Regulatory T Lymphocytes Attenuate Myocardial Infarction-Induced Ventricular Remodeling in Mice

Kazuaki MATSUMOTO,¹ BS, Masahito OGAWA,² MS, Jun-ichi SUZUKI,² MD, Yasunobu HIRATA,² MD, RyoZO NAGAI,³ MD, and Mitsuaki ISOBE,¹ MD

SUMMARY

Downregulation of CD4+CD25+ regulatory T lymphocytes (Treg) has been found in local atherosclerotic lesions and in patients with myocardial infarction (MI). However, the roles of Treg in MI and the following inflammatory response have not yet been well elucidated. Therefore, we hypothesized that adoptive transfer of Treg could attenuate the postinfarction inflammatory response protecting from adverse remodeling, and we attempted to elucidate the mechanism of delayed heart failure after MI. To clarify the role of Treg in MI, we used a murine MI model and administered a single intravenous injection of Treg (1×10^5) (treatment, $n = 6$) or saline (control, $n = 7$) and sacrificed the mice on day 14. Echocardiograms revealed that Treg improved LV contraction after MI. Histopathology also showed that Treg negated MI-induced LV remodeling. RT-PCR demonstrated that the mRNA levels of IFN-gamma in hearts were lower and Foxp3 in spleens were higher in the treatment group than in the control group. We observed that adoptive Treg transfer could attenuate MI-induced cardiac remodeling through the IFN-gamma and Foxp3 alteration. (Int Heart J 2011; 52: 382-387)

Key words: Regulatory T cells, Myocardial ischemia, Cytokine

Recent research has shown that the main cause of coronary arterial disease (CAD) is an inflammatory reaction in which immune mechanisms interact with metabolic risk factors.¹⁾ Activated macrophages, T cells, and mast cells at sites of plaque rupture produce several types of molecules—inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules—that can destabilize lesions.¹⁾ However, little is known regarding the role of lymphocytes, which play an important role in the control of the inflammatory system and in the pathophysiology of acute myocardial infarction (AMI). Furthermore, pro- and anti-inflammatory T lymphocytes as well as regulatory T-cells (Treg) have been barely analyzed in this scenario.²⁾ Adaptive immunity, in particular CD4+ T cells, is highly involved in atherogenesis.³⁾ Up-regulation of T helper cell type 1 (Th1) response has been found in local atherosclerotic lesions and circulating lymphocytes in atherosclerosis-prone models and patients with acute coronary syndrome (ACS). In contrast, T helper cell type 2 (Th2) and related cytokines have rarely been shown in local atherosclerotic lesions. There was no difference in circulation, suggesting that a Th1/Th2 imbalance plays an important role in the development and progression of atherosclerosis and the onset of ACS.⁴⁾ However, evidence is now accumulating for Tregs, a novel functionally distinct subpopulation of T cells that suppress the response of effector T cells and exert important regulatory functions in various inflammatory diseases.⁵⁾

Several subsets of regulatory T cells with distinct phenotypes and distinct mechanisms of action have now been identified. These include T helper cell type 3 (Th3),⁶⁾ which primarily secrete transforming growth factor beta (TGF-beta); T regulatory cell 1 (Tr1),⁷⁾ which secrete high levels of interleukin (IL)-10; and CD4+CD25+ regulatory T cells,⁸⁾ which inhibit immune responses through cell-to-cell contact. Thus, the purpose of our work is to unravel the anti-inflammatory effect of Treg in AMI. We hypothesize that a few Tregs attenuate the broad inflammatory reaction occurring in AMI.

METHODS

Isolation of CD4+CD25+ Tregs: Lymphocytes were taken from the spleen of a C57BL/6 mouse that did not receive any intervention. From the cells, CD4+CD25+ T cells (Tregs) were magnetically isolated in a two-step procedure, according to the protocol of the manufacturer (Miltenyi Biotec, Auburn, CA, USA). First, CD4+ T cells were negatively pre-enriched by depletion of unwanted cells with antibody cocktail and anti-biotin micro beads. Then, CD25+ cells are positively selected from the enriched CD4+ T cell fraction, with CD25-PE antibody and anti-PE micro beads. LS columns were used in these procedures.

Animals and surgical procedure: Eight to 9 week-old C57BL/6

From the ¹ Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Departments of ² Advanced Clinical Science and Therapeutics and ³ Cardiovascular Medicine, The University of Tokyo, Tokyo, Japan.

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Address for correspondence: Jun-ichi Suzuki, MD, Department of Advanced Clinical Science and Therapeutics, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.

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mice weighing 21–24 g and housed and fed under standard laboratory conditions were used. All animals received humane care, with free access to food and water. They were anesthetized with 2,2,2-trichloro-1,1-ethanediol (10 mg/kg, 3.6% diluted with saline) and intubated and mechanically ventilated. The hearts were accessed via an intercostal thoracotomy and the left anterior descending coronary arteries (LAD) were permanently occluded by tying off an 8-0 suture passed under the arteries. The chest was closed and the mice were removed from the ventilator. The mice were allowed to recover on a warmed surface. Sham-operated animals went through all procedures described, except actual occlusion of the LAD. Soon after the chest was closed, the mice were treated with saline (control) or CD4+CD25+ Treg (1×10^5) diluted with saline (treatment) via femoral vein injection. The mice were killed 2 weeks later and the hearts excised.

Next, other mice were operated on without injecting any vehicle; they were sacrificed and their hearts were obtained at 6 hours ($n = 4$), 1 day ($n = 4$), 4 days ($n = 4$), 7 days ($n = 4$) or 14 days ($n = 4$) after operation, and sham mice ($n = 4$) were also sacrificed as control. All animals and organs were analyzed via FACS to determine the changes in the infarct area Treg population over time. The groups were called 6 Hour, Day 1, Day 4, Day 7, Day 14 and Day 0.

For RT-PCR analysis, control group mice and treatment group mice were prepared and sacrificed on day 1 (control: $n = 4$, treatment: $n = 4$) and day 3 (control: $n = 4$, treatment: $n = 4$) after operation, and their spleens and hearts were excised.

As another control group, we prepared mice which were operated on in the same manner as mice in the other groups and were given lymphocytes (1×10^5) via femoral vein injection soon after the operation. The lymphocytes were taken from the spleen of another mouse of the same strain and not purified for Treg, so its fraction of Treg was much smaller than other types of lymphocytes (Miltenyi Biotech). This group was named lymphocyte-transferred control (LT control; $n = 4$).

This investigation conforms with the Guide for the Care and Use of Laboratory Animals of Tokyo Medical and Dental University. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (A5032-01, Foreign Institutions with a PHS Approved Animal Welfare Assurance, Office of Laboratory Animal Welfare).

Stained T lymphocyte injection: CD4+CD25+ Treg cells isolated from lymphocytes obtained from the spleen of an intact mouse as described above were stained red with the specific dye (PKH-26-GL) according to the protocol of the manufacturer (Sigma-Aldrich Corporation, MO). The stained T cells were injected into the femoral vein of another mouse soon after ligation of its LAD. The heart and spleen of the mouse were obtained and sliced 1 day after the operation, and the cell nuclei of each tissue were stained blue. The slices were observed with a confocal microscope.

Heart rate and blood pressure: Heart rate and blood pressure (systolic, diastolic, and mean pressure) were measured in conscious mice using a tail-cuff system (BP-98A, Softron Co., Tokyo) just before operation and sacrifice.

Echocardiography: Mice were anesthetized with 2,2,2-trichloro-1,1-ethanediol (10 mg/kg, 3.6% diluted with saline). Thoracic echocardiography was performed with a commercial-

ly available ultrasound apparatus (Nemio, Toshiba, Tokyo) before sacrifice and 1 week after ligation of LAD. A 14-MHz annular array transducer was used. Hearts were imaged in the two-dimensional mode in short-axis views at the level of papillary muscle. M-mode views were used to measure the LV dimensions according to the American Society for Echocardiography leading edge method.⁹⁾ Left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), and fractional shortening ($FS = ((LVDd - LVDs) / LVDd)$) were calculated from the M-mode recordings.

Flow cytometric analysis: *i) Analyzing the purified Treg population:* The purified CD4+CD25+ T cell populations were analyzed for their purity. For each immunofluorescence staining, 1×10^5 cells were extracted from a mashed spleen and incubated with specific antibodies in PBS containing 0.5% BSA for surface staining. Cell surface markers were measured using anti-CD4-FITC (BD Pharmingen, USA) and anti CD25-PE (Miltenyi Biotech) antibodies. The analysis of the cell populations was based on the acquisition of 10,000 events using CELL Quest software. The results are expressed as a percentage of all cells staining positive for the specific cell-surface proteins. *ii) Analyzing the lymphocyte populations in infarct area:* The ventricles of the hearts from 20 operated mice and 4 sham mice were excised and lymphocytes were isolated from the tissue. The population was then examined in the same way as described above. In addition, the hearts of Treg-treatment mice ($n = 4$) were obtained 1 day after operation and the ratios of CD4+CD25+ Treg were analyzed using FACS as described above.

Histopathology: Hearts were harvested immediately after the mice were killed on day 14. We obtained a midventricular transverse slice from each heart for histopathologic examination. The slices were stained with the hematoxylin eosin (HE)

Figure 1. Purity of Treg used for treatment. CD4+CD25+ Tregs were purified from spleen cells by the MACS procedure and the ratio was measured by FACS.

and Mallory methods. Infarct size and noninfarct size were measured and the infarct ratios (infarct size/ (noninfarct size + infarct size)) were calculated by computer-assisted planimetry (Image-Pro Express) using low power.

RNA isolation and real-time RT-PCR: Total RNA was collected from the hearts and spleens of the sacrificed mice with the TRIzol reagent (Invitrogen, Life Technologies). Real-time RT-PCR was used to determine the mRNA expression of IFN-gamma (Assay ID: Mm00801778_m1) and Foxp3 (Assay ID: Mm00801778_g1) and 18s-rRNA (Assay ID: Hs99999901_s1) as a control. Quantitative data were calculated using the comparative CT ($\Delta\Delta$ CT) method, and the $\Delta\Delta$ CT method using 18s-rRNA as the reference gene was used for relative quantification of IFN-gamma and Foxp3 expression.

Statistical analysis: All values are given as the mean $\pm$ SE. We used Student's *t*-test for comparisons between two groups in the experiment. Differences were considered statistically significant at a value of $P < 0.05$.

Figure 2. Treg improved left ventricular contraction after MI. Fractional shortening (FS) in the control group (control; $n = 7$), lymphocyte-transferred group (LT control; $n = 4$) and Treg-treated group (treatment; $n = 6$) 14 days after MI operation (A). Representative echocardiography of pre- and post-MI operation (control, LT control and treatment group) are shown (B). In the control group and LT control group, anterior wall movement was severely impaired. However, Treg treatment negated MI-induced impairment of LV contraction. * indicates a P value of < 0.05 .

RESULTS

Purity of Treg: FACS analysis revealed that the purity of the CD4+CD25+ Treg cell population was 93.11% (Figure 1).

Treg negated the decline of LV contraction after MI: M-mode echocardiograms showed that Treg injection significantly negated impaired LV contraction and FS decline induced by MI compared to the control and LT control group (Figure 2). There were no statistically significant differences in heart rate and systolic blood pressure between the preoperation mice and

Figure 3. Change in ratio of Tregs. Representative FACS data (A) and statistical analysis (B and C) are shown. There was a statistical difference between day 0 and 6 hours, and between day 1 and day 4 (B). The Treg ratio of the treatment group mice was significantly smaller than that of the no treatment group mice (C). * $P < 0.05$.

Figure 4. Tregs in hearts and spleens. All cell nuclei were stained with DAPI and colored blue. The injected Tregs had red areas around the blue-colored nuclei.

Figure 5. Representative histopathology with Mallory staining (A) and statistical data (B) are shown. Control group exhibited a significantly larger fibrotic area than that of the treatment group mice; the sham-operated mice heart had no fibrosis (Scale bars, 1 mm). The infarct ratios (infarct size/noninfarct size + infarct size) of the control group were larger than that of the treatment group and there was a statistically significant difference * $P < 0.05$.

postoperation mice, or between the control treatment group and LT control group (data not shown).

Treg population in myocardium after MI: The Treg population in hearts with MI was analyzed at each time point. According to the results of FACS, the ratio of CD4+CD25+ Tregs was increased from day 0 (no ligation group) to 6 hours, and then decreased until day 4 and remained constant to day 14. There was a significant difference between day 0 and 6 hours and between day 1 and day 4. FACS analysis revealed that Treg treatment reduced the CD4+CD25+ Treg ratio in infarct myocardium at day 1, and there was a significant difference between the treatment group and non-treatment group (Figure 3).

Injected Treg was predominantly located in spleens: One day

Figure 6. RT-PCR. The mRNA levels of Foxp3 in spleens (A) and IFN-gamma in hearts (B) are shown. The levels of IFN-gamma in hearts on day 3 and Foxp3 in spleens on day 1 were statistically different between the two groups. * $P < 0.05$.

after stained Treg injection, the mice were sacrificed and the hearts and spleens were obtained and sliced. The heart slices were obtained from the infarcted and noninfarcted areas. A few stained Tregs were observed in the infarcted and noninfarcted areas, while many were detected in the spleens. The number of Treg was smaller in the hearts than the spleens (Figure 4).

Treg treatment negated the adverse LV remodeling after MI: Pathological samples with Mallory staining of each group (control, LT control, and treatment) on day 14 showed that broad fibrosis and huge cell infiltration were found in the walls of the hearts in the control group. However, infarct size in the treatment group hearts was significantly less. The infarct ratios (infarct size/noninfarct size + infarct size) in the control and LT control groups were larger than that of the treatment group and there was a significant difference (Figure 5).

Altered IFN-gamma and Foxp3 mRNA levels in hearts and spleens: The hearts and spleens were obtained from control and treatment group mice on day 1 and day 3 after the MI operation. The mRNA levels of IFN-gamma in hearts and Foxp3 in spleens were measured using RT-PCR. The levels of IFN-gamma in hearts on day 3 and Foxp3 in spleens on day 1 were statistically different between the two groups (Figure 6).

DISCUSSION

Inflammatory processes contribute substantially to ischemic cardiac damage. Although the search for new therapies is a focus of current translational cardiovascular research, the pathophysiology of intrinsic immune system modulation is barely understood. In this study, parts of the pathophysiology were clarified.

Foxp3-expressing Treg cells suppress pathology mediated by immune responses against self and sustained expression of the transcription factor Foxp3.¹⁰ In our study, Treg cell recipients exhibited a significant reduction in inflammation. This was characterized by reduced IFN-gamma expression and increased Foxp3 expression, which might lead to suppression of inflammation after MI. In a hypertensive experimental model, inflammation occurred and led to cardiac fibrosis, which was attenuated by adoptive Treg transfer.¹¹ Thus, it suggests that the cardiac fibrosis after MI and following inflammation was attenuated by adoptive Treg transfer, which resulted in improvement of FS. We purified CD4+CD25+ T cells as Tregs from spleen cells and we did not use CD4+CD25+Foxp3+ triple positive T cells. This was because some recent Treg-associated studies also used CD4+CD25+ T cells as Treg and proved it was effective for treating autoimmune and inflammatory disease.^{12,13} Timely resolution of inflammation appears to be essential for prevention of adverse remodeling after MI.^{14,15} Uncontrolled postinfarction inflammation may promote adverse remodeling through several distinct mechanisms. First, proinflammatory cytokines may enhance MMP synthesis,¹⁶ increasing matrix degradation in the infarcted hearts. Second, inflammatory mediators, such as TNF-alpha and IL-1-beta, may enhance cardiomyocyte apoptosis in the remodeling myocardium.¹⁷ Third, proinflammatory cytokine signaling may suppress contractility accentuating ventricular systolic dysfunction.¹⁸ A past cerebral ischemic model study showed IFN-gamma mRNA expression increased after cerebral infarction and was more sustained than TNF-alpha and IL-1-beta mRNA expression.¹⁹ IFN-gamma maintains inflammatory reactions and may influence cerebral tissue necrosis, vascular adhesion molecule expression, and microglial activation.^{20,21} We proved that Treg treatment attenuated mRNA expression of IFN-gamma and that this must have contributed to a reduction of destructive inflammation in hearts.

In a recent study of autoimmunity-mediated diseases, trafficking of Treg cells into inflammatory lesions was found to potentially be crucial for *in vivo* suppression of autoimmunity.²² On the other hand, it was reported that antigen-specific Treg cells expanded in the peripheral lymphoid compartment and readily accumulated in the central nerve system. However, they did not prevent the onset of the disease using experimental autoimmune encephalomyelitis.²³ In our study, we injected stained Treg cells into operated mice, which showed that many of the injected Treg cells migrated to or were captured in the spleen, and fewer Treg cells were found in the heart. Our data demonstrated that the levels of Foxp3 expression were higher in the treatment group than in the control group, but the ratios of CD4+CD25+ Treg cells in hearts after MI were higher in the group without treatment than in the group with treatment. This seemed incompatible but it suggested that attenuation of inflammation in the heart and activation of Foxp3 in the spleen decreased the need for Treg cells to migrate into heart. As a re-

sult, the anti-inflammatory effect on myocardium worked indirectly. The lower ratio of Tregs in the infarcted myocardium could reflect the lower level of inflammation rather than the anti-inflammatory effect. The mechanism of remote anti-inflammatory effect on infarcted myocardium by Tregs in the spleen should be analyzed. It has also been shown that defective control of the postinfarction inflammatory responses in CC chemokine receptor 5 (CCR5) -/- animals were associated with impaired recruitment of CCR5+Foxp3+ Tregs. This suggests that chemokine signaling through CCR5 prevents uncontrolled inflammation in the infarcted myocardium, attenuating matrix degradation and adverse cardiac remodeling.²⁴

In conclusion, we proved that adoptive Treg transfer could attenuate cardiac dysfunction after MI via preventing expansion of inflammation and fibrosis. Further studies should be conducted to explore the clinical utility of Treg for the prevention of chronic adverse LV remodeling after MI.

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