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ORIGINAL ARTICLE

Lag3$^+$ regulatory T lymphocytes in critical carotid artery stenosis

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Abstract
The aim of this study was to evaluate CD25$^+$ and Lag3$^+$ T regulatory subpopulations in patients with critical carotid artery stenosis (CAS) and Stanford-A acute aortic dissection (AAD). CD25$^+$ and Lag3$^+$ were measured in 36 patients affected by CAS and 24 patients with Stanford type A AAD. Based on neurological symptoms, patients affected by CAS were further divided in 25 asymptomatic (CAS-A) and 11 symptomatic (CAS-S) subjects. Twenty-five patients with traditional cardiovascular risk factors (RF), matched for age and sex, were used as control group. Interleukin (IL)-10, IL-6 and transforming growth factor-β-levels were also measured. CD25$^+$ T cells were significantly increased in CAS-S versus CAS-A ($p > 0.05$), AAD ($p > 0.05$) and RF ($p > 0.05$). Moreover, a significant increase in Lag3$^+$ Tregs was observed in CAS e CAS-S versus AAD ($p < 0.05$) and RF ($p < 0.05$), whereas no significant difference was observed between CAS-S and CAS-A. IL-6 was higher in AAD compared to the other groups. Patients with neurological symptoms display a peculiar expansion of CD25$^+$ T cells, strongly confirming a relationship between ischemic brain damage and this regulatory subpopulation, whereas Lag3$^+$ Tregs early distinguish CAS from AAD and probably exert protective actions against aortic wall rupture throughout their anti-inflammatory functions.

Keywords Regulatory T cells · Carotid artery stenosis · Acute aortic dissection

Introduction

Regulatory T cells (Tregs) represent a group of CD4$^+$ T cell subpopulations that play a crucial role in maintaining tolerance to self/foreign-antigens and immunological homeostasis [1, 2]. Several Treg subsets have been identified over the years [3–7], and among them the best characterized are the CD4$^+$CD25$^+$ FOXP3$^+$ regulatory T cells (CD25$^+$ Tregs) [3, 8]. Recently, CD4$^+$ IL-10-producing regulatory T cells (IL-10-producing Tregs) have also been described [4]. CD25$^+$ Tregs develop mainly in the thymus to specifically express the Forkhead box P3 (Foxp3) gene [8] and are identified by the expression of surface markers CD4 and CD25 [9], whereas identifying naturally occurring IL-10-producing Treg cells was difficult due to the lack of suitable surface markers. Compared with traditional CD25$^+$ Tregs, IL-10-producing Tregs (Lag3$^+$ Tregs) normally do not express CD25 and FOXP3 [4, 10]. Recently, lymphocyte activation gene 3 (LAG3) protein, a CD4 homologue, has been identified as a surface marker for IL-10-producing Tregs [11–13] and it is considered a suitable surface marker for these T CD4$^+$ regulatory subpopulations. Tregs play a key role in regulating inflammatory process underlying arterial damage in several cardiovascular diseases including acute aortic dissection (AAD), acute coronary syndromes and critical carotid artery stenosis (CAS) [14–17]. Generally, it is considered that Tregs lower immune response throughout their anti-inflammatory actions, so that a decrease in such regulatory subpopulation has been related to plaque complications

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leading to unstable angina and acute myocardial infarction [14, 17]. Recently, it has been demonstrated that Tregs can also exert proinflammatory actions. In particular, such subpopulation has been strongly related to the extension of brain damage after ischemic injury in mice [15]. Moreover, it has been demonstrated that a $CD25^+$ Tregs expansion occurs in patients with symptomatic carotid artery stenosis (CAS) and is associated with brain injury [16]. Such a surprising opposite behavior of Tregs can be partly explained by the existence of different T subpopulations exerting regulatory actions. On the basis of such premises, the aim of this study was to evaluate $CD25^+$ and $Lag3^+$ Tregs subpopulations in patients with CAS and AAD.

Patients and methods

The present was an observational study.

Study population

The population included in this study was composed of 36 patients with critical CAS selected in a period ranging from September 2015 to September 2017 among those undergoing to carotid thrombo-endo-arteriectomy (TEA) at the Department of Vascular Surgery, Sant'Andrea Hospital, "Sapienza" University of Rome. Patients were selected from about 250 subjects on the basis of the following inclusion criteria: (1) critical carotid stenosis, defined as a narrowing of the carotid lumen > 70% [18]; (2) no cardiac causes of stroke; (3) no history of neoplasm, autoimmune or inflammatory systemic diseases; and (4) no familiar or personal history of aneurysms/dissection. All patients underwent to physical and neurological examinations, carotid artery ultrasound and angiography by magnetic resonance imaging (MRI) or contrast tomography (CT). Brain CT scan or MRI was also performed. Based on neurological symptoms and brain imaging, CAS patients were further divided into two subgroups: (a) group CAS-S (symptomatic CAS) and (b) group CAS-A (asymptomatic CAS). Group CAS-S was composed by 11 patients with evidence of cerebrovascular symptoms such as stroke or transient ischemic attack (TIA) [19]. Patients with (1) doubtful neurological symptoms; (2) negative brain imaging; and (3) symptoms onset > 1 month were excluded. All patients were included in the study in a period ranging from 2 weeks to 1 month from the onset of neurological symptoms; blood and tissue samples were collected at time of surgery. Group CAS-A (asymptomatic CAS) included 25 patients who did not experience any neurological symptoms within the previous 6 months, and their brain MRI was consistent with the diagnosis of chronic cerebrovascular disease.

Twenty-four patients undergoing Stanford-A AAD surgical repair at the Attilio Reale Heart and Great Vessels Department, Policlinico Umberto I, "Sapienza" University of Rome (AAD group) were selected on the basis of the following inclusion criteria: (1) Stanford-A AAD; (2) no history of neoplasm, autoimmune, infectious or inflammatory systemic disease; (3) no presence of genetic syndromes known to be responsible for aortic disease; and (4) no family history of aortic dissection or aneurysm.

Twenty-five patients with traditional cardiovascular risk factors, matched for age and sex, attending to the Department of Atherosclerosis and Dyslipidemia, Sant'Andrea Hospital, "Sapienza" University of Rome, were selected as control group (RF group). Patients were enrolled on the basis of the following criteria: (1) no acute cerebrovascular symptoms or history of cardiovascular disease; (2) no carotid stenosis > 20%; (3) no familiar or personal history of aneurysms/dissection; and (4) no history of neoplasm, autoimmune or inflammatory systemic diseases; routine blood tests, physical and neurological examinations and carotid artery ultrasound were performed in all the controls.

A venous blood sample was withdrawn from each patient (just before surgery) and from each control, in order to evaluate T $CD4^+$, $CD4^+CD25^+/FoxP3^+$, $CD4^+Lag3^+$ lymphocyte subpopulations.

IL-10, transforming growth factor (TGF)-β and IL-6 serum levels were also assessed in all the groups.

Blood samples and peripheral blood mononuclear cells (PBMCs) isolation

Blood samples were collected in tubes containing 0.2-ml sodium heparin, and PBMCs were isolated by density gradient centrifugation (Lympholyte, Cedarlane, Hornby, CA).

Serum was obtained after centrifugation and stored at − 80 °C.

Flow cytometry analysis

Cells were incubated at 4 °C for 20 min with APC-conjugated antihuman CD4 (MiltenyiBiotec, Bergisch Gladbach, Germany), than washed twice with PBS and incubated with FITC-conjugated antihuman CD25 (BD Biosciences) at 4 °C for 20 min. Cells were washed twice with PBS and incubated with PerCP-conjugated antihuman Lag3 (BD Biosciences) at 4 °C for 30 min. After surface staining, cells were fixed and permeabilized by Human FoxP3 buffer set (BD Biosciences) according to the manufacturer's instructions and incubated with PE-conjugated antihuman FoxP3 (BD Biosciences) for 20 min. After washing with PBS, stained cells were resuspended and analyzed with a FACS Calibur cytometer (Becton–Dickinson, San Jose, CA) equipped with Cell Quest/

FlowJosoftware. Isotype controls were used as compensation controls and to confirm antibody specificity (Fig. 1).

Serum IL-10, TGF-β and IL-6 levels

Serum IL-10, TGF-β-levels and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Quantikine ELISA, R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Results for continuous variables were expressed as median and mean ± standard deviation (SD) and compared with the ANOVA multiple comparison test. Categorical variables were expressed in percentage and compared with the Chi-square test. Values of $p < 0.05$ were considered significant. All the statistical procedures were performed by GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA).

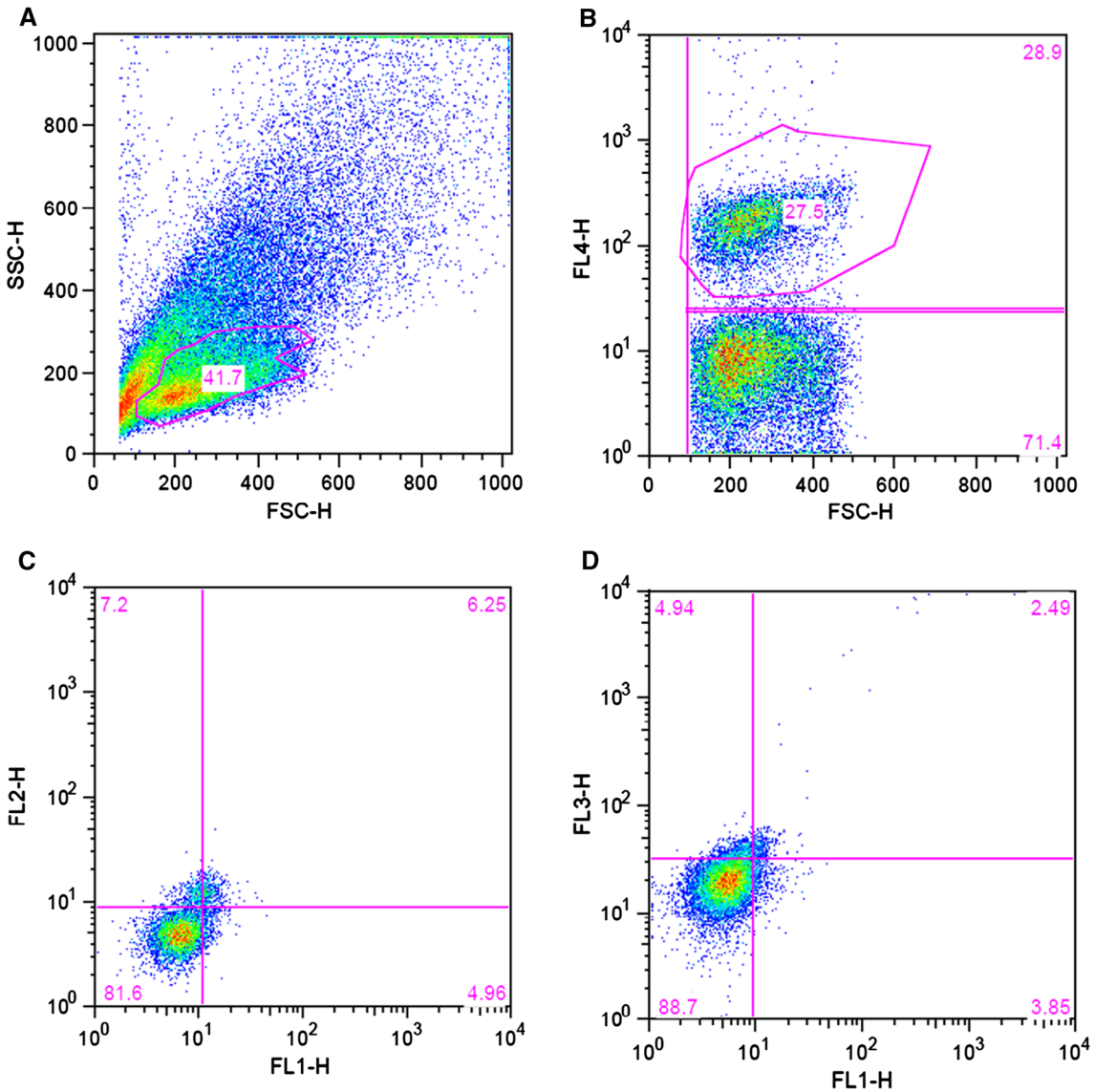

Fig. 1 Representative flow cytometry strategy. Cells are visualized on FSC versus SSC, and an ample gate drawn around the mononucleate cloud (plot **a**). These cells are then viewed on a FSC versus CD4 (APC, FL4-H) plot, and a second gate is drawn around the $CD4^+$ cloud (plot **b**). $CD4^+$ cells are viewed on a CD25 (FITC, FL1-H) versus FoxP3 (PE, FL2-H) plot, and $CD25^+FoxP3^+$ cells were identified in the upper right square (plot **c**). $CD4^+$ cells from plot B are also viewed on a CD25 (FITC, FL1-H) versus Lag3 (PerCP, FL3-H) plot, and $Lag3^+$ Tregs were identified in the upper left square (plot **d**)

Table 1 Demographic and baseline characteristics of patients included in CAS-S, CAS-A, RF and AAD groups

	CAS-A	CAS-S	AAD	RF
Age (years/mean ± SD, median)	71.43 ± 6.50, 73	72.25 ± 7.23, 72	65.00 ± 10.75, 67	64.93 ± 6.34, 65
Female sex	7/25 (28.00%)	3/11 (27.27%)	10/24 (41.67%)	12/25 (48.00%)
Diabetes	9/25 (36.00%)	4/11 (36.36%)	7/24 (29.17%)	4/25 (16.00%)
Dyslipidemia	20/25 (80,00%)	7/11 (63.63%)	4/24 (16.67%)	25/25 (100%)
Hypertension	24/25 (96.00%)	7/11 (63.63%)	13/24 (54.17%)	7/25 (28.00%)
Smoke	12/25 (48.00%)	6/11 (54,55%)	/	8/25 (32.00%)
BMI (*n*)	25.87 ± 4.61, 25.00	24.12 ± 3.79, 24.20	26.81 ± 5.57, 26.82	25.94 ± 3.75, 24.22

Table 2 T $CD4^+$ lymphocyte subpopulations in CAS-S, CAS-A, CAS, RF and AAD patients

	$CD25^+$ Tregs	$Lag3^+$ Tregs	$CD4^+$
CAS-A	2.149 ± 1.788, 1.530	4.506 ± 2.525, 4.490	42.29 ± 18.20, 40.30
CAS-S	5.004 ± 5.206, 2.580	5.862 ± 4.701, 4.350	32.90 ± 15.73, 36.40
AAD	2.096 ± 1.218, 1.755	2.744 ± 1.723, 2.220	39.18 ± 18.89, 41.55
RF	2.026 ± 1.156, 1.790	2.744 ± 1.927, 2.240	46.95 ± 13.08, 47.00
CAS	3.073 ± 3.490, 1.800	4.945 ± 3.371, 4.460	39.04 ± 16.92, 38.80

Data are reported as mean ± SD, median

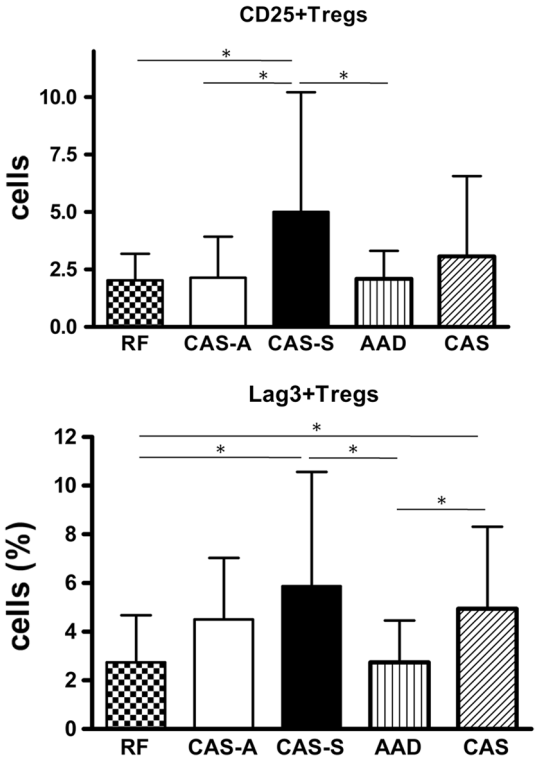

Fig. 2 FACS-analysis of T regulatory lymphocyte subpopulations in CAS-S, CAS-A, CAS, RF and AAD patients. Data are expressed as median and mean ± SD. Statistical analysis: ANOVA multiple comparison test, $*p < 0.05$

Results

Demographic and baseline

Demographic and baseline characteristics of patients included in the study are reported in Table 1.

No significant differences regarding age, sex, diabetes, body mass index (BMI) and smoke among the group were observed. All hypertensive patients were treated with ace inhibitors or sartanic, with or not calcium antagonists or beta-blockers or diuretics.

No significant differences regarding dyslipidemia among the group were observed with the exception of RF versus all other groups. Nevertheless, all RF patients were for a long time treated by statins. All diabetic patients were taking oral blood glucose-lowering drugs and/or subcutaneous insulin, as well as all hypertensive patients were under treatment.

Lymphocyte subpopulations

A reduction in total $CD4^+$ lymphocytes was observed in CAS-A, CAS-S and AAD (Table 2).

A significant increase in $Lag3^+$ Tregs was observed in CAS versus AAD ($p < 0.05$) and versus RF ($p < 0.05$) and in CAS-S versus AAD and RF ($p < 0.05$). No significant differences in $Lag3^+$ Tregs were observed between CAS-S and CAS-A (Table 2, Fig. 2).

A significant increase in $CD25^+$ Tregs was observed in CAS-S versus AAD, CAS-A and RF ($p < 0.05$) (Table 2, Fig. 2).

Table 3 Cytokine serum levels in CAS-S, CAS-A, RF, CAS and AAD patients

	IL-10 (pg/ml)	TGF-β (ng/ml)	IL-6 (pg/ml)
CAS-A	16.12 ± 20.42, 6.56	23.34 ± 14.51, 24.29	3.34 ± 2.49, 3.00
CAS-S	13.20 ± 11.38, 9.29	19.00 ± 11.87, 13.30	6.56 ± 3.16, 3.00
AAD	55.64 ± 77.68, 17.37	24.76 ± 16.6, 20.63	92.39 ± 41.63, 43.5
RF	9.50 ± 10.18, 4.79	35.81 ± 22.90, 33.66	5.34 ± 3.33, 1.50
CAS	15.37 ± 18.37,6.73	22.08 ± 13.75, 20.43	4.20 ± 3.35,3.00

Data are reported as mean ± SD, median

IL-10, TGF-β and IL-6 levels

The value of IL-10 was significantly higher in AAD versus RF ($p < 0.05$), CAS-A ($p < 0.05$) and CAS ($p < 0.01$) (Table 3).

The value of IL-6 was significantly higher in AAD compared to the other groups with p values of $p < 0.001$, $p < 0.05$, $p < 0.001$ and $p < 0.001$ versus CAS-A, CAS-S, CAS and RF, respectively (Table 3).

No significant differences were observed in TGF-β values among the groups.

Discussion

Results of the present study demonstrated an increase in $Lag3^+$ Tregs in CAS versus AAD and RF suggesting that $Lag3^+$ Tregs subpopulation is related, since early phases, to the inflammatory process underlying atherosclerotic plaque formation and can represent a distinguishing cellular marker of athero-occlusive disease. These data seem of particular interest since add new evidences about the immunological differences displayed by the wide spectrum of clinical manifestations related to atherosclerosis (ATS) ranging from critical carotid artery stenosis to Stanford-A AAD. In ADD, indeed, a significant reduction in such subpopulation has been observed. $Lag3^+$ Tregs represent a new regulatory subpopulation that has never been evaluated before in ATS. Despite no data are available about $Lag3^+$ Tregs in atherosclerotic process, their behavior has been studied in some inflammatory diseases. It has been demonstrated that in patients with rheumatoid arthritis (RA), $Lag3^+$ Tregs are inversely related to disease activity [20]. In particular, effective treatment of RA leads to $Lag3^+$ Tregs levels increase and $CD25^+$ Tregs values decrease [20], suggesting that $Lag3^+$ play a key role in lowering inflammation. Thus, it is conceivable that the different regulatory T lymphocyte subpopulations can be activated at different times during inflammation and can exert distinguishing actions driving remodeling of arterial wall remodeling toward a different outcome. In agreement, $Lag3^+$ and $CD25^+$ Tregs show an opposite behavior in CAS and in AAD. In particular, a peculiar expansion of $CD25^+$ T cells was documented in patients with neurological symptoms [16], strongly confirming a relationship between ischemic brain damage and $CD25^+$ T cells, whereas $Lag3^+$ Tregs were significantly decreased in AAD, suggesting that this subpopulation can exert anti-inflammatory protective actions against aortic wall rupture. Results obtained from cytokine measurement further support the hypothesis of a protective role for $Lag3^+$ Tregs. We found, indeed, that in AAD, in which a high proinflammatory state is documented by large amounts of IL-6 [21], and levels of $Lag3^+$ Tregs were low [21].

Interestingly, $Lag3^+$ Tregs levels do not significantly differ in CAS-A versus CAS-S, suggesting that this regulatory subpopulation is not specifically related to plaque instability and brain injury, despite its levels are high in symptomatic CAS.

In conclusion, results of the present study demonstrated that $Lag3^+$ Tregs are related to plaque formation and growth and early distinguish athero-occlusive disease from AAD, whereas $CD25^+$ T cells increase in symptomatic CAS. Moreover, reduction in $Lag3^+$ Tregs observed in AAD strongly suggests that they can exert protective actions against inflammatory pathways underlying to aortic wall rupture.

Compliance with ethical standards

Conflict of interest The authors have no actual or potential conflict of interest to declare, including any financial, personal or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

Ethical approval The study was performed according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Faculty of Medicine.

Informed consent Written informed consent was obtained from each patient or from an authorized family member.

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