Low Levels of Circulating CD4+FoxP3+ T Cells Are Associated With an Increased Risk for Development of Myocardial Infarction But Not for Stroke

Maria Wigren,* Harry Björkbacka,* Linda Andersson, Irena Ljungcrantz, Gunilla Nordin Fredriksson, Margaretha Persson, Carl Bryngelsson, Bo Hedblad, Jan Nilsson

Objective—Regulatory T cells (Tregs) protect against atherosclerosis in experimental models, but their association with cardiovascular disease in humans remains to be elucidated. The aim of the present study was to determine whether circulating Tregs predict the development of acute cardiovascular events in humans.

Methods and Results—The study cohort consisted of a random sample of participants (n=700), aged 68 to 73 years, from the Malmö Diet and Cancer Study. Mononuclear leukocytes, stored at −140°C at the baseline investigation in 1991–1994, were thawed and Tregs, defined by the expression of FoxP3 in CD4+ T cells, were analyzed by flow cytometry. There was no detectable loss of cells during storage, and the viability of thawed leukocytes was 95%. A low fraction of both CD4+FoxP3+ and CD4+CD25+FoxP3+ T cells was associated with a higher release of proinflammatory cytokines from activated mononuclear leukocytes, and this association was strongest for CD4+FoxP3+ cells. Eighty-four coronary events and 66 strokes were registered during follow-up until December 31, 2008. In a Cox proportional hazard regression model adjusting for major risk factors, low levels of baseline CD4+FoxP3+ T cells were associated with an increased risk for the development of acute coronary events but not stroke. There were no associations between CD4+CD25+FoxP3+ T cells and development of an acute coronary event or stroke.

Conclusion—This study provides prospective evidence for the role of Tregs in the development of myocardial infarction. The findings are in accordance with previous experimental studies and provide clinical support for a protective role of Tregs in atherosclerosis. The lack of association between Tregs and stroke may reflect the more heterogeneous cause of this disease. (Arterioscler Thromb Vasc Biol. 2012;32:2000-2007.)

Key Words: coronary disease ■ immune system ■ lymphocytes ■ carotid arteries

Inflammation plays a critical role at all stages of atherosclerosis. Accumulating evidence suggests that this inflammation is modulated by adaptive immune responses against modified self-antigens in the vascular wall and that both pathogenic and protective immunity exist. The concept of atherosclerosis as a disease of partial autoimmune cause is primarily based on studies in hypercholesterolemic mice demonstrating that genetic deletion of components of adaptive immunity significantly alters the progression of disease. Mice deficient for cytokines, costimulatory molecules, and transcription factors required for the maturation and activation of the Th1 subtype of T lymphocytes are all characterized by reduced development of atherosclerosis. Deletion of the CD1d-natural killer T cells pathway required for adaptive immune responses against lipid antigens similarly inhibits atherosclerosis. Support for a role of the immune system also in human atherosclerosis has come from studies demonstrating that the pattern of cytokine expression in human atherosclerotic plaque is characteristic of that of Th1 cells and that as much as 10% of the T cells in plaques are specific for oxidized low-density lipoprotein (LDL).

Earlier studies addressing the role of oxidized LDL immunity unexpectedly showed that immunization with oxidized LDL is associated with inhibition of atherosclerosis, revealing that atheroprotective immune responses also exist. Subsequent studies identified regulatory T cells (Tregs) as a critical part of this atheroprotective immunity. The role of Tregs is to control the activity of autoreactive T-effector cells that have escaped deletion in the thymus. Loss of this control may lead to development of autoimmunity. Natural Tregs, characterized by the expression of the transcription factor FoxP3, are developed in the thymus. These cells migrate to the periphery where they, upon encounter with their self-antigen, secrete inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β or simply deprive effector T cells of the IL-2 that these cells require to proliferate.
second class of inducible Tregs is generated in the periphery and is characterized by the expression of IL-10 (Tr1 cells) or transforming growth factor-β (Th3 cells). Depletion of Tregs through the deletion of CD80/86, CD28, or inducible costimulator, immunization with FoxP3-transfected dendritic cells, and anti-CD25 antibody treatment significantly increase plaque formation in experimental animals. Similarly, inhibition of Th3 cells through the deletion of the T-cell receptor for transforming growth factor-β markedly enhances the progression of the disease, whereas administration of a clone of ovalbumin-specific Tr1 cells together with its cognate antigen inhibits plaque development in ApoE−/− mice. Inhibition of atherosclerosis in ApoE−/− mice has also been observed in response to transfer of CD4+CD25+ Tregs as well as after Treg activation by oral anti-CD3 treatment. There is also evidence that Tregs are involved in the atheroprotective effect of Alum and oxidized LDL/apolipoprotein B antigen immunization.

Despite the strong experimental evidence supporting a protective role for Tregs in atherosclerosis, our understanding of their clinical significance remains limited. Decreased levels of circulating Tregs have been reported in patients with acute coronary syndrome, but prospective studies analyzing the association of Tregs with cardiovascular risk are still lacking. In the present study, we analyzed baseline Tregs in 700 randomly selected subjects in the cardiovascular cohort of the Malmö Diet and Cancer Study (MDCS) and studied the association with the development of acute cardiovascular events during a 15-year follow-up.

Methods

Study Population

The MDCS is a prospective cohort (n=28,449) study examining the association between diet and cancer. Subjects born between 1926 and 1945 and living in Malmö were eligible for inclusion in the study. Between October 1991 and February 1994, every other participant was also invited to take part in a substudy focusing on cardiovascular risk (MDCS Cardiovascular Cohort, n=6103). The present study cohort consists of a random sample of participants (n=700), aged 68 to 73 years, from the MDCS. Participants were followed from baseline examination until the first event of cardiovascular disease (CVD), emigration from Sweden, or death, up to December 31, 2008. Ascertainment of cases and validity of the registries used (the Swedish Discharge Registry, the Stroke Register of Malmö, and the Cause of Death Registry of Sweden) have been proven to be high. A CVD event was defined as a fatal or nonfatal myocardial infarction (MI) (ie, International Classification of Diseases, Ninth Revision, code 410), fatal or nonfatal stroke (International Classification of Diseases, Ninth Revision, codes 430, 431, 432, and 436), or death attributable to underlying coronary heart disease (International Classification of Diseases, Ninth Revision, codes 410–414), whichever came first. Throughout the follow-up period, 150 first CVD cases (84 coronary events and 66 strokes) were identified. For each case, a study participant without event was individually matched for age and sex and used as control. Hypertension was defined as blood pressure ≥140/90 mm Hg or blood pressure lowering medication, high cholesterol as ≥5 mmol/L, and smoking as current smoking. Blood pressure, body mass index, cholesterol, smoking, and lipid levels were determined as previously described. One subject was excluded because of incomplete clinical data. The study was approved by the Regional Ethical Review Board in Lund and was conducted in accordance with the Helsinki Declaration. All subjects gave written consent.

B-Mode Ultrasound

Analysis of common and bulb carotid intima-media thickness (IMT) was performed using an Acuson 128 CT system (Siemens AG, Erlangen, Germany) with a 7-MHz transducer as described previously. Briefly, the right carotid bifurcation was scanned within a predefined window comprising 3 cm of the distal common carotid artery, the bifurcation, and 1 cm of the internal and external carotid arteries. All images for measurement of IMT and plaque thickness were obtained in the longitudinal projection showing the thickest intima-media complex. Plaque was defined as a focal thickening of the IMT exceeding 1.2 mm. The thickness of the common carotid intima-media complex (ie, the mean distance between the leading edges of the lumen-intima and the media-adenititia interfaces of the far wall [mean IMT common carotid artery]), was measured off-line and along 1 cm section in the longitudinal projection using a specially designed computer-assisted image analyzing system based on automated detection of the echo structures, but with the option to make manual corrections by the operator. The maximum thickness of the intima-media (max IMT bifurcation) in the far wall of the carotid bifurcation was also measured off-line.

Isolation of Mononuclear Leukocytes

Blood was collected in heparin-containing BD Vacutainer tubes, (Becton Dickinson, Franklin Lakes, NJ) and the isolation of mononuclear leukocytes was initiated within 2 hours. Mononuclear leukocytes were isolated with FicollPaque Plus (GE Healthcare, Waukesha, WI) density gradient centrifugation according to the instructions of the manufacturer. Briefly, 2.5 mL of FicollPaque Plus was pipetted into a 15-mL centrifugation tube. Five milliliters of the heparinized blood was carefully layered over the FicollPaque Plus, and the tubes were then centrifuged at 1350 g for 10 minutes at room temperature. The cell interface layer was carefully harvested and the cells were then washed twice with 0.9% NaCl (the first centrifugation was at 600 g and the second at 300 g, both 10 minutes, at room temperature). The cells were resuspended in 500 μL autologous serum with 500 μL 20% cold dimethyl sulfoxide in RPMI 1640 medium, transferred to cryo freezing containers (250 μL:tube; Nalgene, Rochester, NY), and then frozen at −80°C for at least 1 hour or overnight. The tubes were then transferred to −140°C (liquid nitrogen) and stored until analysis.

Staining of Mononuclear Leukocytes and Flow Cytometry

The cells were thawed, washed with preheated (37°C) PBS supplemented with 1% human serum, and centrifuged at 330 g for 10 minutes at room temperature. The cells were then resuspended in complete RPMI 1640 media (containing 10% human serum, 1 mM sodium pyruvate, 10 mM HEPES, 50 U penicillin, 50 μg/mL streptomycin, 0.05 mM β-mercaptoethanol, and 2 mM L-glutamine; Gibco; Life Technologies, Bleiswijk, The Netherlands). From each subject included in the study, 400 000 cells were stained with fluorochrome-conjugated antibodies for analysis of Tregs. The antibodies used in this study were PE/Cy7-anti-CD3, Pacific Blue-anti-CD4, PE-anti-CD25, APC-anti-Foxp3, Alexa Fluor700-anti-CD8, and biotinylated-anti-CD45, all from Biolegend except APC-anti-Foxp3 that were from eBioscience. The viability staining 7-aminoactinomycin D (7AAD; Biolegend, San Diego, CA) was added to detect dead or dying cells. Streptavidin coupled to CascadeYellow (Biolegend) was added to detect the biotinylated-anti-CD45. Before Foxp3 staining, cells were incubated and washed with Foxp3 Fixation/Permeabilization buffers (eBioscience, San Diego, CA). Importantly, permeabilization for Foxp3 staining was performed subsequent to the staining for 7AAD to avoid artificial uptake of the viability dye. Stained cells were fixed in 1% paraformaldehyde and measured on a CyAn ADP Flow cytometer (Beckman Coulter, Brea, CA). The analysis was performed with FlowJo7.6 software (Tree Star, Ashland OR). From the forward scatter/side scatter, leukocytes were gated. From the lymphocyte gate, the 7AAD− or 7AAD+/− CD45+ cells were further gated and then the T cells were
gated based on their expression of CD3. From the T-cell gate, the CD4+ and CD8+ cells were gated and the expression of CD25 and Foxp3 on CD4+ cells was determined. Measurements of insufficient technical quality (≤5%) were not included in the statistical analyses. CompBeads (BD) were used to correct for fluorescence spillover in multicolor analyses, and gate boundaries were set by fluorescence-minus-one controls.

Analysis of Cytokines in Cell Supernatants

Cells were cultured in complete RPMI and stimulated with CD3/CD28 beads (MiltenyiBiotec, Bergisch Gladbach, Germany) for 72 hours at 37°C in a cell incubator (5% CO2). Thereafter, the cell supernatants were stored at −80°C until analysis. The concentrations of released cytokines were determined with multiplex technology (MesoScale Discovery, Gaithersburg, MD).

Statistics

An independent sample t test was used to assess normally distributed continuous variables and a χ² test for proportions between subjects with and without a cardiovascular event. Nonparametric test (Mann-Whitney) was used to assess non-normally distributed continuous variables between cases and controls. Skewed variables were logarithmically transformed, and Pearson correlation coefficient was used to examine the relationship among continuous variables. Binary logistic or linear regression models were used to calculate independent associations as appropriate. Cox proportional hazard regression models were used to compare the development of cardiovascular events between Treg tertiles, to calculate linear trends and risk factor–adjusted hazard ratios (95% confidence interval), and to plot survival curves. The adjusted model included potential confounders among the baseline characteristics that differed between cases and noncases.

Results

Mononuclear leukocytes collected and frozen live as part of the cardiovascular MDCS baseline investigation in 1991–1994 provide a unique opportunity to determine whether specific leukocyte populations predict cardiovascular risk. The cells were frozen in autologous serum/dimethyl sulfoxide and stored at −140°C to optimize preservation of viability. To determine whether the cells had remained viable, we analyzed the uptake of 7AAD in thawed CD45+ cells by flow cytometry. 7AAD is excluded by intact cells but is taken up by damaged and dead cells. The results demonstrated that 95% of the cells remained viable (Figure 1A). Furthermore, there was no difference in 7AAD uptake between cells that had been kept frozen for a few weeks only and those that had been kept frozen for at least 15 years (Figure 1B), suggesting that the time the cells were kept at −140°C had limited the impact on cell viability. Comparing cell numbers registered at freezing and thawing also confirmed that no loss of cells had occurred.

The aim of the present study was to investigate whether the level of circulating Tregs predicts risk for cardiovascular events. Tregs were defined based on the expression of CD4+FoxP3+ or CD4+CD25+FoxP3+ as shown in Figure 2A. To specifically determine the ability of Tregs to survive isolation and storage at −140°C, we analyzed the uptake of 7AAD in CD4+CD25+FoxP3+ cells. The fraction of 7AAD-positive CD4+CD25+FoxP3+ cells was markedly higher than for CD45+ cells and represented ≥25% of all CD4+CD25+FoxP3+ cells (Figure 2B), suggesting that Tregs are more sensitive to isolation and storage than other mononuclear leukocytes. The results presented below are based on analysis using total baseline CD4+FoxP3+ and CD4+CD25+FoxP3+ T cells (ie, both 7AAD+ and 7AAD− cells) because this was considered more biologically appropriate than just using cells viable after thawing.

To determine the functional consequences of having high or low frequencies of CD4+CD25+FoxP3+ and CD4+FoxP3+ T cells, we then analyzed the association with cytokine release from anti-CD3/CD28 bead-stimulated mononuclear leukocytes obtained from the same subjects. There were significant inverse associations between the frequency of CD4+CD25+FoxP3+ T cells and the release of IL-2, IL-6, IL-8, interferon-γ, and tumor necrosis factor-α (Table 1). Similar and, in most cases, even stronger inverse associations were observed between CD4+FoxP3+ T cells and these cytokines. In addition, there was also significant inverse association between the frequency of CD4+FoxP3+ T cells and the release of IL-1β (Table 1). There were no significant associations between CD4+CD25+FoxP3+ or CD4+FoxP3+ T cells and fasting plasma glucose, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, the LDL/HDL ratio, triglycerides, or body mass index.

We next analyzed whether baseline CD4+FoxP3+ or CD4+CD25+FoxP3+ T-cell levels predicted the risk for development of a first acute cardiovascular event during follow-up. Eighty-four acute MIs and 66 strokes were registered during follow-up until December 31, 2008. For these analyses, we also identified controls matched for age and sex for each subject with a coronary event or stroke. In the stroke group, we excluded all hemorrhagic strokes (n=11) and 1 stroke of unknown type. Baseline clinical characteristics of the different study cohorts are shown in Table 2. There was a trend toward a lower percent CD4+FoxP3+ T cells in coronary cases than in their matched controls (P=0.05; Table 3). In the next step, CD4+FoxP3+ and CD4+CD25+FoxP3+ T cells were divided into tertiles for each cohort. Entering CD4+FoxP3+ T-cell tertiles into a Cox proportional hazard regression model also including
all variables demonstrating significant difference between cases and noncases (eg, sex, smoking status, HDL, LDL/HDL ratio, systolic blood pressure, hypertension, blood pressure medication, diabetes mellitus, and diabetes mellitus medication), there was a significant association between low levels of CD4+FoxP3+ T cells and the development of MI ($p<0.05$; Figure 3A). The hazard ratio for suffering an MI in the lowest tertile of CD4+FoxP3+ T cells was 1.9 (95% confidence interval 1.1–3.6) compared with the highest tertile of CD4+FoxP3+ T cells. No association was found between CD4+CD25+FoxP3+ T-cell tertiles and development of MI (Figure 3B). There was also a significant association between CD4+FoxP3+ T-cell tertiles and development of coronary events in the coronary case–control cohort ($p<0.005$; Figure 4A), with a hazard ratio for the lowest tertile of 2.6 (95% confidence interval 1.4–5.0) compared with the highest tertile of CD4+FoxP3+ T cells. Again, there was no association between tertiles of CD4+CD25+FoxP3+ T cells and risk for development of MI (Figure 4B). There were no significant associations between CD4+FoxP3+ or CD4+CD25+FoxP3+ T-cell tertiles and risk for development of ischemic stroke (data not shown).

To determine any possible association of CD4+CD25+FoxP3+ and CD4+FoxP3+ T cells with the severity of atherosclerosis, we used measurements of IMT in the carotid artery obtained at the baseline investigation. There were no significant correlations between the percent CD4+CD25+FoxP3+ T cells and the mean IMT in the common carotid artery ($n=666$) or the maximal IMT in the bulb ($n=462$; $r=0.02$, respectively) or between CD4+FoxP3+ T cells and the mean IMT in the common carotid artery or the maximal IMT in the bulb ($r=0.04$ and $r=-0.01$, respectively). There was also no difference in CD4+CD25+FoxP3+ or CD4+FoxP3+ T cells (expressed as percent of all CD4+ T cells) between subjects with (n=302) and without (n=333) a carotid plaque ($0.87±0.57$ versus $0.85±0.55$ and $1.04±0.56$ versus $1.04±0.64$ % of all CD4+ T cells, respectively).

Discussion

There is strong evidence from experimental studies that Tregs have a protective role in atherosclerosis.6 This is not unexpected because atherosclerosis in hypercholesterolemic mice
is partly driven by immune responses against self-antigens, and the main function of Tregs is to control such autoimmunity. However, the possible role of Tregs in human CVD remains to be fully understood. Studies comparing Treg levels in patients with stable angina, acute coronary syndromes, and healthy controls have reported conflicting results, possibly as a result of different strategies in defining Tregs and differences in experimental design. The present study is the first prospective study of the association between circulating Tregs and cardiovascular risk. It involved 700 subjects randomly selected from the cardiovascular cohort of MDCS with an average follow-up of 15 years. The results demonstrated an association between low levels of CD4+/FoxP3+ T cells and risk for development of coronary events, and this association was shown to be independent of other risk factors that differed between cases and noncases in the cohort (eg, sex, smoking status, HDL, LDL/HDL ratio, systolic blood pressure, hypertension, blood pressure medication, diabetes mellitus, and diabetes mellitus medication). This finding is of potential clinical importance because it represents the first evidence from prospective studies that T cells with regulatory properties may have a protective role in CVD and supports the notion that Tregs represent a possible target for development of novel interventions in coronary heart disease. However, it is important to keep in mind that the findings need to be interpreted with caution because of the difficulties in defining human Tregs and because we did not find any association with coronary events using the more established CD4+CD25+ FoxP3+ definition of Tregs.

The lack of distinct phenotypic characterization represents a complicating factor in studying the role of Tregs in human disease. In mice, Tregs are easily identified based on the expression of CD4+CD25+, which characterizes a largely homogenous regulatory population of cells also expressing

Table 1. Association Between FoxP3+ T Cells and Cytokine Release From Anti-CD3/CD28 Bead-Stimulated Mononuclear Leukocytes

<table>
<thead>
<tr>
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<th>FoxP3+ % of CD4+</th>
<th>CD25+FoxP3+ % of CD4+</th>
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<tr>
<td>IL-1β</td>
<td>−0.16***</td>
<td>−0.07</td>
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<tr>
<td>IL-2</td>
<td>−0.08*</td>
<td>−0.08*</td>
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<tr>
<td>IL-4</td>
<td>−0.01</td>
<td>0.00</td>
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<td>IL-5</td>
<td>−0.04</td>
<td>−0.05</td>
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<tr>
<td>IL-6</td>
<td>−0.15***</td>
<td>−0.09*</td>
</tr>
<tr>
<td>IL-8</td>
<td>−0.11**</td>
<td>−0.09*</td>
</tr>
<tr>
<td>IL-10</td>
<td>−0.06</td>
<td>−0.04</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>−0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>INF-γ</td>
<td>−0.12***</td>
<td>−0.14***</td>
</tr>
<tr>
<td>TNF-α</td>
<td>−0.15***</td>
<td>−0.13***</td>
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</table>

Mononuclear leukocytes were cultured and stimulated with anti-CD3/CD28 beads for 72 hours at 37°C, and the concentrations of released cytokines were determined with multiplex technology. The Spearman test was used to calculate r values. *P<0.05, **P<0.01, ***P<0.005. IL indicates interleukin; INF-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

Table 2. Baseline Clinical Characteristic of Study Cohorts

<table>
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<tr>
<th></th>
<th>All Cases (n=150)</th>
<th>All Noncases (n=549)</th>
<th>Coronary Cases (n=84)</th>
<th>Coronary Controls (n=84)</th>
<th>Stroke Cases (n=54)</th>
<th>Stroke Controls (n=54)</th>
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<tr>
<td>Age at screening</td>
<td>65.6±1.2</td>
<td>65.6±1.1</td>
<td>65.7±1.2</td>
<td>65.6±1.2</td>
<td>65.6±1.2</td>
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<td>Sex (% male)</td>
<td>53.3%***</td>
<td>38.3%</td>
<td>53.6%</td>
<td>53.6%</td>
<td>53.7%</td>
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<td>Body mass index</td>
<td>26.5±3.9</td>
<td>26.3±4.0</td>
<td>26.6±4.1</td>
<td>25.8±3.4</td>
<td>26.4±3.8</td>
<td>25.9±4.1</td>
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<td>Current smoker</td>
<td>24.8%*</td>
<td>16.0%</td>
<td>25.3%</td>
<td>9.8%</td>
<td>22.0%</td>
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<tr>
<td>Diabetes mellitus§</td>
<td>21.3%***</td>
<td>11.1%</td>
<td>25.0%*</td>
<td>10.7%</td>
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<td>Hypertension§</td>
<td>87.3%***</td>
<td>79.4%</td>
<td>86.9%</td>
<td>82.1%</td>
<td>87.0%</td>
<td>75.9%</td>
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Medication

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<td>Antidiabetic</td>
<td>7.3%***</td>
<td>2.3%</td>
<td>4.7%</td>
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<td>Lipid lowering</td>
<td>5.6%</td>
<td>2.9%</td>
<td>4.7%</td>
<td>1.1%</td>
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<td>Blood pressure lowering</td>
<td>35.3%***</td>
<td>20.4%</td>
<td>33.3%*</td>
<td>16.6%</td>
<td>38.9%*</td>
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Laboratory parameters

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<tr>
<td>Fasting venous blood glucose</td>
<td>5.6±1.9**</td>
<td>5.3±1.3</td>
<td>5.7±1.8</td>
<td>5.3±1.4</td>
<td>5.9±2.2</td>
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<td>Triglycerides (mmol/L)</td>
<td>1.5±0.8</td>
<td>1.5±0.8</td>
<td>1.5±0.9</td>
<td>1.5±0.8</td>
<td>1.6±0.75</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.3±0.4*</td>
<td>1.4±0.4</td>
<td>1.3±0.4</td>
<td>1.3±0.3</td>
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<tr>
<td>LDL (mmol/L)</td>
<td>4.3±1.1</td>
<td>4.3±1.0</td>
<td>4.4±1.0</td>
<td>4.3±0.9</td>
<td>4.2±1.3</td>
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<td>LDL/HDL ratio</td>
<td>3.7±1.4*</td>
<td>3.4±1.1</td>
<td>3.7±1.4</td>
<td>3.4±1.0</td>
<td>3.6±1.5</td>
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<td>Systolic BP (mm Hg)</td>
<td>154±19*</td>
<td>150±20</td>
<td>156±21*</td>
<td>148±18</td>
<td>153±19</td>
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<td>Diastolic BP (mm Hg)</td>
<td>90±8.8*</td>
<td>88±9.2</td>
<td>89±8</td>
<td>87±109</td>
<td>90±9</td>
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<td>hsCRP (mg/L)</td>
<td>4.1±6.9</td>
<td>2.9±5.0</td>
<td>4.5±8.1</td>
<td>2.4±3.8</td>
<td>3.7±5.4</td>
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Each subject with a coronary event during follow-up was matched for age and sex with subject who did not develop a cardiovascular event during follow-up. *P<0.05, **P<0.01, and ***P<0.005 for case vs control or no event. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein. †Mann-Whitney test or χ² test for categorical data. §History of diabetes mellitus, medication, or fasting glucose ≥6.1 mmol/L.
the transcription factor FoxP3 that is required for Treg development and function. However, the use of CD4+CD25+ is inadequate in humans because also conventional CD4+ T cells express CD25+ in response to activation. One approach has been to identify CD4+CD25high cells as these have been shown to have regulatory properties, but the limitation with this strategy is that it only identifies CD45RO+ memory Tregs and not CD45RA+ naive Tregs. To overcome this problem, the combination of CD25high and CD127low has been used, which identifies a relatively pure population of FoxP3-expressing cells in humans, but this still fails to discriminate between Tregs and conventional T cells that in response to activation upregulate CD25 and downregulate CD127. Also the strategy applied in the present study to define Tregs based on the expression of FoxP3 in CD4+ T cells has similar limitations because some activated conventional CD4+ cells express low levels of FoxP3 in humans. Accordingly, it cannot be excluded that the CD4+FoxP3+ T-cell population studied here also includes some conventional T-effector cells.

In view of the technical difficulties in defining human Tregs based on flow cytometry, we also performed functional studies based on the analysis of cytokines released from mononuclear leukocytes activated with anti-CD3/CD28-coated beads. This leads to a polyclonal activation of all T cells, and factors expressed by these cells can also subsequently activate monocytes and other mononuclear cells in the cell preparation to release cytokines. A high fraction of both CD4+CD25+FoxP3+ and CD4+FoxP3+ T cells was found to be associated with a lower release of proinflammatory cytokines in line with the notion that these cells have regulatory, anti-inflammatory effects. Interestingly, this association was in most cases stronger for CD4+FoxP3+ T cells than for CD4+CD25+FoxP3+ T cells, suggesting that at least in this study the first gating strategy better identified T cells with regulatory properties.

We found no association between Tregs and IMT in the common carotid artery or the carotid bulb. This observation is in line with a recent study by Ammirati et al, who found no association between carotid IMT and circulating Tregs (defined as CD3+CD4+CD25highCD127low cells), mononuclear cell IL-10 mRNA expression, or plasma IL-10 in a subset of 113 subjects participating in the Progressione Leisone Intimale Carotidea (PLIC) study. The authors were
also unable to identify differences in Treg levels between rapid versus slow IMT progressors in this study.

There are some limitations to this study that need to be considered. Most importantly, our analyses were performed on cells that had been stored at −140°C for several years. Compared with initiating new prospective studies, this has the obvious advantage of allowing the studies to be completed within a relatively short period of time. However, it remains to be fully established how well thawed cells are representative of the original cell population. Although we were unable to detect any loss of cells when comparing cell numbers at freezing and thawing, we cannot exclude the possibility of a selective loss of Tregs because these cells represent a small fraction of all mononuclear cells. The observation of a higher fraction of 7AAD+ Tregs than of other CD45+ leukocytes after thawing is in line with the notion that Tregs may be more sensitive to freezing. Another limitation of this study that should be considered is that there is limited knowledge about Treg variability over time and that results based on single samples may not be representative.

In conclusion, the present study provides the first prospective clinical evidence for a role of Tregs in the development of MI. This observation is in line with evidence from experimental studies of an important protective role of Tregs in atherosclerosis. Because Treg activation represents an attractive target for the development of novel therapies for prevention and treatment, it has become important to assess their role in CVD in humans. The lack of association between Tregs and stroke may reflect the more heterogeneous cause of this disease. Although the present findings are encouraging in this respect, they need to be interpreted with due caution in view of the technical difficulties in defining human Tregs.

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Disclosures
J.N. is signed as co-inventor on patents describing the use of immunomodulatory therapy for atherosclerosis and has assigned patent rights to Forskarparten, Sweden. Forskarparten has licensed patent rights to Cardiovas. The other authors have no conflicts to report.

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