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ORIGINAL ARTICLE
Ischemic Heart Disease

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

Takuo Emoto, MD; Naoto Sasaki, MD, PhD; Tomoya Yamashita, MD, PhD; Kazuyuki Kasahara, MD; Keiko Yodoi, MD; Yoshihiro Sasaki, MD; Takuya Matsumoto, MD; Taiji Mizoguchi, MD; Ken-ichi Hirata, MD, PhD

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

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: $CD45RA^+FoxP3^{low}$ resting T_{reg} (Fr1), $CD45RA^-FoxP3^{high}$ activated T_{reg} (Fr2), and $CD45RA^-FoxP3^{low}$ non-T_{reg} (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. T_{reg}/T_{eff} 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 $CD4^+CD28^{null}$ T cells within the $CD4^+$ T-cell population was negatively correlated with T_{reg}/T_{eff} ratio, excluding $CD4^+CD28^{null}$ T cells <0.3% (r=–0.27, P<0.05). High-sensitivity C-reactive protein was also negatively correlated with T_{reg}/T_{eff} ratio (r=–0.22, P<0.05).

Conclusions: CAD patients had reduced T_{reg} and T_{reg}/T_{eff} 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.

Editorial p2843

Recent studies in mice have shown that among $CD4^+$ T-cell subsets, regulatory T cells (T_{reg}) 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 (T_{eff}) response.[6–10] We believe that the balance between T_{eff} and T_{reg} is important for the control of atherosclerotic disease,[11] and that increasing the T_{reg}/T_{eff} ratio, by suppressing T_{eff} response and promoting T_{reg} response, could be a promising therapeutic approach for atherosclerotic disease.[12]

Although there is much experimental evidence supporting a protective role for T_{reg} in atherogenesis, understanding of their clinical importance is still lacking. Some studies investigated the correlation between circulating T_{reg} level and CAD to clarify whether impaired function or reduced numbers of T_{reg} 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 T_{reg} level and CAD may potentially be due to the difference in immune system between humans and mice, or limitations in methodology. The transcription

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Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan
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Mailing address: Naoto Sasaki, MD, PhD, Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. E-mail: sasakin@med.kobe-u.ac.jp
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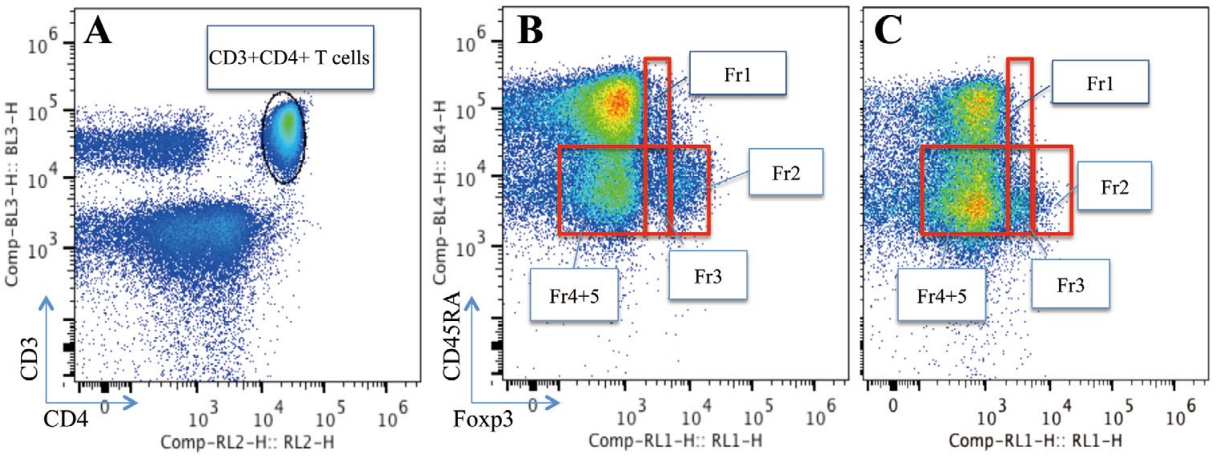

Figure 1. Representative fluorescence-activated cell sorter analysis of T cells. (**A**) Flow cytometry of the expression of CD45RA and FoxP3 in CD3+CD4+ T cells from (**B**) the middle-aged control group and (**C**) the middle-aged coronary artery disease group. Fr1, CD45RA+FoxP3low resting T_{reg}; Fr2, CD45RA-FoxP3high activated T_{reg}; Fr3, CD45RA-FoxP3low non-T_{reg}; Fr4+5, CD45RA-FoxP3- non-T_{reg}. T_{eff}, effector T cell; T_{reg}, regulatory T cell.

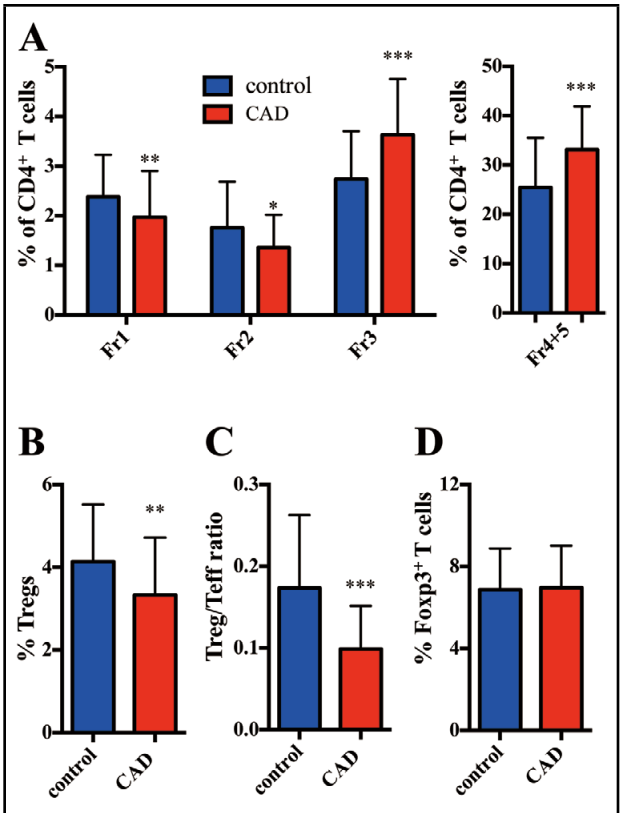

Figure 2. Comparison of T-cell subsets between the age-matched control and coronary artery disease (CAD) groups. (**A**) T-cell fractions; (**B**) regulatory T cells (T_{reg}; defined as Fr1+Fr2) % of CD4+ T cells; (**C**) regulatory T-cell/effector T-cell (T_{reg}/T_{eff}) ratio (Fr1+2/Fr3+4+5); (**D**) FoxP3+ T cells (defined as Fr1+2+3) % of CD4+ T cells. *P<0.05, **P<0.01, ***P<0.001 (middle-aged control and CAD groups; Mann-Whitney U-test or Student's t-test).

factor FoxP3 is the master regulator and the most reliable molecular marker for T_{reg} at least in mice.[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.[17] Thus, further precise clarification for the role of T_{reg} in atherosclerotic diseases is needed using this method.

In the present study, we compared T_{reg} 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. Given that CD4+CD28null T cells have been reported to promote atherosclerosis and plaque vulnerability,[18,19] we also examined the correlation between these cells and T_{reg}. We for the first time identified an imbalance between T_{reg} and T_{eff} and a negative correlation between CD4+CD28null T cells and the T_{reg}/T_{eff} ratio in CAD patients, suggesting the clinical importance of T_{reg} 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,

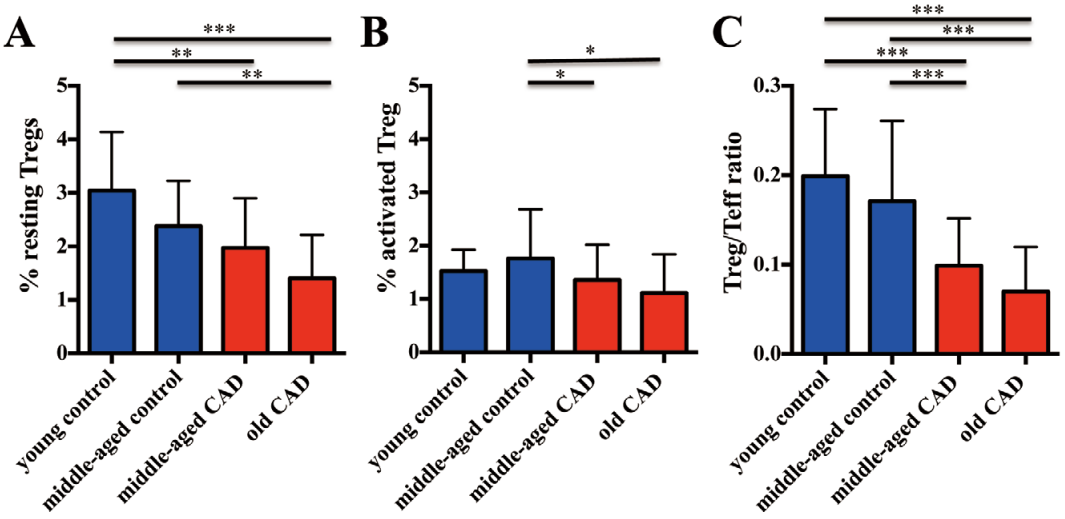

Figure 3. Age-related distribution of regulatory T cells (T_{reg}). (**A**) Resting T_{reg} (Fr1); (**B**) activated T_{reg} (Fr2); (**C**) regulatory T-cell/effector T-cell (T_{reg}/T_{eff}) ratio. *P<0.05, **P<0.01, ***P<0.001 (young control, middle-aged control, middle-aged CAD and old CAD groups;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

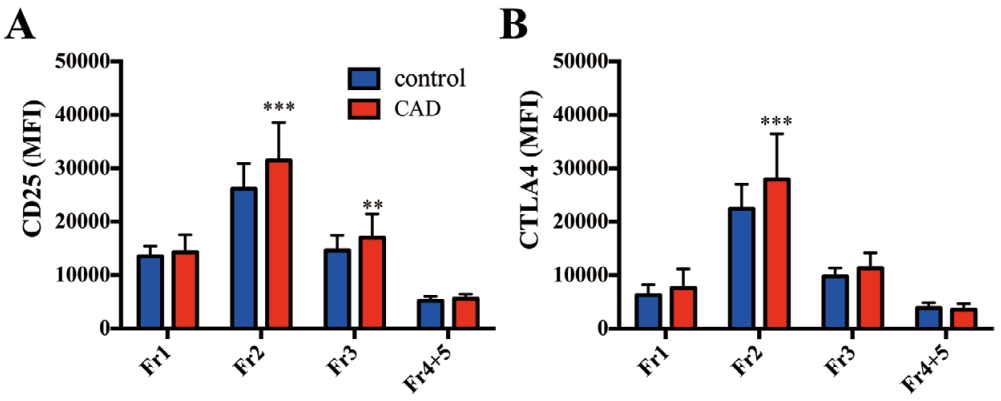

Figure 4. Expression of suppression molecules was increased in activated regulatory T cells (T_{reg}; Fr2). (**A**) CD25 and (**B**) CTLA4 expression of each fraction. *P<0.05, **P<0.01, ***P<0.001 (middle-aged control and CAD groups; Mann-Whitney U-test). CAD, coronary artery disease.

abnormality on electrocardiogram indicating old MI or AP, or abnormality on chest X-ray. Hypertension was defined as blood pressure >140/90 mmHg or use of anti-hypertensive drugs. Diabetes was defined as HbA1c >6.5% (National Glycohemoglobin Standardization Program), use of oral anti-diabetic drugs or insulin. Although they had not been treated, 24 controls with dyslipidemia (low-density lipoprotein cholesterol [LDL-C] >140 mg/dl or triglycerides >150 mg/dl) were included in this control group. We divided the control and CAD groups into 2 groups each: young control group <50 years old, middle-aged control group ≥50 years old, middle-aged CAD group ≤70 years old (middle-aged CAD) and old CAD group >70 years old (old CAD).

This study was approved by the Ethics Committee of Kobe University (No. 1318) and Kenko Life Plaza, Hyogo Health Service Association. All subjects provided oral and written informed consent to participate in this study.

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: PerCPCy5.5-anti-CD3 (clone SK7; BD Biosciences), APCCy7-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 Biosci-

Table. Subject Characteristics

	Young control (n=18)	Middle-aged control (n=49)	Middle-aged CAD (n=55)	Old CAD (n=18)
Characteristics				
Age (years)	45.7±2.43***	59.2±6.0	60.3±8.2	73.5±2.4***
Sex (% male)	72	84	91	83
BMI (kg/m²)	24.4±4.2	22.4±2.7	26.3±4.2***	24.8±8.2
Smoking	78*	48	77**	82*
Dyslipidemia†	22	18	89***	78***
Hypertension‡	0	2	89***	83***
Diabetes§	0	0	44***	44***
No. coronary vessels¶				
1-vessel disease			29	33
2-vessel disease			42	28
3-vessel disease			29	39
Medications				
Anti-diabetes drug (including insulin)	0	0	44	50
Statins	0	0	91	72
ACEI/ARB	0	0	69	67
β-blocker	0	0	60	61
Ca blocker	0	0	69	50
Laboratory data				
AST (U/L)	22.8±8.87	19.8±5.1	24.3±11.4	24.1±8.2
ALT (U/L)	30.0±24.2	19.5±10.3	26.7±18.2	21.8±9.4
BUN (mg/dl)	12.1±2.35	13.8±2.67	17.3±5.2***	17.4±4.7*
Cr (mg/dl)	0.72±0.15	0.80±0.14	0.97±0.27***	0.92±0.25
HDL-C (mg/dl)	57.8±15.6	73.8±32.4	46.5±14.7***	46.9±11.4***
LDL-C (mg/dl)	114.9±31.8	112.5±31.9	93.6±30.5*	91.6±26.2
TG (mg/dl)	142±98*	102±71	200±110***	126±113
HbA1c (NGSP%)	5.29±0.52	5.44±0.30	6.41±1.23***	6.17±0.62*
hsCRP (mg/dl)	0.07±0.12	0.08±0.14	0.09±0.11	0.21±0.24*

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.

ences), PECy7-anti-CD45RA (clone L48; BD Biosciences) and isotype-matched control antibodies.

Statistical Analysis

Data are given as mean±SD. Mann-Whitney U-test or Student's t-test, or Fisher's exact test were used for statistical comparison between 2 groups (Figure 2). One-way and 2-way ANOVA followed by Tukey's post-hoc analysis was used for statistical comparison between 4 groups (Figures 3,4; Table).

Pearson's correlation analysis was used for statistical correlation between 2 parameters (Figures 5,6). All statistical analysis was 2-sided, with P<0.05 considered statistically significant. For statistical analysis, GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) was used.

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).

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 T_{reg} fractions including Fr1+Fr2 was significantly decreased in the middle-aged CAD group compared to the

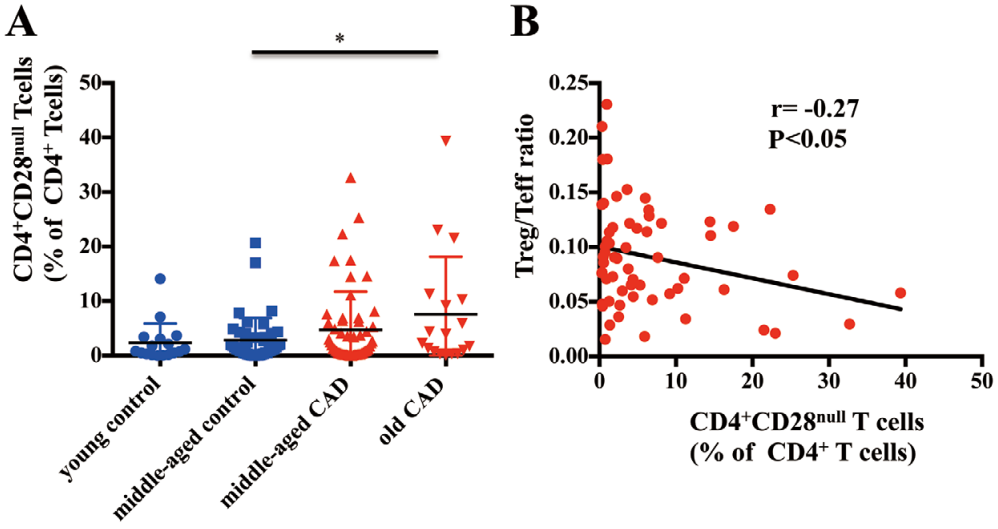

Figure 5. Population of CD4+CD28null T cell was inversely correlated with regulatory T-cell/effector T-cell (Treg/Teff) ratio. (**A**) CD4+CD28null T cell level (1-way ANOVA test followed by Tukey's post-hoc analysis); (**B**) correlation between Treg/Teff ratio and % of CD4+CD28null T cells in the CD4+ T cells in all coronary artery disease (CAD) patients (middle-aged and old CAD groups), excluding % of CD4+CD28null T cells <0.3% in all CAD patients, calculated by Pearson's correlation. *P<0.05.

middle-aged control group (**Figure 2B**). The Treg/Teff ratio (Fr1+2/Fr3+4+5) in middle-aged CAD group (0.10±0.05) was markedly lower than in the middle-aged control group (0.17±0.09, P<0.001; **Figure 2C**), indicating the imbalance between Treg and Teff under atherosclerotic conditions. Notably, we found no difference in FoxP3+ T cell level including Fr1+Fr2+Fr3 between the middle-aged CAD and middle-aged control groups (**Figure 2D**).

Consistent with a previous report,[17] the proportion of resting Treg (Fr1) was decreased in the young control group compared to the middle-aged control group, whereas that of activated Treg (Fr2) was increased (**Figures 3A,B**). Notably, both fractions tended to be decreased in CAD patients. In addition, the Treg/Teff ratio tended to decrease with aging in both the CAD and control groups (**Figure 3C**).

Next, the effects of CAD on the expression of Treg-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−FoxP3high activated Treg (Fr2) and CD45RA−FoxP3low non-Treg (Fr3) from the CAD group expressed higher levels of these molecules compared to those from the control group (**Figure 4**), implying an activated phenotype of Treg and Teff in the presence of CAD.

Recent studies have shown that the percentage of CD4+CD28null T cells is increased in patients with ACS, which may contribute to plaque instability.[18,19] CD4+CD28null T cells tended to be increased in the CAD group compared to the control group (**Figure 5A**). We investigated the association between CD4+CD28null T cells and Treg and found that CD4+CD28null T cells were detected mainly in Fr4 or Fr5 Teff populations, but not in Fr1 or Fr2 Treg populations (data not shown). Interestingly, we observed an inverse correlation between CD4+CD28null T cells and the Treg/Teff ratio in all CAD patients (middle-aged and old CAD groups), when we excluded the population of CD4+CD28null T cells <0.3% of CD4+ T cells (**Figure 5B**), although we found no significant correlation in the whole population of these cells. This suggests that the Treg/Teff balance may modulate expansion but not generation of CD4+CD28null T cells through mechanisms that remain undefined.

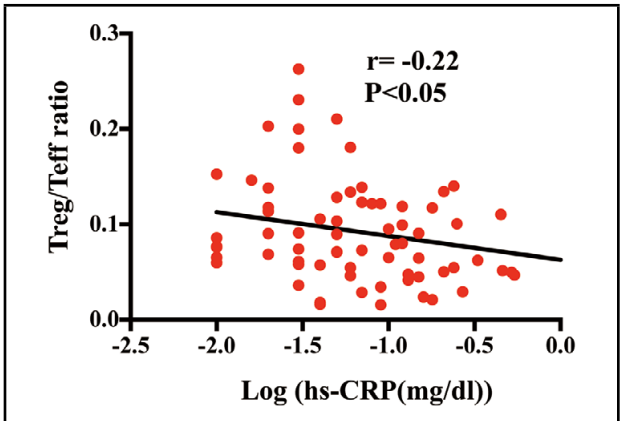

Figure 6. Regulatory T-cell/effector T-cell (Treg/Teff) ratio was inversely correlated with logarithmic transformed high-sensitivity C-reactive protein (hs-CRP) in all coronary artery disease (CAD) patients (middle-aged and old CAD groups; Pearson's correlation test).

Finally, a negative correlation was observed between Treg/Teff ratio and serum high-sensitivity C-reactive protein (hs-CRP) level (**Figure 6**).

Discussion

Several papers investigated the association between Treg level in peripheral blood and atherosclerotic disease, but reported conflicting data possibly due to different methods of defining Treg. In the present study, we precisely defined Treg and Teff on flow cytometry and compared Treg and Treg/Teff ratio in the peripheral blood from CAD patients with those from controls.

We also examined the correlation between $CD4^+CD28^{null}$ T cells, which may contribute to atherosclerosis development and plaque vulnerability, and T_{reg}. We have clearly showed a decreased T_{reg}/T_{eff} ratio and a negative correlation between $CD4^+CD28^{null}$ T cells and the T_{reg}/T_{eff} ratio in CAD patients, suggesting that reduced T_{reg} may contribute to the progression of atherosclerotic disease in humans.

Based on the strong evidence supporting a protective role for T_{reg} in experimental atherosclerosis,[11] several studies explored the role of T_{reg} in clinical atherosclerosis.[13–15,20] Wigren et al defined T_{reg} 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.[15] Their study was the first large-volume and long follow-up study investigating the association between circulating T_{reg}, defined as the expression of FoxP3 in $CD4^+$ T cells, and CAD, and suggests that T_{reg} 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 T_{reg} and T_{eff} more precisely than that using the combination of CD25 and CD127 molecules, it is possible that such a population may still include some T_{eff}.

A recent paper showed that the combination of FoxP3 and CD45RA staining of $CD4^+$ T cells in peripheral blood lymphocytes can identify T_{reg} more precisely than previous methods.[17] Using this staining method, 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). In the present study, we found that both resting T_{reg} and activated T_{reg} were decreased, whereas $CD45RA^-FoxP3^{low}$ non-T_{reg} (Fr3) level was increased in patients with stable AP and old MI compared to controls, which is inconsistent with previous studies showing that peripheral T_{reg} is normal in these patients.[14] Miyara et al showed that the $CD45RA^-FoxP3^{low}$ 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 T_{reg} but T_{eff}.[17] Importantly, in the present study, further analysis by defining T_{reg} as $CD3^+CD4^+FoxP3^+$ cells, without staining CD45RA, showed that there was no difference in T_{reg} level between CAD patients and controls, which could be explained by the inappropriate inclusion of increased T_{eff} Fr3 fraction in the T_{reg} population. Therefore, the present analysis may be an ideal classification for T_{reg} to examine their role in human atherosclerotic disease. Discrepancy between the present findings and previous work with regards to the association between T_{reg} level and coronary atherosclerosis may potentially be due to the difference in the definition of T_{reg}.

It was shown that upon activation through T cell receptor, resting T_{reg} (Fr1) have an ability to easily proliferate, become activated T_{reg} (Fr2), suppress T_{eff}, and undergo cell death.[17] Although both resting T_{reg} and activated T_{reg} are shown to effectively suppress T_{eff} response, the mechanisms underlying the suppression mediated by both T_{reg} types remain to be elucidated. The resting T_{reg} population decreases with aging possibly due to decreased production in the thymus, whereas generation of activated T_{reg} in the periphery in aged individuals may compensate for the decreased T_{reg}. In agreement with a previous study,[17] the decrease of resting T_{reg} and the increase of activated T_{reg} with aging were observed in the control group (**Figure 3**). Similarly, resting T_{reg} tended to decrease with aging in the CAD group, whereas activated T_{reg} was not increased but rather decreased. Notably, we found that the expression of T_{reg} activation markers such as CD25 or CTLA-4 was significantly upregulated in activated T_{reg} of the CAD group compared to the control group, which may promote the death of this population. Taken together, we suppose that decreased activated T_{reg} 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 T_{reg} in the CAD group.

Atherosclerosis is an inflammatory condition of the arterial wall involving innate and adaptive immunity.[4] 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,[21] although its level may be easily changed by systemic inflammatory responses. Interestingly, we observed an inverse correlation between T_{reg}/T_{eff} ratio and serum hs-CRP, suggesting that the T_{reg}/T_{eff} ratio in the peripheral blood could be a useful marker to predict future cardiovascular events. In addition, because the distribution of T_{reg}/T_{eff} ratio is diverse in patients with low serum hs-CRP, measurement of T_{reg}/T_{eff} 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 T_{reg} in circulation are reported to migrate into inflamed tissues to dampen local inflammation,[22] the dynamics of T_{reg} localization should be examined to determine the clinical importance of T_{reg} in atherosclerosis. Finally, it remains unclear whether reduced T_{reg}/T_{eff} ratio is a cause or result of atherosclerotic disease.

Conclusions

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

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Disclosures

The authors have no conflicts of interest to declare.

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