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Original Research

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Effects of an *in vitro* vitamin D treatment on the inflammatory responses in visceral adipose tissue from *Ldlr*$^{-/-}$ mice

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ABSTRACT

BACKGROUND/OBJECTIVES: Atherosclerosis is associated with increased inflammation in the visceral adipose tissue (VAT). Vitamin D has been reported to modulate the inflammatory responses of stromal vascular cells (SVCs) and adipocytes in adipose tissue, but the role of vitamin D in atherosclerosis biology is unclear. This study examined the effects of *in vitro* 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) treatment on the inflammatory responses of SVCs and adipocytes from atherosclerotic mice.
MATERIALS/METHODS: C57BL/6J (B6) mice were divided randomly into 2 groups and fed a 10% kcal fat control diet (control group, CON) or 41% kcal fat, 0.21% cholesterol (high fat + cholesterol, HFC) diet (obese group, OB), and B6.129S7-*Ldlr*^{tm1Her}/J (*Ldlr*$^{-/-}$) mice were fed a HFC diet (obese with atherosclerosis group, OBA) for 16 weeks. SVCs and adipocytes isolated from VAT were pre-incubated with $1,25(OH)_2D_3$ for 24 h and stimulated with lipopolysaccarides for the next 24 h. Proinflammatory cytokine production by adipocytes and SVCs, the immune cell population in SVCs, and the expression of the genes involved in the inflammatory signaling pathway in SVCs were determined.
RESULTS: The numbers of total macrophages and SVCs per mouse were higher in OB and OBA groups than the CON group. The *in vitro* $1,25(OH)_2D_3$ treatment significantly reduced macrophages/SVCs (%) in the OBA group. Consistent with this change, the production of interleukin-6 and monocyte chemoattractant protein 1 (MCP-1) by SVCs from the OBA group was decreased by $1,25(OH)_2D_3$ treatment. The $1,25(OH)_2D_3$ treatment significantly reduced the toll-like receptor 4 and dual-specificity protein phosphatase 1 (also known as mitogen-activated protein kinase phosphatase 1) mRNA levels in SVCs and MCP-1 production by adipocytes from all 3 groups.
CONCLUSIONS: These findings suggest that vitamin D can attribute to the inhibition of the inflammatory response in VAT from atherosclerotic mice by reducing proinflammatory cytokine production.

Keywords: Atherosclerosis; vitamin D; adipose tissue; inflammation

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Conflict of Interest
The authors declare no potential conflicts of interests.

Author Contributions
Conceptualization: Han SN; Formal analysis: Kwon DH; Investigation: Kwon DH, Hwang J, You H, Kim NY, Lee GY; Methodology: Han SN; Supervision: Han SN; Writing - original draft: Kwon DH; Writing - review & editing: Han SN.

INTRODUCTION

Atherosclerosis is no longer considered simply as a degenerative lipid storage disease. Instead, it is an inflammation-driven disease with a diverse risk factor profile [1]. Metabolic syndrome, represented by obesity and insulin resistance, is a major driver of increased cardiovascular disease risk worldwide, and hypertrophic adipose tissue, which contains large numbers of inflammatory cells and produces proinflammatory mediators, contributes to the increased atherosclerotic risk [2].

Adipose tissue is composed of adipocytes and non-adipocytes, known as stromal vascular cells (SVCs) [3]. Previous studies reported that the production of proinflammatory cytokines involved in systemic, low-grade chronic inflammation in obesity is driven largely by adipose tissue-resident macrophages (ATMs) [4-7]. Adipose tissue inflammation leads to the infiltration of large numbers of macrophages and secretion of T helper 1 type cytokines such as interferon-γ, which promotes the M1 phenotype of macrophages. The expression of tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase by M1 macrophages leads to the production of large amounts of reactive oxygen and nitrogen intermediates, causing vascular inflammation and tissue destruction. In this state, anti-inflammatory M2 macrophages, which are responsible for angiogenesis and wound healing, are suppressed [8]. In particular, visceral adipose tissue (VAT) contains more inflammatory immune cells than subcutaneous adipose tissue, and adipocytes in VAT release more free fatty acids and exhibit higher insulin resistance [9]. Furthermore, abdominal body fat distribution measured with the waist/hip circumference ratio is associated with a prevalence of coronary artery disease independent of obesity [10]. Several mechanisms have been proposed to explain how an increased inflammatory response in VAT adversely affects cardiovascular health and atherosclerosis [11-15]. VAT-derived inflammatory products move to the liver through the portal vein and affect the lipoprotein and clotting factor levels or work directly at the vessel wall to regulate gene expression and function of smooth muscle cells and macrophages.

Toll-like receptors (TLRs) are involved in activating the innate immune response through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the mitogen-activated protein kinase (MAPK) inflammatory signaling pathway by recognizing the pathogen-associated molecular patterns (PAMPs) produced by microbes and damage-associated molecular patterns (DAMPs) derived from dead cells or tissue damage [16]. TLR2 and TLR4 are expressed on the immune cells in atherosclerotic plaque and are involved in the development of coronary artery disease [17]. The TLR2 and TLR4 expression levels are higher in the peripheral blood mononuclear cells (PBMCs) and monocytes isolated from obese patients than in non-obese patients. They are activated by free fatty acids and dietary lipids [18,19]. TLR4 recognizes oxidized low-density lipoprotein (ox-LDL) and contributes to the production of inflammatory cytokines and macrophage differentiation into foam cells [20].

Vitamin D has been reported to have anti-inflammatory effects in chronic diseases, such as cancer, obesity, and cardiovascular diseases [21]. The expression of the vitamin D receptor (VDR) and 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1) gene in human and murine adipose tissue suggests that vitamin D is involved in the adipose tissue biology [22]. Previous studies reported that a 100 nM *in vitro* 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) treatment downregulates the protein and mRNA expression of TLR2 and TLR4 in human monocytes [23,24]. In addition, a 10 nM *in vitro* $1,25(OH)_2D_3$ treatment on mouse SVCs or human PBMCs reduced the production of proinflammatory cytokines, such as interleukin-6 (IL-6), monocyte

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chemoattractant protein 1 (MCP-1), and TNF-α, and increased the mRNA levels of dual-specificity protein phosphatase 1 (DUSP1; also known as mitogen-activated protein kinase phosphatase 1, MKP-1) [25,26]. Furthermore, 10 and 100 nM *in vitro* $1,25(OH)_2D_3$ treatments on human preadipocytes/adipocytes decreased the mRNA levels and production of IL-6, IL-1β, and MCP-1 [27-31]. On the other hand, Sun and Zemel [32,33] reported that a 10 nM *in vitro* $1,25(OH)_2D_3$ treatment increased *Mcp1* and *Il6* expression in 3T3-L1 cells and human adipocytes.

In atherosclerosis, vitamin D increases endothelial nitric oxide production and inhibits vascular smooth muscle cell proliferation. A vitamin D deficiency may increase the cardiovascular risk by increasing the expression of vascular adhesion molecules and monocytes/macrophages [34]. Furthermore, $1,25(OH)_2D_3$ inhibited the formation of foam cells during macrophage differentiation by suppressing endoplasmic reticulum stress and leading them into the non-adhesive, anti-atherogenic type in type 2 diabetic patients [35,36]. On the other hand, the effects of $1,25(OH)_2D_3$ on regulating VAT inflammation in atherosclerosis are unclear. Moreover, there is a paucity of research on the effects of vitamin D on atherogenic models other than high-fat diet-induced obese mice. Therefore, this study examined the effects of an *in vitro* $1,25(OH)_2D_3$ treatment on inflammatory responses in VAT from B6.129S7-*Ldlr*^{tm1Her}/J (*Ldlr*$^{-/-}$) mice with high fat + cholesterol (HFC) diet-induced atherosclerosis by determining the changes in proinflammatory cytokine production, inflammation-related gene expression, and immune cell population.

MATERIALS AND METHODS

Animals and diets

Six-week-old male C57BL/6J (B6) and B6.129S7-*Ldlr*^{tm1Her}/J (*Ldlr*$^{-/-}$) mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were housed in a specific pathogen-free room at Seoul National University with an environmentally controlled temperature (23 ± 1°C), humidity (50 ± 10%), and a 12-h light/dark cycle. After 5 days of acclimation, the B6 mice were divided randomly into the control (CON, n = 24) and obese (OB, n = 22) groups. The CON group was fed a 10% kcal fat control diet (D12450B; Research Diets, New Brunswick, NJ, USA), and the OB group was fed a 41% kcal fat, 0.21% cholesterol HFC diet (D12079B, Western diet; Research Diets). The *Ldlr*$^{-/-}$ mice were fed the same diet as the OB group (obese with atherosclerosis, OBA, n = 22). The body weight and food intake were measured weekly and 3 times a week, respectively. Food and water were provided *ad libitum* for 16 weeks.

At the end of the feeding period, the mice were fasted for 12 h and euthanized by CO_2 gas asphyxiation. The blood was collected immediately by cardiac puncture and kept for 2 h at room temperature (RT). The sample was centrifuged at 500 × g for 20 min at 4°C to obtain serum. The thoracic aortas were excised and fixed with 4% paraformaldehyde for oil-red O staining. The perirenal, retroperitoneal, and epididymal fat was weighed and collected in a dish filled with sterile phosphate-buffered saline (PBS) plus 250 ng/mL amphotericin B (Gibco, Grand Island, NY, USA) and 1% bovine serum albumin (BSA). The Institutional Animal Care and Use Committee of Seoul National University approved all experiments (approval No. SNU-210715-3-2).

Serum lipid level analyses

The serum triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) levels were determined using AM157S-K, AM202-K, and AM203-K assay kits (Asan

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Pharm., Seoul, Korea), respectively. The absorbance was measured using the SpectraMax iD3 spectrophotometer (Molecular Devices, San Jose, CA, USA) at 505 nm/570 nm for TC and HDL-C, and 550 nm for TG.

Oil red O staining

After removing the paraformaldehyde, the fixed murine aortas of each group were soaked in 99.0% propylene glycol (Samchun Pure Chemical, Pyeongtaek, Korea) for 2 min. The washed aortas were stained with an oil-red O solution (Sigma-Aldrich, St. Louis, MO, USA). The pathological lesions on the aortas were observed under a microscope (Sunny Optical Tech, Busan, Korea).

Isolation of adipocytes and stromal vascular cells

The collected visceral fat pads were pooled before further experiments (CON: 3, OB: 2, OBA: 2 pooled). The fat tissues were cut into small pieces with scissors and incubated in a shaking water bath for 40 min (37°C, 170 cycle/min) with Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 0.1% collagenase type 2 (Sigma-Aldrich) and 2% BSA. The digested suspension was passed through a 200 μm pore nylon mesh to remove the tissue debris. The filtrate was centrifuged at 500 × g for 5 min at RT, and the top layer was transferred to a new tube as adipocytes. The cell pellet (SVCs) at the bottom was incubated with 3 ml of ammonium-chloride-potassium lysis buffer (Gibco) for 3 min at RT to remove the red blood cells. The isolated adipocytes and SVCs were washed twice with DMEM/10% fetal bovine serum (FBS) and used for flow cytometry or cell culture.

In vitro $1,25(OH)_2D_3$ treatment

The adipocytes were cultured in a 6-well plate (4×10^6 cells/well), and SVCs were cultured in a 12-well plate (5×10^5 cells/well) with DMEM/10% FBS for 48 h in the presence or absence of 10 nM $1,25(OH)_2D_3$ (Sigma-Aldrich). During the final 24 h, the cells were cultured with or without 100 ng/mL lipopolysaccharides (LPS; Sigma-Aldrich). The cells were incubated at 37°C in 5% CO_2 and a 100% humidified atmosphere. The cell culture supernatant was collected and stored at −80°C for cytokine analysis using an enzyme-linked immunosorbent assay (ELISA). The SVCs were collected and used for flow cytometry or stored at −80°C for RNA isolation.

Flow cytometry analysis

The immune cell subpopulation was analyzed by resuspending freshly isolated SVCs from VAT or 48 h cultured SVCs in a FACS-staining buffer (PBS with 0.09% sodium azide [Sigma-Aldrich] and 1% FBS), followed by staining with the following antibodies for 30 min at 4°C: PE-Cy™7 rat anti-mouse cluster of differentiation 45 (CD45), BV786 mouse anti-mouse CD64, APC-R700 rat anti-mouse CD11b, PE hamster anti-mouse CD11c, FITC rat anti-mouse CD3, APC rat anti-mouse CD4, PerCP-Cy™5.5 rat anti-mouse CD8a, and BV605 mouse anti-mouse NK-1.1. After staining, cells were fixed with PBS containing 4% formaldehyde and analyzed using the BD FACSAria™ III and FlowJo™ software version 10. All materials for flow cytometry were obtained from BD Biosciences (Franklin Lakes, NJ, USA). **Table 1** lists the cell surface markers used for the flow cytometry.

Cytokine measurement

The concentrations of MCP-1 and IL-6 in the culture media produced by adipocytes and SVCs were determined using Mouse ELISA MCP-1 and IL-6 kits (#555260 and #555240, respectively; BD Biosciences) according to the manufacturers' instructions. The absorbance was measured at 450 nm using a SpectraMax iD3 (Molecular Devices).

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Table 1. Cell surface markers used for flow cytometry analyses

Cell	Surface markers
Macrophages	PECy7-CD45 BV786-CD64 APCR700-CD11b
M1 macrophages	PECy7-CD45 BV786-CD64 APCR700-CD11b PE-CD11c
$CD4^+$ T cells	PECy7-CD45 FITC-CD3 APC-CD4
$CD8^+$ T cells	PECy7-CD45 FITC-CD3 PerCPCy5.5-CD8a

M1, M1 macrophage; CD, cluster of differentiation.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted from SVCs using the RNAiso Plus (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. The first-strand cDNA was synthesized using the PrimeScript™ RT Master Mix (Takara Bio). qRT-PCR was then performed using StepOnePlus™ Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with TB Green™ Premix Ex Taq (Takara Bio). The relative expression levels of the genes were determined using the $2^{-\Delta\Delta CT}$ method and normalized to the expression of the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). **Table 2** lists the primer sequences used in qRT-PCR.

Statistical analysis

Statistical analyses were performed using SPSS statistical version 26 software (IBM Corp., Armonk, NY, USA). The differences among the 3 groups were determined by one-way analysis of variance (ANOVA) or Kruskal-Wallis test, followed by a Dunnett's *post hoc* test. A paired *t*-test or Wilcoxon signed-rank test was used to evaluate the effects of *in vitro* $1,25(OH)_2D_3$ treatment. All data are presented as the means ± standard error of the means (SEMs), and *P*-values less than 0.05 were considered significant.

RESULTS

Body weight, weight gain, VAT weight, and food intake

The body weights at week 0 were similar in the 3 groups (**Table 3**). After 16 weeks of feeding, the OB and OBA groups had significantly higher body weight and weight gain than the CON

Table 2. Sequences of the primers used in qRT-PCR

Genes	Forward primers	Reverse primers
Tlr2	CTTCATCTACGGGCAGTGGT	TTTGCTGGGCTGACTTCTCT
Tlr4	TTTCACCTCTGCCTTCACTACA	GGGACTTCTCAACCTTCTCAA
Dusp1	TGTGGGAGCATTTAGAGAGACTG	CCAAAACGACAGCCAAAAGT
Dusp10	CCAAGGAGTTGTTTCCGTTAGC	AGTGGAGCAGGTGAAGAGTGA
Ikbα	CAGCATCTCCACTCCGTCCT	ACATCAGCCCCACATTTCA
Gapdh	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

qRT-PCR, quantitative reverse transcription polymerase chain reaction; *Tlr2*, Toll-like receptor 2; *Tlr4*, Toll-like receptor 4; *Dusp1*, dual specificity protein phosphatase 1 (also known as mitogen-activated protein kinase phosphatase 1, MKP-1); *Dusp10*, dual specificity protein phosphatase 10; *Ikbα*, nuclear factor of kappa light polypeptide gene enhanced in B-cells inhibitor alpha; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

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Table 3. Body weight, weight gain, VAT weight, and food intake

Variables	CON (n = 24)	OB (n = 22)	OBA (n = 21)	*P*-value
Body weight at week 0 (g)	19.3 ± 0.5	19.4 ± 0.4	19.0 ± 0.6	0.897
Body weight at week 16 (g)	27.5 ± 0.5[a]	34.7 ± 0.6[b]	32.4 ± 0.9[b]	< 0.001
Weight gain (g)	8.2 ± 0.5[a]	15.4 ± 0.6[b]	13.5 ± 1.0[b]	< 0.001
VAT weight (g)[*]	0.82 ± 0.06[a]	2.35 ± 0.12[c]	1.83 ± 0.17[b]	< 0.001
Average food intake (g/day)	2.46 ± 0.04[b]	2.32 ± 0.02[a]	2.28 ± 0.02[a]	< 0.001
Average energy intake (kcal/day)	9.45 ± 0.17[a]	10.88 ± 0.10[b]	10.70 ± 0.09[b]	< 0.001

Data are presented as the means ± SEMs. One-way ANOVA (body weight at week 16 and VAT weight) or Kruskal-Wallis test (body weight at week 0, weight gain, average food intake, and average energy intake) was performed to determine the significant difference among the 3 groups.
VAT, visceral adipose tissue; CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr* $^{-/-}$ mice, high fat + cholesterol diet; SEMs, standard error of the means; ANOVA, analysis of variance.
[a,b]Different superscripts indicate significant differences ($P < 0.05$) by Dunnett's test or Bonferroni correction.
[*]VAT weight includes perirenal, retroperitoneal, and epididymal fat.

group, but there was no difference between the OB and OBA groups. The VAT weight was significantly different among the 3 groups. The OBA group had a higher VAT weight than the CON group but lower than the OB group. The CON group had a significantly higher average food intake (g/day) but a lower average energy intake (kcal/day) than the other 2 groups. The food and energy intakes were similar in the OB and OBA groups.

Serum lipid levels and the atherosclerotic lesions in the aorta

The OBA group exhibited significantly higher serum TG and TC levels but lower HDL-C levels than the OB and CON groups (**Table 4**). The OB group had higher serum TC and HDL-C levels than the CON group, but the TG levels were similar.

Atherosclerotic lesions on the aortic arch only developed in the OBA group (**Fig. 1**).

CD45$^+$ immune cell population in SVCs

Fig. 2 shows the total cell numbers of SVCs, ATMs, CD4$^+$ T cells, and CD8$^+$ T cells freshly isolated from VAT. The cell numbers were calibrated by dividing by the pooled number of mice in each group (CON: 3, OB: 2, OBA: 2) because the fat pads were pooled before cell counting. The total numbers of SVCs, ATMs, and CD4$^+$ T cells were higher in the OBA group than in the CON group. In the OB group, however, only the number of ATMs was higher than in the CON group. The total number of CD8$^+$ T cells was similar in the 3 groups.

The percentages of ATMs/SVCs and M1 macrophages (M1)/ATMs were similar in the 3 groups (**Fig. 2**). The effects of the *in vitro* $1,25(OH)_2D_3$ treatment on macrophages and M1 population in LPS-stimulated SVCs were determined (**Fig. 3**). Only the OBA group showed a decrease in percentage of macrophages/SVCs after the $1,25(OH)_2D_3$ treatment (7.7% less). The *in vitro* $1,25(OH)_2D_3$ treatment did not affect the percentage of M1 in the macrophages in all 3 groups.

Table 4. Serum TC, TG, and HDL-C levels

Variables	CON (n = 8)	OB (n = 8)	OBA (n = 8)	*P*-value
TG (mg/dL)	100.1 ± 14.7[a]	76.8 ± 5.2[a]	359.0 ± 57.7[b]	< 0.001
TC (mg/dL)	108.4 ± 6.1[a]	185.5 ± 6.2[b]	1,717.3 ± 166.6[c]	< 0.001
HDL-C (mg/dL)	67.1 ± 3.5[b]	107.4 ± 2.7[c]	54.4 ± 3.1[a]	< 0.001

Data are presented as the means ± SEMs. One-way ANOVA (TC and HDL-C) or Kruskal-Wallis test (TG) was performed to determine the significant difference among the 3 groups.
TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr*$^{-/-}$ mice, high fat + cholesterol diet; SEMs, standard error of the means; ANOVA, analysis of variance.
[a,b,c]Different superscripts indicate significant differences ($P < 0.05$) by Dunnett's test or Bonferroni correction.

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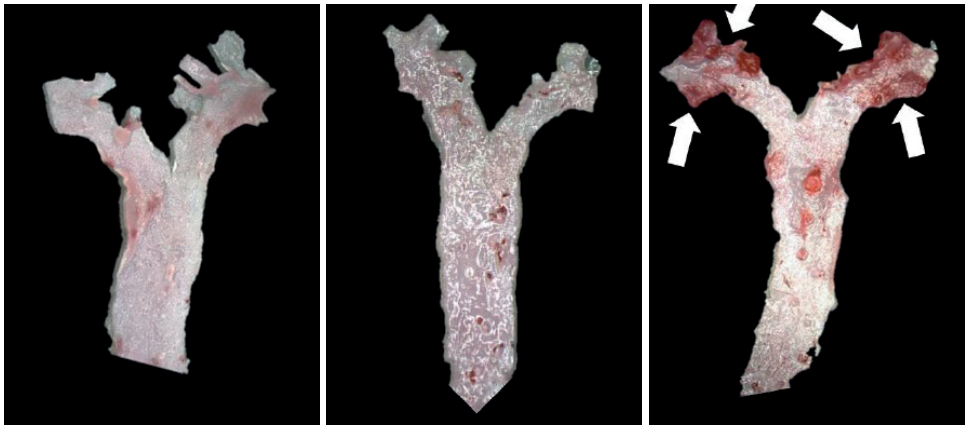

Fig. 1. Oil-red O-stained atherosclerotic lesions in the aorta. Atherosclerotic lesions (directed by white arrows) were developed only in the OBA group. The aortas were prepared from mice with the *en face* method, and atherosclerotic lesions were stained with an oil-red O solution. The mice were sacrificed after 16 weeks of feeding. A representative aorta image of each group is shown.
CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr* $^{-/-}$ mice, high fat + cholesterol diet.

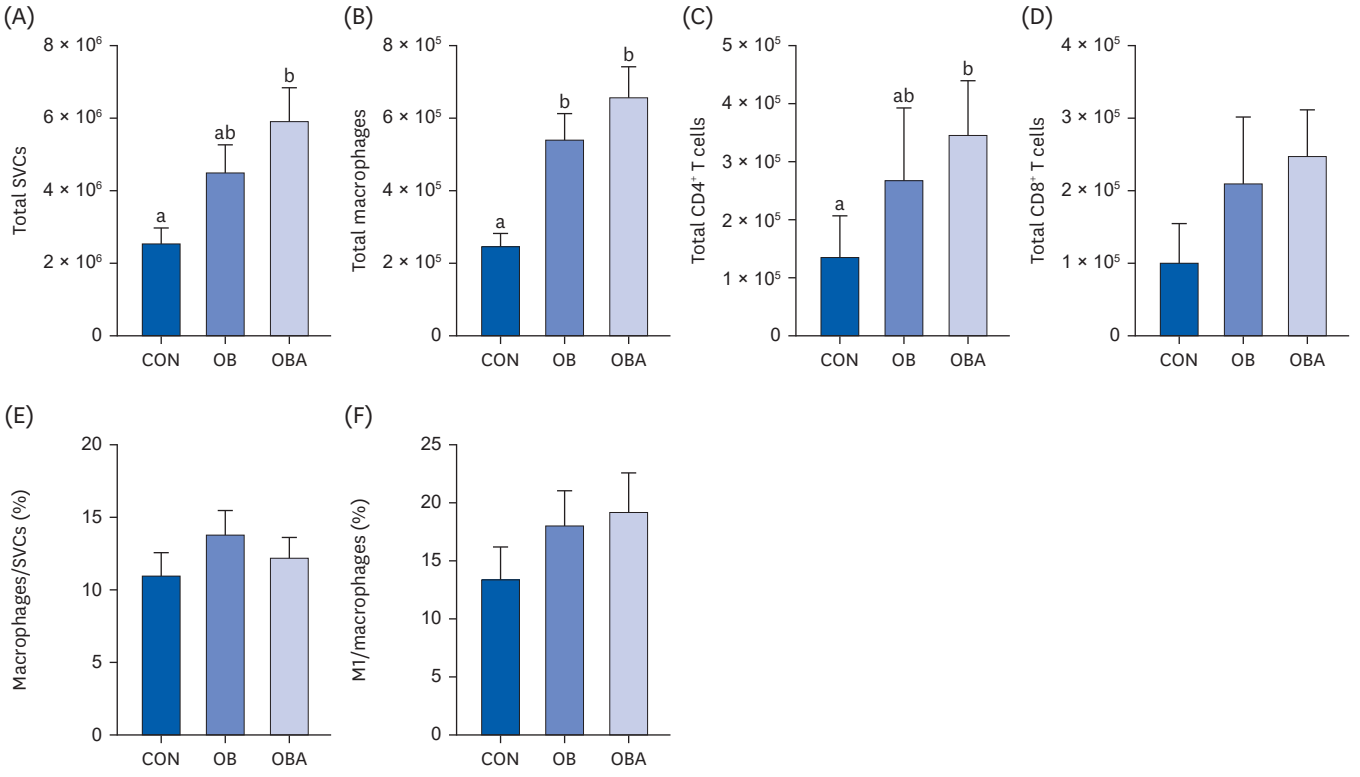

Fig. 2. Total cell numbers per mouse of (A) SVCs, (B) macrophages, (C) $CD4^+$ T cells and (D) $CD8^+$ T cells and percentages of (E) macrophages/SVCs, (F) M1/macrophages in SVCs from CON, OB, and OBA groups. The SVCs were freshly isolated from the murine visceral adipose tissue and analyzed by flow cytometry. The total cell numbers were calibrated by dividing by the pooled number of mice in each group (CON: 3, OB: 2, OBA: 2). The data are presented as the means ± SEMs, n = 9–12 for each group. One-way ANOVA (A, B, E, and F) or Kruskal-Wallis test (C and D) was performed to determine the difference among the 3 groups.
SVCs, stromal vascular cells; M1, M1 macrophages; CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr*$^{-/-}$ mice, high fat + cholesterol diet; SEMs, standard error of the means; ANOVA, analysis of variance.
[a,b]The different superscripts indicate significant differences ($P < 0.05$) by a Dunnett's test or Bonferroni correction.

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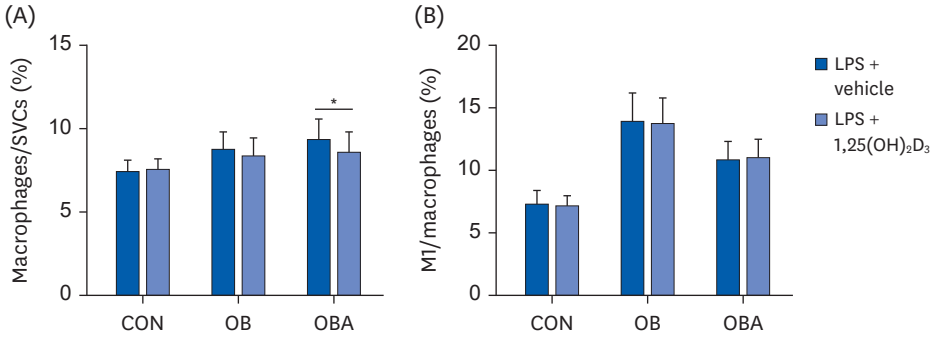

Fig. 3. Percentages of (A) macrophages/SVCs, (B) M1/macrophages in SVCs from CON, OB, and OBA groups. The SVCs were analyzed by flow cytometry after incubation with $1,25(OH)_2D_3$ (10 nM) or vehicle (0.1% ethanol) for 24 h and then stimulated with LPS (100 ng/mL) for another 24 h. The data are presented as the means ± SEMs, n = 7–10 for each group. One-way ANOVA (A) or Kruskal-Wallis (B) test was performed to determine the difference among the groups, and a paired Student's *t*-test was performed to determine the effects of *in vitro* $1,25(OH)_2D_3$ treatment in each group.
SVCs, Stromal vascular cells; M1, M1 macrophages; CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr* $^{-/-}$ mice, high fat + cholesterol diet; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; LPS, lipopolysaccharides; SEMs, standard error of the means; ANOVA, analysis of variance.
*Significant difference ($P < 0.05$) between the $1,25(OH)_2D_3$ treated and vehicle-treated group.

Proinflammatory cytokine production by SVCs and adipocytes

The levels of IL-6 and MCP-1 produced by SVCs and adipocytes were measured to determine if the *in vitro* $1,25(OH)_2D_3$ treatment affected the production of proinflammatory cytokines in VAT (**Fig. 4**). In SVCs with or without LPS stimulation, there was no difference in the IL-6 and MCP-1 production levels among 3 groups. The *in vitro* $1,25(OH)_2D_3$ treatment decreased the IL-6 and MCP-1 production significantly in the LPS-stimulated SVCs (7.8% and 8.8%

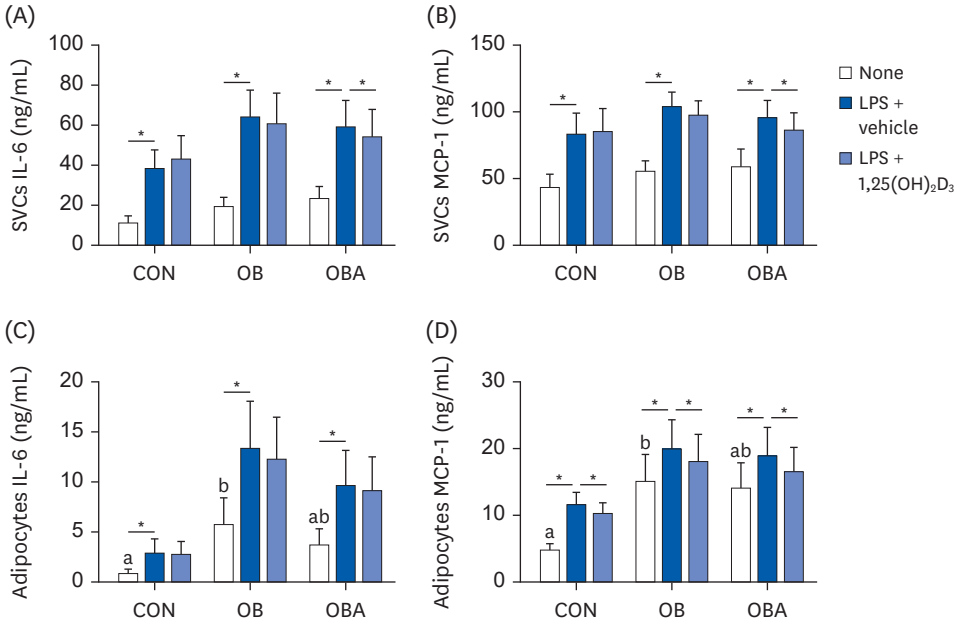

Fig. 4. Production of (A) IL-6 and (B) MCP-1 by SVCs, and (C) IL-6 and (D) MCP-1 by adipocytes from the CON, OB, and OBA groups. SVCs and adipocytes were cultured with $1,25(OH)_2D_3$ (10 nM) or the vehicle (0.1% ethanol) for 24 h and stimulated or unstimulated with LPS (100 ng/mL) for another 24 h. The supernatants were obtained to measure the IL-6 and MCP-1 levels using ELISA. The data are presented as the means ± SEMs, n = 7–12 for the SVCs from each group, n = 8–11 for adipocytes. A Kruskal-Wallis test was performed to determine the difference among the groups, and a paired Student's *t*-test or Wilcoxon signed-rank test was performed to determine the effects of the *in vitro* $1,25(OH)_2D_3$ treatment in each group.
IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein 1; SVCs, stromal vascular cells; CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr*$^{-/-}$ mice, high fat + cholesterol diet; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; LPS, lipopolysaccharides; SEMs, standard error of the means; ANOVA, analysis of variance.
[a,b]The different superscripts indicate a significant difference ($P < 0.05$) by a Bonferroni correction. *Significant difference ($P < 0.05$).

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decrease, respectively) only in the OBA group. In adipocytes, the OB group showed higher IL-6 and MCP-1 production than the CON group. MCP-1 production by LPS-stimulated adipocytes was reduced by the *in vitro* $1,25(OH)_2D_3$ treatment in all 3 groups. The production of IL-6 by adipocytes was unaffected by the $1,25(OH)_2D_3$ treatment.

mRNA expression of genes involved in inflammatory signaling pathways in SVCs

The expression of the genes involved in inflammatory signaling pathways was measured to determine if the decreased production of proinflammatory cytokines by $1,25(OH)_2D_3$ treatment in SVCs from the OBA group was due to the regulation of genes involved in the MAPK and NF-κB signaling pathways (**Fig. 5**). *Dusp1* and *Dusp10* are phosphatases that

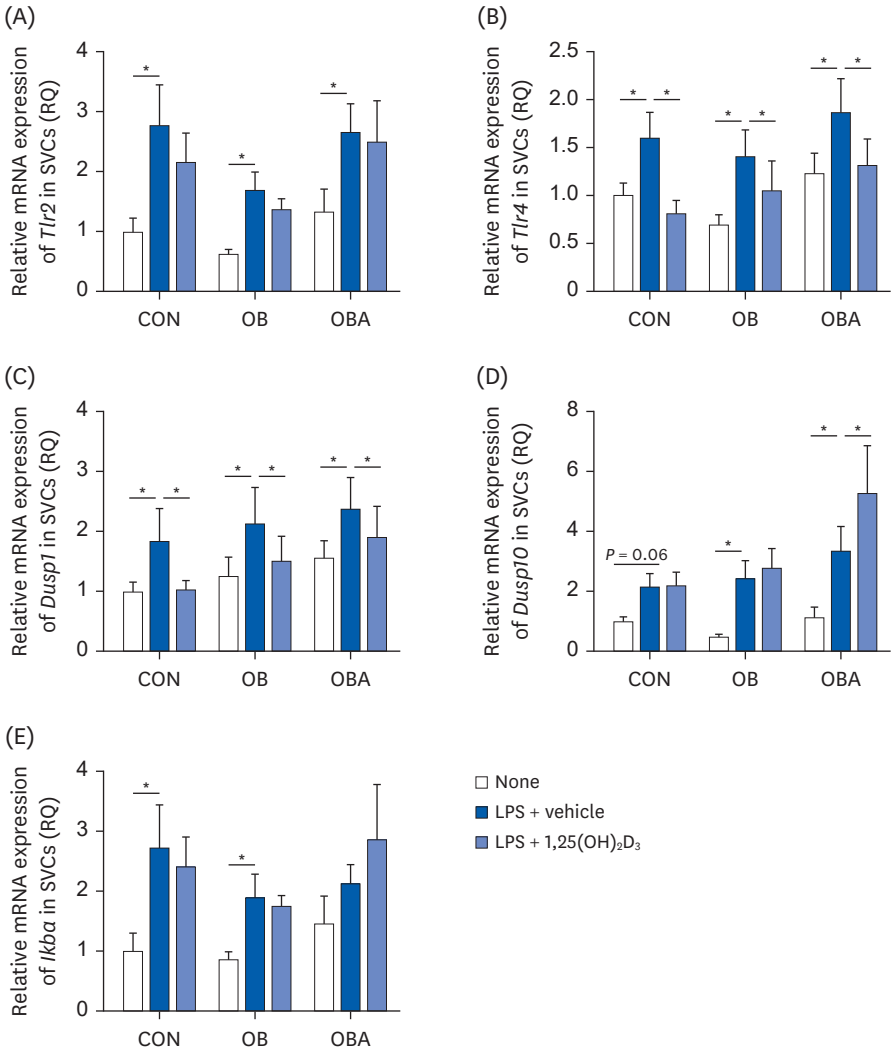

Fig. 5. Expression of the genes related to inflammatory signaling pathway in SVCs from CON, OB, and OBA groups. The relative mRNA levels of (A) *Tlr2*, (B) *Tlr4*, (C) *Dusp1*, (D) *Dusp10*, and (E) *Ikba* were analyzed by qRT-PCR. The SVCs were cultured with $1,25(OH)_2D_3$ (10 nM) or vehicle (0.1% ethanol) for 24 h and stimulated or unstimulated with LPS (100 ng/mL) for another 24 h. The data are presented as the means ± SEMs, n = 8–9 for each group. A one-way ANOVA (B and D) or Kruskal-Wallis test (A, C, and E) was performed to determine the difference among the groups, and a paired Student's *t*-test or Wilcoxon signed-rank test was performed to determine the effects of *in vitro* $1,25(OH)_2D_3$ treatment in each group.
SVCs, stromal vascular cells;*Tlr2*, Toll-like receptor 2; *Tlr4*, Toll-like receptor 4; *Dusp1*, dual-specificity protein phosphatase 1; *Dusp10*, dual-specificity protein phosphatase 10; *Ikbα*, nuclear factor of kappa light polypeptide gene enhanced in B-cells inhibitor alpha; CON, C57BL/6J, control diet; OB, C57BL/6J, high fat + cholesterol diet; OBA, *Ldlr* $^{-/-}$mice, high fat + cholesterol diet; qRT-PCR, quantitative reverse transcription polymerase chain reaction; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3; LPS, lipopolysaccharides; SEMs, standard error of the means; ANOVA, analysis of variance.
*Significant difference ($P < 0.05$).

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inactivate MAPK, and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (*Ikbα*) is an inhibitor of NF-κB transcription factor. The mRNA levels of *Tlr2*, *Tlr4*, *Dusp1*, *Dusp10*, and *Ikbα* in the SVCs, regardless of LPS stimulation, were similar in all 3 animal groups. The *in vitro* $1,25(OH)_2D_3$ treatment had no significant effect on *Tlr2* expression in all 3 groups, but it increased the *Dusp10* expression level significantly in the LPS-stimulated SVCs in the OBA group (56% higher). Moreover, the *Tlr4* and *Dusp1* mRNA levels in the LPS-stimulated SVCs from all 3 groups were significantly decreased by the $1,25(OH)_2D_3$ treatment. The mRNA expression of *Ikbα* was unaffected by the $1,25(OH)_2D_3$ treatment.

DISCUSSION

This study showed that vitamin D can exert anti-inflammatory effects on the adipose tissue of atherosclerotic mice. The *in vitro* treatment with $1,25(OH)_2D_3$ reduced MCP-1 production in adipocytes from the control, obese, and atherosclerotic mice and inhibited the production of inflammatory cytokines (IL-6 and MCP-1) by decreasing the macrophage ratio in the SVCs of atherosclerotic mice.

Adipose tissue is an energy reservoir and a regulator of inflammation in the body, leading to a systemic inflammatory state by increasing immune cell infiltration and proinflammatory cytokine secretion in obese individuals [37]. In the present study, HFC diet-fed mice in the OB and OBA groups had more SVCs and macrophages (per mouse) in VAT than in the CON group. There was no significant difference between the OB and OBA groups, a finding consistent with a report showing that the degree of macrophage infiltration in adipose tissue correlates with adiposity and not with hyperlipidemia and atherosclerosis development [38]. In addition, the OBA group had a larger number of total $CD4^+$ T cells than the other groups, suggesting that atherosclerosis development may be an independent factor promoting $CD4^+$ T cell infiltration into VAT.

The *in vitro* $1,25(OH)_2D_3$ treatment reduced MCP-1 production by adipocytes in all 3 groups, but the levels of MCP-1 and IL-6 production by SVCs were reduced only in the OBA group. The levels of MCP-1 and IL-6 production, which are involved in the pathogenesis of atherosclerosis, are related to the number of adipose tissue-resident macrophages [39]. Consistent with this, the percentage of macrophages in SVCs was reduced only in the OBA group. Sixteen weeks of vitamin D supplementation (10,000 IU/kg diet) with a high-fat diet has been reported to reduce the expression of M1 macrophage polarization-related genes in whole epididymal fat of obese mice [40]. In the present study, however, M1/macrophages (%) analyzed by flow cytometry in isolated SVCs was not changed by the *in vitro* $1,25(OH)_2D_3$ treatment. *Dusp10* expression increased after the *in vitro* $1,25(OH)_2D_3$ treatment in SVCs from the OBA group. *Dusp10* is involved in p38 and JNK dephosphorylation, which can inhibit IL-6 and MCP-1 production [41]. This suggests that vitamin D may help alleviate adipose tissue inflammation by reducing the production of proinflammatory cytokines in adipocytes and the SVCs of atherosclerotic mice.

On the other hand, adipose tissue-resident macrophages in obese mice increased the circulating TNF concentrations but had no direct effect on the development of atherosclerotic plaque [42]. In addition, DUSP10 plays an essential role in foam cell formation via ox-LDL-stimulated NF-κB activation in macrophages, and *Dusp10*-deficient $Ldlr^{-/-}$ mice showed attenuated atherosclerotic plaque formation, suggesting that DUSP10 is a novel target for the

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treatment of atherosclerosis [43,44]. The expression of *Ikbα*, a subunit that inhibits NF-κB activation, was increased by a $1,25(OH)_2D_3$ treatment [45], but the present study showed no change in *Ikbα* expression after the $1,25(OH)_2D_3$ treatment. Moreover, our study did not evaluate the effects of *Dusp10* on activating the ox-LDL/NF-κB pathway. Therefore, further investigation is required to determine the contribution of vitamin D on plaque development in atherosclerosis.

Activating TLR signaling through the recognition of fatty acids or LPS released from lipolysis of hypertrophic adipocytes can induce inflammatory cytokine production in adipose tissue via the NF-κB and MAPK downstream signaling pathways [46,47]. The subtypes of TLRs are involved in different aspects of the atherosclerotic inflammatory response, with TLR4, in particular, being closely associated with ox-LDL-induced inflammatory cytokine production and foam cell differentiation of macrophages [20]. Adipocyte hypertrophy, macrophage accumulation, and local inflammation were not attenuated in *Tlr4* and *Ldlr* double knockout mice, but the blood lipid levels and atherosclerosis were improved [48]. In the present study, the *Tlr4* expression levels in SVCs were decreased significantly by the $1,25(OH)_2D_3$ treatment in all 3 groups. TLR4 is a critical receptor that mediates the inflammatory response to LPS, and $1,25(OH)_2D_3$ downregulates *Tlr4* mRNA in monocytes in a time- and dose-dependent manner, which can modulate the expression of proinflammatory cytokines [21]. The *in vitro* $1,25(OH)_2D_3$ treatment decreased the *Dusp1* mRNA levels in all 3 groups. *Dusp1* rapidly dephosphorylates and inactivates activated MAPKs in mammalian cells [49]. In addition, *Dusp1* was upregulated by vitamin D in human PBMCs and mouse SVCs, a mechanism for inhibiting the MAPK signaling pathway downstream of the TLR-mediated pathway by LPS stimulation [25,26]. On the other hand, reduced response to LPS stimulation in SVCs because of a decrease in *Tlr4* expression by the $1,25(OH)_2D_3$ treatment may have limited its inhibitory mechanism. *Dusp1* also plays an important role in the lipid metabolism. Because PPARα, a key mediator of lipid oxidation, is a target of p38 MAPK, DUSP1 can indirectly attenuate PPARα activity [50,51]. DUSP1 is overexpressed in obese humans [52]. On the other hand, mice lacking DUSP1 exhibited increased MAPK activity and fatty acid oxidation and were resistant to high-fat diet-induced obesity [51]. This suggests that the vitamin D treatment can affect the lipid metabolism in SVCs by inhibiting *Tlr4* expression and preventing *Dusp1* overexpression.

Overall, the *in vitro* $1,25(OH)_2D_3$ treatment of SVCs decreased the percentage of macrophages/SVCs and increased the *Dusp10* mRNA level only in the OBA group, possibly leading to the downregulation of proinflammatory cytokine production. This might be driven by specific cholesterol metabolic differences in the OBA group. On the other hand, the *in vitro* $1,25(OH)_2D_3$ treatment decreased MCP-1 production by adipocytes and *Tlr4* expression in SVCs in all 3 groups. *Tlr4* expression does not appear to have a major $Ldlr^{-/-}$ mice-specific impact on alleviating adipocyte hypertrophy, macrophage accumulation, and local inflammation because the vitamin D treatment reduced *Tlr4* expression in all 3 groups. Increased *Dusp10* expression in the OBA group might have affected macrophage apoptosis, resulting in reduced proinflammatory cytokine production. In conclusion, vitamin D can be beneficial in decreasing macrophage recruitment and alleviating the immune response in atherosclerosis.

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