Circulating CD4⁺CD25hiCD127lo Regulatory T-Cell Levels Do Not Reflect the Extent or Severity of Carotid and Coronary Atherosclerosis

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Objective—Regulatory T (Treg) cells play a protective role in experimental atherosclerosis. In the present study, we investigated whether the levels of circulating Treg cells relate to the degree of atherosclerosis in carotid and coronary arteries.

Methods and Results—We studied 2 distinct populations: (1) 113 subjects, selected from a free-living population (carotid study), in which we measured the intima-media thickness of the common carotid artery, as a surrogate marker of initial atherosclerosis; and (2) 75 controls and 125 patients with coronary artery disease (coronary study): 36 with chronic stable angina, 50 with non-ST-elevation acute coronary syndrome, 39 with ST-elevation acute myocardial infarction. Treg-cell levels were evaluated by flow cytometry (Treg cells identified as CD4⁺CD25hiCD127lo and by mRNA expression of forkhead box P3 or of Treg-associated cytokine interleukin 10). In the carotid study, no correlation was observed between Treg-cell levels and intima-media thickness. No differences in Treg-cell levels were observed comparing rapid versus slow intima-media thickness progressors from a subgroup of patients (n=65), in which prospective data on 6-year intima-media thickness progression were available. In the coronary group, Treg-cell levels were not altered in chronic stable angina patients. In contrast, nonunivocal variations were observed in patients suffering an acute coronary syndrome (with a Treg-cell increase in ST-elevation acute myocardial infarction and a Treg-cell decrease in non-ST-elevation acute coronary syndrome patients).

Conclusion—The results suggest that determination of circulating Treg-cell levels based on flow cytometry or mRNA assessment is not an useful indicator of the extent or severity of atherosclerosis. (Arterioscler Thromb Vase Biol. 2010; 30:00-00.)

Key Words: regulatory T cells ■ coronary artery disease ■ flow cytometry ■ carotid artery intima-media thickness ■ acute coronary syndrome

T cells play a role in atherosclerosis and in acute manifestation of plaque destabilization.1 The activation of inflammatory pathways in atherosclerosis and in coronary artery disease (CAD) is not confined to coronary lesions but involves the activation of neutrophils, monocytes, and lymphocytes (ie, CD69+, HLA-DR+, and CD 137+ T cells) in peripheral blood in particular during acute coronary syndromes (ACSs).2-4 On the other hand, regulatory T (Treg) cells reduce the development of experimental atherosclerosis acting both systemically and within the lesion.5,6 Immuno-staining of human atherosclerotic plaques showed that Treg cells are present during all stages of plaque development in the intima and adventitia.7 In general, Treg cells play a key role in the maintenance of immunologic self-tolerance and negative control of a variety of pathologic immune responses.8 Several subsets of Treg cells with distinct phenotypes and distinct mechanisms of action have been described (see Sakaguchi et al,8 Roncarolo and coworkers,9 and O’Garra and Vieira10 for review). Treg cells mediate the immunoregulatory function by producing cytokines, such as interleukin

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(IL)-10 and transforming growth factor-β (mainly adaptive CD4+CD25+ Tregs or Tr1 cells induced in the periphery) and by cell-cell contacts (mainly natural occurring CD4+CD25hi T cells, originated in the thymus that express the forkhead box P3 [FoxP3] transcription factor that is required for Treg development and function).10,11 In the context of human atherosclerosis, a reduction in Treg cells could promote development and progression of the plaque.5

The few human studies on circulating Treg cells’ levels, defined as CD4+CD25+ T cells in patients with CAD, reported contrasting results.12–15 The discrepancy may be due, at least in part, to the experimental approach and the quality of cytomtery-based identification of Tcell. This issue is less relevant in murine models, where CD4+CD25+ phenotype identifies a largely homogenous regulatory population. In humans, a sizeable population of activated CD4+ T cells expresses CD25 (the IL-2 receptor α-chain), in particular, in inflammatory conditions, and a clear distinction between activated and Treg cells can be challenging.16 The human CD4dimCD25hi population is the most efficient in terms of immune-response suppression.17,18 Recent studies have demonstrat that downregulation of the IL-7 receptor α-chain (CD127) distinguishes T cells from activated nonregulatory CD25+ T cells, facilitating the identification both of naturally occurring FoxP3+ and adaptive T cells,19–21 and enables improved Treg-cell quantification.17,21 Furthermore, the chemokine receptor CCR5 (CD195), identifies highly suppressive CCR5+ Treg cells, which have increased ability of homing and trafficking to infiltrated nonlymphoid tissues.22,23

Treg-cell levels are reduced in atherosclerotic animal models.6,24 Furthermore, approaches resulting in increased Treg-cell levels, such as administration of anti-CD3 antibody, aluminum hydroxide adjuvant, or replacing CD31 in mouse models of atherosclerosis decreased atherogenesis.25–28 To clarify the role of these cells on plaque formation and destabilization in humans, we investigate the Treg-cell levels related to the degree and severity of atherosclerosis in patients. In this, might help to identify whether this T-cell subpopulation could represent a suitable biomarker for future diagnostic or prognostic approaches.

Therefore, we first investigated the association of Treg-cell levels with a surrogate marker of atherosclerosis, ie, the intima-media thickness (IMT) of the common carotid artery in a free-living population. Next, we examined Treg-cell levels in patients with CAD comparing those with stable stenotic coronary atherosclerosis (patients with chronic stable angina [CSA]) with patients with unstable manifestations (patients with non-ST-elevation ACS [NSTACS] and ST-elevation acute myocardial infarction [STEMI]).

Methods

Carotid and Coronary Study Populations

Institutional Ethics Committees approved the study, and informed written consent was obtained from all participating subjects. The study was conducted according to the standards of the Declaration of Helsinki. For the study aimed at addressing the role of Treg on carotid atherosclerosis (carotid study), blood samples were obtained from a subset of subjects (n = 113) randomly selected from those enrolled in the progression of lesions in the intima of the carotid (PLIC) study.29 Forty-eight subjects had a single IMT evaluation, whereas for 64 subjects, a 6-year follow-up evaluation of IMT was available (supplemental Figure IA, available online at http://arabiahajournals.org). We further divided the 64 subjects into slow or rapid IMT progressors, based on IMT progression values being below or above the mean 6-year IMT progression observed in the PLIC study (0.07 mm in 6 years). Characteristics of patients in the carotid group are summarized in Table 1.

For the coronary study, blood samples were obtained from patients on admission to San Raffaele Scientific Institute, Centro Cardiologico Monzino, Multimedica Istituto Di Ricovero e Cura a Carattere Scientifico, S.S. Giovanni, and Istituto Clinico Città Studi, all in Milan, and Azienda Ospedaliera di Lodi, Lodi, all in Italy, before angiography from December 2007 to June 2009. A total of 226 controls/cases entered the study, of whom 200 completed the analysis (the coronary group; supplemental Figure IB). The control group was made of 75 healthy donors without clinical and electrocardiographic signs of CAD. One hundred and twenty-five patients with CAD were divided in a CSA group, including 36 patients with effort angina (lasting >3 months and without previous history of unstable angina or myocardial infarction) and angiographic evidence of coronary artery stenosis (>50%), a NSTACS group, including 50 patients, and a STEMI group, including 39 patients. Exclusion criteria were creatinine levels >1.5 mg/dL, cirrhosis, acute heart failure or acute pulmonary edema, chronic left ventricle dysfunction with ejection fraction <40%, cancer (active or in the last 5 years), infectious/inflammatory disease or current assumption of immunosuppressive drugs, recent surgery or major trauma (in the last month), ACSs due to in-stent thrombosis/restenosis or occlusion of arterious and venous bypass grafts (confirmed after angiography), admission >6 hours from the onset of symptoms for STEMI, or admission >24 hours from the onset of symptoms for NSTACS. NSTACS and STEMI samples were obtained early after the onset of symptoms when the elevation of troponin I were still minimal (median: 0.8 and 97.1 ng/mL, respectively), thus minimizing the possible confounding effect of myocardial necrosis. Clinical characteristics and biological parameters of patients and healthy individuals are reported in Table 2. A new sample was obtained from 26 healthy individuals after a

<table>
<thead>
<tr>
<th>n</th>
<th>Carotid Study</th>
<th>Slow IMT Progressors</th>
<th>Rapid IMT Progressors</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>113</td>
<td>22</td>
<td>43</td>
<td></td>
<td>0.57</td>
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<tr>
<td>Age, years</td>
<td>58±13</td>
<td>62±8</td>
<td>60±12</td>
<td>0.07</td>
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<td>Male sex, no. (%)</td>
<td>65 (57.5)</td>
<td>11 (50)</td>
<td>19 (44)</td>
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<tr>
<td>Current smoker</td>
<td>13 (11.5)</td>
<td>2 (10)</td>
<td>4 (10)</td>
<td>0.70</td>
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<tr>
<td>Hypertension</td>
<td>29 (25.7)</td>
<td>6 (27)</td>
<td>12 (28)</td>
<td>0.95</td>
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<tr>
<td>Diabetes mellitus</td>
<td>10 (8.8)</td>
<td>0 (0)</td>
<td>6 (14)</td>
<td>0.06</td>
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<tr>
<td>Hypercholesterolemia</td>
<td>22 (19.5)</td>
<td>8 (36)</td>
<td>10 (23)</td>
<td>0.36</td>
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<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>127±17</td>
<td>130±13</td>
<td>127±19</td>
<td>0.52</td>
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<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>77±9</td>
<td>78±17</td>
<td>75±17</td>
<td>0.36</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6±3.6</td>
<td>26.7±2.6</td>
<td>27.1±4.2</td>
<td>0.65</td>
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<tr>
<td>Glicæmia (mg/dL)</td>
<td>103±23</td>
<td>97±12</td>
<td>106±20</td>
<td>0.18</td>
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<td>Total cholesterol (mg/dL)</td>
<td>220±40</td>
<td>233±38</td>
<td>236±35</td>
<td>0.77</td>
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<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>148±56</td>
<td>157±40</td>
<td>158±31</td>
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<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>53±11</td>
<td>51±10</td>
<td>56±13</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>109±61</td>
<td>125±52</td>
<td>110±77</td>
<td>0.43</td>
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<tr>
<td>Creatinine (mg/dL)</td>
<td>0.89±0.18</td>
<td>0.90±0.14</td>
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<tr>
<td>IMT (mm)</td>
<td>0.71±0.14</td>
<td>0.74±0.15</td>
<td>0.79±0.23</td>
<td>0.13</td>
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</table>

P is referred to rapid vs slow IMT progressors. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
Flow Cytometry

Whole blood from each patient at admission was collected in EDTA anticoagulated vacutainer tube. Samples were stained and fixed within the day of collection. We verified that there were no significant differences in the investigated marker levels in samples stained immediately after collection in comparison with samples stained up to a maximum 24-hour time interval postsampling and stored at room temperature. These results allowed the enrollment also from other centers within 30 km from the central laboratory. To reduce cellular loss and analysis sampling bias in the specimen, the one providing the fluorescence minus one gating control. Two panels were used, consisted of: (1) CD3, CD4, CD25, and CD127 (for the carotid study); and (2) CD3, CD4, CD25, CD69, and CCR5 (for the carotid and the coronary studies). Fluorescence intensity for each signal measured was standardized using multiple peak Rainbow calibration particles (Code RCP-30 to 5A; Spherotech) to allow reproducible and comparable median fluorescence intensity throughout the study period, of particular relevance for markers, such as CD25-PC5, used for identifying Treg cells on the basis of its high fluorescence in comparison with intermediate CD25-PC5 fluorescence in activated T cells (Figure 1A through 1E).

We used 2 different and recognized strategies of Treg identification based on surface markers: (1) gating on CD3 \( \text{dim/low} \) CD25 \( \text{hi} \) Treg cells, CD3 \( \text{dim/low} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and activated CD3 \( \text{hi} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and (2) gating on CD25 \( \text{lo} \) cells.17,20,21 We reported very high correlation between the 2 approaches in the evaluation of the number of circulating Treg cells in the 113 subjects in the carotid study, where we used both modalities (range: 0.89 to 0.94). We used 2 different and recognized strategies of Treg identification based on surface markers: (1) gating on CD3 \( \text{dim/low} \) CD25 \( \text{hi} \) Treg cells, CD3 \( \text{dim/low} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and activated CD3 \( \text{hi} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and (2) gating on CD25 \( \text{lo} \) cells.17,20,21 We reported very high correlation between the 2 approaches in the evaluation of the number of circulating Treg cells in the 113 subjects (range: 0.89 to 0.94). We used 2 different and recognized strategies of Treg identification based on surface markers: (1) gating on CD3 \( \text{dim/low} \) CD25 \( \text{hi} \) Treg cells, CD3 \( \text{dim/low} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and activated CD3 \( \text{hi} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and (2) gating on CD25 \( \text{lo} \) cells.17,20,21 We reported very high correlation between the 2 approaches in the evaluation of the number of circulating Treg cells in the 113 subjects (range: 0.89 to 0.94). We used 2 different and recognized strategies of Treg identification based on surface markers: (1) gating on CD3 \( \text{dim/low} \) CD25 \( \text{hi} \) Treg cells, CD3 \( \text{dim/low} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and activated CD3 \( \text{hi} \) CD4 \( \text{dim} \) CD25 \( \text{hi} \) Treg cells, and (2) gating on CD25 \( \text{lo} \) cells.17,20,21 We reported very high correlation between the 2 approaches in the evaluation of the number of circulating Treg cells in the 113 subjects (range: 0.89 to 0.94).
Peripheral Blood Mononuclear Cells
mRNA Analysis
Peripheral blood mononuclear cells were collected from 41 patients enrolled in the carotid study (for details, see supplemental material). Total RNA was extracted and underwent reverse transcription as described. Two microliters of cDNA were amplified by real-time quantitative polymerase chain reaction with SYBR green universal polymerase chain reaction mastermix (Bio-Rad). The specificity of the SYBR green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. The primers used are presented as supplemental Table I. The melting peaks of the amplicons were as expected (data not shown). Each sample was analyzed in duplicate using the IQ-Cycler (Bio-Rad). The polymerase chain reaction amplification was related to a standard curve ranging from $10^{-11}$ to $10^{-14}$ mol/L. Relative number of cDNA copies of the target gene for each $10^6$ cDNA copies of the housekeeping gene after log-transformation is reported. The following genes were analyzed: FoxP3, IL-10, and the housekeeping gene RPLP13a.

Biochemical Analysis
For details, please see supplemental material.

IMT Measurement
For details, please see supplemental material.

Statistical Analysis
The datasets did not conform to a normal distribution. Therefore, Mann–Whitney U test and Kruskall–Wallis test with Dunn’s multiple comparison test were used as appropriate. Wilcoxon matched paired test was used for repeated measures in the time. Spearman’s rank test was used to test correlations between variables. GraphPad Prism 4 and GraphPad Instat 3 softwares were used for analysis. A probability value $<0.05$ was considered significant. Age-adjusted IMT data were used for all the analysis.

Results
Correlation Between Circulating Treg-Cell Levels, Cardiovascular Risk Factors, and Pharmacological Treatment in the General Population
Circulating Treg-cell levels were significantly increased in male subjects compared with female (5.3 versus 4.8%, $P=0.03$) but were not correlated to age and renal function and were unaffected by major cardiovascular risk factors (supplemental Figure II). Only a significant inverse correlation between Treg-cell levels and high-density lipoprotein levels was found (Spearman’s rank test, $r=-0.24; P=0.03$) (supplemental Figure III). No significant changes of Treg-cell levels were observed in patients under chronic therapy with aspirin, statin, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, or βblockers (supplemental Figure IV).

Correlation Among Cytometry-Based and mRNA Treg-Markers
We found a significant correlation between $CD3^{+}CD4^{+}CD25^{hi}-CD127^{lo}$ Treg-cell identified by flow cytometry and mRNA levels of the transcription factor FoxP3 (Spearman’s rank test, $r=0.32; P=0.039$) and the Treg-associated cytokine IL-10 (Spearman’s rank test, $r=0.31; P=0.046$) (supplemental Figure VA and VB). Similarly, Treg cells identified as $CD3^{+}CD4^{dim}CD25^{hi}$ correlate both with FoxP3 and IL-10 mRNA levels (data not shown). As expected, a signif-
ificant correlation between FoxP3 and IL-10 mRNA levels was observed (Spearman’s rank test, \( r = 0.87; P < 0.0001 \)) (supplementary Figure VC). No correlation among serum circulating IL-10 levels and levels of Treg cells, FoxP3, or IL-10 mRNA was detected (data not shown).

**Treg-Markers Do Not Correlate With IMT and Are Not Associated With Altered IMT Progression**

No correlation was observed between Treg cells defined by flow cytometry or by mRNA levels and carotid IMT (Figure 2). Similarly, IMT levels were not correlated with mRNA IL-10 or circulating IL-10 levels (Figure 2). Within the 65 subjects with a 6-year follow-up evaluation of IMT, the rapid IMT progressor group (defined as those subjects having IMT progression higher than the mean observed for the general population in the PLIC study, i.e., \( \geq 0.07 \) mm in 6 years) had similar levels of circulating Treg-markers compared with the slow IMT progressor group (Figure 2). These findings suggest that circulating Treg-cell levels do not correlate with the extent and progression of initial atherosclerosis. This does not
exclude the presence of a proinflammatory imbalance between Treg cells and activated T cells, despite the absence of significant difference in circulating Treg-cells levels. To evaluate this hypothesis, the activated T-cells counterpart defined by CD3⁺CD4⁻CD69⁺ T cells was also investigated. Activated T-cell levels did not correlate with IMT levels, and the same was true also for the Treg/CD69⁺ T-cell ratio. Finally, rapid versus slow IMT progressors had similar Treg/CD69⁺ T-cell ratio (supplemental Figure VI).

**Treg-Cell Level Perturbation During Acute Manifestation of CAD**

Next, the levels of Treg cells defined as CD3⁺CD4dimCD25hiT cells were investigated in patients with stable and unstable manifestations of CAD compared with age- and sex-matched controls. Treg cells were not altered in CSA patients with stable stenotic coronary plaques compared with controls, whereas significant perturbation of Treg-cell levels was registered in patients with STEMI and NSTACS in comparison with controls and CSA patients (P<0.0001). The percentage of Treg cells was significantly increased in STEMI whereas reduced in NSTACS (Figure 3). No significant changes in Treg-cell levels were observed in the coronary circulation in 3 STEMI patients (supplemental results). When CD3⁺CD4⁺CD69⁺ T cells were investigated, a significant (P=0.003) increase was observed in NSTACS (median range 0.64% [interquartile range 0.31 to 1.00%]) and STEMI patients (0.61% [0.39 to 0.93%]) compared with controls (0.37% [0.23 to 0.68%], both P<0.05) but not in comparison with CSA patients (0.60% [0.31 to 0.82%]) (supplemental Figure VIIA). The analysis of the ratio Treg/activated T cells showed a significant proinflammatory imbalance only in NSTACS but not in STEMI patients (P<0.001) (supplemental Figure VIIB).

Next, to exclude the possibility that the small significant perturbations in Treg cells observed in NSTACS and STEMI patients compared with controls were the consequence of intraindividual variations, the stability during time of Treg-cell population was investigated in the control population (Figure 4A). Of note, the increase observed in STEMI patients was higher (more than doubled) as compared with the expected intraindividual variations of Treg cells in normal volunteers. Furthermore, Treg-cell levels were similar in patients with STEMI sampled during the acute phase before percutaneous coronary intervention and after 24 hours from the revascularization (5.6% [4.6 to 6.9%] to 5.2% [4.4 to 8.3%], n=7), suggesting that the increase in Treg-cell observed during the acute phase could not be secondary to the inflammatory response induced by myocardial injury, at least in the early hours from the onset of STEMI (Figure 4B). Accordingly, no significant differences were detected in samples from NSTACS patients obtained within 6 hours compared with samples obtained between 6 and 24 hours from symptoms’ onset. This observation reduces the likelihood that differences between NSTACS and STEMI are due to differences in time of sampling. Finally, Treg cells were significantly increased in both NSTACS and STEMI groups after 55 days of follow-up (Figure 4C through 4E). No significant changes were observed in activated T-cell levels and in the Treg/CD69⁺ ratio in patients with NSTACS and STEMI during the follow-up (supplemental Figure VIII).

**Association of the Pro- and Antiinflammatory Cytokine Profile With T-Cell Subsets in CAD Patients**

The levels of the proinflammatory IL-6 and the antiinflammatory IL-10 cytokines were investigated in CAD patients (supplemental Figure IX). STEMI patients had significantly increased IL-10 levels compared with other groups in agreement with the difference observed in the Treg-cell levels. Of note, no differences were observed in NSTACS and CSA patients compared with controls. IL-6 levels were significantly increased in all 3 CAD groups compared with controls. This resulted in a decreased IL10/IL6 ratio in NSTACS.
Treg cells. Therefore, we investigated the levels of Treg cells not only drives effector T cells, but also Treg cells’ homing and trafficking in inflamed nonlymphoid tissue, suggesting that CCR5 Treg cells constituted a subgroup of “effector” Treg cells. Therefore, we investigated the levels of CCR5 Treg cells in the carotid and coronary group. In humans, Treg cells were present with an increase expression of CCR5 (P<0.0001) (supplemental Figure X), CCR5 Treg cells were not significantly correlated with IMT (supplemental Figure XIA). Although rapid IMT progressors had lower levels of CCR5 Treg cells (1.1% [0.8 to 1.5%]) compared with slow IMT progressors, this difference was of borderline statistical significance (1.4% [1.0 to 2.1%], P=0.057) (supplemental Figure XIB). Furthermore, no significant change between controls (1.0% [0.6 to 1.7%]), CSA (0.8% [0.4 to 1.4%]), NSTACS (1.0% [0.5 to 1.6%]), and STEMI patients (0.8% [0.6 to 1.4%], overall P=0.43) (supplemental Figure XIC) were found.

Discussion

Several studies have suggested an important role of Treg cells in the modulation of cardiovascular disease. Although animal model studies consistently show that an increase in T-cell level and function was associated with reduced atherosclerosis, results from human studies are less clear. Here, we investigate the Treg-cell levels, mainly based on an accurate flow cytometry analysis, and verify their possible association with the degree and severity of atherosclerosis in patients. We show that circulating Treg-cell levels are not significantly associated with IMT progression or altered in patients with stenotic stable coronary plaques. Minimal perturbations of Treg-cell subsets are observed in ACS with different behaviors in patients with NSTACS and in patients with STEMI (results are summarized in supplemental Figure XII).

Figure 4. Treg-cell variations in controls and in patients with unstable CADs in the time. Dots represent individual patient data, and continuous lines show the temporal changes between the first sampling and the second one at the follow-up. A, No significant changes in the Treg population in the same subjects were observed in the follow-up (median 5.4% at baseline vs 5.1% after median time of 111 days; Wilcoxon matched-paired test was used for repeated measures in the time). Median (interquartile) variation between the 2 measures of Treg cells in the same subject was 0.4% (0.2 to 1.0%). B, Treg-cell levels were similar in patients with STEMI sampled during the acute phase before percutaneous coronary intervention (PCI) and after 24 hours from the revascularization (5.6% [4.6 to 6.9%] to 5.2% [4.4 to 6.3%], n=7). C and D, Treg cells were significantly increased in both NSTACS (4.3% [3.3 to 5.4%] to 6.1% [4.8 to 7.8%], P=0.0009) and STEMI groups (6.2% [5.5 to 6.8%] to 6.8% [5.8 to 8.4%], P=0.03) after 55 days of follow-up. E, Representative color dot plots from a patients with NSTACS (showed with the arrow) during the acute event (left sided) and at the follow-up (right sided).

Circulating CCR5 Treg-Cell Levels Do Not Correlate With IMT Levels and Are Unchanged in the Spectrum of Clinical CAD

Recent animal studies showed that chemokine receptor CCR5 not only drives effector T cells, but also Treg cells’ homing and trafficking in inflamed nonlymphoid tissue, suggesting that CCR5 Treg cells constituted a subgroup of “effector” Treg cells. Therefore, we investigated the levels of CCR5 Treg cells in the carotid and coronary group. In patients compared with controls and STEMI patients. The cytokine profiles were consistent with the changes observed for T-cell subsets.

Treg Cells, Cardiovascular Risk Factors, and Pharmacological Treatments

In agreement with previous studies, Treg-cell levels in healthy controls were 5 to 6% with a median variability with time in the same subject <0.5% as percentage of total CD3 CD4 T-cells. Women had lower levels of Treg cells. A reverse correlation between Treg cells and high-density lipoprotein levels was also observed. Furthermore, current smokers had increased Treg levels, but this difference was not statistically significant. These findings might suggest that subjects with increased inflammatory status have also increased Treg-cell levels. When the role of pharmacological treatment was taken into account, neither subjects under angiotensin blocker treatment nor those under statin treatment had altered Treg-cell levels in contrast to results reported by others.

Treg Cells and Preclinical and Stable Atherosclerosis

Circulating Treg-cell levels were not associated with the extent and progression of carotid IMT through 2 independent approaches (flow cytometry and FoxP3 mRNA analysis) and were not significantly altered in patients with CSA. These findings support and extend the observations from 2 smaller studies. This observation is further supported by the analysis of a specific Treg-cell subpopulation with a functional role, such as CCR5 Treg cells (defined as effector Treg cells). Recently, the value of carotid IMT in predicting cardiovascular risk underwent scrutiny.
Treg Cells and Unstable CAD

Previous reports showed decreased levels of Treg cells in patients with ACS without distinguishing between NSTACS and STEMI patients. We observed in NSTACS patients decreased Treg-cell levels and in STEMI patients increased Treg-cell levels compared with controls and patients with CSA. Both NSTACS and STEMI patients had increased activated CD69+ T cells, although only STEMI patients had a concomitant Treg-cell increase. A follow-up analysis showed that Treg-cell levels were increased in NSTACS and in STEMI patients also after 55 days, thus suggesting the hypothesis of a compensatory role for Treg cells due to either the myocardial damage or the return to a steady state balance between regulator- and effector-cell activity. Speculatively, differences between Treg-cell behavior in NSTACS and STEMI might depend on the pathological mechanisms sustaining lymphocyte activation. For instance, the increase of CD28null T-cell levels could be related to unstable angina. CD28null T cells, although only STEMI investigated, lead to a minimal Treg-cell decrease in NSTACS. Antigen exposure can modify the composition of T-cell subset such CD28null T-cells and Treg-cell compartment. A temporally limited Treg-cell reduction few days after antigen exposure was reported in a previous study, and also our preliminary results on changes in Treg cells after tetanic vaccination (supplemental Figure XIII) mimic the levels of reduction in Treg-cell levels in NSTACS patients. A more prominent activation in STEMI patients might be the consequence of preserved expression of costimulatory molecules, such as CD28, that account for a proportional compensatory Treg-cell counterbalance.

Consistent with our results, previous studies showed increased levels of activated CD69+ T cells, Treg cells, and IL-10 in patients with ACS. This is consistent with what has been described in other immune-mediated diseases, such as rheumatoid arthritis, where increased Treg-cell levels in synovial fluid of inflamed joints were observed. Furthermore, it has been described relatively high Treg-cell levels, using CD127 as a Treg-cell marker, in some patients with type 1 diabetes mellitus, suggesting that Treg-cell numbers might increase during some stages of the disease in an attempt to regulate effector-cell activity.

Study Limitation

The combination of CD4, CD25, and CD127 used in the present study to assess the Treg-cell levels by flow cytometry and mRNA FoxP3 expression represents a widely accepted and used approach to identify Treg cells, and by this cytometry-based strategy, highly purified population of functional Treg cells can be obtained (eg, see Reference21). Although the inverse association between Treg-cell levels and the extent of atherosclerosis has been unequivocally observed in experimental models,6 the human Treg-cell population is more heterogeneous than that of mouse, regarding cell surface phenotype and functional capability.16 Therefore, the lack of association between circulating Treg-cell levels and the extent of atherosclerosis may be due to the difficulty to define Treg cells in the human setting or to the fact that systemic changes in Treg-cells are too small in patients with atherosclerosis to be detected by flow cytometry. Indeed, the currently used surface markers for the definition of Treg-cell (as well as the expression markers IL-10 and FoxP3) may not be enough to unequivocally discriminate T cells with regulatory functions, and indeed, we were not able to detect subsets of Treg cells, such as adaptive Treg or Tr1, which, to date, lack specific surface marker molecules.

Conclusions

Studies addressing accurately and reproducibly Treg-cell quantification in human blood are central to define Treg-cell reference ranges and the relevance of their variability in patients with subclinical and clinical manifestations of atherosclerosis. In our large dataset, we did not find significant changes in the circulating Treg-cell levels, identified by CD3+CD4+CD25highCD127low expression on flow cytometry or by FoxP3 mRNA assessment, in relation to the extent or progression of human atherosclerotic disease at carotid and coronary sites. The reason may lie in the difficulty to define Treg cells in humans, in which the distinction with active T cells is challenging. Finally, the possibility that the increase in Treg-cell levels, as well as IL-10 observed only in STEMI patients, might represent the hallmark of a counterregulatory response aimed at limiting the cardiovascular damage or alternatively a proinflammatory state preceding the acute coronary event should be further investigated.

Acknowledgments

We thank Prof Attilio Maseri (Heart Care Foundation, Florence, Italy) for his support and suggestion; Prof Ziad Mallat, Cardiovascular Research Center, Institut National de la Sainté et de la Recherche Médicale and Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France) for critically reading the manuscript; and BD Biosciences for support and assistance, in particular Gianluca Rotta.

Sources of Funding

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Disclosures

None.

References


Circulating CD4+CD25hiCD127lo Regulatory T-Cell Levels Do Not Reflect the Extent or Severity of Carotid and Coronary Atherosclerosis

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Flow cytometry

**Antibodies.** The staining panels consisted on the following: CD3-Pacific Blue (Clone UCHT1, Code 558117, BD Pharmingen); CD4-APC Cy7 (Clone SK3, Code 341115, BD Bioscience); CCR5-PE (Clone 2D7, Code 555993, BD Bioscience); CD25-PC5 (Clone B1.49.9, Code IM2646, Beckman Coulter, Immunotech); CD69-FITC (Code 555530, BD Bioscience); CD127-PE (Clone hIL-7R-M21, Code 557938, BD Bioscience). To optimize fluorescence compensation settings, spectral overlap determinations we used the Compbeads Compensation Particles antimouse Ig, k set (Code 552843, BD Bioscience).

**Data collection and analysis.** Lymphocytes were identified and electronically gated on forward and orthogonal light scatter signals. The fluorescent signals for phenotype analyses were accumulated for the gated lymphocytes. The instrument raw data were stored electronically to a server for archiving and data processing. Data were processed and analyzed using FCS Express V3 Research edition (De Novo Software, Inc; [www.denovosoftware.com](http://www.denovosoftware.com)). Cell viability was >99%, assessed using the Molecular Probes Patented LIVE/DEAD Viability (Invitrogen) according to the manufacturer instructions. Compared to previous studies therefore Treg cells were identified with a more accurate cytometry-based approach by surface markers (using and comparing two novel strategies based on CD4$_{dim}$CD25$_{high}$ and CD25$^+$CD127$_{low}$), the data on assay inter-variability of Treg was reported and the fluorescence intensity of CD25 and CD127 along two years of enrollment was standardized using the rainbow calibration particles, thus limiting systematic errors. Furthermore the use of fresh blood samples limited the bias linked to cellular loss, whereas in previous studies flow cytometry analysis were performed on PBMC, and fine Treg variations (within the order of 1%) were documented.
Peripheral blood mononuclear cells (PBMC) mRNA analysis.

Briefly blood diluted 1:3 in 15 ml phosphate buffer saline (PBS) was layered onto 4 ml of Ficoll Hipaque (Amersham) and centrifuged at 1500 rpm for 35 minutes. PBMC were removed from the interface and washed twice (1500 rpm for 10 minutes) in PBS before being counted.

Biochemical analysis

After an overnight fast (only for carotid group), blood was collected into tubes containing NaEDTA (0.1 mg/mL), and plasma was separated by low-speed centrifugation at 4°C. The measurement of plasma lipids was performed by standard enzymatic techniques (ABX for Cobas Mira Plus, Montpellier, France); HDL-C was determined after precipitation of apoB-containing lipoproteins and LDL-C was calculated using the Friedewald formula. Serum concentration analysis of IL-6 and IL-10 were performed using CBA Flex Set (BD) following manufacturer’s instructions. Ten replicates of human serum pool used as internal control were made and respective intra-assay variability for IL-6 and IL-10 concentration were respectively 5.3% and 3.2%. The IL-6 and IL-10 detection limit, as for BD data sheets were respectively 0.20 pg/ml and 0.13 pg/ml.

IMT measurement

Ultrasound scanning and reading of carotid arteries were performed by a single expert sonographer, using an 8-MHz transducer (Biosound 2000 II sa, Indianapolis, USA) with an axial and lateral resolution of 0.385 and 0.500 mm, respectively. The sonographer was blinded to the subject's identity. B-mode evaluations are obtained from echographic images of the far wall in the first centimeter of common carotid arteries, proximal to the bulb dilation, in lateral projection. Five standardized points 5, 10, 20, 25 and 30 mm from bulb were measured in both arteries and averaged to calculate the mean IMT (mIMT) for each subject. In two scans performed on 65 subjects by the
same operator the mean difference in IMTm was 0.005 ± 0.002 mm and the variation coefficient equal to 1.9%. The correlation between two scans was significant with \( r=0.96 \) (\( p<0.0001 \)).

**SUPPLEMENTAL RESULTS**

The analysis of Treg cells in blood samples upstream (from the aorta) and downstream (from the coronary sinus) of the culprit atherosclerotic lesion before PCI and in samples obtained after revascularization (from the coronary sinus) resulted in similar levels of Treg cells, thus excluding a local Treg-cell reduction or accumulation in the coronary circulation during the acute phase (data not shown).
A) Carotid Study
113 subjects

Six-year longitudinal data
65 subjects

Single evaluation
48 subjects

Slow IMT progressors
22 subjects

Rapid IMT progressors
43 subjects

B) Coronary Study
226 subjects

77 Controls
39 CSA
65 NSTACS
45 STEMI

75 Analyzed
36 Analyzed
50 Analyzed
39 Analyzed

26 with follow-up
3 Excluded
15 with follow-up
17 with follow-up

2 Excluded
1 Technical issue
1 Excluded
15 Excluded

Technical issues
1 Technical issue
1 without stenosis
5 Excluded
7 not confirmed NSTACS
5 Excluded
1 RA

1 EF<40%
1 without stenosis
5 Excluded
1 EF<40%
1 RA

1 CRF
1 stent thrombosis
Supplemental Figure I. Enrolment flow chart of the Carotid Study (A) and the Coronary Study (B).

IMT = Intimal media thickness, CSA = chronic stable angina, NSTACS = Non-ST-elevation Acute Coronary Syndrome, STEMI = ST-Elevation acute Myocardial Infarction, EF = ejection fraction, CRF = chronic renal failure defined as creatinine levels > 1.5 mg/dl, RA = rheumatoid arthritis.
A) 

**BMI (Kg/m²)**

- Male (n=64)
- Female (n=48)

$P=0.03$

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

5.3% 4.8%

B) 

**Creatininemia (mg/dl)**

$P=0.88$

$P=0.87$

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

C) 

**r= 0.02**

**P=0.87**

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

D) 

**r= 0.11**

**P=0.26**

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

E) 

**P=0.95**

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

5.2% 5.2%

F) 

**P=0.44**

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

5.2% 5.1%

G) 

**P=0.09**

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

5.1% 5.7%

H) 

**P=0.81**

CD$^{25}$CD$^{127}$ Treg/CD$^{3+}$CD$^{4+}$ (%)

5.2% 5.1%
Supplemental Figure II. Circulating Treg cells, cardiovascular risk factors in 113 subject in the Carotid Study. Dots represent individual patient data; dashed lines show median value and continuous lines show 25th and 75th percentiles. (A) Male subjects showed significant (Mann-Whitney U test) higher levels of Treg cells (expressed as percentage of total CD3+CD4+ T cells). No correlations (Spearman’s rank test) between circulating Treg cells and age (B), body max index (BMI, C) and creaninin levels (D). No differences in Treg-cell levels between based on the presence of hypercholesterolemia (E), diabetes (F), smoking habit (G) and arterious hypertension (H).
A) $r = -0.24$  
$P = 0.01$

B) $r = 0.02$  
$P = 0.37$

C) $r = -0.10$  
$P = 0.29$

D) $r = -0.24$  
$P = 0.01$
Supplemental Figure III. Circulating Treg cells and lipid profile in 113 subject in the Carotid Study. No correlations (Spearman’s rank test) between circulating Treg cells (expressed as percentage of total CD3⁺CD4⁺ T cells and total cholesterol (A), low density lipoprotein (LDL) cholesterol (B) and triglyceride levels (C) levels. Significant inverse correlation between Treg-cell levels and high density lipoprotein (HDL) levels (C).
A) P=0.70

B) P=0.14

C) P=0.76

D) P=0.61

Aspirin

Statin

ACEi/ARB

Beta-blocker

CD25^hiCD127^loTreg/CD3^+CD4^+ (%)
Supplemental Figure IV. Circulating Treg cells and ongoing drug treatment in 113 subjects in the Carotid Study. No differences (Mann-Whitney U test) in Treg levels between subjects under or without aspirin (A), statin (B), angiotensin-converting enzyme inhibitors (ACEi)/angiotensin receptor blockers (ARB) (C) or beta blockers (D).
Supplemental Figure V. Correlation among cytometry-based and mRNA FoxP3 and IL-10 in 41 subject in the Carotid Study. Significant correlations (Spearman’s rank test) between CD3+CD4+CD25hiCD127loTreg-cell identified by flow cytometry and mRNA levels of the transcription factor forkhead box P3 (FoxP3) (A) and of the anti-inflammatory cytokine interleukin (IL)-10 (B). High correlation between mRNA levels of the transcription factor FoxP3 and of the IL-10 (C).
Supplemental Figure VI. (A) No correlation (Spearman’s rank test) between intima media thickness (IMT) and the activated T cells defined by CD3+CD4+CD69+ T cells. (B) Rapid (≥0.07 mm in 6 years) versus slow IMT progressors did not differ concerning the Treg/CD69+ T-cell ratio (Mann-Whitney U test). Dots represent individual patient data; dashed lines show median value and continuous lines show 25th and 75th percentiles.
A) CD69⁺/CD3⁺CD4⁺ (%)

B) Treg/CD69⁺ Ratio

Overall P=0.003

P<0.05

P<0.05

Controls (n=75)  CSA (n=36)  NSTEMI (n=50)  STEMI (n=39)

0.37

0.60

0.62

0.61

P=0.001

13.6

8.5

6.5

9.6
Supplemental Figure VII. Activated T cells and Treg/activated T cells ratio in Coronary Artery group. Dots represent individual patient data; dashed lines show median value and continuous lines show 25\textsuperscript{th} and 75\textsuperscript{th} percentiles. (A) Activated CD3\textsuperscript{+}CD4\textsuperscript{+}CD69\textsuperscript{+} T cells were significantly increased in NSTACS (median 0.64\% interquartile 0.31-1.00\%) and STEMI patients (0.61\%; 0.39-0.93\%) compared to controls. (B) The analysis of the ratio Treg/activated T cells showed a significant proinflammatory imbalance only in NSTACS but not in STEMI patients (Kruskall-Wallis and Dunn’s test were performed). CSA = chronic stable angina, NSTACS = Non-ST-elevation Acute Coronary Syndrome, STEMI = ST-Elevation acute Myocardial Infarction.
A) CD69^+CD3^-CD4^- (%)

During NSTACS Follow-up
(n=15)

P=0.46

0.74% 0.64%

B) CD69^+CD3^-CD4^- (%)

During STEMI Follow-up
(n=17)

P=0.70

0.46% 0.63%

C) Treg/CD69+ (ratio)

During NSTACS Follow-up

P=0.15

6.2% 8.8%

D) Treg/CD69+ (ratio)

During STEMI Follow-up

P=0.99

10.7% 12.3%

E) CD69^+ T cells:

During NSTACS
CD69^+ T cells:
0.71%

Follow up
CD69^+ T cells:
0.77%
Supplemental Figure VIII. Activated-T-cell and Treg/activated-T variations in patients with unstable Coronary Artery Disease in the time. (A-D) Dots represent individual patient data and continuous lines show the temporal changes between the first sampling and the second one at the follow-up. No significant changes were observed in activated CD3⁺CD4⁺CD69⁺ T-cell levels and in the Treg/CD69⁺ ratio in patients with NSTACS and STEMI during the follow up (Wilcoxon matched paired test was used for repeated measures in the time). (E) Representative color dot plots from a patients with NSTACS (showed with the arrow) during the acute event (left-sided) and at the follow-up (right-sided). Percentage is referred to the total number of CD3⁺CD4⁺T cells.
Supplemental Figure IX. Pro- and anti-inflammatory cytokine profile in Coronary Artery Disease (CAD) patients. Dots represent individual patient data; dashed lines show median value and continuous lines show 25th and 75th percentiles. Pro-inflammatory IL-6 and the anti-inflammatory IL-10 cytokines were investigated in CAD patients. (A) STEMI (ST-Elevation acute Myocardial Infarction) patients had significantly increased IL-10 levels compared to other groups in agreement with the difference observed in the Treg-cell levels (Kruskall-Wallis and Dunn’s test was performed). (B) IL-6 levels were significantly increased in all three CAD groups compared to controls. (C) The IL10/IL6 ratio decreased in NSTACS (Non-ST-elevation Acute Coronary Syndrome) patients compared to controls and STEMI patients. The cytokine profiles were consistent with the changes observed for T cell subsets. CSA = chronic stable angina.
Gated on CD3+ T cells

Gated on CD25high Treg cells

Gated on CD25+ T cells

Gated on CD25- T cells

E) Overall P<0.0001

<table>
<thead>
<tr>
<th>CCR5+ (%)</th>
<th>CD25high Treg</th>
<th>CD25+ T</th>
<th>CD25- T</th>
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<tr>
<td>25.9%</td>
<td>12.4%</td>
<td>10.6%</td>
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Supplemental Figure X. Expression of the chemokine receptor CCR5 in Treg cells. (A) Color dot plot of a representative subject. We gated CD3⁺CD4⁻CD25⁻Treg cells, activated CD3⁺CD4⁺CD25<sup>intermediate</sup> and CD3⁺CD4⁺CD25⁻T cells and then we evaluated the expression of CCR5 respectively in (B) CD3⁺CD4⁻CD25<sup>hi</sup>Treg cells, (C) CD3⁺CD4⁺CD25<sup>intermediate</sup>-T and (D) CD3⁺CD4⁺CD25⁻T cells. (E) Treg cells showed an increase expression of CCR5 (median 25.9%, interquartile range 15.2 to 36.0%) in average compared to other T-cell subsets such as not regulatory CD3⁺CD4⁺CD25<sup>intermediate</sup> (12.4%; 6.7 to 18.1%) and CD3⁺CD4⁺CD25⁻T (10.6%; 6.8 to 17.3%) T cells ($P<0.0001$, Kruskall-Wallis and Dunn’s test was performed). Dots represent individual patient data; dashed lines show median value and continuous lines show 25<sup>th</sup> and 75<sup>th</sup> percentiles.
A) 

$\text{CCR5}^+\text{Treg/CD3}^+\text{CD4}^+ (\%)$

- $r = 0.15$
- $P = 0.12$
- $n = 113$

B) 

$\text{CCR5}^+\text{Treg/CD3}^+\text{CD4}^+ (\%)

- Overall $P = 0.43$
- Slow progressors: $1.4\%$ (n=22)
- Rapid progressors: $1.1\%$ (n=43)

C) 

$\text{CCR5}^+\text{Treg cells/CD3}^+\text{CD4}^+

- Controls (n=75): $1.0\%$
- CSA (n=36): $0.8\%$
- NSTACS (n=50): $0.8\%$
- STEMI (n=39): $1.0\%$

Overall $P = 0.43$
Supplemental Figure XI. Circulating CCR5^+Treg cell levels did not correlated with IMT levels and were unchanged in the spectrum of clinical CAD. (A) No correlation (Spearman’s rank test) between intima media thickness (IMT) and the circulating CCR5^+Treg cells (B) Rapid (≥0.07 mm in 6 years) versus slow IMT progressors did not differ concerning the CCR5^+Treg-cell levels (median 1.1%; interquartile range 0.8 to 1.5% versus 1.4%; 1.0 to 2.1%, P=0.057; Mann-Whitney U test). (C) In the coronary group, we did not observed any significant change between controls (1.0%; 0.6 to 1.7%), CSA (chronic stable angina, 0.8%; 0.4 to 1.4%), NSTACS (Non-ST-elevation Acute Coronary Syndrome, 1.0%; 0.5 to 1.6%) and STEMI (ST-Elevation acute Myocardial Infarction) patients (0.8%; 0.6 to 1.4%; overall P=0.43, Kruskall-Wallis and Dunn’s test was performed). Dots represent individual patient data; dashed lines show median value and continuous lines show 25th and 75th percentiles.
<table>
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<tr>
<th></th>
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<th>NSTACS</th>
<th>STEMI</th>
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<td>↑</td>
</tr>
<tr>
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<td>=</td>
<td>=</td>
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<tr>
<td>IL-10/IL-6</td>
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Figure XII. Summary of the main results concerning circulating T-cell subsets and interleukins in comparison to controls. Arrows show the statistically significant variations of the levels of the specific T-cell subset or interleukin in comparison to controls. CSA: chronic stable angina, NSTACS: Non-ST-elevation Acute Coronary Syndrome, STEMI: ST-Elevation acute Myocardial Infarction.
Supplemental Figure XIII. Treg-cell (upper graph) and activated CD69+T-cell (lower graph) variations before and at several time points after tetanic vaccination in three healthy subjects. Dots represent subject and continuous lines show the temporal changes between the first sampling (before the tetanic vaccination) and the following ones at several time-point after the antigen exposure (after 2, 7, 14 and 21 days). We observed a temporally limited Treg-cell reduction (*P<0.05) at two days after antigen exposure with a subsequent increase of Treg cells to pre-vaccination levels after one and three weeks after the exposure, whereas we did not observed significant changes in activated T-cell levels. Percentage are referred to the total number of CD3⁺CD4⁺T cells.
Supplemental Table I. Primer sequences.

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