs. without diabetes	30.48 ± 10.48 vs.39.65 ± 11.07	*0.644*	179.52 ± 14.14 vs. 182.18 ± 9.26	*0.704*
with vs. without hypertension	36.75 ± 8.47 vs.29.70 ± 10.55	*0.543*	181.33 ± 7.97 vs. 177.79 ± 25.73	*0.865*

Italic values were mean that there no statistically significant differences between the tested groups.

all other plaques containing large, lipid-rich necrotic core with a thin and inflamed cap (< 65 μm of thickness) and other features such as expansive remodelling, plaque haemorrhage, neovascularization, adventitial inflammation, and 'spotty' calcifications were designated as unstable plaques.

Immunohistochemical analyses were performed on frozen sections for IL-33, ST2, CD-68 and α-SMA expression. Frozen sections from 28 atherosclerotic plaques were incubated with primary anti-IL-33 antibody (Dako A/S, Denmark), primary anti-ST-2 antibody (Dako A/S, Denmark), primary anti-CD68 antibody (Dako A/S, Denmark M0876) and primary anti–smooth muscle α-actin antibody (Dako A/S, Denmark).

The evaluation of plaque inflammation, stability and the level of cytokine expression were performed by two independent investigators in a blinded manner (S.M., I.T.).

2.3. Quantification of cytokines in sera

Blood samples were collected from patients prior to surgery, and from control subjects by a single needle stick, between 09:00 and 09:30 a.m., before morning meal. Sera were separated, aliquoted and stored at −20 °C until thawed for the assays of IL-33 and sST2. Cytokine levels were measured using highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN) specific for the human IL-33 and human sST2, according to the manufacturer's instruction. Absorbance was measured on ZENYTH 3100 apparatus, at 450 nm, and obtained data was then processed by Software for Anthos Multimode Detectors. The results are expressed in pg/ml [18].

Serum levels of IL-33 in CEA patients and healthy subjects were determined using Quantikine® ELISA Human IL-33 Immunoassay (R&D Systems Minneapolis, MN; Cat No D3300B). Blood samples were allowed to clot for 30 min at room temperature before centrifugation for 15 min at 1000 × *g*. Serum was removed, aliquoted and stored at ≤ −20 °C for further use. Serum was diluted 1:5. A standard curve was generated for each set of samples assayed for IL-33. Minimum detectable dose (MDD) of human IL-33 ranged from 0.069 to 1.51 pg/mL (the mean MDD was 0.357 pg/mL) and the higher detection limit for hIL-33 was 200 pg/mL as indicated in manufacturer's instructions. The lower and higher detection limits for IL-33 in our experiments were similar to those indicated above. In our repeated experiments, intra-assay (CV 6.4%) and inter-assay (CV 7.1%) precision were satisfactory.

The levels of ST2 (IL-33R) in sera of patients and controls were determined using Quantikine® ELISA Human ST2/IL-33 R Immunoassay (R&D Systems Minneapolis, MN; Cat No DST200). Blood samples were allowed to clot for 30 min at room temperature before centrifugation for 15 min at 1000 × *g*. Serum was removed, aliquoted and stored at ≤ −20 °C for further use. A standard curve was generated for each set of samples assayed for ST2. Serum was diluted 1:20 as recommended. Minimum detectable dose (MDD) of human ST2 ranged from 2.45 to 13.5 pg/mL (the mean MDD was 5.1 pg/mL) and the higher detection limit for hST2 was 2000 pg/mL as indicated in manufacturer's instructions. The lower and higher detection limits for ST2 in our experiments were similar to those indicated above.

In our repeated experiments intra-assay (CV 4.4%) and inter-assay (CV 7.6%) precision were satisfactory.

2.4. Statistics analyses

Results are expressed as mean ± SE. Clinical and histological results were compared between groups (symptomatic vs. asymptomatic) by $\chi 2$ test. The Kruskal-Wallis and Mann–Whitney non-parametric tests or independent Student *t*-test were used to investigate the significance of differences between groups depending on the normality of the data. Correlations were assessed by Spearman's method. A P value < 0.05 was considered statistically significant. All statistical calculations were performed with the IBM SPSS statistics version 20.

3. Results

3.1. Demographic and clinical characteristics of patients with carotid atherosclerosis

Demographic, clinical and histological characteristics of patients undergoing carotid endarterectomy is shown in Table 1. When cross-analysed for the presence of concomitant coronary disease, we observed that 19 symptomatic patients had coronary artery disease (CAD) in previous medical history (19/63, 30.16%), while 44 symptomatic patients were without CAD (44/63, 69.84%). On the other hand, 65 asymptomatic patients had CAD at the moment of surgery (65/128, 50.78%), and 63 were both asymptomatic and without CAD in 49.22% (63/128 patients, P = 0.007).

Further, cholesterol serum levels were slightly elevated in the group of patients with symptomatic carotid artery disease, when compared to

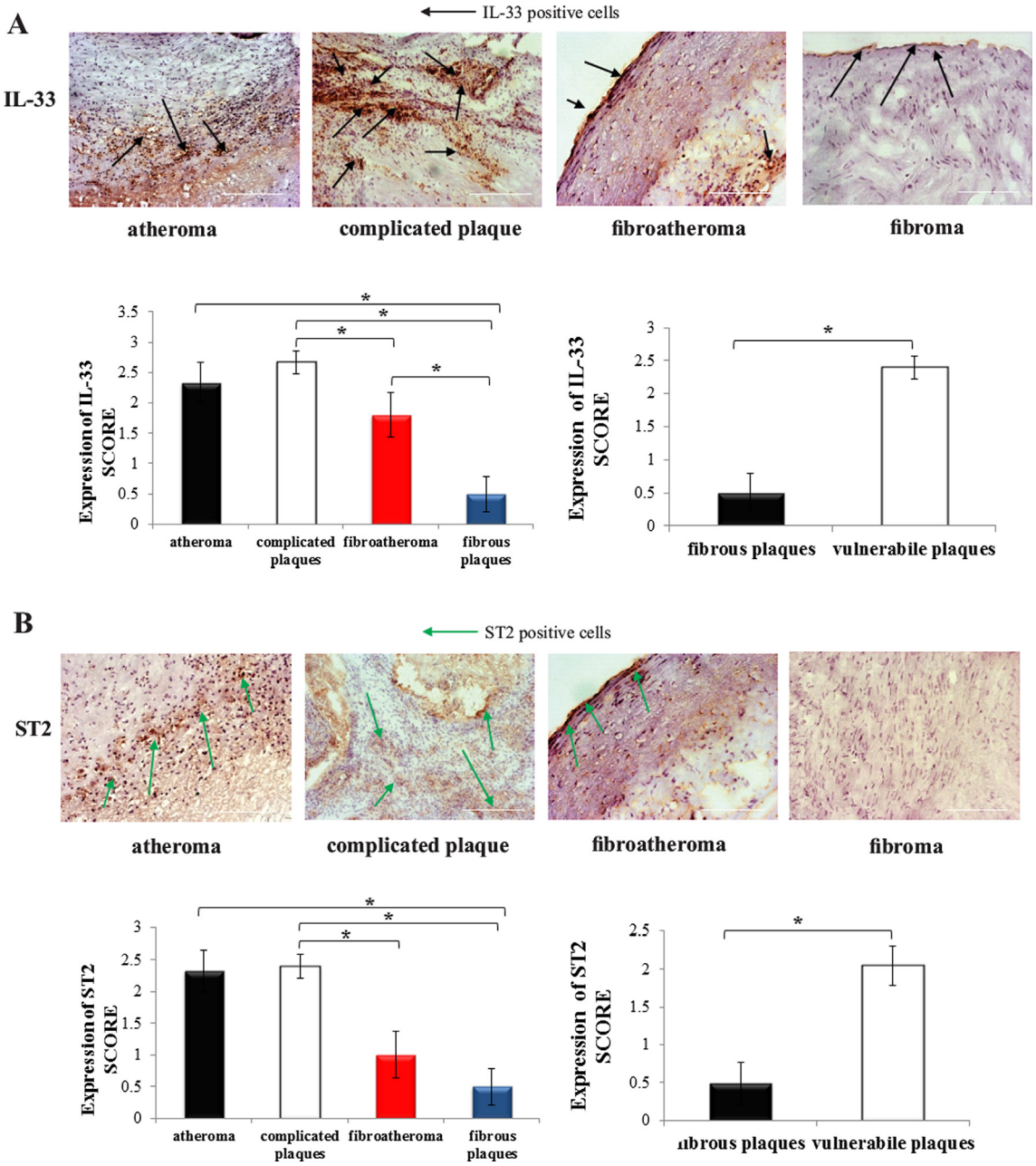

Fig. 3. IL-33 and IL-33R expression in carotid atherosclerotic plaques. (A) Representative photomicrographs of IL-33 and IL-33R immunostaining of four carotid plaque types (n = 28). Numerous IL-33 and IL-33R positive cells were mainly localized in the lipid-rich and necrotic core, inflammatory infiltrate and foam cells as seen in atheromas and complicated plaques (arrows), while less abundant IL-33 and IL-33R positive cells were observed in fibroatheromas and fibromas (arrows). (B) IL-33 and IL-33R expression levels (score) in stable (n = 14) and unstable plaques (n = 14). Results are expressed as mean + SD **P < 0.01. Magnification 200X, scale bar 500 μm.

asymptomatic patients (5.08 ± 0.10 mmol/L vs. 4.88 ± 0.10 mmol/L, P = 0.053).

Other clinical and laboratory parameters showed no statistically significant differences between these groups of patients.

3.2. Histologic plaque characteristics in patients with carotid atherosclerosis

Based on carotid plaque histological characteristics, patients were classified into groups IV, V and VI according to AHA classification of human atherosclerotic plaques, that is, all atherosclerotic lesions in this study were advanced atherosclerotic lesions [36].

Out of 191 analyzed carotid plaques (Fig. 1), 13 were atherosclerotic plaques in the stage of atheroma (6.7%, type IV lesion), 43 were at the stage of fibroatheroma (22.4%, type V lesion), 66 were fibrous plaques (34.4%, type Vc lesion), 69 were at the stage of ruptured atheroma with calcifications, atheroma complicated by previous rupture, thrombosis or haemorrhage, thus designated as type VI lesion (36.5%). Regarding to the histological features defining stability of atherosclerotic plaques, patients can be divided into two groups, the first group comprising patients with stable atherosclerotic lesions (66 patients, 34.56%), and in second group were patients with vulnerable plaques (125 patients, 65.44%).

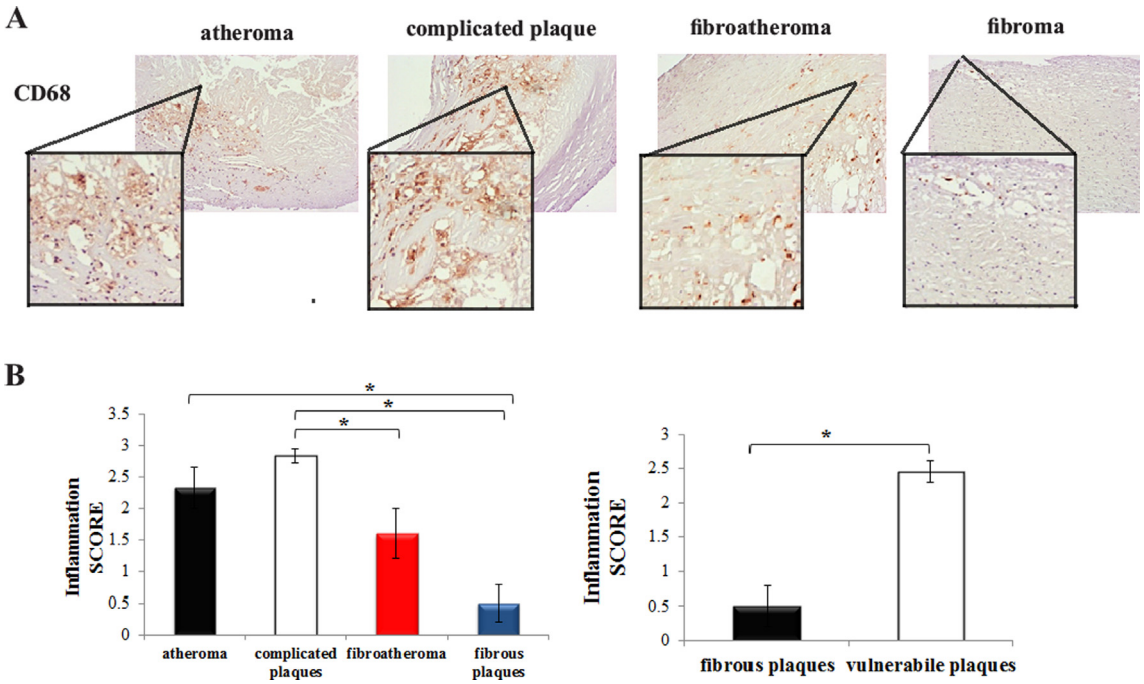

Fig. 4. Inflammation in carotid atherosclerotic plaques. (A) Representative photomicrographs showing tissue expression of CD68 in carotid artery plaques (n = 28). Expression of CD68 is the highest in complicated and atheroma plaques. In complicated plaques inflammation is hallmark of histological image. In atheroma, expression of CD68 is confined to tissue macrophages. Low level of CD68 expression is detected in fibroatheroma, proportionally to low number of tissue macrophages. The lowest level of CD68 expression is detected in fibroma plaques. (B) Comparison of inflammation score in four carotid plaque types and in unstable (vulnerabile) and stable (fibrous) plaques (stable plaques n = 14; unstable plaques n = 14). Results are expressed as mean + SD ** P < 0.01. Magnification 200X, scale bar 500 μm.

3.3. Serum levels of IL-33 and sST2 are increased in patients undergoing carotid endarterectomy

Our data show a significant difference in the serum levels of IL-33 in patients with carotid atherosclerosis compared with healthy subjects (Fig. 2). Higher IL-33 levels were observed in CEA patients compared to healthy controls (35.86 ± 7.93 pg/ml vs. 12.29 ± 1.8 pg/ml, P < 0.05) (Fig. 2**A**). Similar results were obtained when sera were analyzed for sST2 levels. Patients enrolled in this study had higher sST2 levels compared to healthy controls (183 ± 8.03 vs. 122.31 ± 15.89 pg/ml, P < 0.05) (Fig. 2**B**).

We tested circulating levels of IL-33 in patients without and those with diabetes mainly treated with metformin and no statistically significant difference in the serum levels of IL-33 was found (P = 0.644). Similarly patients without hypertension and those with hypertension mainly treated with ACE inhibitors had similar levels of circulating IL-33 (P = 0.543).

We tested circulating levels of ST2 in patients without and those with diabetes mainly treated with metformin and no statistically significant difference in the serum levels of ST2 was found (P = 0.704). Similarly patients without hypertension and those with hypertension mainly treated with ACE inhibitors had similar levels of circulating ST2 (P = 0.865) (Table 2).

3.4. Interleukin 33 and ST2 tissue expression in different types of atherosclerotic plaques

The expression of both IL-33 and ST2 in different types of atherosclerotic lesions show similar patterns and closely correlate. Expression of IL33 and ST2 is abundant and similar in atheromatous, fibroatheromatous and complicated atherosclerotic lesions, whereas significantly lower in fibrous plaques, as shown in Fig. 3**A and B**. Thus, highest level of in-plaque IL-33 and ST2 expression is confined to vulnerable plaques, while the lowest level of expression was observed in stable, fibrous plaques (Fig. 3**A and B**).

3.5. Interleukin 33 and ST2 tissue expression correlate to in-plaque inflammation

Furthermore, this pattern of IL-33 and ST2 expression closely relates to inflammation score in examined atherosclerotic plaques (Fig. 4**A**). Vulnerable atherosclerotic lesions showed abundant presence of inflammatory cells, and, presenting with higher inflammatory score, while fibrous, stabile plaques characterize dominance of extracellular matrix over scattered and sporadic cellular elements. The degree of inflammatory cell infiltration was associated with IL-33 and ST2 expression levels within the plaques. Plaques with less abundant inflammatory infiltrate had lower levels of IL-33 and ST2 expression, such as fibrous plaques. Thus, the highest level of in-plaque CD68 positive macrophages is confined to vulnerable plaques, while the lowest level of expression was observed in stable, fibrous plaques (Fig. 4**B**).

3.6. Expression of CD68 correlates with expression of IL-33 in atherosclerotic plaques

Expression of IL-33 is confined to different cell types in atherosclerotic lesion, mostly to foam cells, but is also observed to be abundant in endothelial cells, mononuclear cells and smooth muscle cells in blood vessel media layer (Fig. 5**A**). In order to confirm that the macrophages within the plaques express IL-33, consecutive serial sections were stained with CD68 and α-SMA. Pattern of cellular CD68 expression correlates with IL-33 expression in different types of atherosclerotic plaques. We also found that proportion of IL-33 positive foam cells expressed α-SMA, a marker of smooth muscle cells (Fig. 5**A**). Thus, the significant correlation between the levels of IL-33 expression and inflammatory score was observed in analyzed plaques (R = 0.579, P = 0.049) (Fig. 5**B**).

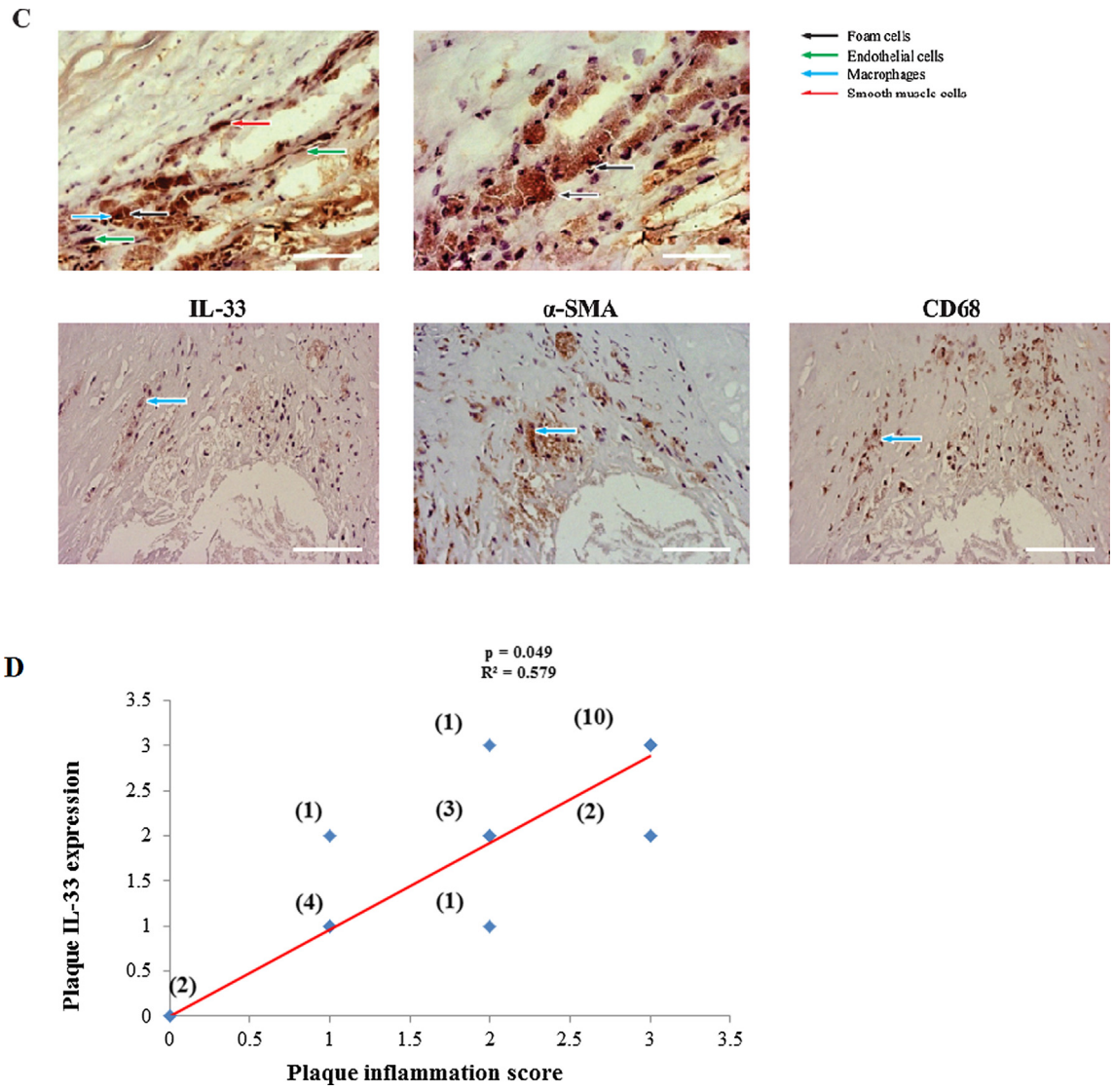

Fig. 5. Correlation between plaque IL-33 expression and plaque inflammation score. (A) Cellular origin of IL-33 within the carotid plaques. Representative photomicrographs of IL-33 stained foam cells, endothelial cells, macrophages and vascular smooth muscle cells in carotid plaques (top panels, arrows). Serial sections of carotid atherosclerotic lesions (n = 28) showing co-staining of IL-33 with α-SMA and CD68 positive cells (bottom panels, arrows). (B) Positive correlation between the levels of IL-33 plaque expression and plaque inflammation score (Spearman correlation coefficient $\mathbf{R^2}$ = 0.579,*p < 0.05).

4. Discussion

In the present study we show significantly elevated IL-33 and sST2 serum levels in CEA patients as compared to healthy subjects. Moreover, CEA patients with vulnerable plaques show higher tissue expression of both IL-33 and IL-33R (ST2) than patients with stable, fibrous plaques. Additionally, our data show that the source of IL-33 is primarily CD68 positive cells. A significant positive correlation between the levels of IL-33 expression and the degree of inflammation in carotid atherosclerotic lesions was observed.

The influence of serum levels of various cytokines on the outcome and the prediction of disease progression is indicated by the many studies carried out on patients suffering from cardiovascular diseases [26–30]. High serum level of sST2 was designated as a predictor of poor outcome in patients with NSTEMI and STEMI. Recently, Dhillon et al. [29] have shown that high levels of sST2 correlate with poor outcome in patients with NSTEMI and STEMI, but high IL-33 levels are associated only with increased mortality of patients with STEMI [34]. However, study of Willems et al. [40] showed no predictive value of serum sST2 level for cardiovascular events in patients with severe carotid artery stenosis.

On the other hand, correlation between IL and 33 levels and stage and progression of atherosclerotic carotid artery disease in patients with rheumatoid arthritis has been reported [30]. This finding partially concurs with our results, that patients with atherosclerotic disease have higher circulating levels of both IL-33 and sST2, when compared to healthy individuals.

When significantly higher serum levels of both IL-33 and sST2 in CEA patients were detected, we looked at the local expression of these molecules in the atherosclerotic plaques. Here, we demonstrate that IL-33 and IL-33R (ST2) are expressed in all four types of atherosclerotic plaques and that their expression is the highest in the atheroma and complicated plaques. As both of these pathologies belong to vulnerable plaques, we conclude that plaque stability negatively correlates to the expression of IL-33 and IL-33R (ST2) in the lesions. The highest level of IL-33 expression was detected in the complicated plaques which are characterized by large areas of necrosis, haemorrhage, and/or thrombosis (Fig. 3**A**). This finding is expected knowing that IL-33 is released and activated during necrotic cell death, which is associated with tissue damage during infection or trauma, but kept intracellular during cell

apoptosis [41–44].

Inflammation fundamentally contributes to the formation and progression of atherosclerotic lesions, and the development of atherothrombotic complications clinically presents as acute myocardial infarction, unstable angina, or stroke [45–47]. Therefore, we further examined the degree of inflammation and type of inflammatory cells in the atherosclerotic plaques and their association with the expression of IL-33. Here we noticed that complicated plaques have abundant inflammatory infiltrate, mostly involving CD68 positive macrophages, while the lowest inflammatory score and the low number of CD68-positive macrophages were found in fibrous plaques (Fig. 4**A**). This finding is not surprising, due to the fact that complicated plaques, belonging to the group of advanced atherosclerotic plaques, show progression depending on the degree of inflammation [48].

It has been demonstrated that IL-33 is expressed in inflammatory conditions, but also that its expression is associated with the degree of inflammation [31]. Thus, we investigated a possible link between expression levels of IL-33 and the inflammatory scores in carotid plaques. We found a significant positive correlation between IL and 33 expression in plaques and inflammatory score as shown in Fig. 5**B**. This concurs with recent study, that IL-33 acts mainly in pro-inflammatory manner in atherosclerotic disease of carotid artery [49].

IL-33 is not only expressed in necrotic cells, but also in various tissue cells and endothelial cells of blood vessels [43–44]. Results obtained in this study indicate that the source of IL-33 are foam cells, endothelial cells, macrophages and smooth muscle cells. It is known that all these types of cells actively participate in the initiation and progression of atherosclerotic plaques and the presence and number of these cells increases with the advancement of plaque pathology [37], indicating that the expression of IL-33 correlates with the stage of atherosclerotic plaque progression.

5. Conclusion

In summing up, our data show the increased presence and possible roles of IL-33 in carotid atherosclerosis, as this cytokine hallmarks progressive and vulnerable atherosclerotic plaques. This is reflected in higher levels of circulating full-length form of IL-33 in patients prior to carotid endarterectomy as compared to healthy subjects, and also in its marked expression in advanced atherosclerotic lesions, particularly those known to be prone to undesired and potentially harmful incident, like rupture, thrombosis, or both.

The practical gain of examining the role of IL-33 in atherosclerosis as a possible therapeutic target remains to be elucidated.

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Conflict-of-interest

The authors declare no other conflict of interests.

Author contribution

MS, BLJ, NA, DJR, NP and MLL substantially contributed to the conception or design of the work; MS, BLJ, SB, VMS, SM, DJR, NP and MLL acquired, analyzed, or interpreted the data; MS, BLJ, SB, VMS, SM, DJR, NP and MLL drafted the manuscript or revised it critically for important intellectual content; MS, BLJ, SB, VMS, SM, NA, DJR, NP and MLL finally approved the version to be published; BLJ, NP and MLL provide agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy of integrity of any part of the work are appropriately investigated and resolved.

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