Regulatory/Effector T-Cell Ratio Is Reduced in Coronary Artery Disease

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Background: The protective function of regulatory T cells (Treg) has been identified in experimental atherosclerosis, but the contribution of Treg to the pathogenesis of human coronary artery disease (CAD) remains poorly understood. We investigated Treg and regulatory T-cell/effector T-cell (Treg/Teff) ratio in peripheral blood samples from CAD patients using a new strategy for precise identification of Treg.

Methods and Results: Peripheral blood samples were collected from 73 stable CAD patients (55 middle-aged CAD patients and 18 old CAD patients) and 64 controls (47 middle-aged controls and 17 young controls). CD3+CD4+FoxP3+ T cells were divided into 3 fractions: CD45RA+FoxP3low resting Treg (Fr1), CD45RA+FoxP3high activated Treg (Fr2), and CD45RA-FoxP3low non-Treg (Fr3). CAD patients had lower percentages of Fr1 and Fr2 and higher percentages of Fr3 and CD45RA-Foxp3+ T eff (Fr4+5) within the CD3+CD4+ T-cell population compared to age-matched controls. Treg/Teff ratio (Fr1+2/Fr3+4+5) in CAD patients was also markedly lower than in controls (middle-aged control, 0.17±0.09 vs. middle-aged CAD, 0.10±0.05; P<0.001). The percentage of CD4+CD28null T cells within the CD4+ T-cell population was negatively correlated with Treg/Teff ratio, excluding CD4+CD28null T cells <0.3% (r=–0.27, P<0.05). High-sensitivity C-reactive protein was also negatively correlated with Treg/Teff ratio (r=–0.22, P<0.05).

Conclusions: CAD patients had reduced Treg and Treg/Teff ratio compared to healthy controls. The present findings may be helpful when developing immunotherapy for the prevention of CAD.  (Circ J 2014; 78: 2935–2941)

Key Words: Coronary artery disease; Immune system; Regulatory T cell

Coronary artery disease (CAD) is one of the life-threatening manifestations of atherosclerosis in humans. It is now widely accepted that vascular wall inflammation is an important hallmark of atherosclerosis and contributes to severe clinical events including acute coronary syndrome (ACS) and stroke.1–3 It is well known that in addition to innate immunity, adaptive immunity involving T-cell-mediated pathogenic immune response plays an important role in the inflammatory process during atherogenesis in humans and mice.4,5 Thus, therapeutic interventions targeting the inflammatory response in atherogenesis represent a promising therapeutic strategy to improve cardiovascular outcome.

Recent studies in mice have shown that among CD4+ T-cell subsets, regulatory T cells (Treg) expressing CD25 (interleukin [IL]-2 receptor α-chain) molecule and the transcription factor FoxP3 (forkhead box P3), play a protective role in atherogenesis by dampening pathogenic effector T cell (Teff) response.6–10 We believe that the balance between T eff and Treg is important for the control of atherosclerotic disease,11 and that increasing the Treg/Teff ratio, by suppressing T eff response and promoting Treg response, could be a promising therapeutic approach for atherosclerotic disease.12

Although there is much experimental evidence supporting a protective role for Treg in atherogenesis, understanding of their clinical importance is still lacking. Some studies investigated the correlation between circulating Treg level and CAD to clarify whether impaired function or reduced numbers of Treg may contribute to the progression of atherosclerotic diseases in humans, but the results are still controversial.13–15 Discrepancy among previous reports with regards to the association between circulating Treg level and CAD may potentially be due to the difference in immune system between humans and mice, or limitations in methodology. The transcription
factor FoxP3 is the master regulator and the most reliable molecular marker for Treg at least in mice.\textsuperscript{16} Miyara et al, however, showed that human CD4\(^+\)FoxP3\(^+\) T cells are heterogeneous in function by separating FoxP3\(^+\) cells into 3 subsets based on the expression of FoxP3 and CD45RA.\textsuperscript{17} Thus, further precise clarification for the role of Treg in atherosclerotic diseases is needed using this method.

In the present study, we compared Treg level in the control group with that in the CAD group by separating CD4\(^+\)FoxP3\(^-\) T cells into 3 functionally and phenotypically different subpopulations based on the expression of FoxP3 and CD45RA.\textsuperscript{18,19} We also examined the correlation between these cells and Treg. We for the first time identified an imbalance between Treg and T eff and a negative correlation between CD4\(^+\)CD28\(^{null}\) T cells and the Treg/Teff ratio in CAD patients, suggesting the clinical importance of Treg for the prevention of atherosclerotic diseases.

**Methods**

**Subjects**

Sixty-four patients with CAD were recruited from Kobe University Hospital. We included only stable angina pectoris (AP) and old myocardial infarction (MI) patients who had undergone percutaneous coronary intervention or coronary artery bypass graft surgery ≥6 months earlier; ACS patients were excluded. Patients with systemic disease including hepatic disease, renal disease (serum creatinine >2.0 mg/dl), collagen disease and malignancy were also excluded. Blood samples were collected after overnight fast.

Sixty-four controls without cardiovascular health problems were recruited as an age- and gender-matched control group from a health medical center, Kenko Life Plaza, Hyogo Health Service Association. Blood samples were also collected after overnight fast. The criteria for inclusion in the control group were no history of vascular disease, hypertension, diabetes, or treatment for dyslipidemia. No history of vascular disease was defined as no documented vascular disease, symptoms of AP,
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**Flow Cytometry**

Human peripheral blood mononuclear cells of CAD patients and healthy volunteers were obtained in EDTA-coated tubes and prepared by Ficoll gradient centrifugation. Cells were stained in phosphate-buffered saline containing 2% fetal calf serum. Fluorescence-activated cell sorter analysis (Figure 1) was done using an Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA) using FlowJo10.0.6 software (Tree Star). The antibodies used were as follows: PerCP-Cy5.5-anti-CD3 (clone SK7; BD Biosciences), APC-Cy7-anti-CD4 (clone RPA-T4; BD Biosciences), FITC-anti-CD25 (clone MA251; BD Biosciences), PE-anti-CD28 (clone CD28.2; BD Biosciences), APC-anti-FoxP3 (clone 236A/E7; BD Biosciences), PE-anti-CTLA4 (clone BNI3; BD Biosciences), PE-anti-CD25 (clone SK7; BD Biosciences), APC-Cy7-anti-CD4 (clone RPA-T4; BD Biosciences), FITC-anti-CD25 (clone MA251; BD Biosciences), PE-anti-CD28 (clone CD28.2; BD Biosciences), APC-anti-FoxP3 (clone 236A/E7; BD Biosciences), PE-anti-CTLA4 (clone BNI3; BD Biosciences).

**Figure 3.** Age-related distribution of regulatory T cells (T$_{\text{reg}}$). (A) Resting T$_{\text{reg}}$ (Fr1); (B) activated T$_{\text{reg}}$ (Fr2); (C) regulatory T-cell/effectort cell (T$_{\text{reg}}$/T$_{\text{eff}}$) ratio. *P<0.05, **P<0.01, ***P<0.001 (young control, middle-aged control, middle-aged CAD and old CAD groups; 1-way ANOVA test followed by Tukey’s post-hoc analysis). CAD, coronary artery disease.

**Figure 4.** Expression of suppression molecules was increased in activated regulatory T cells (T$_{\text{reg}}$; Fr2). (A) CD25 and (B) CTLA4 expression of each fraction. *P<0.05, **P<0.01, ***P<0.001 (middle-aged control and CAD groups; Mann-Whitney U-test). CAD, coronary artery disease.
lower, and triglycerides and HbA1c were higher in the middle-aged CAD group compared to age-matched controls (Table). Based on the expression of FoxP3 and CD45RA, we separated the T-cell subpopulations into fractions (Fr1, Fr2, Fr3 and Fr4+5; Figure 1) on flow cytometry. Representative analyses of each T-cell fraction from the control and CAD groups are shown in Figures 1B, C. In accordance with a previous report, human T reg were divided into CD45RA + FoxP3 low resting T reg (Fr1) and CD45RA –FoxP3 high activated T reg (Fr2), and activated T eff were defined as CD45RA –FoxP3 low T cells (Fr3) and CD45RA –FoxP3 – T cells (Fr4+5). The middle-aged CAD group had a lower percentage of resting T reg (2.38 ± 0.84% in the control vs. 1.97 ± 0.93% in the CAD group, P<0.01) and activated T reg (1.76±0.93% in the control vs. 1.36±0.66% in the CAD group, P<0.05) and a higher percentage of Fr3 (2.74±0.96% in the control vs. 3.63±1.13% in the CAD group, P<0.001) and Fr4+5 (25.3±10.2% in the control group, 33.1±8.8% in the CAD group, P<0.001) within the CD4 + T cell population compared to the middle-aged control group (Figure 2A). The percentage of Treg fractions including Fr1+Fr2 was significantly decreased in the middle-aged CAD group compared to the

Table. Subject Characteristics

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<th>Characteristics</th>
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<td>BUN (mg/dl)</td>
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<td>93.6±30.5*</td>
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<td>TG (mg/dl)</td>
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<td>102±71</td>
<td>200±110***</td>
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<td>HbA1c (NGSP%)</td>
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<td>5.44±0.30</td>
<td>6.41±1.23***</td>
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<td>0.08±0.14</td>
<td>0.09±0.11</td>
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Data given as mean±SD or %. *P<0.05, **P<0.01, ***P<0.001 (1-way ANOVA test followed by Tukey’s post-hoc analysis; natural logarithmic transformation used for comparison of TG and hs-CRP). †LDL-C >140 mg/dl. TG >150 mg/dl or use of anti-dyslipidemic drugs. ‡Blood pressure >140/90 mmHg or use of anti-hypertensive drugs. §HbA1c >6.5% (NGSP), use of oral anti-diabetic drugs, or insulin. *No. major coronary vessels with >75% stenosis on diagnostic coronary angiography and requiring treatment. ACEI, angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; CAD, coronary artery disease; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides.

Statistical Analysis

The baseline characteristics, medications and laboratory data of the CAD and control groups are listed in Table. The CAD group had relatively low LDL-C because most of these patients took statins. High-density lipoprotein cholesterol was lower, and triglycerides and HbA1c were higher in the middle-aged CAD group compared to age-matched controls (Table).

Based on the expression of FoxP3 and CD45RA, we separated the T-cell subpopulations into fractions (Fr1, Fr2, Fr3 and Fr4+5; Figure 2) on flow cytometry. Representative analyses of each T-cell fraction from the control and CAD groups are shown in Figures 1B, C. In accordance with a previous report, human T reg were divided into CD45RA+FoxP3low resting Treg (Fr1) and CD45RA-FoxP3high activated Treg (Fr2), and activated Treg were defined as CD45RA-FoxP3low T cells (Fr3) and CD45RA-FoxP3+ T cells (Fr4+5). The middle-aged CAD group had a lower percentage of resting Treg (2.38±0.84% in the control vs. 1.97±0.93% in the CAD group, P<0.01) and activated Treg (1.76±0.93% in the control vs. 1.36±0.66% in the CAD group, P<0.05) and a higher percentage of Fr3 (2.74±0.96% in the control vs. 3.63±1.13% in the CAD group, P<0.001) and Fr4+5 (25.3±10.2% in the control group, 33.1±8.8% in the CAD group, P<0.001) within the CD4+ T cell population compared to the middle-aged control group (Figure 2A). The percentage of Treg fractions including Fr1+Fr2 was significantly decreased in the middle-aged CAD group compared to the

Results

The baseline characteristics, medications and laboratory data of the CAD and control groups are listed in Table. The CAD group had relatively low LDL-C because most of these patients took statins. High-density lipoprotein cholesterol was lower, and triglycerides and HbA1c were higher in the middle-aged CAD group compared to age-matched controls (Table).
The T<sub>reg</sub>/T<sub>eff</sub> ratio was reduced in CAD patients. Several papers investigated the association between T<sub>reg</sub> level in peripheral blood and atherosclerotic disease, but reported conflicting data possibly due to different methods of defining T<sub>reg</sub>. In the present study, we precisely defined T<sub>reg</sub> and T<sub>eff</sub> on flow cytometry and compared T<sub>reg</sub> and T<sub>reg</sub>/T<sub>eff</sub> ratio in the peripheral blood from CAD patients with those from controls.

Consistent with a previous report, the proportion of resting T<sub>reg</sub> (Fr1) was decreased in the young control group compared to the middle-aged control group, whereas that of activated T<sub>reg</sub> (Fr2) was increased. Notably, both fractions tended to be decreased in CAD patients. In addition, the T<sub>reg</sub>/T<sub>eff</sub> ratio tended to decrease with aging in both the CAD and control groups.

Next, the effects of CAD on the expression of T<sub>reg</sub>-associated molecules such as CD25 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) were determined in each T-cell fraction on flow cytometry. Notably, CD45RA<sup>-</sup>FoxP3<sup>high</sup> activated T<sub>reg</sub> (Fr2) and CD45RA<sup>-</sup>FoxP3<sup>low</sup> non-T<sub>reg</sub> (Fr3) from the CAD group expressed higher levels of these molecules compared to those from the control group, implying an activated phenotype of T<sub>reg</sub> and T<sub>eff</sub> in the presence of CAD.

Recent studies have shown that the percentage of CD<sup>+</sup>CD28<sup>null</sup> T cells is increased in patients with ACS, which may contribute to plaque instability. CD<sup>+</sup>CD28<sup>null</sup> T cells tended to be increased in the CAD group compared to the control group. We investigated the association between CD<sup>+</sup>CD28<sup>null</sup> T cells and T<sub>reg</sub> and found that CD<sup>+</sup>CD28<sup>null</sup> T cells were detected mainly in Fr4 or Fr5 T<sub>eff</sub> populations, but not in Fr1 or Fr2 T<sub>reg</sub> populations. Interestingly, we observed an inverse correlation between CD<sup>+</sup>CD28<sup>null</sup> T cells and the T<sub>reg</sub>/T<sub>eff</sub> ratio in all CAD patients, middle-aged and old CAD groups, when we excluded the population of CD<sup>+</sup>CD28<sup>null</sup> T cells <0.3% of CD<sup>+</sup> T cells (Figure 5B), although we found no significant correlation in the whole population of these cells. This suggests that the T<sub>reg</sub>/T<sub>eff</sub> balance may modulate expansion but not generation of CD<sup>+</sup>CD28<sup>null</sup> T cells through mechanisms that remain undefined.

Finally, a negative correlation was observed between T<sub>reg</sub>/T<sub>eff</sub> ratio and serum high-sensitivity C-reactive protein (hs-CRP) level (Figure 6).

**Discussion**

Several papers investigated the association between T<sub>reg</sub> level in peripheral blood and atherosclerotic disease, but reported conflicting data possibly due to different methods of defining T<sub>reg</sub>. In the present study, we precisely defined T<sub>reg</sub> and T<sub>eff</sub> on flow cytometry and compared T<sub>reg</sub> and T<sub>reg</sub>/T<sub>eff</sub> ratio in the peripheral blood from CAD patients with those from controls.
We also examined the correlation between CD4+CD28null T cells, which may contribute to atherosclerosis development and plaque vulnerability, and Treg. We have clearly shown a decreased Treg/Teff ratio and a negative correlation between CD4+CD28null T cells and the Treg/Teff ratio in CAD patients, suggesting that reduced Treg may contribute to the progression of atherosclerotic disease in humans.

Based on the strong evidence supporting a protective role for Treg in experimental atherosclerosis, several studies explored the role of Treg in clinical atherosclerosis. Wigen et al defined Treg as CD4+FoxP3+ or CD4+CD25+FoxP3+ cells and showed that there was an association between low baseline CD4+FoxP3+ T cells and an increased risk for the development of acute coronary events but not stroke. Their study was the first large-volume and long follow-up study investigating the association between circulating Treg, defined as the expression of FoxP3 in CD4+ T cells, and CAD, and suggests that Treg may play a protective role in human atherosclerosis and therefore are of potential clinical importance. Although the staining method using the Foxp3 molecule can discriminate between Treg and Teff more precisely than that using the combination of CD25 and CD127 molecules, it is possible that such a population may still include some Teff.

A recent paper showed that the combination of FoxP3 and CD45RA staining of CD4+ T cells in peripheral blood lymphocytes can identify Treg more precisely than previous methods. Using this staining method, human Treg were divided into CD45RAFoxP3high resting Treg (Fr1) and CD45RAFoxP3high activated Treg (Fr2), and activated Treg were defined as CD45RAFoxP3low T cells (Fr3) and CD45RAFoxP3low T cells (Fr4+5). In the present study, we found that both resting Treg and activated Treg were decreased, whereas CD45RAFoxP3low non-Treg (Fr5) level was increased in patients with acute AP and old MI compared to controls, which is inconsistent with previous studies showing that peripheral Treg is normal in these patients. Miyara et al showed that the CD45RA-FoxP3low T cell (Fr3) population produces high amounts of pro-inflammatory cytokines such as IL-2 and interferon-γ (IFN-γ) and does not have suppressor function, indicating that this population does not seem to be real Treg but Teff. Importantly, in the present study, further analysis by defining Treg as CD3+CD4+FoxP3+ cells, without staining CD45RA, showed that there was no difference in Treg level between CAD patients and controls, which could be explained by the inappropriate inclusion of increased Teff Fr3 fraction in the Treg population. Therefore, the present analysis may be an ideal classification for Treg to examine their role in human atherosclerotic disease. Discrepancy between the present findings and previous work with regards to the association between Treg level and coronary atherosclerosis may potentially be due to the difference in the definition of Treg.

It was shown that upon activation through T cell receptor, resting Treg (Fr1) have an ability to easily proliferate, become activated Treg (Fr2), suppress T eff, and undergo cell death. Although both resting Treg and activated Treg are shown to effectively suppress T eff response, the mechanisms underlying the suppression mediated by both Treg types remain to be elucidated. The resting Treg population decreases with aging possibly due to decreased production in the thymus, whereas generation of activated Treg in the periphery in aged individuals may compensate for the decreased Treg. In agreement with a previous study, the decrease of resting Treg and the increase of activated Treg with aging were observed in the control group (Figure 3). Similarly, resting Treg tended to decrease with aging in the CAD group, whereas activated Treg was not increased but rather decreased. Notably, we found that the expression of Treg activation markers such as CD25 or CTLA-4 was significantly upregulated in activated Treg of the CAD group compared to the control group, which may promote the death of this population. Taken together, we suppose that decreased activated Treg in the CAD group might be due to the increased cell death after activation. Further studies are needed to identify the molecular mechanisms for the decrease in activated Treg in the CAD group.

Atherosclerosis is an inflammatory condition of the arterial wall involving innate and adaptive immunity. It is well recognized that serum hs-CRP concentration is one of the most popular and established inflammation markers and independently predicts future cardiovascular events, although its level may be easily changed by systemic inflammatory responses. Interestingly, we observed an inverse correlation between Treg/Teff ratio and serum hs-CRP, suggesting that the Treg/Teff ratio in the peripheral blood could be a useful marker to predict future cardiovascular events. In addition, because the distribution of Treg/Teff ratio is diverse in patients with low serum hs-CRP, measurement of Treg/Teff ratio may enable more detailed evaluation of immune-inflammatory status in CAD patients in combination with hs-CRP.

The present study had some limitations that should be considered when interpreting the results. First, the number of patients was small, and so additional larger trials are needed to validate these observations. Second, we examined only peripheral blood samples, and analysis of the local immune response in atherosclerotic lesions was not performed. Given that Treg in circulation are reported to migrate into inflamed tissues to dampen local inflammation, the dynamics of Treg localization should be examined to determine the clinical importance of Treg in atherosclerosis. Finally, it remains unclear whether reduced Treg/Teff ratio is a cause or result of atherosclerotic disease.

Conclusions
Reduced Treg/Teff ratio is closely related with the pathophysiology of coronary atherosclerosis, suggesting that peripheral Treg/Teff ratio may be a useful marker for the evaluation of severity of atherosclerosis. The present data imply that enhancing a Treg-mediated immune response could be a possible therapeutic approach to treat human atherosclerosis, although prospective clinical studies are required to ascertain whether reduced Treg promotes atherosclerosis in humans.

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Disclosures
The authors have no conflicts of interest to declare.

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References