Oral Administration of an Active Form of Vitamin D₃ (Calcitriol) Decreases Atherosclerosis in Mice by Inducing Regulatory T Cells and Immature Dendritic Cells With Tolerogenic Functions

Masafumi Takeda, Tomoya Yamashita, Naoto Sasaki, Kenji Nakajima, Tomoyuki Kita, Masakazu Shinohara, Tatsuro Ishida, Ken-ichi Hirata

Objective—To determine whether the administration of an active form of vitamin D₃ (calcitriol) could prevent atherosclerosis through anti-inflammatory actions.

Methods and Results—Recent clinical studies have shown that lack of vitamin D₃ is a risk factor for cardiovascular events. Oral calcitriol administration decreased atherosclerotic lesions, macrophage accumulation, and CD4⁺ T-cell infiltration at the aortic sinus, when compared with the corresponding observations in control mice. We observed a significant increase in Foxp₃⁺ T-regulatory cells and a decrease in CD80⁺CD86⁺ dendritic cells (DCs) in the mesenteric lymph nodes, spleen, and atherosclerotic lesions in oral calcitriol–treated mice in association with increased interleukin 10 and decreased interleukin 12 mRNA expression. CD11c⁺ DCs from the calcitriol group showed reduced proliferative activity of T lymphocytes, suggesting the suppression of DC maturation. Neutralization of CD25 in vivo revealed that calcitriol inhibited atherosclerosis mainly in a T-regulatory cell–dependent manner but also partly because of a decrease in DC maturation.

Conclusion—Oral calcitriol treatment could prevent the development of atherosclerosis by changing the function or differentiation of DCs and T-regulatory cells. These findings suggest that intestinal and systemic immune modulation by calcitriol may be a potentially valuable therapeutic approach against atherosclerosis. (Arterioscler Thromb Vasc Biol. 2010;30:00–00.)

Key Words: atherosclerosis ■ regulatory T cells ■ dendritic cells ■ immune system ■ calcitriol

The active form of vitamin D₃, 1,25(OH)₂-dihydroxyvitamin D₃, is a secosteroid hormone that not only plays a central role in bone and calcium metabolism but also modulates the immune response. Recent epidemiological studies have shown a relationship between low plasma levels of vitamin D₃ and a predisposition to cardiovascular events.¹⁻³ This finding is supported by a meta-analysis showing that oral vitamin D₃ treatment contributes to the improvement of mortality from all causes, in part by decreasing cardiovascular deaths.⁴ Transgenic rats constitutively expressing vitamin D-24-hydroxylase, a model of vitamin D₃ deficiency, showed aggravated atherosclerosis under a high-fat and high-cholesterol diet, when compared with control rats.⁵ However, there are no reports about the direct effects of an orally administered active form of vitamin D₃ on atherosclerosis.

It is widely recognized that atherosclerosis is a complex inflammatory disease of the arterial wall,⁶⁻⁷ in which the T-lymphocyte–mediated pathogenic immune response plays a critical role. Clinical strategies developed to modulate the immune response have been insufficient for preventing atherosclerosis. Cumulative data based on experimental animal models suggest that CD4⁺ T cells are present within plaques from the initial stages of the disease in mice, and adaptive transfer of these cells is potentially proatherogenic.⁸ Accumulating evidence has revealed novel functions of several subsets of T-regulatory cells (Tregs), which maintain immunologic tolerance to self-antigens and inhibit atherosclerosis development by suppressing the inflammatory response of effector T cells.⁹⁻¹² These studies have provided new insights into the immunopathogenesis of atherosclerosis and imply that promotion of regulatory immune responses may have therapeutic potential for suppression of atherosclerotic diseases.

In addition to Tregs, dendritic cells (DCs) are also reportedly involved in maintaining immune tolerance to self-antigens. DCs are the most potent antigen-presenting cells.
They efficiently stimulate the differentiation of effector T cells from naïve T-cell precursors. DCs are also thought to perform the important function of presenting antigens to T cells, which leads to peripheral tolerance by inducing Tregs or inhibiting effector T cells. DCs can be tolerogenic and immunogenic. In particular, immature DCs, which down-regulate major histocompatibility complex class II molecules and costimulatory molecules (eg, CD40, CD80, and CD86) have had tolerogenic properties associated with decreased interleukin (IL) 12 and enhanced IL-10 production. Recently, the contribution of DCs to atherogenesis has been extensively examined. Furthermore, DCs and Tregs may be beneficial in the treatment of atherosclerotic disease.

Recently, several articles have reported that 1,25(OH)2-dihydroxyvitamin D3 induced reciprocal differentiation and/or expansion of Tregs and induced tolerogenic DCs characterized by downregulation of costimulatory molecules. Calcitriol and its analogues have inhibited autoimmune disease, allergy, and the rejection of transplanted organs in animal models via induction of tolerogenic DCs and Tregs. However, the interaction between DCs and Tregs remains to be fully elucidated. Given this background, we examined whether orally administered calcitriol would induce Tregs and immature DCs via the intestinal immune system as therapeutic targets for disease.

We fed 6-week-old female ApoE−/− mice 20 or 200 ng of calcitriol dissolved in carboxymethylcellulose or vehicle alone by gastric intubation with a plastic tube twice a week for 12 weeks. Mice under the same protocol as previously described were injected with either 100 μg of neutralizing CD25 monoclonal antibody (clone PC61) to deplete CD4+CD25+ Tregs or 100 μg of isotype-matched control rat IgG once every 4 weeks at the age of 6, 10, and 14 weeks. Mice were euthanized at the age of 18 weeks, and atherosclerotic lesions were examined as previously described. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine, Kobe, Japan.

**Methods**

Detailed Methods are provided in the supplemental data (available online at http://atvb.ahajournals.org).

**Experimental Design**

We fed 6-week-old female ApoE−/− mice 20 or 200 ng of calcitriol dissolved in carboxymethylcellulose or vehicle alone by gastric intubation with a plastic tube twice a week for 12 weeks. Mice under the same protocol as previously described were injected with either 100 μg of neutralizing CD25 monoclonal antibody (clone PC61) to deplete CD4+CD25+ Tregs or 100 μg of isotype-matched control rat IgG once every 4 weeks at the age of 6, 10, and 14 weeks. Mice were euthanized at the age of 18 weeks, and atherosclerotic lesions were examined as previously described. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine, Kobe, Japan.

**Cell Isolation Using In Vitro Cell Functional Experiments and Flow Cytometry Analyses**

Purified CD4+ T cells and CD11c+ DCs were isolated from mesenteric lymph nodes (MLNs) and spleens. A cell proliferation assay was performed by assessing [3H]thymidine incorporation.

**Results**

**Effects of Oral Calcitriol Treatment on General Conditions and Plasma Examination Values**

Calcitriol at various doses inhibits autoimmune disease (0.03 μg/kg PO per day), allergy (0.5 μg/kg SC per day), and rejection of transplanted organs (5 μg/kg PO 3 times per week) in animal models. Compared with previous reports, the dose used in our study (10 μg/kg [200 ng] PO 2 times per week) was somewhat higher. However, severe adverse effects, such as loss in body weight and hypercalcemia, were not observed in the present study. Notably, 200 ng of calcitriol (2 times per week) significantly increased its plasma level without affecting plasma levels of 25(OH)−hydroxyvitamin D3, intact parathyroid hormone, calcium, or phosphorus. Although plasma 25(OH)−hydroxyvitamin D3 levels were inversely associated with renin activity in hypertensive patients, and calcitriol administration led to suppression of renin in an animal model, in the present study, calcitriol administration affected neither blood pressure nor renin mRNA expression in the kidney (Table). The effect of vitamin D3 supplementation on blood pressure may be limited only to subjects under hypertensive conditions or vitamin D3 insufficiency. Contrary to our expectations, the administration of

<table>
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†P<0.05 vs control mice.

Fluorescence-activated cell sorter (FACS) analysis was performed. Total RNA was extracted from these cells for real-time RT-PCR.

**Statistical Analysis**

Data were expressed as mean±SEM. To detect significant differences between 2 groups or among 3 groups, an unpaired Student t test, a Mann–Whitney U test, or a 1-way ANOVA with a post hoc test was used when appropriate. P<0.05 was considered statistically significant.
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Effects of Calcitriol on Tregs in the Small Intestine, MLNs, and Spleen

To investigate the effects of 200 ng of calcitriol treatment on Treg induction in the small intestine by immunohistochemistry and revealed that there was a significant increase in the number of Foxp3⁺ Tregs in the calcitriol group (P<0.05, Figure 2E). Next, to confirm the induction of Tregs, we applied quantitative RT-PCR to assess mRNA levels of Treg-associated markers in CD4⁺ T cells from MLNs after positively separating them with anti-CD4⁺ microbeads (Figure 2F). We found a significant increase in mRNA levels of Treg-associated markers (ie, CD25, Foxp3, and cytotoxic lymphocyte antigen 4 [CTLA4]) in the calcitriol group (P<0.05). In addition, mRNA expression of IL-10 and transforming growth factor (TGF)-β tended to increase in CD4⁺ T cells from the MLNs of calcitriol-treated mice (P=0.0816 and P=0.510, respectively), suggesting the possibility of Treg induction. To determine whether a calcitriol-induced increase in Tregs contributes to the suppression of T-cell function, we performed in vitro proliferation assays of CD4⁺ T cells from MLNs and spleen of the calcitriol or control group. As shown in Figure 2G, CD4⁺ T-cell proliferation was suppressed in the calcitriol group, suggesting an increased number of Tregs by calcitriol might contribute to the suppressive potential of T lymphocytes.

Effects of Calcitriol on DCs in the Small Intestine, MLNs, and Spleen

CD80 and CD86 are recognized as important costimulatory molecules related to the maturation of DCs. We performed FACS analyses using MLN cells (Figure 3A) and splenocytes and found a significant decrease in CD80⁺/CD86⁺ mature DCs within the CD11c⁺ DC population from calcitriol-treated mice (P<0.05, Figure 3B). To confirm the effects of calcitriol on inhibition of DC maturation, we assessed mRNA levels of DC-associated markers in CD11c⁺ DCs from spleens after positive selection with anti-CD11c⁺ microbeads (Figure 3C). RT-PCR analyses revealed that CD80 and CD86 mRNA levels tended to decrease in the calcitriol group (P=0.098 and P=0.1023, respectively). Furthermore, we found a significant decrease in IL-12p40 mRNA levels and an increase in those of IL-10 in the calcitriol group (P<0.05). Notably, we also found a significant increase in CC-chemokine ligand (CCL)17 and CCL22 mRNA levels, which were recognized as Treg-attracting chemokines, in the calcitriol group (P<0.05). Consistent with previous reports, these results indicate that calcitriol induced immature DCs, which interact with Tregs to suppress activation of immune reactions via their tolerogenic properties. To determine whether a calcitriol-induced increase in the number of tolerogenic DCs contributes to the suppression of T lymphocytes, we performed in vitro proliferation assays of CD4⁺ T cells from the MLNs of calcitriol-treated mice (Figure 3D). CD4⁺ T-cell proliferation was suppressed to a greater extent when cocultured with DCs from the calcitriol group than when cultured with DCs from the control group. These results indicated that DCs from calcitriol-treated mice resulted in reduced proliferation activity of T lymphocytes. Taken together, tolerogenic DCs induced by oral calcitriol administration suppressed T-cell immune responses and worked as antiinflammatory factors mainly by changing their cytokine and chemokine productions. We further evaluated the number of CD11c⁺/CD86⁺ mature DCs in the small intestine by immunohistochemistry and revealed that there was a significant increase in intestinal Foxp3⁺ Tregs by calcitriol treatment with vehicle (control), 20 ng of calcitriol (D₃ 20 ng), or 200 ng of calcitriol (D₃ 200 ng) for 12 weeks. At the age of 18 weeks, the mice were euthanized; and cryosections of the intestine, MLNs, and Spleen were stained with oil red O and immunohistochemistry in the calcitriol group (P<0.05, Figure 2A–2D). A further detailed investigation just after starting calcitriol treatment revealed that the number of Tregs first increased in MLNs (but not thymus), suggesting that the active form of vitamin D₃ mainly induces Tregs peripherally (supplemental Figure I). We also demonstrated a significant increase in intestinal Foxp3⁺ Tregs by immunohistochemistry in the calcitriol group (P<0.05, Figure 1).
were no significant differences in the number of CD11c+/H11001 DCs and CD11c+/H11001 CD86+/H11001 mature DCs between the 2 groups (Figure 3E). However, the percentage of CD86+/H11001 cells in CD11c+/H11001 DCs was lower in the calcitriol group.

**Effects of Calcitriol on Tregs, DCs, and Inflammatory Cells in Atherosclerotic Plaques**

To evaluate the effects of calcitriol on Tregs and DCs in atherosclerotic plaques, we conducted immunohistochemical studies and RT-PCR in atherosclerotic lesions. The immunohistochemical analyses of Foxp3+/H11001 Tregs in atherosclerotic lesions showed a significantly increased number of Foxp3+/H11001 Tregs in the calcitriol group ($P<0.05$, Figure 4A and 4B). We further examined the expression of Treg-associated markers, such as CD25, Foxp3, and CTLA4, in atherosclerotic lesions and demonstrated that mRNA levels of all Treg-associated markers significantly increased in the calcitriol group when compared with the control group ($P<0.05$, Figure 4C).

Recent studies have suggested that antigen presentation may occur within atherosclerotic plaques and in lymphoid organs and that Tregs migrate to atherosclerotic lesions to suppress local immune responses.$^{25}$ Our finding supports this notion and implies that Tregs could be a promising target in treating atherosclerotic plaques. Next, we investigated the effect of calcitriol on DCs in atherosclerotic lesions and found that the numbers of CD11c+/H11001 DCs and CD11c+/H11001 CD86+ mature DCs were significantly decreased in the calcitriol group ($P<0.05$, Figure 4D and 4E). These results indicated that calcitriol decreased the number of DCs recruited into the plaque and that they were maintained in an immature state in atherosclerotic lesions. To reveal the effects of oral calcitriol treatment on inflammatory cells, such as macrophage and CD4+ cells, we conducted immunohistochemical studies of the atherosclerotic lesions (Figure 4F and 4G). Interestingly, the 200-ng calcitriol group showed a 29.0% reduction in the accumulation of macrophages ($P<0.01$) and also a 26.4% decrease in CD4+ T-cell infiltration ($P=0.02$), compared with the values recorded for the control group.

Next, to reveal the mechanisms of reduced atherosclerotic plaque development and inflammatory cell recruitment, we examined mRNA expression of the proinflammatory mole-
cules in atherosclerotic plaques by quantitative RT-PCR (Figure 4H). We found that adhesion molecule and proinflammatory chemokine and cytokine, such as vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, and interferon-γ, were markedly reduced (P<0.05) and that anti-inflammatory cytokines, such as TGF-β and IL-10, were significantly increased (P<0.05) in the calcitriol group when compared with the control group. The expression of DC-derived chemokine CCL22 mRNA was significantly increased (P<0.05); and the expression of its receptor, CC-chemokine receptor (CCR) 4, tended to increase in atherosclerotic lesions of the calcitriol group (P=0.0983).

Taken together, these results indicated that calcitriol might inhibit the migration of effector T cells and macrophages into the plaques by increasing the proportion of immature DCs and Tregs systemically and locally in atherosclerotic plaques.

**Inhibition of Tregs by Injection of Neutralizing Anti–CD25 Antibodies**

To clarify the interaction between Tregs and DCs in preventing atherosclerosis after calcitriol treatment, we conducted an in vivo CD25 neutralization study with the injection of anti–CD25 antibody. According to a previous report,9 the effect of a single intraperitoneal injection of 100 μg of CD25-depleting PC61 antibody was maintained for 4 weeks. In our experiment, we injected CD25-depleting PC61 antibody or isotype-matched control antibody into mice once every 4 weeks at the ages of 6, 10, and 14 weeks. Both the calcitriol and control groups receiving anti–CD25 antibody showed significantly increased atherosclerotic lesion formation when compared with the calcitriol group receiving isotype-matched control antibody (P<0.05, Figure 5A).

These results suggested that Tregs had pivotal and major roles in inhibiting atherosclerosis after calcitriol treatment. Notably, when mice were injected with neutralizing antibody, there remained significant differences in atherosclerotic lesion formation between calcitriol-treated and control mice, indicating that calcitriol partially inhibited atherosclerotic lesion formation independent of Tregs (P<0.05). We also measured the percentage of Tregs by FACS in the MLNs and spleens after the injection of anti–CD25 antibody (Figure 5B–5E). In contrast with a previous report,9 we observed a 60% to 80% depletion in CD4+CD25+ Tregs and CD4+CD25+Foxp3+ Tregs in MLNs and spleens at 1 week after a single intraperitoneal injection of 100 μg of CD25-depleting PC61 antibody. However, all changes had reversed by 2 weeks after injection (supplemental Figure II).

We further analyzed DC maturation after partial depletion of CD4+CD25+ Tregs and observed that the rates of maturation were increased by anti–CD25 antibody administration and that the calcitriol group receiving anti–CD25 antibody still showed a decrease in the number of CD80+CD86+ mature DCs when compared with the control group receiving anti–CD25 antibody injection (Figure 5F). These data suggest that DC maturation was highly associated with the induction of Tregs, although calcitriol may partially decrease DC maturation independent of Tregs. It is likely that calcitriol mainly inhibits the progression of atherosclerosis via a Treg-dependent pathway but also a partly DC-mediated and Treg-independent manner.

**Discussion**

Recent clinical studies and experimental investigations have indicated that vitamin D₃ insufficiency increases the incidence of cardiovascular events.1–3 Supplementation with an
active form of vitamin D3 (calcitriol) should have beneficial effects on cardiovascular disease (CVD) through anti-inflammatory and vasculoprotective actions. However, there have been no clinical or animal studies showing the beneficial actions of vitamin D3 in the treatment of CVD, including atherosclerosis. In the present study, we showed, for the first time to our knowledge, that oral calcitriol administration inhibited atherosclerosis in an animal model, suggesting a beneficial effect of vitamin D3 on clinical CVD. At least 2 different cells (ie, Tregs and immature DCs) were involved in the antiatherogenic mechanisms of vitamin D3 treatment. Although the antiatherogenic mechanism of Tregs’ effect and the functional role of DCs in atherogenesis still remain to be determined, we have revealed, for the first time to our knowledge, that calcitriol-induced tolerogenic DCs and Tregs might have antiatherogenic properties.

Accumulating evidence has revealed that several subsets of Tregs have beneficial effects on atherogenesis. Both naturally occurring CD4+CD25+ Tregs and IL-10–producing Tregs (Tr1) have inhibited atherosclerosis in mouse models. Recently, it was demonstrated that an orally administrated immunomodulatory agent, anti-CD3 antibody, inhibits atherosclerosis by inducing Tregs, especially latency-associated peptide Tregs. VDR ligands induce the differentiation of Foxp3+ Tregs and IL-10–producing Tregs (Tr1) in the presence of dexamethasone, and both types of Tregs contribute to T-cell immune response inhibition. In the present study, FACS analyses in MLNs and spleens documented that Foxp3+ Tregs were significantly increased in the calcitriol group. However, it is unlikely that Tr1 and latency-associated peptide Tregs play central roles in Treg induction by calcitriol. Indeed, intraplaque Tregs in atherogenesis have not yet been clarified, a previous study suggested that increasing numbers of Tregs might suppress pathogenic T-cell immune responses or macrophage activation in atherosclerotic lesions.
molecules, such as CD80 and CD86; decreased IL-12 production; and enhanced secretion of IL-10 and CCL22, resulting in T-cell hyporesponsiveness. The presence of such costimulatory molecules on DCs is required for T-cell activation and for differentiation from naïve T lymphocytes into effector T cells. In the absence of costimulation, T cells interacting with DCs undergo anergy or apoptosis. IL-12p70, a heterodimeric cytokine consisting of p35 and p40, is released mainly by activated macrophages and DCs. IL-12p70 is a key mediator in inducing T-helper type 1 response and stimulates the production of interferon-γ from T-helper type 1. Inhibition of DC-derived IL-12p70 production is followed by a downregulated response of T-helper type 1. Furthermore, the anti-inflammatory cytokine IL-10 also inhibits T-cell immune response by acting on antigen-presenting cells, such as macrophages and DCs. Our FACS analysis revealed that CD80⁺/CD86⁺ DCs are decreased in MLNs and spleens of calcitriol-treated mice, indicating augmentation of the immature DC phenotype. Expression of IL-12p40 mRNA in splenic DCs was decreased, and expression of IL-10 was increased. We clearly demonstrated that DCs from the calcitriol treatment group had less T-cell proliferation activity, which might indicate that DCs changed their phenotypes to tolerogenic. Recently, the functional importance of DCs in atherosclerosis has been highlighted in several animal and human studies. Accumulating evidence suggests that DCs in atherosclerosis are involved in antigen presentation to T cells within plaques. DCs present in normal arteries are immature and become activated during atherogenesis, and DCs in vessels have been involved in the initiation and progression of atherosclerosis.

Regarding the interactions between Tregs and tolerogenic DCs, immature DCs lacking sufficient expression of costimulatory molecules have induced Tregs and affected suppressive immunoresponses through the induction of anti-inflammatory cytokines, such as IL-10 and TGF-β. In our study, we demonstrated that expression of IL-10 mRNA in DCs was significantly increased, whereas expression of TGF-β mRNA was not increased. In accordance with previously published findings that naïve T cells in the periphery can differentiate into Foxp3⁺ Tregs in the presence of IL-10, the increase of IL-10 in tolerogenic DCs might contribute to the induction of Tregs, including Tr1. Tregs play critical roles in the differentiation of immature DCs through the interaction of CTLA4 and CD80/CD86. Tregs constitutively express high levels of CTLA4 that bind to CD80 and CD86 with high affinity. CTLA4 activity is important for Treg-induced tolerance in several animal models. The interaction of CTLA4 on Tregs with CD80/CD86 on CD11c⁺ DCs conveyed a negative signal to DCs and reduced the expression of these costimulatory molecules on DCs in vitro. In our study, CTLA4 mRNA expression in CD4⁺ T cells was significantly increased in the calcitriol group. Taken together, we conclude that Tregs and tolerogenic DCs might interact via CTLA4 and CD80/CD86 to induce each other, resulting in effective inhibitory immuno-
responses in both cell types. We also found another important interaction between Tregs and tolerogenic DCs: expression of CCL17 and CCL22 mRNA in the splenic DCs was enhanced in the calcitriol group in this study. These chemokines are ligands for CCR4 and CCR8 expressed on Tregs. DCs are the major source of CCL22 in vitro and in vivo, and transcriptional changes and production of CCL22 in human myeloid DCs have been induced by calcitriol. In the present study, calcitriol increased CCL22 expression in DCs, suggesting that DCs released Treg-attracting chemokines and attracted Tregs via CCR4. Tregs were also promoted in vivo by calcitriol administration. In addition, the percentage of immature DCs was increased in atherosclerotic lesions in the calcitriol group. Interestingly, the expression of CCL22 mRNA was significantly increased and its chemokine, CCR4, tended to increase in atherosclerotic lesions of the calcitriol group. It is likely that tolerogenic DCs release CCL22 to interact with CCR4-expressing Tregs in spleen and within atherosclerotic plaques; thus, DCs may function as antiatherogenic agents.

To clarify the roles of Tregs in the antiatherogenic effects of calcitriol and DC maturation, we examined the effect of CD25-neutralizing monoclonal antibody. Tregs characteristically express Foxp3 and, in most cases, CD25. Functional studies in which CD4+CD25+ Tregs were depleted with anti–CD25 antibodies in ApoE−/− mice resulted in an increase of atherosclerotic lesions. In contrast to the previous study, in our model, Tregs were not completely deleted by anti–CD25 antibodies after 4 weeks; however, partial inhibition of Tregs by injection of anti–CD25 antibodies partially reversed the beneficial effects of calcitriol on atherogenesis and slightly increased DC maturation at 1 week after anti–CD25 antibody injection. These data suggest that Tregs induced by oral calcitriol administration may have an important role in inhibiting atherogenesis and that calcitriol directly decreased DC maturation and inhibited the progression of atherosclerosis, independent of Tregs; however, partly immature DCs were induced via a Treg-dependent pathway.

In the present study, oral administration of calcitriol definitely regulated and affected the function and proportion of DCs and Tregs in MLNs and reduced atherosclerotic lesion formation. It is likely that modulation of both intestinal and systemic immune systems is critical to calcitriol-induced antiatherogenic properties. In a previous article, it was reported that oral administration of anti–CD3 antibody induced latency-associated peptide T cells in MLNs and spleens. We also confirmed that the maturation of DCs was inhibited and that the proportion of CD11c+CD80+CD86+ DCs in MLNs was increased at the same time, suggesting that an orally administered small amount of antibody reached the intestine (but not in the blood), possibly regulated intestinal immunity, and resulted in the mutual differentiation of tolerogenic DCs and Tregs in MLNs (unpublished data). In the present study, we demonstrated that oral calcitriol administration increased the number of Tregs in MLNs but not in the thymus (supplemental Figure I). Taken together, although we should be careful about the interpretation of our results, the intestinal immune system might be a novel therapeutic target for treatment of CVDs and atherosclerosis. This possibility must be further investigated.

In summary, we have demonstrated that oral administration of the active form of vitamin D3 inhibits atherosclerosis development by inducing tolerogenic DCs and Tregs. We report herein, for the first time to our knowledge, that Tregs and tolerogenic DCs work as antiatherogenic agents and that both cells may play key roles in the beneficial effects of calcitriol on atherogenesis. It is likely that tolerogenic DCs recruit Tregs through chemokine CCL22 and its receptor, CCR4; both cells interact through the cell-to-cell contact of CTLA4 and CD80/CD86 at lymphoid organs and atherosclerotic lesions. These data indicate that calcitriol could be used clinically as a promising therapy for preventing atherosclerotic CVD. Clinical studies in humans are required to identify the efficacy of oral calcitriol in the prevention of atherosclerotic diseases and to evaluate the relationship between plasma levels of calcitriol and CVDs.

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**Disclosures**

None.

**References**

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### Supplement Methods

#### Animals

*ApoE<sup>−/−</sup>* mice (offspring of homozygous *ApoE<sup>−/−</sup>* mice, backcrossed onto the C57BL/6 background)<sup>1</sup> were kept in a specific pathogen-free animal facility at Kobe University institute, were fed a normal chow (Oriental Yeast, Tokyo, Japan) and water ad libitum, and were maintained on a 12:12-h light-dark cycle in the animal facility. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine.

#### Experimental Design

We fed six-week-old female *ApoE<sup>−/−</sup>* mice 20ng or 200ng calcitriol dissolved in carboxymethylecellulose or vehicle alone by gastric intubation with a plastic tube twice a week for 12 weeks. Mice under the same protocol as described above were injected with either 100 μg of neutralizing CD25 monoclonal antibody (clone PC61; Bio Express Inc., West Lebanon, NH) to deplete CD4<sup>+</sup>CD25<sup>+</sup> Tregs or 100 μg of isotype-matched control rat IgG (Bio Express Inc.) once every 4 weeks at 6, 10 and 14 week-old. Mice were killed at 18 weeks of age, and atherosclerotic lesions were examined.

#### Assessment of Biochemical and Physiological Parameters

Blood was obtained on the day after the last feeding with calcitriol or vehicle under overnight fasting condition and was collected by the cardiac puncture under anesthetic conditions using pentobarbital sodium (80mg/kg intraperitoneal injection). Plasma was obtained through centrifugation and stored at -80°C. Concentrations of plasma...
total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglyceride, calcium and phosphorus were determined enzymatically by using an automated chemistry analyzer (SRL, Tokyo, Japan). 1,25(OH)$_2$-dihydroxyvitamin D$_3$ and 25(OH)-hydroxyvitamin D$_3$, the principal circulating form of vitamin D$_3$, were analyzed by radioimmunoassay (SRL). Plasma intact parathyroid hormone (PTH) was determined using a commercial ELISA kit (Alpco Diagnostics, Salem, NH). Systolic and diastolic blood pressure was measured with the tail cuff method (Softron blood pressure-98A, Tokyo, Japan).

**Atherosclerotic Lesions Assessments**

At 18 weeks of age, mice were anesthetized and the aorta was perfused with saline. For aortic root lesion analysis, samples were cut from the ascending aorta, and the proximal samples containing the aortic sinus were embedded in OCT compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Five consecutive sections (thickness, 10 $\mu$m), spanning 550 $\mu$m of the aortic sinus, were collected from each mouse and stained with Oil Red O (Sigma, St Louis, MO). For quantitative analysis of atherosclerosis, the total lesion area of 5 separate sections from each mouse was obtained and quantitatively analyzed with the use of the Image J software (National Institutes of Health) as previously described.$^{1,2}$ We assessed the aortic sinus mean plaque area in comparison among the groups. Lesion analysis was conducted by a single observer blinded to the group of the mice.

**Immunohistochemical Analyses of Atherosclerotic Lesions**

Immunohistochemistry was performed on acetone-fixed cryosections (10 $\mu$m) of mouse
aortic roots using antibodies to identify macrophages (MOMA-2 at a dilution of 1:400; BMA Biomedicals, Augst, Switzerland), and T cells (CD4, 1:100; BD Biosciences, San Jose, CA), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. For natural Tregs, acetone-fixed cryosections of mouse aortic roots were incubated with rat anti-Foxp3 antibody (clone FJK-16s, 1:100; eBioscience, San Diego, CA), followed by Alexa Fluor 588 anti-rat secondary antibody (1:500; Molecular Probes, Eugene, OR) as described. For mature DCs, slides were stained by using TSA™- kit (PerkinElmer LAS, Inc., Boston, MA) according to the manufacturer’s instructions. In brief, endogenous peroxidase activity was quenched with 0.5% H2O2 for 30 minutes. Sections were blocked with TNB buffer (TSA™- kit) and hamster anti-CD11c antibody (clone N418, 1:200; BioLegend, San Diego, CA) and rat anti-CD86 (clone PO3.1, 1:200; eBioscience, San Diego, CA) of primary antibodies were applied for 1 hour at room temperature. Slides were washed and incubated with HRP-labeled goat-anti-rat IgG (American Qualex, San Clemente, CA) for 30 minutes. CD86 were detected with Cy3-Tyramide. Next, primary HRP was deactivated by treatment with 0.5% H2O2 for 30 minutes, and the sections were then incubated with biotinylated anti-hamster IgG (Vector Laboratories, Inc., Burlingame, CA). Slides were washed and incubated with streptavidine-HRP (TSA™- kit). Staining by hamster anti-CD11c was visualized by amplification of the signal with FITC-Tyramide. Nuclei were counterstained with DAPI (Molecular Probes). The appropriate fixation reagent depending on the primary antibodies was used. Negative controls were prepared with substitution with an isotype control antibody. Staining with Masson’s trichrome was used to delineate the fibrous area as previously described. Sections were observed under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence,
Osaka, Japan) using BZ Analyzer Software (Keyence). Stained sections were digitally captured, and the percentage of the stained area (the stained area per total atherosclerotic lesion area) was calculated. Quantification of CD4$^+$ T cells, Foxp3$^+$ Tregs, CD11c$^+$ DCs and CD11c$^+$CD86$^+$ DCs in atherosclerotic lesions was done by counting positively stained cells, which was divided by total plaque area.

**Cell Isolation and Flow Cytometry Analyses**

Purified CD4$^+$ T cells and CD11c$^+$ DCs were isolated from mesenteric lymph nodes (MLNs) and spleens. CD4$^+$ T cells and CD11c$^+$ DCs were positively selected with anti-CD4$^+$ antibody microbeads and with anti-CD11c$^+$ microbeads (Miltenyi Biotec, Inc., Auburn, CA) using an AutoMACS separator (Miltenyi Biotec) according to the manufacturer’s instructions. For fluorescence-activated cell sorter (FACS) analyses of lymphoid organs, MLN cells and splenocytes were isolated at 18 weeks of age. Cells were stained in PBS containing 2% FCS. FACS analysis was performed with a FACSCalibur using CellQuest Pro software (BD Bioscience). The antibodies used were as follows; anti-CD16/CD32 (clone 2.4G2; BD Bioscience), FITC-conjugated anti-CD4, Peridinin Chlorophyll Protein cyanin 5.5 (PerCP Cy5.5)-conjugated anti-CD4 (clone H129.19; BD Bioscience), PE-conjugated anti-CD8 (clone 53-6.7; BD Bioscience), phycoerythrin (PE)-conjugated anti-CD25, allophycocyanin (APC)-conjugated anti-CD25 (clone PC61; BD Bioscience), (APC)-conjugated Foxp3 (clone FJK-16s; eBioscience), PE-conjugated anti-IL-4 (clone BVD4-1D11; BD Bioscience), PE-conjugated anti-IL-10 (clone JES5-16E3; BD Bioscience), PE-conjugated anti-IFN-γ (cloneXMG1.2; BD Bioscience),PE-conjugated streptavidin (BD Bioscience), biotinylated anti-human LAP (BAF 246; R&D Systems),
FITC-conjugated anti-CD3e (clone 17A2; BD Bioscience), PE-conjugated anti-NK1.1 (clone PK136; eBioscience), APC-conjugated CD80 (clone 16-10A1; eBioscience), PE-conjugated CD86 (clone PO3.1; eBioscience), and isotype matched control antibodies. For CD80 and CD86 staining, CD11c+ DCs were positively selected with anti-CD11c+ microbeads as described above and were stained. Intracellular staining of Foxp3 or cytokines such as IL-4, IL-10 and IFN-γ was performed using the Foxp3 staining buffer set (eBioscience) or intracellular cytokine staining kit (BD Bioscience) and APC-conjugated Foxp3, PE-conjugated anti-IL-4, PE-conjugated anti-IL-10, PE-conjugated anti-IFN-γ or antibody as described above according to the manufacturer’s instructions. All staining procedures were performed after blocking Fc receptor with anti-CD16/CD32 antibody. Surface staining was performed according to standard procedures at a density of 1x10^6 cells per 100 μl, and volumes were scaled up accordingly.

**Cell Proliferation Assays and Cytokine assays**

In all cell culture experiments, we used RPMI 1640 medium (Sigma) supplemented with 10% FCS, 10mM Hepes, 50 μ mol/L 2 β -mercaptoethanol and antibiotics. For analysis of in vitro suppressive function of CD11c+ DCs, CD11c+ cells from spleens of calcitriol-treated or control mice were cultured with 1x10^5 of CD4+ T cells from spleens of Balb/c mice at various ratios (total volume, 200 μ L/well). These cells were cultured at various ratios in flat-bottomed 96-well plates at 37°C with 5% CO2 for 72 hours. In these experiments, CD11c+ DC were irradiation with 18.5 Gy before co-culture. The cells were pulsed with 1 μ Ci of [3H]-thymidine (GE Healthcare, Buckinghamshire, UK) for the last 16 hours, and thymidine incorporation was assessed
with a LS 6500 liquid scintillation counter (Beckman Coulter, Inc, Brea, CA). For measurement of cytokine such as IL-17, IL-10, IFN-γ, and IL-6, splenocytes were cultured at a concentration of 1×10^6 cells/ml for 72 hours with 2 μg/ml concanavalin A (Con A; Sigma). Culture supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) using paired antibodies specific for corresponding cytokines according to the manufacturer’s instructions (R&D Systems).

**RT-PCR Analysis**

At 18 weeks old, mice were anesthetized and the aortic roots were excised as described above. Total RNA was extracted from CD4^+^ T cells, CD11c^+^ DCs, and mouse aortic roots after perfusion with RNA-later (Ambion, Austin, TX) using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using One Step SYBR PrimeScript RT-PCR Kit (Takara, Shiga, Japan) and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol as described previously.² The following primers were used to amplify renin, CD25, Foxp3, Cytotoxic lymphocyte antigen 4 (CTLA4), CD11c, CD80, CD86, interleukin (IL)-6, IL-10, IL-12p35, IL12p40, interferon (IFN)-γ, transforming growth factor (TGF)-β, intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, monocyte chemoattractant protein (MCP)-1, thymus and CC-chemokine ligand (CCL)17, CCL22, CC-chemokine receptor (CCR)4, CCR8 and GAPDH: Renin, 5’-CTC CGT GCA GAT CAC GAT GAA G-3’ and 5’-GGA GCT CGT AGT AGC CGA GAT A-3’; CD25, 5’-CTG ATC CCA TGT GCC AGG AA G-3’ and 5’-AGG GCT TTG AAT GTG GCA TTG-3’; Foxp3, 5’-CTG ATG ATA GTG GCA TTG-3’; CTLA4,
5'-CCT CTG CAA GGT GGA ACT CAT GTA-3' and 5'-AGC TAA CTG CGA CAA GGA TCC AA-3'; **CD11c**, 5'-AGA CTG GCC AGT CAG CAT CAA C-3' and 5'-CTA TTC CGA TAG CAT TGG GTG AGT G-3'; **CD80**, 5'-AGT TTC CAT TGG GTT TA-3' and 5'-TTG TAA CGG CAA GGC AGC AAT A-3'; **CD86**, 5'-AGA CGT GCC AGT CAG CAT CAA C-3' and 5'-TTA TTC CGA TAG CAT TGG GTG AGT G-3'; **IL-6**, 5'-CCA CTT CAC AAG TCG GAG GCT TA-3' and 5'-CCA AGG TAA CTG CGA CAA GGA TCC AA-3'; **CD11c**, 5'-AGA CGT GCC AGT CAG CAT CAA C-3' and 5'-CTA TTC CGA TAG CAT TGG GTG AGT G-3'; **IL-6**, 5'-CCA CTT CAC AAG TCG GAG GCT TA-3' and 5'-CCA AGG TAA CTG CGA CAA GGA TCC AA-3'; **IL-12p35**, 5'-TGT CTT AGC CAG TCC CGA AAC C-3' and 5'-CTA TTC CAT GTC CAA GGC TCA TTC-3'; **IL-12p40**, 5'-GCT CGC AGC AAA GCA AGG TAA-3' and 5'-CCA TGA GTG GAG ACA CCA GCA-3'; **IFN- y**, 5'-CGG CAC AGT CAT TGA AAG CCT A-3' and 5'-GTT GCT GAT GAT GGC CTG ATT GTG-3'; **TGF-β**, 5'-GTG TGG AGC AAC ATG TGG AAC TCT A-3' and 5'-TTG GCT CCA CTG CCG TA-3'; **ICAM-1**, 5'-CTA TTC ACA CTG AAT GCC AGC TC-3' and 5'-CCA CTT CAC AAG TCG GAG GCT TA-3' and 5'-CCA AGG TAA CTG CGA CAA GGA TCC AA-3'; **VCAM-1**, 5'-TGC TCG CCT TGT TTC AGA CTG CTT GCC ATG GTC TTG-3' and 5'-AGC TTG CCC TGG ACA GTC AGA-3'; **CCL17**, 5'-CCG AGA GTG CTG CCT GGA TTA-3' and 5'-AGC TTG CCC TGG ACA GTC AGA-3'; **CCL22**, 5'-GGC ACC TAT CCA GTG CCA CA-3' and 5'-TGG TGG ACC AGC CTG AAA CTC-3'; **CCR4**, 5'-TGC TCG CCT TGT TTC AGT CAG-3' and 5'-AGC CAT CTT GCC ATG GTC TTG-3'; **CCR8**, 5'-CAG ACC CAC AAC CTG CTG GA-3' and 5'-GAC AGC GTG GAC AAT AGC CAG A-3'; **GAPDH**, 5'-TGT GTC CGT CTG GGA TCT GA-3' and 5'-TTG CTG AGG TCA-3'. Amplification reactions were performed in duplicate and fluorescence curves were analyzed with included software. GAPDH was used as an
endogenous control reference.

**Statistical Analysis**

Data were expressed as means±SEM. To detect significant differences between 2 groups or among 3 groups, unpaired Student t test, Mann-Whitney U test, or one way ANOVA with post hoc test was used when appropriate. Statistical values of \( p<0.05 \) were considered statistically significant. For statistical analysis, GraphPad Prism 4.0 was used.

**Supplemental References**


Supplemental Figure I

Thymus

MLNs

Spleen

CD25+ cells / CD4+ cells (%)

CD25+ cells / CD4+ cells (%)

CD25+ cells / CD4+ cells (%)

CD25+ cells / CD4+ cells (%)

CD25+ Foxp3+ cells / CD4+ cells (%)

CD25+ Foxp3+ cells / CD4+ cells (%)

CD25+ Foxp3+ cells / CD4+ cells (%)

Base (4 weeks of age)
Control
D₃ 200ng

*
Supplemental Figure II

**MLNs**

![Graph showing CD25+ cells/CD4+ cells (%) in MLNs.](image1)

- Control + mCD25Ab
- D₀ 200ng + mCD25 Ab
- D₀ 200g + Rat IgG

**Spleen**

![Graph showing CD25+ cells/CD4+ cells (%) in Spleen.](image2)

- Control + mCD25Ab
- D₀ 200ng + mCD25 Ab
- D₀ 200g + Rat IgG

* and † indicate significant differences.
Supplemental Figure III

A

IFN-γ / CD4+ cells (%)

IL-4 / CD4+ cells (%)

Control

D3 200ng

MLN Spleen

B

IL-10 / CD4+ cells (%)

LAP+/CD4+ cells (%)

CD25+LAP+ / CD4+ cells (%)

MLN Spleen

C

(pg/ml) IL-10

(pg/ml) IL-17

(pg/ml) IFN-γ

(pg/ml) IL-6

MLN Spleen

D

CD3+ NK.1.1+ (%)

CD3+ NK.1.1+ (%)

MLN Spleen

Supplemental Figure III
Supplemental Figure Legends

Supplemental Figure I. Effects of Calcitriol on stimulating the expansion of thymus and peripherally induced Tregs.
We fed 4 week-old female ApoE−/− mice 200ng calcitriol dissolved in carboxymethylcellulose or vehicle alone by gastric intubation with a plastic tube twice a week for 4 weeks. For FACS analyses of lymphoid organs, thymus cells, MLN cells and splenocytes were isolated at 4, 6 and 8 weeks of age. Thymus cells were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and APC-conjugated anti-Foxp3. MLN cells and splenocytes cells were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD25 and APC-conjugated anti-Foxp3. For all FACS analyses, data represent means ± SEM of 3 mice in each group. Horizontal bars represent means. *p<0.05 vs. control.

Supplemental Figure II. The dynamics of the alteration of Tregs after a single intraperitoneal injection of 100 µg of CD25-depleting PC61 antibody.
MLN cells and splenocytes from calcitriol with anti-CD25 antibodies (D3 200ng+anti-CD25 Ab; closed circle), control with anti-CD25 antibodies (Control+anti-CD25 Ab; open circle), and calcitriol with isotype-matched control antibodies (D3 200ng+rat-IgG; gray circle) were isolated 1, 2, and 4 weeks after a single intraperitoneal injection of 100 µg of CD25-depleting PC61 antibody. Data represent means ± SEM of 3 mice in each group. *p<0.05 vs. D3 200ng+anti-CD25. †p<0.05 vs. control+anti-CD25 Ab.
Supplemental Figure III. Effects of calcitriol on the phenotypes of T lymphocytes and other cells in MLNs and spleen.

For FACS analyses of lymphoid organs, MLN cells and splenocytes were isolated at 18 weeks of age. CD4⁺ T cells were stained with PE-conjugated anti-IFN-γ for Th1 lymphocytes, PE-conjugated anti-IL-4 for Th2 lymphocytes, PE-conjugated anti-IL-10 for Tr1 cells, APC-conjugated anti-CD25 and PE-conjugated anti-LAP for Th3, or FITC-conjugated anti-CD3ε and PE-conjugated anti-NK1.1 for Natural Killer (NK) cells or NKT cells (A, B, and D). There was no significant differences in IFN-γ producing CD4⁺ T cells (Th1) and IL-4 producing CD4⁺ T cells (Th2) in the calcitriol-treated group and control group (A). Furthermore, there was no obvious induction of IL-10 producing CD4⁺ T cells (Tr1) and LAP⁺ Tregs (mainly Th3) in calcitriol-treated mice (B). Furthermore, there were no significant difference in NK cells (CD3⁻NK1.1⁺) and NKT cells (CD3⁺ NK1.1⁺) between the two groups (D). Data represent means ± SEM of 3 mice in each group. Horizontal bars represent means. *p<0.05 vs. control.

Splenocytes were stimulated with Con A in vitro for 72 hours. IL-17, IL-10, IFN-γ, and IL-6 productions in supernatants were measured by ELISA (C). The productions of IL-10 and IL-17 from splenocytes were similar between the two groups, suggesting it is unlikely calcitriol affected Tr1 and Th17 in the spleen. Productions of inflammatory cytokine such as IFN-γ and IL-6 from splenocytes were significantly decreased in calcitriol-treated mice. Data represent means±SEM of at least 6 mice in each group. Horizontal bars represent means. *p<0.05 vs. control.