Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice

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Aims
Although recent animal studies have investigated the cellular and molecular mechanisms underlying the process of atherosclerosis regression, it remains unknown whether adaptive immune responses including T cells are involved in this process. We investigated the role of T cells in atherosclerosis regression.

Methods and results
LDL receptor-deficient mice were fed a high-cholesterol diet for 8 weeks to form atherosclerotic lesions and were then changed to a standard diet, and atherosclerosis was assessed 4 weeks later. Just before changing the diet, the mice received an iv injection of anti-CD3 antibody (CD3-Ab) or control immunoglobulin G for 5 consecutive days. CD3-Ab treatment regressed atherosclerosis and decreased the accumulation of macrophages and CD4+ T cells in the plaques. CD3-Ab treatment also dramatically reduced CD4+ T cells and increased the proportion of regulatory T cells (Tregs). Depletion of Tregs by anti-CD25 antibody injection abolished the regression of atherosclerosis seen in CD3-Ab-treated mice, indicating the essential role for Tregs in this process.

Conclusion
CD3-Ab treatment induced rapid regression of established atherosclerosis via reducing CD4+ T cells and increasing the proportion of Tregs. These findings suggest that therapeutic intervention for T-cell-mediated immune responses may represent a novel strategy to induce atherosclerosis regression in combination with lipid-lowering therapy.

Keywords
Atherosclerosis • Regression • Inflammation • Lymphocytes • Regulatory T-cell

1. Introduction
Recent clinical trials and animal studies have demonstrated that lowering LDL-cholesterol levels, increasing HDL-cholesterol levels, or dampening of chronic inflammation result in atherosclerosis regression.1–8 in association with a reduction in cardiovascular events. Atherosclerosis is an inflammatory condition of the arterial wall involving cells of the innate and adaptive immune systems9 and therapeutic intervention of inflammatory responses including various immune cells could represent a promising therapeutic strategy to improve cardiovascular outcome. The mechanism of plaque development has been extensively examined, but mechanisms that reverse the disease are not well understood. Although several studies in mouse models of atherosclerosis regression have investigated the cellular and molecular mechanisms underlying the process of regression during the lipid-lowering therapy,2,3,5,7 the mechanisms that promote lesion regression have not been fully clarified. A thorough understanding of these mechanisms could lead to new therapeutic methods to enhance plaque regression and reduce cardiovascular events.

Previous studies in mouse models of transplant atherosclerosis regression have shown that decreasing plasma non-HDL-cholesterol or increasing HDL promotes the emigration of macrophages from the atherosclerotic plaques, which may contribute to promoting atherosclerosis regression.2,3 In addition to macrophage activation, adaptive immunity, such as T cell-mediated pathogenic immune responses, plays an important role in the inflammatory process during atherogenesis.10 However, the role of T cells in atherosclerosis regression remains completely unknown. After stimulation by several cytokines in the presence of specific antigens, naive CD4+ T cells differentiate into effector T cells (Teffs) such as the T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) lineages.9 Recent evidence indicates that several subsets of regulatory T cells (Tregs), which actively suppress excessive immune responses, inhibit atherosclerosis development or progression by down-regulating Teff responses, suggesting that
the balance between pathogenic Teffs and Tregs may be important for controlling atherosclerotic diseases. 11–13

Anti-CD3-specific antibody (CD3-Ab) strongly suppresses immune responses by antigenic modulation of the T-cell receptor–CD3 complex, resulting in antigenic modulation, anergy, and apoptosis of activated T cells. 14 Notably, iv administration of FcR-non-binding CD3-Ab has been shown to be effective for suppressing atherosclerosis development and progression in mice, 15 autoimmune diabetes in mice and humans, and acute transplant rejection in humans through downregulation of Teff responses and induction of transforming growth factor (TGF)-β-producing CD4+CD25+ Tregs. 14 In addition, recent studies have demonstrated that oral or nasal administration of CD3-Ab, with or without the Fc portion, induces Tregs expressing a latency-associated peptide that suppresses several autoimmune diseases and atherosclerosis development in mice through a TGF-β-dependent mechanism. 12,16,17 These data suggest that the beneficial effects of CD3-Ab may involve suppressing Teff responses and increasing Treg-to-Teff ratios at later time points, implying that this therapy could be used in treating established atherosclerosis in the clinic.

Based on this background information, we tested the impact of T-cell modulation by FcR-non-binding CD3-Ab on atherosclerosis regression in a recently established mouse atherosclerosis regression model. 4 We demonstrated for the first time that iv administration of CD3-Ab could induce rapid regression of atherosclerosis by favourably modulating the balance between CD4+ T cells and Tregs in both lymphoid organs and atherosclerotic plaques. Our findings indicate that modulation of adaptive immune responses, such as reduction of CD4+ T cells and induction of Tregs, could be a promising therapeutic strategy in combination with aggressive lipid-lowering therapies for regressing established plaques.

2. Methods

2.1 Animals and experimental design

We used a previously reported atherosclerosis regression mouse model. 4 Six-week-old male LDL receptor knockout (LDLR−/−) mice on C57BL/6 background were fed a high-fat and -cholesterol diet for 8 weeks to form atherosclerotic lesions in the aortic sinus. At 14 weeks of age, the diet was changed to a normal chow, and 50 μg of anti-CD3 antibody (F(ab)2 (145-2C11; Bio X cell, West Lebanon, NH, USA) or 50 μg of isotype-matched Hamster immunoglobulin G (F(ab)2)2 (control IgG) (Bio X cell) was injected into the mice for five consecutive days. In several experiments, mice received ip injection of 100 μg of anti-CD25 antibody (CD25-Ab) (clone PC61; Bio X cell) to deplete CD4+CD25+Foxp3+ Tregs or 100 μg of isotype-matched rat IgG (Bio X cell) a week after the first injection of CD3-Ab or control IgG. Mice were euthanized under anaesthesia using 2,2,2-tribromoethanol (250 mg/kg ip injection) at 14, 16, and 18 weeks of age, and atherosclerotic lesions were assessed as previously described. 4 Blood was taken for chemical analyses. All animal experiments were conducted in accordance to the Guidelines for Animal Experiments at Kobe University School of Medicine.

2.2 Atherosclerotic lesions assessments

Atherosclerotic lesion size was analysed as previously described. 4 Immunohistochemistry was performed to identify macrophages (MOMA-2, BMA Biomedicals, Augst, Switzerland), CD4+ T cells (CD4, BD Biosciences), and Foxp3+ cells (Foxp3, eBioscience). Staining with Masson’s trichrome was used to delineate the fibrous area. Sections were observed with a microscope (BZ-8000; Keyence, Osaka, Japan).

2.3 Flow cytometric analysis

Flow cytometric analyses were performed to determine the phenotype of T cells, dendritic cells (DCs), and monocytes. Furthermore, intracellular cytokine staining was performed for phenotypic assessment of CD4+ T cells. These analyses were done by a FACSCalibur (BD Bioscience, Franklin Lakes, NJ, USA) or an Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA). Used antibodies and more precise methods are described in Supplementary material online. Methods.

2.4 Quantitative real-time reverse transcriptase polymerase chain reaction (real-time qRT–PCR) analysis

Total RNA was extracted from the aortas after perfusion with RNA later (Ambion, Austin, TX, USA) using the TRIzol reagent (Life Technologies). Real-time qRT–PCR was performed using the One Step SYBR PrimeScript RT–PCR Kit (Takara, Shiga, Japan) and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol.

2.5 Statistical analysis

Data were expressed as the means ± SEM. The Mann–Whitney U test was used to detect significant differences between two groups. The Kruskal–Wallis test was used to detect significant differences when comparing more than three groups. A P < 0.05 was considered statistically significant. Detailed methods are provided in Supplementary material online.

3. Results

3.1 CD3-Ab induced regression of established atherosclerosis

Recently, we and another group simultaneously developed a nearly identical atherosclerosis regression mouse model, 5,6 and the experimental design is outlined in Figure 1A. Briefly, 6-week-old LDLR−/− mice were fed a high-cholesterol diet for 8 weeks to form atherosclerosis in the aortic sinus. We defined 14-week-old mice as the baseline and assessed the atherosclerotic lesion size (baseline: 610.9 ± 30.9 × 103 μm2, Figure 1B and C). At 14 weeks, we changed the diet to a standard diet, and CD3-Ab or control IgG was intravenously injected for five consecutive days. Change of the diet decreased the plasma non-HDL-cholesterol level in both groups compared with the baseline as shown in Table 1, while no significant differences were observed between the two groups. To evaluate whether regression of atherosclerosis was achieved, the lesion area at the aortic sinus was assessed at 16 and 18 weeks for each group (Figure 1C). No significant decrease in the plaque area was observed in the control group at both 16 and 18 weeks (control at 18 weeks: 260.7 ± 28.4 × 103 μm2) compared with the baseline. Conversely, the established atherosclerosis was significantly regressed in the CD3-Ab group at 18 weeks (CD3-Ab at 18 weeks: 459.8 ± 15.2 × 103 μm2; −24.7% vs. the baseline; −25.9% vs. the control, P < 0.01, Figure 1B and C). Consistent with this, the lipid content of the established plaques was also significantly decreased in the CD3-Ab group compared with the baseline (Figure 1D). On the other hand, neither regression nor decrease in atherosclerosis progression was observed in CD3-Ab-injected mice under a high-fat and -cholesterol diet (see Supplementary material online, Figure S1).
3.2 Effects of CD3-Ab injection on CD4⁺ T cells in lymphoid organs

Peripheral leucocyte counts were examined at 16 weeks and no statistical differences in blood leucocyte, lymphocyte, platelet, or erythrocyte counts were found among the three groups (see Supplementary material online, Figure S2). We performed flow cytometric analysis to investigate the effects of CD3-Ab on the number and function of CD4⁺ T cells in the spleen and peripheral lymph nodes (LNs) (Figure 2). In the control group, the total number of CD4⁺ T cells in the spleen and LNs was significantly reduced at 16 weeks compared with the baseline \( (P < 0.01; \text{Figure 2A}) \). Thus, normalization of plasma cholesterol may decrease the number of CD4⁺ T cells under hypercholesterolaemia. However, a further decrease in number of CD4⁺ T cells in the CD3-Ab group was seen at 16 weeks compared with the control group \( (P < 0.01; \text{Figure 2A}) \). At 18 weeks, the number of CD4⁺ T cells was still significantly reduced in the CD3-Ab group in the spleen \( (P < 0.01) \) and LNs \( (P < 0.05) \) compared with the baseline but not the control group \( (P < 0.01; \text{Figure 2A}) \). Similar phenomena were observed for the number of CD4⁺ CD25⁺ Foxp3⁺ Tregs \( (P < 0.01) \) and LNs \( (P < 0.05) \) compared with the baseline but not the control group \( (P < 0.01; \text{Figure 2A}) \). At 18 weeks, the number of CD4⁺ T cells was still significantly reduced in the CD3-Ab group in the spleen \( (P < 0.01) \) and LNs \( (P < 0.05) \) compared with the baseline but not the control group \( (P < 0.01; \text{Figure 2A}) \). Similar phenomena were observed for the number of CD4⁺ CD25⁺ Foxp3⁺ Tregs \( (P < 0.01) \) and LNs \( (P < 0.05) \) compared with the baseline but not the control group \( (P < 0.01; \text{Figure 2A}) \). However, the proportion of CD4⁺ CD25⁺ Foxp3⁺ Tregs was significantly increased in the CD3-Ab group at 16 weeks in both the spleen and LNs \( (P < 0.01; \text{Figure 2C and D}) \). This result supports the idea that Tregs are more resistant to CD3-Ab than Teffs, resulting in an increased Treg-to-CD4⁺ T-cell ratio, despite a reduction in absolute number of Tregs. Next, the effects of CD3-Ab on the expression of Treg-associated molecules in CD25⁺ Foxp3⁺ Tregs were determined by flow cytometry. Notably,
Figure 2  Effects of CD3-Ab on CD4+ T cells in the spleen and LNs. (A and B) Quantitative analysis of the number of CD4+ T cells (A) and CD4+CD25+Foxp3+ Tregs (B) in the spleen and LNs. n = 9 for baseline, n = 6 for control, and n = 7 for the CD3-Ab group at 16 and 18 weeks. (C) Representative dot plot of the percentage of CD4+ T cells and CD4+CD25+Foxp3+ T cells by flow cytometry at 14 weeks (baseline), 16 weeks (control and CD3-Ab) mice. (D) Quantitative analysis of the proportion of CD25+Foxp3+ Tregs within CD4+ T cells. (E–G) Graphs represent the percentage of IFN-γ- (E), IL-4- (F), and IL-10- (G) producing cells within the CD4+ T-cell population in the spleen of 14-week-old (baseline) and 16- and 18-week-old (control and CD3-Ab) mice. n = 8 for all groups. *P < 0.05, **P < 0.01 vs. control at the same week, and *#P < 0.05, **##P < 0.01 vs. baseline. (H–I) Graph represents the percentage of Th1-specific transcription factor, T-Bet+ (H) or Th2-specific transcription factor, GATA3+ (I) cells within CD4+ T cells. n = 8 for baseline, control and CD3-Ab groups at 16 weeks and n = 6 for control and CD3-Ab groups at 18 weeks. *P < 0.05, **#P < 0.01 vs. baseline and *P < 0.05 vs. control at the same week.
CD25⁺Foxp3⁺ Tregs from CD3-Ab-treated mice expressed higher levels of CD25, CD103, glucocorticoid-induced TNF receptor family-related gene/protein, cytotoxic T lymphocyte-associated protein 4 compared with those from the baseline and control groups (see Supplementary material online, Figure S3A–C), implying an activated phenotype of Tregs after CD3-Ab treatment.

We also measured proliferation of Tregs and Teffs by Ki-67 staining. As expected, the number of Ki-67-positive T cells was markedly increased in the CD3-Ab group at 16 weeks, indicating that both Tregs and Teffs are vigorously expanding after transient depletion by CD3-Ab injection (see Supplementary material online, Figure S3D and E). Lipid lowering by diet change did not change the number of naive (CD62L⁺CD44⁻) or effector (CD62L⁻CD44⁺) T cells in the spleen and LNs, whereas CD3-Ab treatment significantly decreased the number of naive T cells and the naive/effect T-cell ratio, suggesting that CD3-Ab treatment induces T-cell activation (see Supplementary material online, Figure S3F–H). Next, we investigated the detailed phenotype of CD4⁺ T cells using intracellular cytokine staining. The staining revealed that the percentage of IFN-γ-producing (Th1) cells was not changed by CD3-Ab treatment (Figure 2E), although the number of Th1 cells was markedly reduced in the CD3-Ab group (data not shown). In contrast, the percentage of IL-4-producing (Th2) cells and immunosuppressive cytokine IL-10-producing cells significantly increased (P < 0.05, Figure 2F and G). By the use of flow cytometric analysis, we also examined lineage-restricted transcription factors such as T-box expressed in T cells (T-Bet) or GATA-binding protein 3 (GATA3), which are specifically expressed in Th1 or Th2 lineage cells, respectively. We found that the percentage of T-Bet-expressing cells tended to be decreased, whereas the percentage of GATA3-expressing cells was markedly increased within the CD4⁺ T-cell population in the CD3-Ab group (Figure 2H and I). These results are in line with the data obtained by intracellular cytokine staining.

In summary, CD3-Ab considerably reduced the number of CD4⁺ T cells, increased the proportion and anti-inflammatory capacity of CD4⁺CD25⁺Foxp3⁺ Tregs, and shifted the Th1/Th2 balance to Th2, which may affect the anti-atherogenic effects of CD3-Ab.

Figure 3 Effects of CD3-Ab on CD4⁺ T cells in atherosclerotic lesions. (A) Representative sections of the aortic sinus stained with antibodies specific for CD4⁺ T cells and Foxp3⁺ Tregs in male LDLR⁻/⁻ mice at 14 weeks (baseline) and 18 weeks (control and CD3-Ab). The black bars represent 200 μm in CD4 staining and 100 μm in Foxp3 staining. (B) Quantitative analysis of the immunohistochemical staining for CD4 and Foxp3. Data are presented as means ± SEM; with n = 9 for baseline at 14 weeks, n = 6 for control and CD3-Ab groups at 16 weeks, n = 5 for control group, and n = 8 for the CD3-Ab group at 18 week for CD4 staining and with n = 8 for all groups for Foxp3 staining. (C) Expression of various cytokines (IFN-γ, IL-4, IL-10, and IL-17), T-cell-associated markers (CD4, CD8), and Treg-associated markers (TGF-β, Foxp3) in atherosclerotic aortas was quantified by qRT–PCR and normalized to GAPDH. Fold changes relative to each baseline group are shown. n = 5 for baseline, n = 7 for control and CD3-Ab groups. *P < 0.05 vs. control at the same age, and #P < 0.05 vs. baseline.

3.3 Effects of CD3-Ab on CD4⁺ T cells in atherosclerotic lesion

We investigated the effect of CD3-Ab treatment on CD4⁺ T-cell number in the atherosclerotic lesions by immunohistochemistry (Figure 3). We found that normalization of plasma cholesterol levels significantly decreases the number of CD4⁺ T cells in the lesions (P < 0.05, Figure 3A–C).
To determine whether increased accumulation of Tregs in plaques is involved in regression of atherosclerotic lesions, Treg numbers in the lesions were determined by immunohistochemical analysis using anti-Foxp3 antibody and a marked increase in the number of Foxp3+ Tregs within the plaques was detected in both the control IgG and CD3-Ab groups compared with the baseline (Figure 3A and B). Notably, CD3-Ab treatment further promoted the accumulation of Tregs in atherosclerotic plaques in the diet reversal mice. We also analysed the expression of both Teff- and Treg-associated markers in the lesions by real-time qRT–PCR. Pro-inflammatory cytokine such as IFN-γ was significantly decreased in the CD3-Ab group compared with the baseline, whereas no significant differences in Teff-related cytokines, such as IL-4 or IL-17, were found among the three groups. In addition, no differences in the relative mRNA expressions of Treg-associated molecules such as Foxp3, IL-10, and TGF-β in the plaques were found among the three groups, whereas the expression levels of CD4 and CD8 were significantly decreased in both the control and CD3-Ab groups compared with the baseline (Figure 3C). Taking together with the observation that CD3-Ab-treated mice had less plaque in the aorta compared with control IgG-treated mice, these findings were consistent with the immunostaining results shown above.

These findings suggest that reversal of hypercholesterolaemia by changing the diet promotes accumulation of Tregs and inhibits infiltration of CD4+ T cells in atherosclerotic plaques, and that CD3-Ab treatment may augment these beneficial effects associated with diet reversal. We speculate that induction of Tregs in plaques, as well as in lymphoid organs, might also contribute to atherosclerosis regression in our study.

### 3.4 Effects of CD3-Ab on immune cells other than CD4+ T cells and atherosclerotic plaque components

Flow cytometric analysis revealed that CD3-Ab did not alter surface maturation markers CD80 and CD86 in CD11c+ DCs in the spleen and LNs (Figure 4A). We also examined the phenotype of monocytes in the blood. Lymphocyte antigen 6 complex, locus C (Ly6C)high, monocytes constitute a more pro-inflammatory subtype and likely exacerbate atherosclerosis.19 The CD3-Ab injection significantly reduced the number of Ly6Chigh monocytes compared with the baseline at 16 weeks (P < 0.05) (Figure 4B). We next performed immunohistochemical analysis for the atherosclerotic plaque components and found that macrophage accumulation assessed by MOMA2-immunostaining was significantly reduced in the CD3-Ab group compared with the baseline and control groups (Figure 4C and D). Relative smooth muscle cell content in the plaques was significantly increased in the CD3-Ab group compared with the baseline and control groups (Figure 4C and D). Mason’s trichrome staining revealed a significant increase in the collagen content in atherosclerotic lesions in CD3-Ab groups compared with other groups (P < 0.05, Figure 4C and D). We also examined the mRNA expression of cytokines, chemokines, matrix metalloproteinases (MMPs), and adhesion molecules in the atherosclerotic aortas by real-time qRT–PCR (Figure 4E) and found that the mRNA expression of CD68 was significantly reduced in the CD3-Ab group compared with the baseline and control groups, although the expression of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, and monocyte chemoattractant protein (MCP)-1 remained unchanged with CD3-Ab treatment. Neither the mRNA expression of C–C chemokine receptor (CCR) 7, which is associated with emigration of macrophages from atherosclerotic lesion,3 nor the mRNA expression of M2 macrophage marker arginase I18 was affected by the CD3-Ab injection. The mRNA expression of α-actin, which is relevant to smooth muscle cells and stability of atherosclerotic lesion, was significantly increased in both the control group and the CD3-Ab group compared with baseline (Figure 4F). The mRNA expression of MMP2 was similar among the three groups, but that of MMP9 was significantly decreased in the CD3-Ab group (Figure 4G). These results suggest that atherosclerosis regression was associated with reduced macrophage accumulation in the plaques and increased collagen content.

### 3.5 Depletion of Treg abolished CD3-Ab-mediated atherosclerosis regression

Injection of CD3-Ab markedly reduced CD4+ T cells and increased the percentage of athero-protective Tregs not only in lymphoid organs but also in atherosclerotic lesion, resulting in the regression of atherosclerosis. Next, we examined whether a reduction of CD4+ T cells, relative increase in Tregs, or both were critical for regression. It has been reported that administration of anti-CD25 antibody (CD25-Ab) depletes Tregs in vivo, and this effect is maintained for 4 weeks after a...
Figure 5 Depletion of CD4+CD25+Foxp3+ Tregs by ip injection of CD25-Ab abolished the beneficial effects of CD3-Ab on the regression of atherosclerosis. (A) Treg depletion study design. (B) Representative photomicrographs of oil red O staining. The black bar represents 200 μm. (C) Quantitative analysis of atherosclerotic lesion size in the aortic sinus of LDLR-/- mice at 14 and 18 weeks of age. (D) Graphs represent the number of CD4+ T cells in the spleen and LNs. (E) Quantitative analysis of immunohistochemical CD4 staining in atherosclerotic lesions. (F) Graphs represent the percentage of CD25+ Foxp3+ Tregs within the CD4+ T-cell population in the spleen and LNs. (G) Quantitative analysis of immunohistochemical Foxp3 staining in atherosclerotic lesions. All data in Figure 5 are presented as means ± SEM; n = 6 in (C), (E), and (G), and n = 5 in (D) and (F) for each group. #P < 0.05 vs. baseline. *P < 0.05 vs. Hamster IgG plus rat IgG. †P < 0.05 vs. Hamster IgG plus CD25-Ab. ‡P < 0.05 vs. CD3-Ab plus rat IgG. §P < 0.05 vs. CD3-Ab plus CD25-Ab.
single administration. We conducted an in vivo Treg depletion study with the injection of CD25-Ab. We injected 100 μg of CD25-Ab or isotype-matched rat IgG as the control intraperitoneally at 15 weeks (1 week after the first CD3-Ab or control IgG administration) (Figure 5A). Interestingly, CD25-Ab injection abolished the CD3-Ab-mediated atherosclerosis regression, while rat IgG injection did not affect the beneficial effects of CD3-Ab (Figure 5B and C). CD25-Ab did not affect the number of CD4^+ T cells in the spleen, LNs (Figure 5D), and atherosclerotic lesion (Figure 5E) of CD3-Ab-treated mice. We confirmed that CD25-Ab nearly depleted the Tregs as detected by flow cytometry (Figure 5F). Furthermore, immunohistochemical analysis revealed that injection of CD25-Ab nearly abolished the increase of Treg number in the plaques of the CD3-Ab group. Thus, we believe that Tregs play an indispensable role in CD3-Ab-mediated atherosclerosis regression in our mouse model.

4. Discussion

In the current study, we showed that therapeutic intervention of T lymphocytes using CD3-Ab could induce rapid regression of established atherosclerosis and reduce the macrophage content in the atherosclerotic lesion. These beneficial effects are associated with a reduced number of CD4^+ T cells and pro-inflammatory Ly6C^hi monocytes as well as the conversion of Th1 immune responses into Th2 and an increased proportion of Tregs within the CD4^+ T-cell population in the periphery. Atherosclerosis regression was accompanied with an increased proportion of Tregs and a decreased number of CD4^+ T cells in atherosclerotic plaques, as well as in the spleen and LNs. This is the first report of a possible role of CD4^+ T cells linking Tregs to atherosclerosis regression. Interestingly, we observed that Treg depletion by CD25-Ab injection abolished the regression of established atherosclerosis in CD3-Ab-treated mice, despite a significant reduction in CD4^+ T-cell numbers, indicating that in addition to a dramatic reduction of CD4^+ T cells, induction of Tregs may be essential for the regression of atherosclerosis in this model. In summary, these data establish T cell as an attractive therapeutic target for atherosclerosis regression.

We previously reported that eicosapentaenoic acid (EPA) induces atherosclerosis regression by changing DCs to an immature phenotype and reducing CD4^+ T cells. We suppose that the beneficial effects of EPA on the regression of atherosclerosis in this model were mediated at least in part by induced indoleamine 2,3-dioxygenase (IDO) activity in DCs because administration of IDO-inhibitor abolished the anti-atherogenic effects of EPA. IDO is an enzyme involved in tryptophan catabolism and breaks down tryptophan, a critical amino acid for T-cell proliferation. Increasing IDO activity resulted in decreased tryptophan metabolism and breaks down tryptophan, a critical amino acid for T-cell proliferation. Although our previous study suggested the possible involvement of T cells, which are known to play critical roles in atherogenesis, there is no evidence that adaptive immunity including T cells is directly involved in the regression of atherosclerotic plaques. In the present study, using CD3-Ab, we examined the effects of CD4^+ T-cell depletion on the regression of established atherosclerosis. In our mouse atherosclerosis regression model, switching to a standard diet after formation of atherosclerotic lesions at the aortic sinus from a high-cholesterol diet leads to a reduction in the plasma non-HDL-cholesterol level. Although changing the diet inhibited atherosclerosis lesion progression, the plaques did not regress. We found that iv injection of CD3-Ab with lowering plasma cholesterol markedly reduced lesion size, plaque macrophage content, and CD4^+ T-cell content. We also found that the relative collagen content in the aortic sinus plaques increased remarkably in CD3-Ab-treated mice, suggesting the down-regulation of plaque inflammation and increased plaque stability. These data imply that modulation of T lymphocyte number and function by CD3-Ab therapy in combination with lipid-lowering therapy may have beneficial effects on atherosclerosis regression.

A recent study has demonstrated that hypercholesterolaemia impairs Treg but not Teff accumulation in atherosclerotic lesions, resulting in a decreased Treg-to-Teff ratio in the plaques. Moreover, reversal of hypercholesterolaemia was shown to maintain Treg numbers and Treg-to-Teff ratio in atherosclerotic aortas. These results may imply that increasing the proportion of Tregs in atherosclerotic lesions might suppress plaque inflammation and consequently reduce atherosclerosis, although the actual roles of intraplaque Tregs in atherogenesis have not been clarified yet. Consistent with a previous report, we found that reversal of hypercholesterolaemia by change in diet resulted in an increase in Tregs in atherosclerotic plaques, whereas such effect was not observed in lymphoid organs such as the spleen and LNs. In our study, reversal of hypercholesterolaemia did not induce atherosclerosis regression, suggesting that increasing the proportion of Tregs alone in atherosclerotic lesions might not be effective for regressing atherosclerosis. On the other hand, CD3-Ab treatment in the diet reversal mice significantly induced atherosclerosis regression, along with a dramatically increased proportion of Tregs in both lymphoid organs and atherosclerotic plaques. Furthermore, we conducted an in vivo Treg depletion study using CD25-Ab and found that its injection nearly depleted CD4^+CD25^+Foxp3^+ Tregs in the spleen and LNs. Importantly, the increase in Treg numbers and plaque regression from CD3-Ab treatment were abolished with CD25-Ab administration, while we still observed a significant reduction in CD4^+ T-cell numbers in atherosclerotic lesions. These findings suggest that induction of Tregs is essential for the initiation of rapid atherosclerosis regression in our model, although decreased accumulation of CD4^+ T cells in plaques may also contribute to atherosclerotic plaque regression. As the limitation of our Treg depletion study using CD25-Ab, it is possible that administration of this antibody might deplete not only CD25^+ Tregs but also myeloid-derived suppressor cells, which may be one possible explanation for the result of abolished atherosclerosis regression by CD25-Ab treatment. A recent study using a genetic approach has shown that depletion of Foxp3^+ Tregs leads to a substantial increase in atherosclerosis and plasma cholesterol levels. However, in our study, induction of Tregs by CD3-Ab treatment did not affect the plasma lipid profile (Table 1). A few recent papers showed that Tregs inhibit the pro-inflammatory activation of human umbilical vein endothelial cells and also suppress macrophage foam-cell formation through a cell-contact-dependent manner or producing anti-inflammatory cytokines such as IL-10 and TGF-β. Thus, Tregs may directly inhibits the activation or accumulation of macrophages in plaques, which could partly contribute to promoting atherosclerotic lesion regression.

Treatment with IL-2 and anti-IL-2 monoclonal Ab complexes (IL-2 complexes) has been reported to systemically increase Tregs, although it remains unknown whether this treatment can also increase Tregs in atherosclerotic lesions. Recently, Foks et al. demonstrated that expansion of Tregs using this method did not induce atherosclerosis regression in LDLR~/-~ mice fed a western-type diet. To investigate the effect of Treg expansion on atherosclerosis regression without changing the number of CD4^+ T cells, we employed IL-2 complexes in our regression model. Although IL-2 complexes treatment significantly increased the proportion of CD25^+Foxp3^+ Tregs in the CD4^+ T cells in the spleen and LNs without affecting the total CD4^+ T-cell number, atherosclerosis...
regression was not observed (see Supplementary material online, Figure S4), which is consistent with the data of Foks et al.22 On the other hand, this treatment reduced the number of CD4+ T cells in the atherosclerotic plaques of the diet reversal mice, while changes in Treg numbers were absent. These results indicate that treatment with IL-2 complexes does not induce the accumulation of Tregs into plaques despite systemic expansion in the periphery, and may suggest that increasing Tregs only in lymphoid organs may not be effective for regressing atherosclerosis in our model. Furthermore, a mouse autoimmune disease model has demonstrated the importance of controlling local inflammatory responses in target tissues to maintain the suppressive function of Tregs.21 Based on the above findings and previous data, we suppose that preceding suppression of CD4+ T-cell-mediated immune responses before expanding Tregs may be essential for the induction of Tregs in plaques, maintenance of their anti-inflammatory effects, and subsequent induction of atherosclerosis regression. A recent study demonstrated that the C–C-chemokine ligand (CCL) 17 producing monocyte-derived CD11b+ DCs plays pro-atherogenic roles by limiting the expansion of Tregs in lymphoid organs and their accumulation in atherosclerotic plaques and suggests CCL17 as a potential therapeutic target for atherosclerosis.24 Based on our findings, we suppose that blocking this molecule may be effective for regressing atherosclerosis in combination with suppression of CD4+ T-cell-mediated immune response. However, it thus far remains unclear whether Tregs not only in lymphoid organs but also in atherosclerotic lesions play an important role in atherogenesis including plaque regression. Further studies are required to more precisely define the relationship between expansion of Tregs in atherosclerotic plaques and regression of atherosclerosis.

Many reports have focused on monocytes, macrophages, and cholesterol efflux to elucidate the mechanisms of atherosclerosis regression.2,3,5–7 Particularly, the macrophage volume in plaques is a critical factor for regressing atherosclerosis. Monocyte recruitment into plaques occurs during plaque progression, depending on the cholesterol level, or monocyte content in the blood.19,25 In the present study, we found that CD3-Ab treatment significantly reduced pro-inflammatory Ly6Ghigh monocytes in the blood compared with the baseline, 2 weeks after changing the diet. This result represents one possible explanation for the beneficial effect of CD3-Ab treatment on atherosclerosis regression. Furthermore, we performed immunohistochemical analysis to assess changes of the atherosclerotic plaque components before and after regression and found that accumulation of macrophages was markedly reduced in CD3-Ab-treated mice compared with baseline and control groups. There is a report which suggests that reduced monocyte recruitment into the lesions is responsible for the decreased macrophage content in the plaques and atherosclerosis regression,7 whereas others report that atherosclerotic lesion regression occurs through macrophage egression from the arterial wall.3,5 However, it remains to be determined which is more critical in macrophage reduction in the plaques. Recent studies in other models of atherosclerosis regression, using genetic manipulations, have demonstrated that increasing HDL or dramatically decreasing LDL-cholesterol induces atherosclerosis regression, along with reduced markers of M1 macrophages and enriched markers of M2 macrophages: hallmarks of regressing plaques.2,3,5 However, in our study, mRNA expressions of M1 or M2 macrophages markers in atherosclerotic aortas were similar among the three groups, suggesting that macrophage phenotype change in the plaques might not be involved in atherosclerosis regression in this study.

In conclusion, we demonstrated that iv administration of CD3-Ab induced rapid regression of established atherosclerosis via CD4+ T-cell reduction and increased proportion of Tregs in both lymphoid organs and atherosclerotic plaques. A Treg depletion experiment using the depleting antibody CD25-Ab clearly revealed that induction of Tregs is crucial in this process. Our data imply that in combination with aggressive lipid-lowering therapies, therapeutic intervention aimed at enhancing a Treg-mediated immune response may represent a novel therapeutic approach for atherosclerosis regression. Further investigations are required to elucidate a more precise role of the adaptive immune system, including the T-cell-mediated immune response, in atherosclerosis regression.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References


