Research article

Depletion of Foxp3$^+$ regulatory T cells augments CD4$^+$ T cell immune responses in atherosclerosis-prone hypercholesterolemic mice

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ARTICLE INFO

ABSTRACT

Compelling evidence suggests a crucial role for Foxp3$^+$ regulatory T cells (Tregs) in the control of atherosclerosis. Although suppression of pro-inflammatory CD4$^+$ T cell immune responses is supposed to be important for athero-protective action of Foxp3$^+$ Tregs, few studies have provided direct evidence for this protective mechanism. We investigated the impact of Foxp3$^+$ Treg depletion on CD4$^+$ T cell immune responses and the development of atherosclerosis under hypercholesterolemia. We employed DEREG (depletion of regulatory T cells) mice on an atherosclerosis-prone low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) background, which carry a diphtheria toxin (DT) receptor under the control of the *foxp3* gene locus. In these mice, DT injection led to efficient depletion of Foxp3$^+$ Tregs in spleen, lymph nodes and aorta. Depletion of Foxp3$^+$ Tregs augmented CD4$^+$ effector T cell immune responses and aggravated atherosclerosis without affecting plasma lipid profile. Notably, the proportion of pro-inflammatory IFN-γ-producing T cells were increased in spleen and aorta following Foxp3$^+$ Treg depletion, implying that Foxp3$^+$ Tregs efficiently regulate systemic and aortic T cell-mediated inflammatory responses under hypercholesterolemia. Unexpectedly, Foxp3$^+$ Treg depletion resulted in an increase in anti-inflammatory IL-10-producing T cells, which was not sufficient to suppress the augmented proinflammatory T cell immune responses caused by reduced numbers of Foxp3$^+$ Tregs. Our data indicate that Foxp3$^+$ Tregs suppress pro-inflammatory CD4$^+$ T cell immune responses to control atherosclerosis under hypercholesterolemia.

1. Introduction

Ischemic heart disease and stroke caused by atherosclerosis are major causes of mortality worldwide [1]. Clinical and experimental evidence suggests that vascular wall inflammation is critical for the development of atherosclerosis [2]. Hypercholesterolemia promotes accumulation and retention of low-density lipoprotein (LDL) in the arterial intima, which is considered to be the initial inflammatory response in atherosclerosis. Monocytes adhere to the activated endothelium, enter the arterial intima, differentiate into macrophages, and become lipid-laden foam cells after taking up modified LDL particles, leading to activation of other immune responses including T cells and atherosclerotic plaque formation [3]. After antigen-presenting cells present suspected antigens such as LDL or its major component apolipoprotein B-100 [2] to naïve CD4$^+$ T cells, they differentiate into several subsets of CD4$^+$ effector T cells (Teffs) including

T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) lineage. Th1 cells are found in atherosclerotic lesions and drive atherosclerosis by producing pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ [3]. Immune responses mediated by Th2 and Th17 cells differentially affect atherosclerosis depending on their producing cytokines or experimental models [3].

Experimental evidence suggests that several subsets of regulatory T cells (Tregs), which potently regulate excessive immune responses, play a critical role in preventing atherosclerosis by dampening immunoinflammatory responses [4, 5, 6, 7, 8]. Clinical studies reported the association between decreased proportion of Tregs and an increased incidence of coronary atherosclerotic disease [9, 10]. Naturally occurring CD4$^+$ Tregs highly express CD25 (interleukin-2 receptor α-chain) molecule [11], though some Teffs also express this molecule upon activation under inflammatory conditions. A number of previous experimental

studies used the CD25 molecule to examine the role of naturally occurring $CD4^+$ Tregs in the development of atherosclerosis and its underlying mechanisms. Antibody-mediated depletion of $CD4^+CD25^+$ Tregs exacerbated experimental atherosclerosis [5]. In addition, transfer of $CD4^+CD25^+$ Tregs into immune-deficient hyperlipidemic mice protected against atherosclerosis, associated with decreased pro-inflammatory IFN-γ production and increased anti-inflammatory interleukin (IL)-10 production from $CD4^+$ T cells [5]. This report provided the first evidence that $CD4^+CD25^+$ Tregs play athero-protective roles through the regulation of $CD4^+$ T cell-mediated inflammatory responses.

Since $CD4^+CD25^+$ T cell population partly contains Teffs as described above, the data obtained using $CD4^+CD25^+$ Tregs should be carefully interpreted. Natural Tregs specifically express the transcription factor Foxp3 which is essential for their development and functions and is considered to be the most reliable molecular marker [12]. Expansion of $Foxp3^+$ Tregs by administration of IL-2/anti-IL-2 monoclonal antibody complex [13], an anti-CD3 antibody [6, 14], or ultraviolet B (UVB) irradiation [15] has been shown to prevent or regress atherosclerosis by regulating pro-inflammatory T cell immune responses. Klingenberg *et al.* transplanted bone marrow from DEREG (depletion of regulatory T cells) mice, in which $Foxp3^+$ Tregs can be selectively depleted upon diphtheria toxin (DT) administration, into lethally irradiated atherosclerosis-prone LDL receptor-deficient ($Ldlr^{-/-}$) mice and demonstrated that depletion of $Foxp3^+$ Tregs exacerbated atherosclerosis associated with elevated plasma cholesterol levels with an atherogenic lipoprotein profile, providing direct evidence for the role of $Foxp3^+$ Tregs in controlling lipoprotein metabolism and atherosclerosis [7]. Although suppression of pro-inflammatory $CD4^+$ T cell responses is supposed to be important for athero-protective action of $Foxp3^+$ Tregs, few studies have provided direct evidence for this protective mechanism. In the present study, using hypercholesterolemic DEREG mouse model on a $Ldlr^{-/-}$ background (DEREG/$Ldlr^{-/-}$ mice), we investigated the mechanisms of $Foxp3^+$ Treg-mediated athero-protection by focusing on $CD4^+$ T cell immune responses under hypercholesterolemia without invasive procedures such as bone marrow transplantation or lethal irradiation.

2. Materials and methods

2.1. Animals

All mice were on a C57BL/6 background. $Ldlr^{-/-}$ mice are previously described [16]. All mice were fed a normal chow (CLEA, Tokyo, Japan) and water ad libitum and switched to a high-cholesterol diet containing 1.25% cholesterol (CLEA, Tokyo, Japan) at 8 weeks of age which was continued until the sacrifice day. For *in vivo* selective depletion of $CD4^+Foxp3^+$ Tregs, we used DEREG mice [17] on a LDL receptor-deficient background (DEREG/$Ldlr^{-/-}$ mice) which express a DT receptor under the control of the *foxp3* gene locus. To deplete $CD4^+Foxp3^+$ Tregs, DEREG/$Ldlr^{-/-}$ mice were intraperitoneally injected with DT (125 ng/mouse; Sigma) diluted in endotoxin-free phosphate-buffered saline (PBS) at the specified timing in each experiment. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Kobe University Graduate School of Medicine (Permit Numbers: P091004-R1, P120502, P120502-R1) and by the Animal Care Committee of Kobe Pharmaceutical University (Permit Numbers: 2016-051, 2017-022, 2018-001, 2019-006, 2020-045) and conform to the NIH guidelines.

2.2. Assessment of biochemical parameters

After overnight fasting for 12 h, blood was collected by the cardiac puncture using a syringe with 26 gauge needle under anesthesia and plasma was obtained by centrifugation. Concentrations of total cholesterol, LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglyceride in plasma were analyzed enzymatically using an automated chemistry analyzer (SRL, Tokyo, Japan).

2.3. Atherosclerotic lesion assessment

The mice were euthanized by blood loss under anesthesia, and the aorta was perfused with saline and dissected for the examination of atherosclerotic lesions in the aortic root. The aortic sinus samples were embedded in OCT compounds (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Five consecutive sections (10 μm thickness), spanning 600 μm of the aortic sinus, were collected from each mouse and stained with Oil Red O (Sigma) or hematoxylin-eosin. Stained sections were digitally captured using an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence, Osaka, Japan). For quantitative analysis of atherosclerosis, the total lesion area of 5 separate sections from each mouse was obtained with the use of the ImageJ (National Institutes of Health) as previously described [15].

2.4. Immunohistochemical analysis of atherosclerotic lesions

Immunohistochemistry was performed on cryosections (10 μm) of aortic roots using antibodies to identify macrophages (MOMA-2, 1:400; #T-2007, BMA Biomedicals) or T cells (CD4, 1:100; #550280, BD Biosciences), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Smooth muscle cells were identified by immunostaining with fluorescein isothiocyanate (FITC)-conjugated primary antibody against α-smooth muscle actin (clone 1A4, 1:400; #F3777, Sigma), followed by anti-FITC biotin-conjugated secondary antibody (1:400; #B0287, Sigma). The appropriate fixation reagent depending on the primary antibodies was used. Sections were incubated with the primary antibodies overnight at 4 °C and with the secondary antibodies at room temperature for 1 h. The fibrous area was determined by staining with Masson's trichrome. Stained sections were digitally captured using an All-in-one Type Fluorescence Microscope, and the percentage of the stained area (the stained area per total atherosclerotic lesion area) was calculated.

2.5. Flow cytometry

For fluorescent-activated cell sorter analysis of lymphoid tissues, inguinal and axillary lymph node (LN) cells, splenocytes, and aortic immune cells were isolated and stained in PBS containing 2% fetal calf serum. For isolation of immune cells within aorta, the aorta was digested with Librase TM (Roche Diagnostics) in plane RPMI 1640 medium (Sigma) at 37 °C for 1 h with vortex and a cell suspension was obtained. For intracellular cytokine staining, splenocytes or aortic immune cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 1 mmol/L ionomycin (Sigma) for 5 h in RPMI 1640 medium in the presence of GolgiStop (BD Biosciences). After staining for surface antigens, intracellular cytokine staining was performed using an intracellular cytokine staining kit (BD Biosciences) and anti-cytokine antibodies according to the manufacturer's instructions. Flow cytometric analysis was performed by Attune Acoustic Focusing Cytometer (Life Technologies) using FlowJo software (Tree Star). The antibodies used were as follows; anti-CD3 (clone 145-2C11; #552774, BD Biosciences), anti-CD4 (clone H129.19, clone GK1.5; #553651, #563933, BD Biosciences), anti-CD25 (clone PC61; #553866, BD Biosciences), anti-CD45 (clone 30-F11; #550994, BD Biosciences), anti-CD49b (clone HM alpha 2; #558295, BD Biosciences), anti-CD223 (clone C9B7W; #552380, BD Biosciences), anti-Foxp3 (clone FJK-16s; #17-5773-82, Thermo Fisher Scientific), anti-Ki-67 (clone B56; #556027, BD Biosciences), anti-T-box expressed in T cells (T-bet) (clone O4-46; #561268, BD Bioscience), anti-retinoic acid-related orphan receptor gamma t (RORγt) (clone Q31-378; #562682, BD Bioscience), anti-GATA3 (clone L50-823; #560074, BD Bioscience), anti-IL-4, (clone BVD4-1D11; #12-7041-82, Thermo Fisher Scientific), anti-IL-10 (clone JES5-16E3; #17-7101-82, Thermo Fisher Scientific), anti-IL-17 (clone 17-B7; #17-7177-81, BD Bioscience), anti-IFN-γ (cloneXMG1.2; #12-7311-82, Thermo Fisher Scientific), and isotype matched control antibodies. Intracellular staining of Foxp3 was

performed using the Foxp3 staining buffer set (Thermo Fisher Scientific) according to the manufacturer's instructions. All staining procedure was performed after blocking Fc receptor with anti-CD16/CD32 (clone 2.4G2; #553142, BD Bioscience). Surface staining was performed according to standard procedures at a density of $5–10 \times 10^5$ cells per 50 μL, and volumes were scaled up accordingly. Anti-CD4 antibodies were used at 1:200 dilution and other antibodies were used at 1:100 dilution.

2.6. Cytokine assay

In cell culture experiments, we used RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μmol/L 2β-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin. For the culture of splenocytes, whole isolated cells were cultured in 24-well plates in RPMI 1640 medium at a concentration of 4×10^6 cells/mL and stimulated with 2 μg/mL concanavalin A (Sigma). Culture supernatants were collected at 72 h and analyzed by ELISA for IL-4, IL-10, IL-17, and IFN-γ using paired antibodies specific for corresponding cytokines (#MAB404-500, #BAF404, #MAB417-500, #BAF417, #MAB721-500, #BAF421, #MAB785-500, #BAF485) according to the manufacturer's instructions (R & D Systems).

2.7. Statistical analysis

Unpaired *t*-test was used to detect significant differences between 2 groups. A value of $P < 0.05$ was considered statistically significant. The data on body weight and plasma lipid profile in DEREG/*Ldlr*$^{-/-}$ and control *Ldlr*$^{-/-}$ mice treated with DT or PBS are expressed as box-and-whisker plots where the boxes represent the median values and the interquartile ranges and the whiskers represent the minimum and maximum values. The other data are expressed as mean ± s.d. For statistical analysis, GraphPad Prism version 7.0 (GraphPad Software Inc.) was used. Randomization was used whenever possible. Investigators were not blinded to data collection and outcome assessment.

3. Results

3.1. Efficient depletion of Foxp3$^+$ Tregs in DEREG/Ldlr$^{-/-}$ mice

To investigate the efficacy of Treg depletion in hypercholesterolemic DEREG/*Ldlr*$^{-/-}$ mice, we injected DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice with a single dose of DT and evaluated the percentage of Foxp3$^+$ Tregs in spleen and aorta on the next day. Consistent with previous reports using DEREG mice on wild-type or *Ldlr*$^{-/-}$ background [7, 17], DT injection in DEREG/*Ldlr*$^{-/-}$ mice led to efficient depletion of Foxp3$^+$ Tregs in spleen and aorta (Figures 1A–C). However, the effect of DT treatment on aortic Tregs at this time point was not examined in the previous studies. To further investigate the long-term effect of DT treatment on the percentage of Foxp3$^+$ Tregs in lymphoid tissues, we injected DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice with DT twice per week for 4 weeks and examined the percentage of CD4$^+$ T cells and Foxp3$^+$ Tregs in spleen and peripheral LNs. We found no difference in the percentage of CD4$^+$ T cells in spleen and peripheral LNs in these mice (Figure 1D). Long-term Treg depletion was modestly achieved both in spleen and peripheral LNs (Figure 1E), which may be due to a compensatory replenishment of DT-resistant Tregs following depletion [7].

3.2. Accelerated development of atherosclerosis following Foxp3$^+$ Treg depletion

To investigate the effect of Foxp3$^+$ Treg depletion on the development of atherosclerosis under hypercholesterolemia, we injected DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice with DT or PBS twice per week for 4 weeks and examined atherosclerotic lesions in the aortic root. Foxp3$^+$ Treg depletion did not affect plasma lipid profile, while it modestly decreased body weight (Figure 2A). We found a marked increase in atherosclerotic lesion formation in the aortic root of Foxp3$^+$ Treg-depleted DEREG/*Ldlr*$^{-/-}$ mice compared with control *Ldlr*$^{-/-}$ mice treated with DT (Figure 2B). No significant differences in body weight,
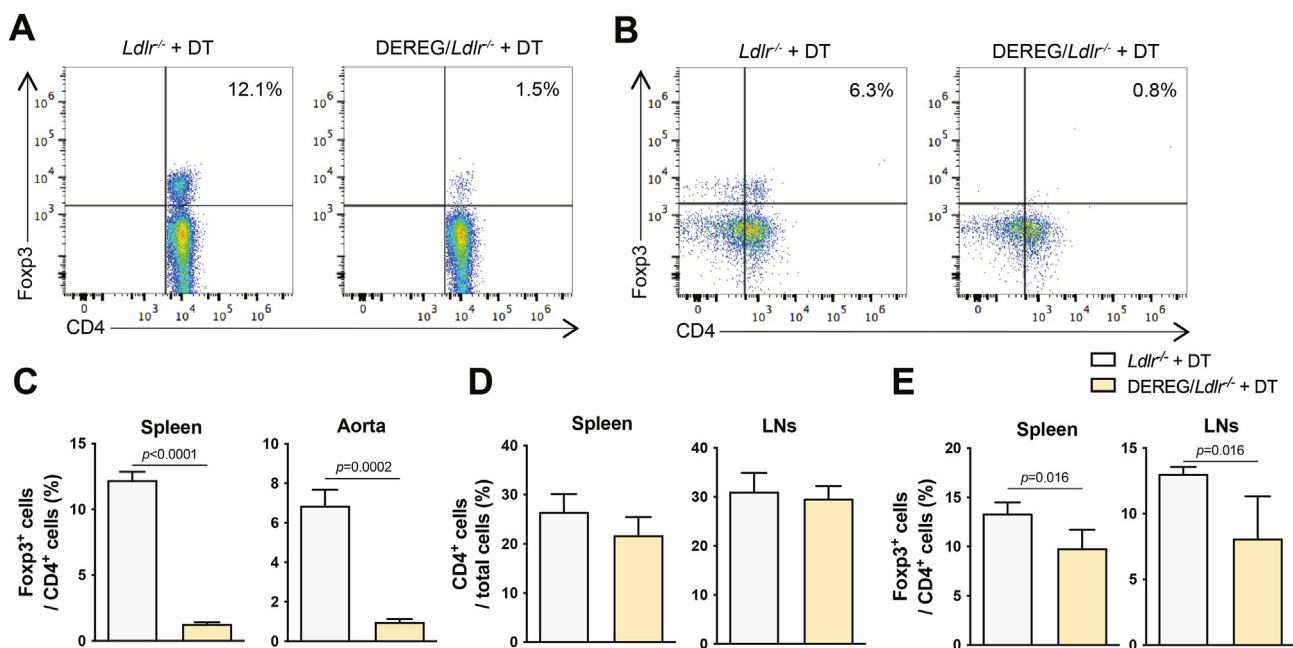

Figure 1. DT administration leads to the efficient depletion of Foxp3$^+$ Tregs in DEREG/*Ldlr*$^{-/-}$ mice. (A–C) Ten-week-old male DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice fed a high-cholesterol diet were injected with DT once and immune responses were evaluated by flow cytometry on the next day. Representative results of CD4 and Foxp3 expression in spleen (A) and aorta (B). Each number shown in the dot plot means the percentage of Foxp3$^+$ Tregs within the CD4$^+$ population in spleen and aorta. (C) The graphs represent the percentage of Foxp3$^+$ Tregs within the CD4$^+$ population in spleen and aorta. n = 4 per group. (D and E) Ten-week-old male DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice fed a high-cholesterol diet were injected with DT twice per week for 4 weeks and immune responses were evaluated by flow cytometry. The graphs represent the percentage of CD4$^+$ T cells within total cells (D) and Foxp3$^+$ Tregs within the CD4$^+$ population (E) in spleen and LNs. n = 4 to 5 mice per group.
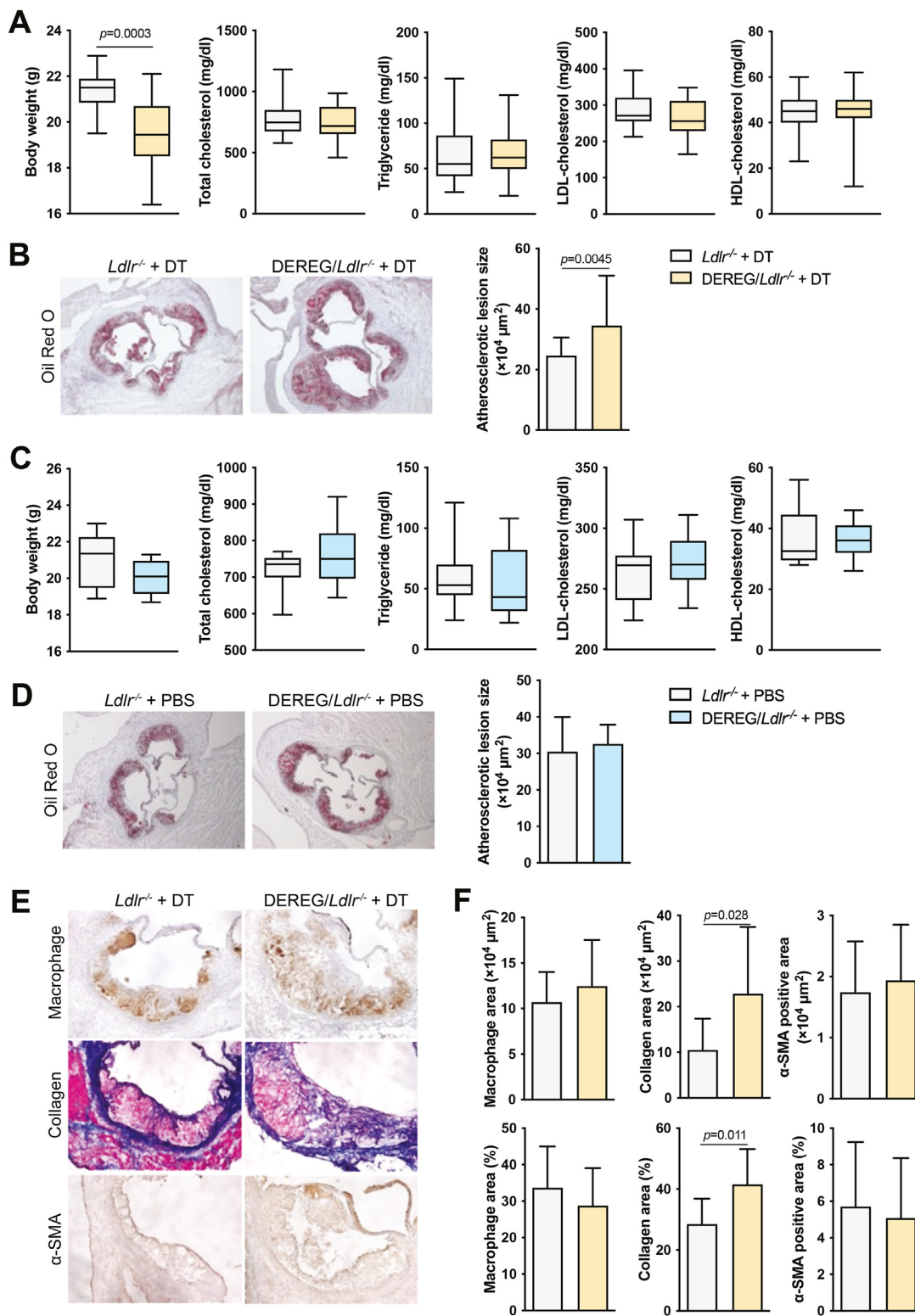
Figure 2. Depletion of Foxp3^{+} Tregs accelerates the development of atherosclerosis in DEREG/$Ldlr^{-/-}$ mice. Ten-week-old male DEREG/$Ldlr^{-/-}$ or control $Ldlr^{-/-}$ mice fed a high-cholesterol diet were injected with DT or PBS twice per week for 4 weeks, and body weight, plasma lipid profile, and atherosclerotic lesions were assessed at 14 weeks of age. (A) Body weight and plasma lipid profile in DEREG/$Ldlr^{-/-}$ or control $Ldlr^{-/-}$ mice treated with DT. n = 21 to 22 per group. (B) Representative photomicrographs of Oil Red O staining and quantitative analysis of atherosclerotic lesion area in the aortic sinus of DEREG/$Ldlr^{-/-}$ mice or control $Ldlr^{-/-}$ mice treated with DT. n = 21 to 22 per group. (C) Body weight and plasma lipid profile in DEREG/$Ldlr^{-/-}$ or control $Ldlr^{-/-}$ mice treated with PBS. n = 9 to 11 per group. (D) Representative photomicrographs of Oil Red O staining and quantitative analysis of atherosclerotic lesion area in the aortic sinus of DEREG/$Ldlr^{-/-}$ mice or control $Ldlr^{-/-}$ mice treated with PBS. n = 10 to 11 per group. (E and F) Representative photomicrographs (E) and quantitative analyses (F) of MOMA-2^{+} macrophages, collagen, and α-smooth muscle actin[+] smooth muscle cells in the aortic sinus of DEREG/$Ldlr^{-/-}$ mice or control $Ldlr^{-/-}$ mice treated with DT. n = 8 to 10 per group. HDL, high-density lipoprotein; LDL indicates low-density lipoprotein.

plasma lipid profile, or atherosclerotic lesions in the aortic root were observed between DEREG/$Ldlr^{-/-}$ and $Ldlr^{-/-}$ mice treated with PBS (Figures 2C and D). To determine the effects of Foxp3$^+$ Treg depletion on plaque component, we performed immunohistochemical studies of atherosclerotic lesions in the aortic sinus. We found no changes in the accumulation of macrophages and smooth muscle cell content in the aortic sinus plaques, whereas a significant increase in collagen content in the lesions was observed under Foxp3$^+$ Treg-depleted conditions (Figures 2E and F). These data provide evidence that Foxp3$^+$ Tregs play a critical role in limiting atherosclerotic lesion development with minor effects on plaque components.

3.3. Effect of Foxp3$^+$ Treg depletion on the activation of aortic CD4$^+$ T cells

We examined CD4$^+$ T cell infiltration into the aortic sinus plaques of DEREG/$Ldlr^{-/-}$ or control $Ldlr^{-/-}$ mice injected with DT twice per week for 4 weeks. Foxp3$^+$ Treg depletion for 4 weeks led to a marked increase in CD4$^+$ T cell accumulation into the atherosclerotic lesions (Figure 3A). We also analyzed the percentage of CD4$^+$ T cells including Foxp3$^+$ Tregs by FACS analysis of collagenase-digested atherosclerotic aortas. Consistent with the immunohistochemical analysis data on CD4$^+$ T cells, the percentage of CD4$^+$ T cells in atherosclerotic aorta had a tendency toward increase in DT-treated DEREG/$Ldlr^{-/-}$ mice, although this did not reach a statistical significance (Figure 3B). As expected, the percentage of Foxp3$^+$ Tregs in atherosclerotic aorta was markedly decreased in

DEREG/$Ldlr^{-/-}$ mice following DT treatment for 4 weeks (Figure 3B). Notably, the expression levels of activation marker CD25 and cell proliferation marker Ki-67 in aortic CD4$^+$Foxp3$^-$ T cells were markedly upregulated in these mice (Figure 3C). To determine whether Foxp3$^+$ Treg depletion affects aortic T cell responses such as cytokine secretion, we performed intracellular cytokine staining of lymphocytes isolated from collagenase-digested atherosclerotic aortas. Interestingly, Foxp3$^+$ Treg depletion resulted in a significant increase in the percentage of T cells producing Th1-related cytokine IFN-γ, but had no effect on the fractions of T cells producing anti-inflammatory cytokine IL-10 or Th17-related cytokine IL-17 in DT-treated DEREG/$Ldlr^{-/-}$ mice (Figure 3D). T cells producing Th2-related cytokine IL-4 could not be detected in both DT-treated DEREG/$Ldlr^{-/-}$ and control $Ldlr^{-/-}$ mice. Taken together, these results indicate that repetitive injections of DT to DEREG/$Ldlr^{-/-}$ mice resulted in reduced accumulation of Foxp3$^+$ Tregs into atherosclerotic aorta, leading to activation and enhanced accumulation of CD4$^+$ effector T cells in atherosclerotic aortas and subsequent exacerbated atherosclerosis.

3.4. Effect of Foxp3$^+$ Treg depletion on the activation of splenic CD4$^+$ T cells

To reveal the effect of Foxp3$^+$ Treg depletion on systemic CD4$^+$ T cell immune responses, we performed intracellular cytokine staining of splenic lymphocytes. The fractions of splenic IFN-γ, IL-4, and IL-10 producing CD4$^+$ T cells were significantly increased upon Foxp3$^+$ Treg
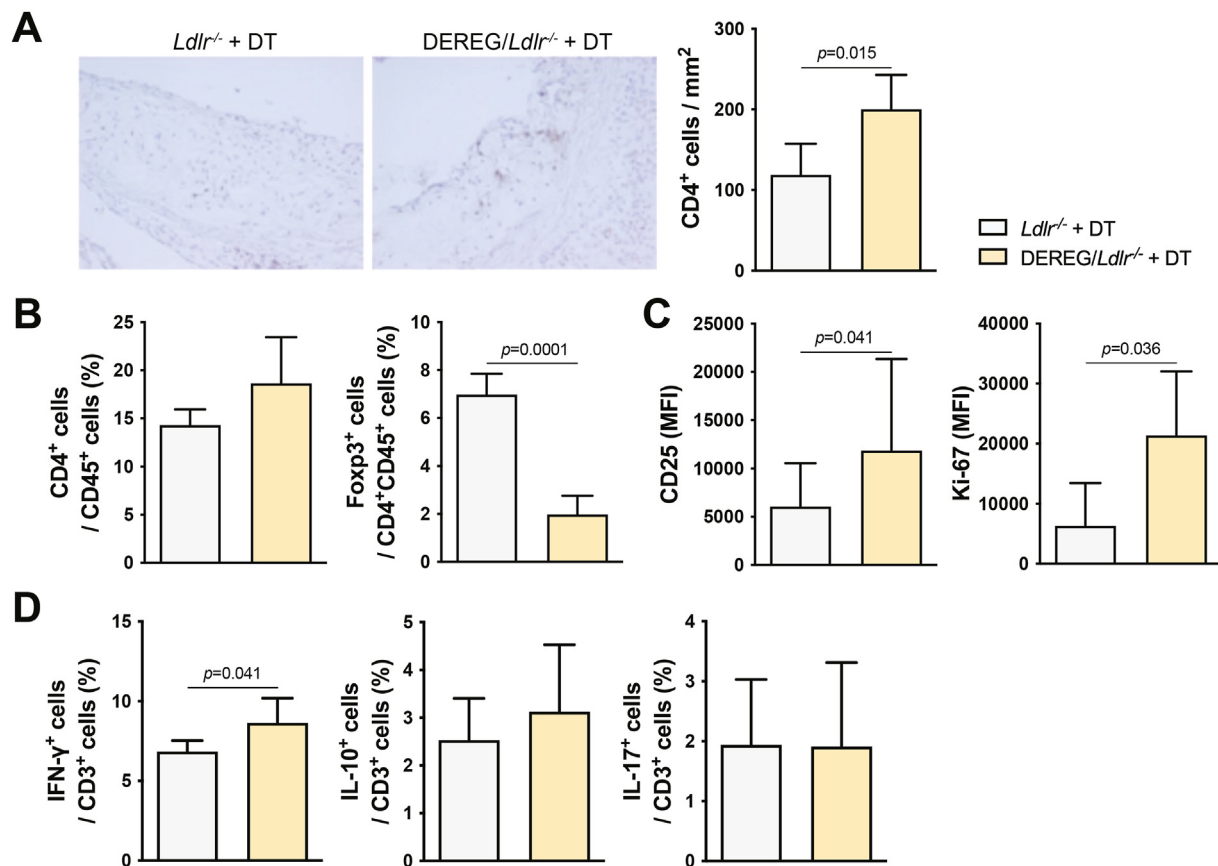

Figure 3. Depletion of Foxp3$^+$ Tregs enhances the activation of aortic CD4$^+$ T cells. (A) Ten-week-old male DEREG/$Ldlr^{-/-}$ or control $Ldlr^{-/-}$ mice fed a high-cholesterol diet were injected with DT twice per week for 4 weeks and atherosclerotic lesions were assessed at 14 weeks of age. Representative sections and quantitative analysis of CD4$^+$ T cells in the aortic sinus. n = 8 to 10 per group. (B–D) Ten-week-old male DEREG/$Ldlr^{-/-}$ or control $Ldlr^{-/-}$ mice were injected with DT twice per week for 2 (D) or 4 weeks (B and C), and cell suspension of collagenase-digested aorta from each mouse was analyzed by flow cytometry. n = 5 to 6 per group. (B) The graphs represent the percentage of CD4$^+$ T cells within the CD45$^+$ cells and Foxp3$^+$ Tregs within the CD4$^+$ cells in aorta. (C) The expression levels of CD25 and Ki-67 were analyzed gating on CD45$^+$CD4$^+$Foxp3$^-$ T cells in aorta. The data are shown as mean fluorescence intensity (MFI). (D) The graphs represent the frequencies of IFN-γ$^+$, IL-10$^+$, and IL-17$^+$ T cells in aortic CD45$^+$CD3$^+$ cells.

depletion (Figure 4A). The fraction of splenic IL-17 producing CD4$^+$ T cells tended to be increased (Figure 4A). In line with these data, all Th cell fractions such as CD4$^+$ T cells expressing Th1-specific transcription factor T-bet, Th2-specific transcription factor GATA3, and Th17-specific transcription factor RORγt were markedly increased in Foxp3$^+$ Treg-depleted DEREG/*Ldlr*$^{-/-}$ mice compared with control *Ldlr*$^{-/-}$ mice (Figure 4B). Intriguingly, Tr1 cells, defined as CD4$^+$Foxp3$^-$CD49b$^+$CD223$^+$ population and known to massively produce anti-inflammatory cytokine IL-10 [18], were also increased in the spleen of Foxp3$^+$ Treg-depleted DEREG/*Ldlr*$^{-/-}$ mice (Figure 4B), which is consistent with the data obtained by intracellular cytokine staining for IL-10. There was no difference in the production of IFN-γ, IL-4, and IL-17 from splenic lymphocytes stimulated with concanavalin A *in vitro* (Figure 4C). Importantly, splenic lymphocytes from Foxp3$^+$ Treg-depleted DEREG/*Ldlr*$^{-/-}$ mice stimulated with concanavalin A secreted much more anti-atherogenic Tr1-related cytokine IL-10 (Figure 4C). These results collectively suggest that Foxp3$^+$ Treg depletion systemically augments not only all Th immune responses but also anti-atherogenic Tr1 immune responses in hypercholesterolemic DEREG/*Ldlr*$^{-/-}$ mice.

4. Discussion

In this study, using hypercholesterolemic Foxp3$^+$ Treg-depleted mice newly established in this study, we clearly demonstrated that Foxp3$^+$ Tregs play a critical role in suppressing pro-inflammatory CD4$^+$ T cell immune responses in lymphoid tissues and aorta and preventing atherosclerotic plaque development without affecting plasma lipid profile in hypercholesterolemic mice. Unexpectedly, we found that Foxp3$^+$ Treg depletion resulted in a marked increase in splenic Tr1 cells, known as IL-10 producing T cells with anti-atherogenic properties [4], although this action was insufficient to reverse the exacerbated atherosclerosis caused by substantial Foxp3$^+$ Treg depletion.

We performed detailed analyses of CD4$^+$ T cell immune responses in lymphoid tissues and atherosclerotic aorta upon Foxp3$^+$ Treg depletion under hypercholesterolemia. We found that Th cell subsets such as Th1, Th2, and Th17 cells were significantly increased in the lymphoid tissues of Treg-depleted hypercholesterolemic DEREG/*Ldlr*$^{-/-}$ mice. Importantly, Th1 cells were also increased in the atherosclerotic aorta of these mice. Th1 cells are a major T cell subset that produces pro-inflammatory
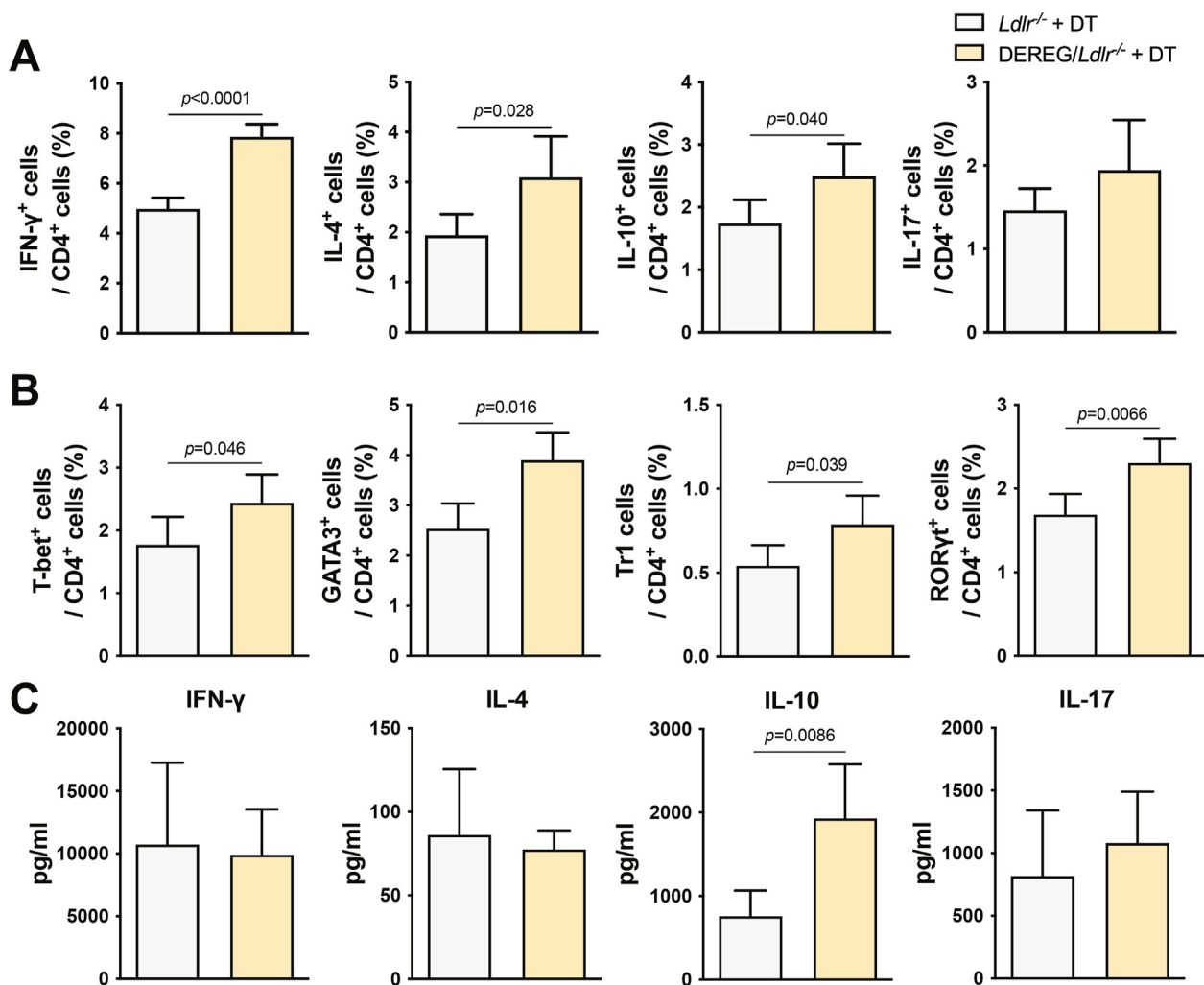

Figure 4. Depletion of Foxp3$^+$ Tregs increases anti-inflammatory Tr1 cells as well as all Th cell fractions in spleen. (A and B) Ten-week-old male DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice were injected with DT twice per week for 2 weeks, and splenocytes were analyzed by flow cytometry. (A) The graphs represent the frequencies of IFN-γ$^+$, IL-4$^+$, IL-10$^+$, and IL-17$^+$ T cells in splenic CD4$^+$ T cells. (B) The graphs represent the frequencies of T-bet$^+$, GATA3$^+$ cells, T regulatory type 1 (Tr1) cells, and RORγt$^+$ cells in splenic CD4$^+$ T cells. Tr1 cells were defined as CD4$^+$Foxp3$^-$CD49b$^+$CD223$^+$ population. (C) Ten-week-old male DEREG/*Ldlr*$^{-/-}$ or control *Ldlr*$^{-/-}$ mice were injected with DT twice per week for 2 weeks, and splenocytes were stimulated with concanavalin A *in vitro*. The graphs represent the cytokine production from splenic lymphocytes analyzed by ELISA. n = 4 to 5 per group.

cytokines and contributes to atherosclerosis development [3]. In consideration of exacerbated atherosclerosis following Foxp3$^+$ Treg depletion, our data suggest that Foxp3$^+$ Tregs regulate systemic and aortic Th1-mediated immune responses, which may contribute to the prevention of atherosclerosis. However, because the roles of Th2 and Th17 cells in atherosclerosis have not been consistently defined [3], it remains unclear whether in addition to Th1 immune response, immune responses mediated by Th2 and Th17 affected the atherosclerosis phenotype observed in our study. Interestingly, we found that IL-10 producing CD4$^+$ T cells, which would be Tr1 cells with anti-atherogenic properties [4], were markedly increased upon Foxp3$^+$ Treg depletion, raising an intriguing possibility that Foxp3$^+$ Tregs and Tr1 cells would cooperatively inhibit atherosclerosis under hypercholesterolemia. We suppose that upregulated Tr1 immune responses might compensatively contribute to the attenuation of pro-inflammatory T cell responses caused by dramatically reduced numbers of Foxp3$^+$ Tregs in DEREG/*Ldlr*$^{-/-}$ mice.

To examine the impact of Foxp3$^+$ Treg depletion on the development of atherosclerosis, Klingenberg *et al.* performed bone marrow transplantation experiments using DEREG mice [7]. Because we thought that invasive procedures such as bone marrow transplantation and lethal irradiation could have substantial effects on immune responses and atherosclerosis, in the present study we generated DEREG mice on a LDL receptor-deficient background by crossing DEREG mice with *Ldlr*$^{-/-}$ mice. Consistent with the finding in the previous report [7], we found that depletion of Foxp3$^+$ Tregs resulted in acceleration of the development of atherosclerotic lesions. However, we observed no changes in plasma cholesterol and triglyceride levels in Treg-depleted DEREG/-*Ldlr*$^{-/-}$ mice, although the previous report showed an increase in plasma cholesterol levels and a decrease in triglyceride levels upon Treg depletion [7]. Therefore, we suppose that in our model, the athero-protective action of Foxp3$^+$ Tregs is independent of modulation of lipoprotein metabolism. The differences in experimental protocols and performance of bone marrow transplantation and lethal irradiation might lead to the discrepant findings in the lipid profile between our study and the previous report.

A number of animal studies have demonstrated that the activation and expansion of Foxp3$^+$ Tregs can prevent atherosclerosis by regulating pathogenic immunoinflammatory responses. Several strategies such as pharmacological approaches, vaccination with atherosclerosis-related antigens, and UVB irradiation are reported to increase Treg numbers and attenuate the development of atherosclerosis in atherosclerosis-prone mice [19], although athero-protective action of Foxp3$^+$ Tregs has not been completely clarified yet. Our findings may help to better understand the mechanisms of Foxp3$^+$ Treg-mediated athero-protection under hypercholesterolemia. Notably, previous clinical studies in patients with chronic graft-versus-host disease or hepatitis C virus-related vasculitis demonstrated that the administration of low-dose IL-2 expanded Tregs and improved these diseases without severe adverse effects [20, 21]. Based on definitive experimental [13] and these clinical data, a clinical study has been conducted to assess the safety and efficacy of low-dose IL-2 in patients with coronary artery disease [22]. We expect that this trial will provide useful information about the efficacy of Treg therapy in preventing atherosclerotic disease. Considering that recent clinical trials provided the possibility of anti-inflammatory therapies as potentially feasible strategies to reduce cardiovascular events [23, 24, 25], promotion of Treg immune responses could be a possible therapeutic approach to atherosclerosis. A thorough understanding of the mechanisms of Treg-mediated athero-protection could lead to the establishment of novel therapeutic approaches to prevent and treat atherosclerotic cardiovascular disease.

In conclusion, we show that Foxp3$^+$ Tregs regulate immune responses of various Th cell subsets such as Th1, Th2, and Th17 cells in lymphoid tissues and pro-atherogenic Th1-mediated immune responses in atherosclerotic aorta to control atherosclerosis under hypercholesterolemia, and that augmented anti-atherogenic Tr1 immune responses might compensatively attenuate pro-atherogenic immune responses caused by dramatically decreased Foxp3$^+$ Tregs. Our data indicate that therapeutic intervention aimed at augmenting Foxp3$^+$ Treg-mediated protective immunity may represent an attractive therapeutic approach to control T cell-induced inflammation and prevent atherosclerotic cardiovascular disease.

Declarations

Author contribution statement

Kazuyuki Kasahara and Naoto Sasaki: conceived and designed the experiments; performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Toru Tanaka, Hilman Zulkifli Amin, Sayo Horibe, Tomoya Yamashita, Ken-ichi Hirata, and Yoshiyuki Rikitake analyzed and interpreted the data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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