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Original Article

Reblastatins Inhibit Phenotypic Changes of Monocytes/Macrophages in a Milieu Rich in 27-Hydroxycholesterol

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ABSTRACT

We investigated effects of reblastatins on phenotypic changes in monocytes/macrophages induced by 27-hydroxycholesterol (27OHChol). Treatment of THP-1 monocytic cells with reblastatin derivatives, such as 17-demethoxy-reblastatin (17-DR), 18-dehydroxyl-17-demethoxyreblastatin (WK88-1), 18-hydroxyl-17-demethoxyreblastatin (WK88-2), and 18-hydroxyl-17-demethoxy-4,5-dehydroreblastatin (WK88-3), resulted in blockage of CCL2, CCL3, and CCL4 expression at the transcription and protein levels, which, in turn, impaired migration of monocytes/macrophages and Jurkat T cells expressing CCR5, and almost complete inhibition of transcription of M1 marker cytokines, like CXCL10, CXCL11, and TNF-α. Reblastatins also downregulated surface CD14 as well as soluble CD14 along with inhibition of LPS response and matrix metalloprotease-9 expression. Surface levels of mature dendritic cell (mDC)-specific markers, including CD80, CD83, CD88, CD197, and MHC class I and II molecules, were remarkably down-regulated, and 27OHChol-induced decrease of endocytic activity was recovered following treatment with 17-DR, WK88-1, WK88-2, and WK88-3. However, 15-hydroxyl-17-demethoxyreblastatin (DHQ3) did not affect the molecular or functional changes in monocytic cells induced by 27OHChol. Furthermore, surface levels of CD105, CD137, and CD166 were also down-regulated by 17-DR, WK88-1, WK88-2, and WK88-3, but not by DHQ3. Collectively, results of the current study indicate that, except DHQ3, reblastatins regulate the conversion and differentiation of monocytic cells to an immunostimulatory phenotype and mDCs, respectively, which suggests possible applications of reblastatins for immunomodulation in a milieu rich in oxygenated cholesterol molecules.

Keywords: Reblastatin; Monocytes; Macrophages; 27-Hydroxycholesterol

INTRODUCTION

Heat shock protein 90 inhibition leads to attenuated formation of atherosclerotic plaques by reducing inflammatory responses and suppressing migration of vascular smooth muscle cells into atherosclerotic plaque lesions in mouse model of atherosclerosis (1-3). Reblastatins

Conflicts of Interest

The authors declare no potential conflicts of interest.

Abbreviations

17-DR, 17-demethoxy-reblastatin; 27OHChol, 27-hydroxycholesterol; DHQ3, 15-hydroxyl-17-demethoxyreblastatin; ER, estrogen receptor; mCD14, membrane CD14; mDC, mature dendritic cell; MMP, matrix metalloprotease-9; PAMP, pathogen-associated molecular patterns; sCD14, soluble CD14; WK88-1, 18-dehydroxyl-17-demethoxyreblastatin; WK88-2, 18-hydroxyl-17-demethoxyreblastatin; WK88-3, 18-hydroxyl-17-demethoxy-4,5-dehydroreblastatin

Author Contributions

Conceptualization: Choi J, Kim BY, Lee D, Hong YS, Kim MS, Kim K; Data curation: Choi J, Kim K; Formal analysis: Choi J, Kim BY, Son Y, Kim K; Investigation: Choi J, Son Y; Resources: Lee D, Hong YS; Visualization: Choi J, Kim BY; Writing - original draft: Choi J; Writing - review & editing: Kim BY, Kim MS, Kim K.

inhibit heat shock protein 90 ATPase involved in the folding, activation, stabilization, and complex formation of proteins, thereby affecting various biological functions related to cell growth, differentiation and survival under normal and stress conditions (4,5). This inhibitory activity renders their derivatives effective against cancer cells. Both 17-demethoxy-reblastatin (17-DR) and 15-hydroxyl-17-demethoxyreblastatin (DHQ3) inhibit cell proliferation, induce apoptosis, and suppress cell invasion and migration of human breast cancer cells (6,7). 18-dehydroxyl-17-demethoxyreblastatin (WK88-1), 18-hydroxyl-17-demethoxyreblastatin (WK88-2), and 18-hydroxyl-17-demethoxy-4,5-dehydroreblastatin (WK88-3) suppress proliferation of gefitinib-resistant non-small cell lung cancer cells (8). However, it is unknown whether reblastatins exert their effects in a milieu rich in oxygenated cholesterol molecules.

27-Hydroxycholesterol (27OHChol) is an endogenous oxysterol with distinct effects on different cell types. 27OHChol regulates cholesterol biosynthesis in multiple types of cells (9), activates estrogen receptors (ERs) α target genes and stimulates breast cancer cell growth (10), decreases bone mineral density, potentially contributing to osteopenia (11), and antagonizes ER α/β, thereby inhibiting ER-mediated nitric oxide production by endothelium and vascular smooth muscle cells (12). Experimental results indicate that 27OHChol has multiple effects on immune cells. It enhances adhesion of monocytes to endothelial cells by promoting expression of TNF-α via the ERα-dependent process (13) and induces differentiation of monocytic cells to mature dendritc cells (mDCs) (14). Furthermore, monocytic cells activated by 27OHChol produce chemokines involved in inflammation and immune cell migration (15,16). This oxysterol also enhances responses to pathogen-associated molecular patterns (PAMPs) by up-regulating pattern recognition receptors (17,18). These findings suggest that 27OHChol alters the phenotype of monocytic cells and thereby modifies inflammation and immune responses. However, little is known regarding the bioactive metabolites from *Streptomyces* spp. that affect the phenotypic alteration induced by 27OHChol.

This study was undertaken to investigate whether reblastatins isolated from the culture of *Streptomyces* spp. influence the effects of 27OHChol on monocytic cells at molecular and cellular levels. We report a novel pharmacological action of reblastatins, which encompass inhibition of the 27OHChol-induced activation and differentiation of monocytic cells, as indicated by downregulation of inflammatory and cell surface molecules and functional modifications. We also determined effects of reblastatins on the expression of cell surface molecules whose levels are associated with atherosclerosis.

MATERIALS AND METHODS

Reagents

17-DR and DHQ3 were purified from the culture broths of *Streptomyces hygroscopicus* AC11 and from a genetically engineered strain (AC15) of *S. hygroscopicus*, respectively, as previously described (4,19). WK88-1, WK88-2, and WK88-3 were purified from a culture of *S. hygroscopicus* AC2, whose AHBA synthase gene was disrupted by the kanamycin-resistance gene, supplemented with 3-aminobenzoic acid (4). 27OHChol and Abs against CD14, CD80, CD83, CD88, CD197, β-actin, and MHC I and II molecules were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Phospho-specific Akt (Ser473) and Akt Abs were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-p65 and -phosphorylated p65 Abs were purchased from Santa Cruz Biotechnology. LPS from *Escherichia coli* K12 was purchased from InvivoGen (San Diego, CA, USA).

Cell culture and serum-starvation

THP-1 human monocytic cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C in RPMI 1640 medium supplemented with 10% FBS in the presence of penicillin and streptomycin. Jurkat T cells stably expressing CCR5 were maintained in RPMI 1640 medium supplemented with 10% FBS in the presence of geneticin (20).

Serum-starvation and treatment

THP-1 cells (2.5×10^5 cells/ml) were serum-starved by incubating for 24 h in RPMI 1640 medium supplemented with 0.1% BSA (endotoxin-free). 27OHChol and reblastatins, which were dissolved in ethanol and DMSO, respectively, were added. After incubation for indicated time periods, cells were centrifuged (500×g, 5 min). Supernatants were collected in fresh tubes and used in chemotaxis assay, ELISA, and zymography.

RT-PCR

Total RNA was reverse-transcribed to produce cDNA for 1 h at 42°C with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR was performed in triplicate using the LightCycler® 96 Real-Time PCR System (Roche Life Science, Penzberg, Germany) following a previously reported protocol; briefly, each 20 μl reaction consisted of 4 μl of cDNA template, 10 μl of SYBR Green Master Mix, and 2 μl of 10 pM forward and reverse primers of the gene to be quantified (21). Thermal cycling conditions were as follows: 95°C for 10 min, and 45 cycles at 95°C for 10 s, 50°C for 10 s, and 72°C elongation period for 10 s. The relative expression of each gene was calculated as the ratio to as the housekeeping gene of GAPDH using the LightCycler® 96 software (version 1.1.0.1320; Roche Life Science). Target gene mRNA levels were normalized to those of GAPDH using the $2^{-\Delta\Delta Ct}$ method (22). The primers used for real-time PCR were as follows: GAPDH, 5′-GAAGGTGAAGGTCGGAGT-3′ (forward) and 5′-GAAGATGGTGATGGGATTTC-3′ (reverse); CXCL10, 5′-TGTACGCTGTACCTGCATCA-3′ (forward) and 5′-GGACAAAATTGGCTTGCAGGA-3′ (reverse); CXCL11, 5′-AAGCAGTGAAAG TGGCAGAT-3′ (forward) and 5′-TAAGCCTTGCTTGCTTCGAT-3′ (reverse); TNF-α, 5′-CCCAG GGACCTCTCTCTAATC-3′ (forward) and 5′-ATGGGCTACAGGCTTGTCACT-3′ (reverse); CCL2, 5′-CAGCCAGATGCAATCAATGCC-3′ (forward) and 5′-TGGAATCCTGAACCCACTTCT-3′ (reverse); CCL3, 5′-AGTTCTCTGCATCACTTGCTG-3′ (forward) and 5′-CGGCTTCGCTTGG TTAGGAA-3′ (reverse); CCL4, 5′-CTGGGTCCAGGAGTACGTGT-3′ (forward) and 5′-GCGGA GAGGAGTCCTGAGTA-3′ (reverse); CD80, 5′-TGGTGCTGGCTGGTCTTTC-3′ (forward) and 5′-CTGTGCCACTTCTTTCACTTCC-3′ (reverse); CD83, forward 5′-TCCTGAGCTGCGCCTAC AG-3′ and 5′-GCAGGGCAAGTCCACATCTT-3′ (reverse); CD88, 5′-GTGGTCCGGGAGGAGT ACTTT-3′ (forward) and 5′-GCCGTTTGTCGTGGCTGTA-3′ (reverse); matrix metalloprotease-9 (MMP-9), 5′-GCACGACGTCTTCCAGTACC-3′ (forward) and 5′-CAGGATGTCATAGGTCACG TAGC-3′ (reverse).

Chemotaxis assay

Transwell Permeable Supports (Costar, Cambridge, MA, USA) were used to measure cell migration, as previously described (15). Briefly, THP-1 cells and $CCR5^+$ Jurkat T cells were loaded in 5-μm-pore polycarbonate transwell inserts which were placed in wells filled with supernatant isolated from THP-1 cells treated with 27OHChol and/or reblastatins. Following incubation at 37°C for 1 h, the number of cells in the bottom chamber was counted using a Vi-Cell XR cell counter (Beckman Coulter, Indianapolis, IN, USA).

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ELISA

The levels of CCL2, CCL3, CCL4, soluble CD14 (sCD14), and MMP-9 secreted in the culture media were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

MMP-9 gelatinolytic activity in cell supernatants

MMP-9 activity was assessed by gelatin zymography as previously reported (18). Briefly, supernatants isolated from the cultured THP-1 cells in serum-free media were concentrated 30-fold using the Vivaspin 2 centricon (Satorius Lab Instruments, Goettingen, Germany). The concentrated media were electrophoretically separated on 8% polyacrylamide gels containing 0.15% gelatin, following which the gels were washed and activated for 18 h at 37ºC. The activated gels were then stained with 0.2% Coomassie brilliant blue R-250. After destaining with methanol/acetic acid (20/10%) solution, the gels were photographed using ZoomBrowser EX 5.0 (Canon, Tokyo, Japan).

Flow cytometric analysis

Following treatment with reblastatins and 27OHChol, THP-1 cells were incubated for 2 h at 4ºC with Abs against CD14, CD80, CD83, CD88, CD197, CD105, CD137, and CD166 as well as with anti-MHC class I (rabbit polyclonal) and -MHC class II (mouse monoclonal IgG_1 kappa chain) Abs. Cells were washed and incubated with fluorescent dye-conjugated secondary Abs. After washing twice with PBS, cells were resuspended in 1% paraformaldehyde in PBS. Fluorescence was analyzed in the FACSCanto™II using the FACS Diva™ 6.0 software (BD Bioscience, Franklin Lakes, NJ, USA).

FITC-dextran uptake assay

After treatment with reblastatins and 27OHChol, THP-1 cells were re-suspended in culture medium containing 1 mg/ml FITC-conjugated dextran (40 kDa) (Sigma Aldrich, St. Louis, MO, USA) and incubated for 30 min at 37ºC. Resultant fluorescence was analyzed using a flow cytometer.

Western blot analysis

Cell lysates were separated by 10% SDS-PAGE followed by transfer of resolved proteins to nitrocellulose membranes. After blocking for 1 h in 1% skim milk in TBS (pH 7.4) containing 0.05% Tween-20, membranes were incubated with primary Abs against phospho-specific Akt (Ser473), Akt or β-actin at 4°C overnight. After 3 washes with 0.05% Tween 20/TBS for 10 min each, membranes were incubated for 1 h with HRP conjugated secondary Abs (1:8,000) at room temperature. Following washing with 0.05% Tween 20/TBS, membranes were exposed to chemiluminescent detection reagents (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL, USA). Chemiluminescence images were captured by using an Amersham Imager 600 (GE Healthcare Life Scicences, Pittsburgh, PA, USA).

Statistical analysis

Statistical analysis was performed via 1-way ANOVA, followed by Dunnett's multiple comparison test, using PRISM (version 5.0; GraphPad Software Inc., San Diego, CA, USA). A p-value of less than 0.05 is considered statistically significant.

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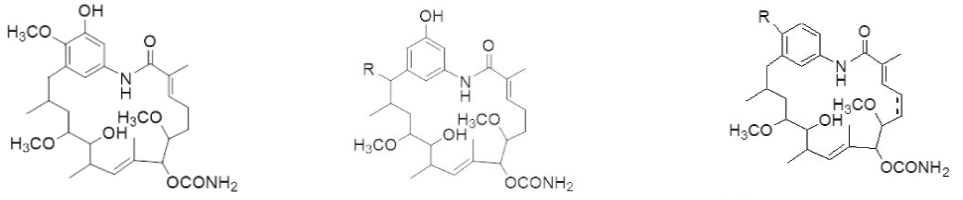
RESULTS

Reblastatins impair CCL2 expression and monocytic cell migration

Molecular structures of reblastatin derivatives used in this study are presented in **Fig. 1**, and effects of the reblastatins on monocytic cell activation were investigated by examining their effects on CCL2 expression. Transcription of the CCL2 gene and production of the gene product markedly increased in the presence of 27OHChol, and the increases were almost completely suppressed by treatment with 17-DR, WK88-1, WK88-2, and WK88-3. In contrast, DHQ3 did not attenuate mRNA and protein levels of CCL2 elevated by 27OHChol (**Fig. 2A and B**). We determined whether the reblastatins affect migration of monocytic cells by performing the chemotaxis assay (**Fig. 2C**). Enhanced monocytic cell migration was observed in response to the supernatant isolated from cells stimulated with 27OHChol. But when supernatants were isolated from cells stimulated in the presence of 17-DR, WK88-1, WK88-2, or WK88-3, the migration was reduced to basal level or almost completely inhibited. DHQ3, however, did not influence cell migration. These results indicate that 17-DR, WK88-1, WK88-2, and WK88-3, but not DHQ3, inhibit CCL2 expression and migration of monocytic cells induced by 27OHChol.



Compounds	MW	Name
17-DR	518	17-demethoxy-reblastatin
DHQ3	534	15-hydroxyl-17-demethoxyreblastatin
WK88-1	502	18-dehydroxyl-17-demethoxyreblastatin
WK88-2	518	18-hydroxyl-17-demethoxyreblastatin
WK88-3	516	18-hydroxyl-17-demethoxy-4,5-dehydroreblastatin

Figure 1. Structure of reblastatin derivatives used in this study. Reblastatin has a unique chemical structure. The chromophore is neither a benzoquinone nor a hydroquinone but a phenol, and the double bond at position 4 is hydrogenated.

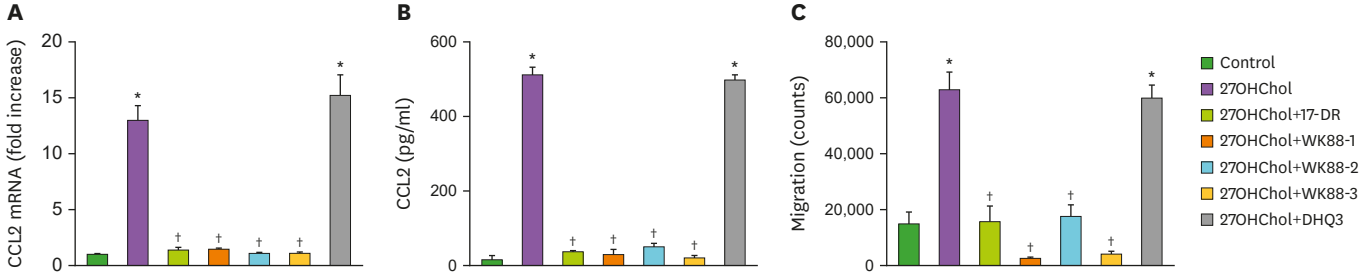


Figure 2. Inhibition of CCL2 expression and monocytic cell migration. After serum-starvation, THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatin derivatives (1 μg/ml each). (A) Levels of CCL2 transcript were assessed by real-time PCR. The y-axis values represent fold increases in CCL2 mRNA levels normalized to GAPDH levels, relative to that of the non-treated THP-1 cells (control). (B) The levels of CCL2 protein in the media were measured by ELISA. (C) Monocytic cells were exposed to the conditioned media isolated above, and numbers of migrated monocytic cells were counted after incubation for 3 h.Data are expressed as the means±SD (n=3 replicates for each group).
*p<0.001 vs. control; †p<0.001 vs. 27OHChol.

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We examined concentration effects of the reblastatin derivatives. Among the reblastatins used in this study, WK88-3 inhibited 27OHChol-induced expression of CCL2 in a dose-dependent manner with an ED50 of 0.02 μg/ml (**Supplementary Fig. 1**).

Reblastatins inhibit expression of CCR5 ligands and migration of CCR5-positive T cells

Monocytic cells stimulated with 27OHChol secrete CCR5 ligands, like CCL3 and CCL4, thereby enhancing migration of CCR5-expressing T cells (15). We, therefore, examined whether reblastatins affect expression of the CCR5 ligands and migration of the T cells. We observed that treatment with 17-DR, WK88-1, WK88-2, and WK88-3 resulted in profound suppression of both transcription and secretion of CCL3 and CCL4 (**Fig. 3A and B**). The supernatant isolated from THP-1 cells stimulated with 27OHChol enhanced migration of the CCR5-positive T cells (**Fig. 3C**). This migration was impaired when supernatants were isolated in the presence of 17-DR, WK88-1, WK88-2, or WK88-3. However, the supernatant isolated from the THP-1 cells stimulated with 27OHChol in the presence of DHQ3 did not influence migration of the Jurkat cells although decreased secretion of the CCR5 ligands was observed (**Fig. 3**). Overall, these results indicate that reblastatins, except DHQ3, inhibit the production of CCL3 and CCL4 and migration of CCR5-positive T cells induced by 27OHChol.

Reblastatins regulate expression of M1 cytokines

CCL2 is associated with classically activated/M1 macrophages. Therefore, we examined whether reblastatins influence the expression of cytokines expressed by pro-inflammatory M1-macrophages. Transcript levels of CXCL10, CXCL11, and TNF-α were significantly elevated following stimulation with 27OHChol, but these increases were blocked by 17-DR, WK88-1, WK88-2, and WK88-3, but not by DHQ3 (**Fig. 4**). Taken together with data of CCL2, these results indicate that 17-DR, WK88-1, WK88-2, and WK88-3 inhibit activation of cells to an inflammatory phenotype.

Reblastatins down-regulate CD14 and LPS response

27OHChol upregulates pattern recognition receptors and enhances the innate immune response (17,18). We evaluated effects of reblastatins on the CD14 protein expressed by monocytic cells. The level of cellular CD14 protein increased following stimulation with 27OHChol, but the increase was suppressed by treatment with reblastatins, as demonstrated via Western blot analysis (**Fig. 5A**). To evaluate the effect of individual reblastatin derivatives

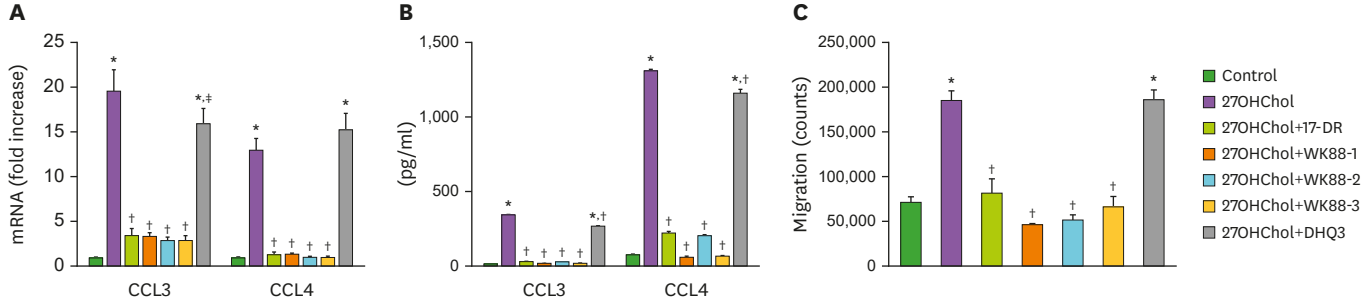

Figure 3. Inhibition of CCL3 and CCL4 production and migration of CCR5-expressing T cells. Following serum-starvation, THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each). (A) Transcript levels of the CCL3 and CCL4 genes were determined by real-time PCR. (B) The amount of CCL3 and CCL4 proteins released from THP-1 cells was calculated by ELISA. (C) CCR5-positive Jurkat T cells were exposed to the conditioned media isolated from THP-1 cells treated with or without reblastatins, and tested for their ability to migrate by chemotaxis assay. Data are expressed as the means±SD (n=3 replicates for each group).
*$p<0.001$ vs. control; †$p<0.001$ vs. 27OHChol; ‡$p<0.05$ vs. 27OHChol.

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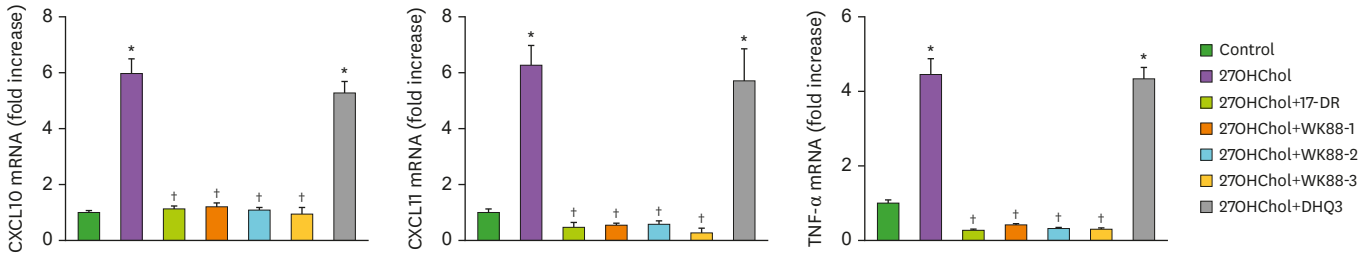

Figure 4. Blockage of transcription of M1 cytokines. Serum-starved THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each), after which transcript levels of CXCL10, CXCL11, and TNF-α were assessed by real-time PCR. The results are representative of 3 independent experiments. Data are expressed as mean±SD (n=3 replicates for each group).
*p<0.001 vs. control; †p<0.001 vs. 27OHChol.

on CD14 expression, the levels of membrane CD14 (mCD14) and sCD14 were determined by flow cytometry and ELISA, respectively. Stimulation of THP-1 cells with 27OHChol resulted in increased percentage of cells expressing mCD14, from 13.4% to 35.5%, as compared to untreated cells; this increase was reduced to 7.2%, 7.7%, 7.1%, 8.3%, and 22.5%, following treatment with 17-DR, WK88-1, WK88-2, WK88-3, and DHQ3, respectively (**Fig. 5B**) (for information of time course effects of reblastatins on the level of surface CD14; **Supplementary Fig. 2**). Similarly, the significantly increased levels of sCD14 secreted from THP-1 cells by stimulation with 27OHChol were suppressed after treatment with 17-DR, WK88-1, WK88-2, and WK88-3, but not with DHQ3 (**Fig. 5C**). We also investigated whether reblastatins affect transcription of CD14. Transcript levels of the CD14 were elevated in the presence of 27OHChol, and the elevation remain unchanged following treatment with the reblastatins (data not shown).

CD14 mediates responses to bacterial LPS (23). We, therefore, investigated whether reblastatins influence the LPS response by determining CCL2 expression. Transcript levels of the CCL2 gene increased to 16.7- and 5.5-folds in the presence of 27OHChol and LPS alone, respectively (**Fig. 5D**). Addition of LPS to 27OHChol-stimulated cells resulted in a 31.3-fold elevation of the CCL2 transcript levels, but the elevation was significantly suppressed by treatment with 17-DR, WK88-1, WK88-2, and WK88-3, but not by DHQ3. Reblastatins also affected the LPS-mediated production of CCL2 in a similar pattern as observed in its transcription (**Fig. 5E**). Stimulation of THP-1 cells with 27OHChol resulted in increased secretion of CCL2, and addition of LPS to 27OHChol-stimulated cells further enhanced CCL2 secretion. The LPS-enhanced CCL2 secretion was impaired after treatment with 17-DR, WK88-1, WK88-2, and WK88-3. DHQ3, however, did not inhibit the CCL2 production. Collectively, these results indicate that 17-DR, WK88-1, WK88-2, and WK88-3 suppress expression of CD14 and response to LPS stimulus.

Reblastatins regulate MMP-9 activity

Proteolytic cleavage of mCD14 by MMP-9 is one of the mechanisms generating sCD14 (24). Therefore, we examined the effects of reblastatins on MMP-9 activity. Stimulation of THP-1 cells with 27OHChol resulted in increased MMP-9 activity, which was attenuated by treatment with 17-DR, WK88-1, WK88-2, and WK88-3, as visualized by gelatin zymography (**Fig. 6A**). Quantification of the effects of reblastatins on MMP-9 revealed that 27OHChol increased the transcript levels of the MMP-9 gene by 3.9-fold, but MMP-9 gene transcription was completely inhibited by treatment with 17-DR, WK88-1, WK88-2, and WK88-3, and decreased to 3.06 fold by DHQ3 (**Fig. 6B**). These results indicate that 17-DR, WK88-1, WK88-2, and WK88-3 effectively regulate the expression of MMP-9 induced by 27OHChol.

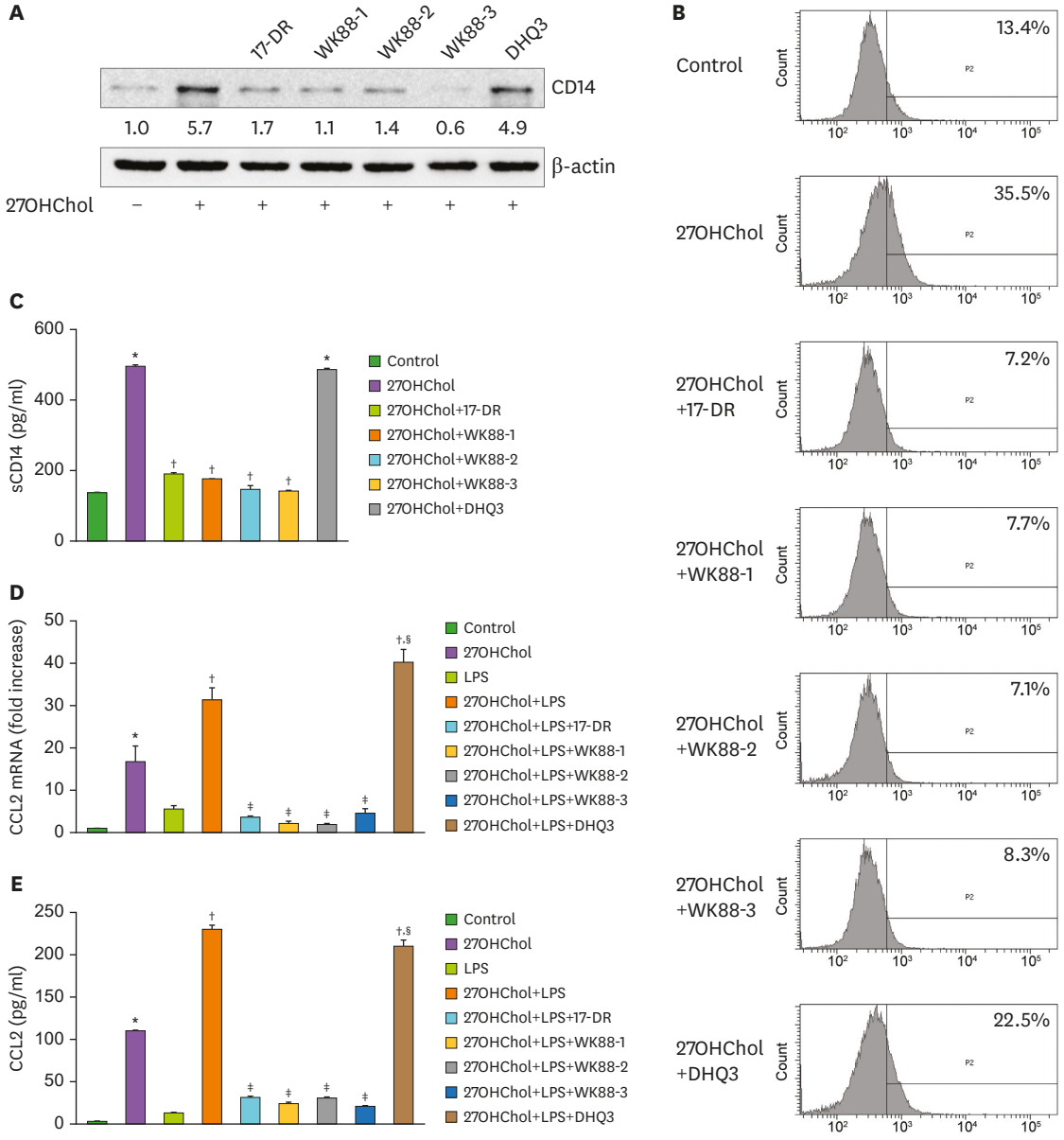

Figure 5. Down-regulation of CD14 expression and suppression of LPS response. (A-C) After serum-starvation, THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each). (A) Cell extracts were obtained from the cells, followed by detection of CD14 and β-actin via Western blot analysis. (B) Cells were immunostained for surface CD14, and fluorescence was analyzed by flow cytometry. (C) Culture media were isolated, and the amount of soluble CD14 protein secreted into the culture media was measured by ELISA. The results are representative of 3 independent experiments. (D, E) Serum-starved THP-1 cells were incubated for 24 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each), followed by stimulation for 9 h with or without LPS (100 ng/ml). (D) Levels of CCL2 transcript were assessed by real-time PCR. Data are expressed as the means±SD (n = 3 replicates for each group). (E) Culture media were isolated, and the amount of CCL2 protein secreted into the culture media was measured by ELISA. Data represent a representative experiment (from 3 independent experiments).
*$p<0.001$ vs. control; †$p<0.001$ vs. 27OHChol; ‡$p<0.001$ vs. 27OHChol plus LPS; §$p<0.01$ vs. 27OHChol plus LPS.

Reblastatins attenuates Akt phosphorylation

Akt is involved in the 27OHChol-induced expression of CCL2 (16). Therefore, to understand molecular mechanisms underlying their inhibitory effects, we investigated whether reblastatins affect the Akt phosphorylation (**Fig. 7**). We observed that the 27OHChol-enhanced Akt phosphorylation was suppressed following treatment with 17-DR, WK88-1, WK88-2, and WK88-3. DHQ3, however, did not affect phosphorylation of Akt.

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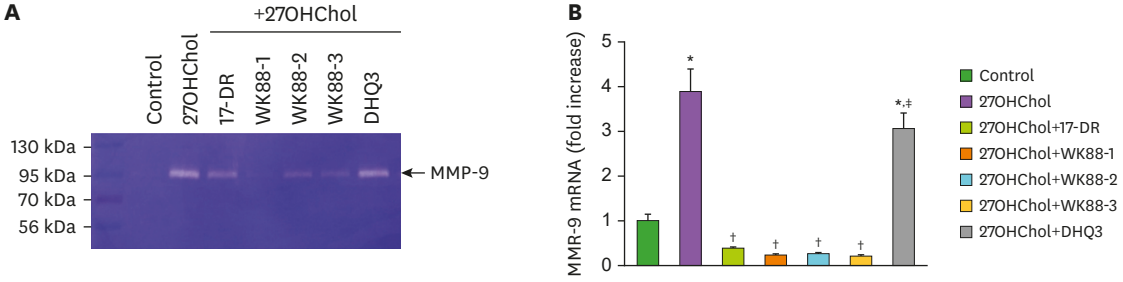

Figure 6. Decreases in MMP-9 activity. Following serum-starvation, THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each). (A) Culture media were isolated, and the activity of MMP-9 secreted from cells was assessed by gelatin zymography. Clear zones against the blue background indicate gelatinolytic activity. Control cells were cultured for 48 h in the medium alone. (B) Levels of MMP-9 transcript were assessed by real-time PCR. Data are expressed as the means±SD (n = 3 replicates for each group). The results are representative of 3 independent experiments.
*$p<0.001$ vs. control; †$p<0.001$ vs. 27OHChol; ‡$p<0.05$ vs. 27OHChol.

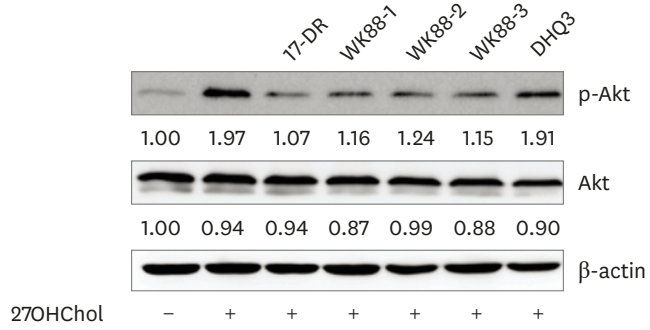

Figure 7. Suppression of Akt phosphorylation. Cell lysates were obtained after exposure of THP-1 cells for 4 h to 27OHChol (2 μg/ml) and indicated reblastatins (1 μg/ml each). Following determination of protein concentration, an equal amount of protein was analyzed by western blotting using Abs against Akt and phosphorylated Akt. Data represent a representative experiment (from 3 independent experiments).

Reblastatins down-regulate mDC-specific markers

To examine whether reblastatins affect monocytic cell differentiation, we examined their effects on expression of mDC-specific markers by real-time PCR and flow cytometry (**Fig. 8**). Transcript levels of CD80, CD83, and CD88 genes were elevated after stimulation with 27OHChol. However, treatment with 17-DR, WK88-1, WK88-2, and WK88-3 resulted in significantly attenuated transcription of the CD molecules (**Fig. 8A**). In agreement with results of the real-time PCR, we observed down-regulation in the cell surface levels of CD molecules after reblastatins exposure (**Fig. 8B**). The percentage of CD80 positive cells increased from 4.3% to 25.2% in the presence of 27OHChol, but was suppressed to 1.9%, 3.5%, 2.3%, and 2.1% after treatment with 17-DR, WK88-1, WK88-2, and WK88-3, respectively. The reblastatins also decreased the surface level expression of CD83 and CD88 in a pattern similar to that of CD80. However, DHQ3 did not significantly affect the transcription and surface expression of these molecules. Overall, these results indicate that reblastatins, except DHQ3, down-regulate the transcription and surface expression of mDC markers induced by 27OHChol. These results suggest that reblastatins inhibit differentiation of monocytic cells to mDC phenotype induced by 27OHChol.

Reblastatins down-regulate MHC molecules and homing factor

We investigated effects of reblastatins on levels of MHC molecules and CD197 (CCR7), a homing factor. The percentage of cells exhibiting high levels of MHC class I and II molecules increased following stimulation with 27OHChol, which was abrogated by treatment with

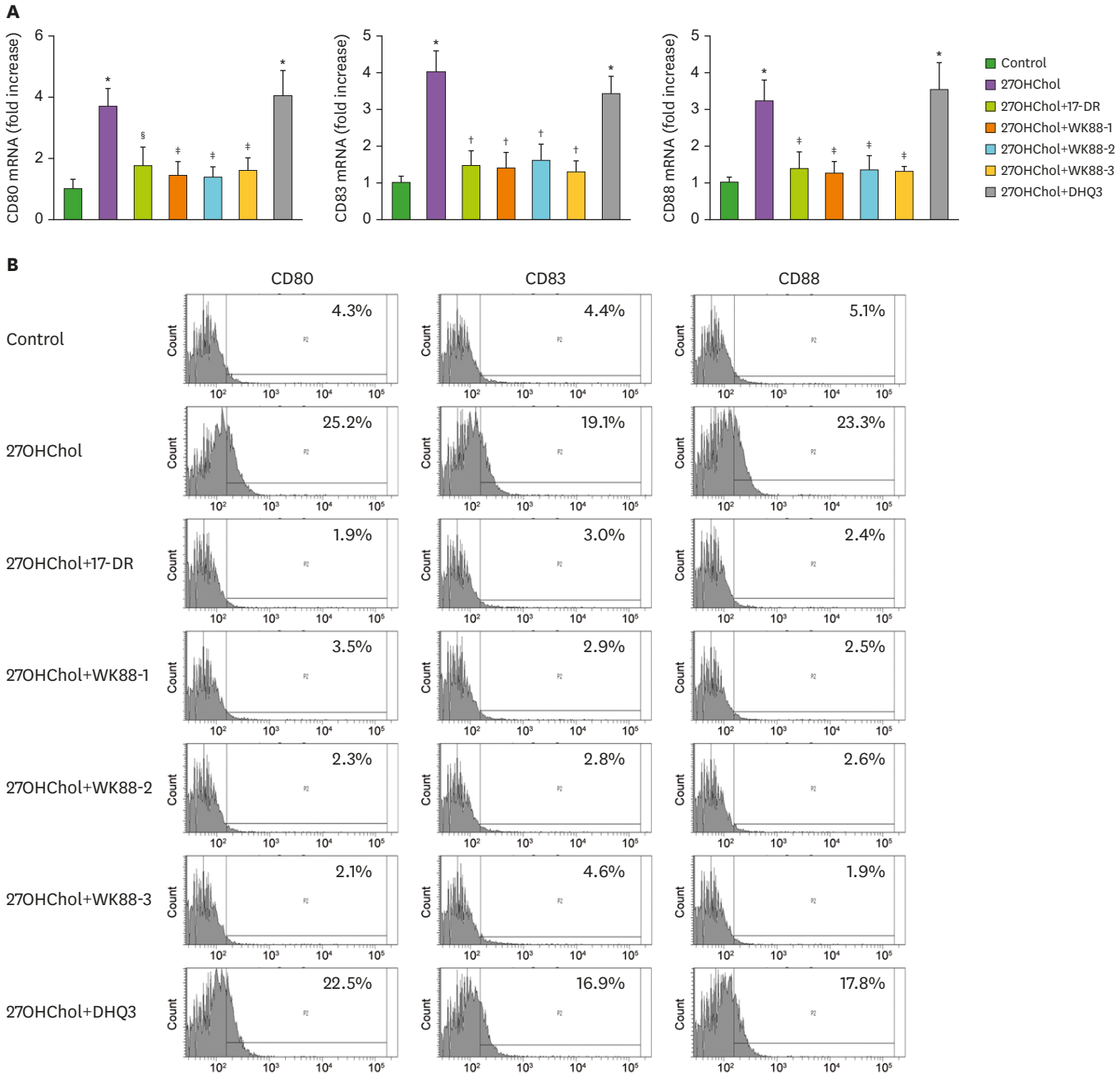

Figure 8. Down-regulation of mDC markers. Following serum-starvation, THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each). (A) Transcription of CD80, D83, and CD88 genes was assessed by real-time PCR. Data are expressed as mean±SD (n=3 replicates for each group). (B) After immunostaining of CD80, CD83, and CD88 with fluorophore-conjugated Abs, cells were analyzed by flow cytometry. The results are representative of 3 independent experiments.
*p<0.001 vs. control; †p<0.001 vs. 27OHChol; ‡p<0.01 vs. 27OHChol; §p<0.05 vs. 27OHChol.

17-DR, WK88-1, WK88-2, and WK88-3 (**Fig. 9A and B**). DHQ3, however, did not affect the expression of MHC molecules. CD197 is a cell surface molecule important for migration of mDC. 27OHChol elevated the percentage of cells positive for CD197 from 2.5% to 19.9%, but the percentage was decreased to 1.5%, 1.1%, 1.9%, and 1.5% following treatment with 17-DR, WK88-1, WK88-2, and WK88-3, respectively. In contrast, DHQ3 did not influence the CD197 expression (**Fig. 9C**).

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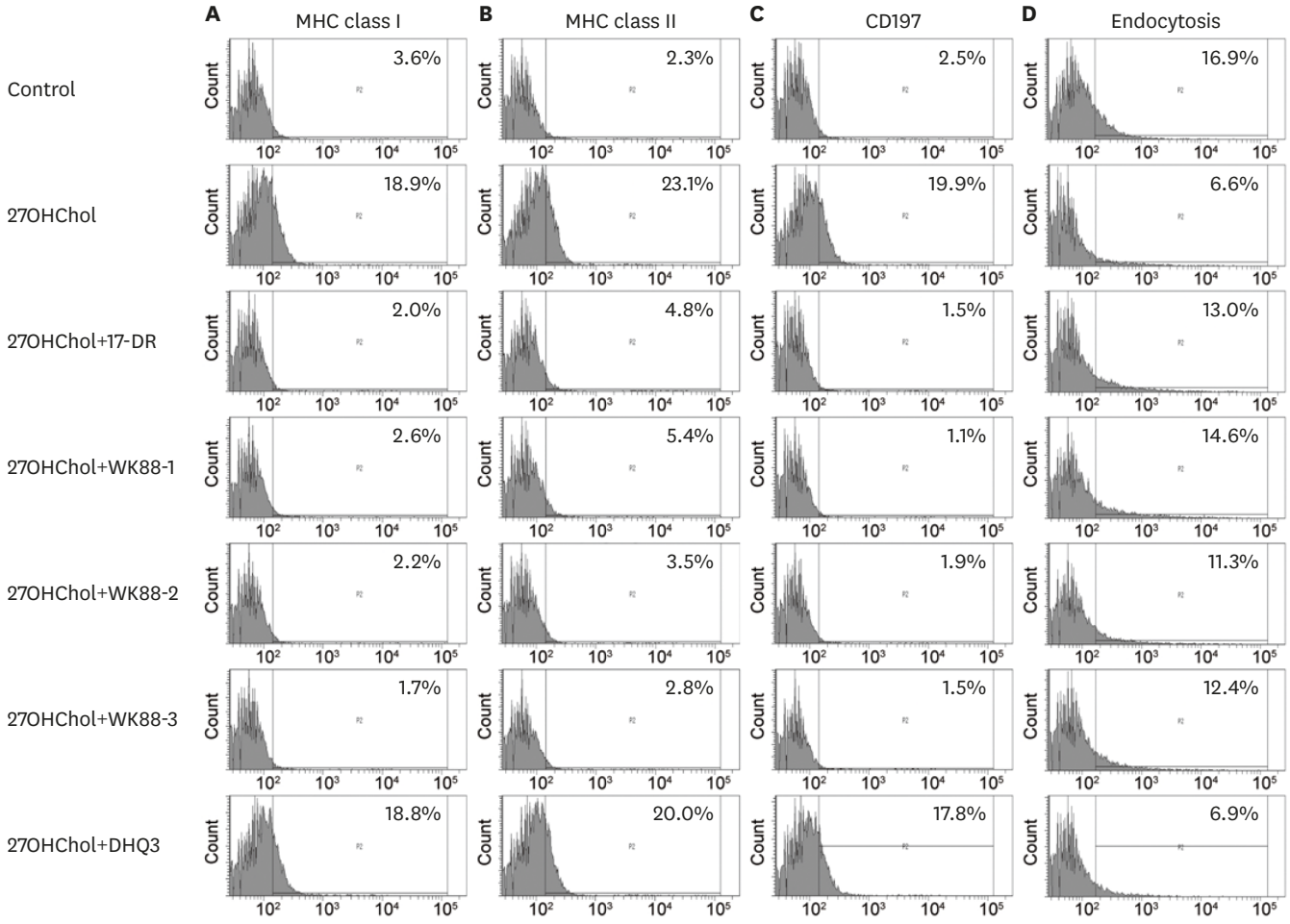

Figure 9. Down-regulation of MHC molecules as well as CD197 and recovery of endocytic function. (A-C) After serum-starvation, THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each). Cells were immunostained for MHC I (A), MHC II (B), and CD197 (C) with fluorophore-conjugated Abs, and fluorescence was analyzed by flow cytometry. The results are representative of 3 independent experiments. (D) Following treatment for 48 h with 27OHChol in the absence or presence of the indicated reblastatins, THP-1 cells were incubated with for 1 h with 1 mg/ml FITC-conjugated dextran. Fluorescence was analyzed by flow cytometry. Data represent a representative experiment (from 3 independent experiments).

Reblastatins suppress alteration of endocytic function

We investigated whether reblastatins affect endocytic function of monocytic cells (**Fig. 9D**). The percentage of control cells exhibiting endocytic activity reduced from 16.9% to 6.6% following stimulation with 27OHChol, but it recovered to 13.0%, 14.6%, 11.3%, and 12.4% in the presence of 17-DR, WK88-1, WK88-2, and WK88-3, respectively. Similar to other results, DHQ3 did not affect the endocytic activity altered by 27OHChol. The data indicate that reblastatins, except DHQ3, restore the functional alteration of monocytic cells modified by 27OHChol. Collectively, these results suggest that 17-DR, WK88-1, WK88-2, and WK88-3 regulate differentiation and function of monocytic cells in a milieu rich in 27OHChol.

Reblastatins down-regulate CD molecules involved in atherosclerosis

27OHChol promotes atherosclerosis and upregulates CD molecules involved in atherosclerosis, like CD105, CD137, and CD166 (13,25). Therefore, by applying flow cytometry, we determined whether reblastatins influence the expression of CD molecules (**Fig. 10**). The percentage of cells expressing CD105, CD137, and CD166 increased from 5.5%, 5.2%, and 4.6% to 42.8%, 39.3%, and 34.9%, respectively, following stimulation with

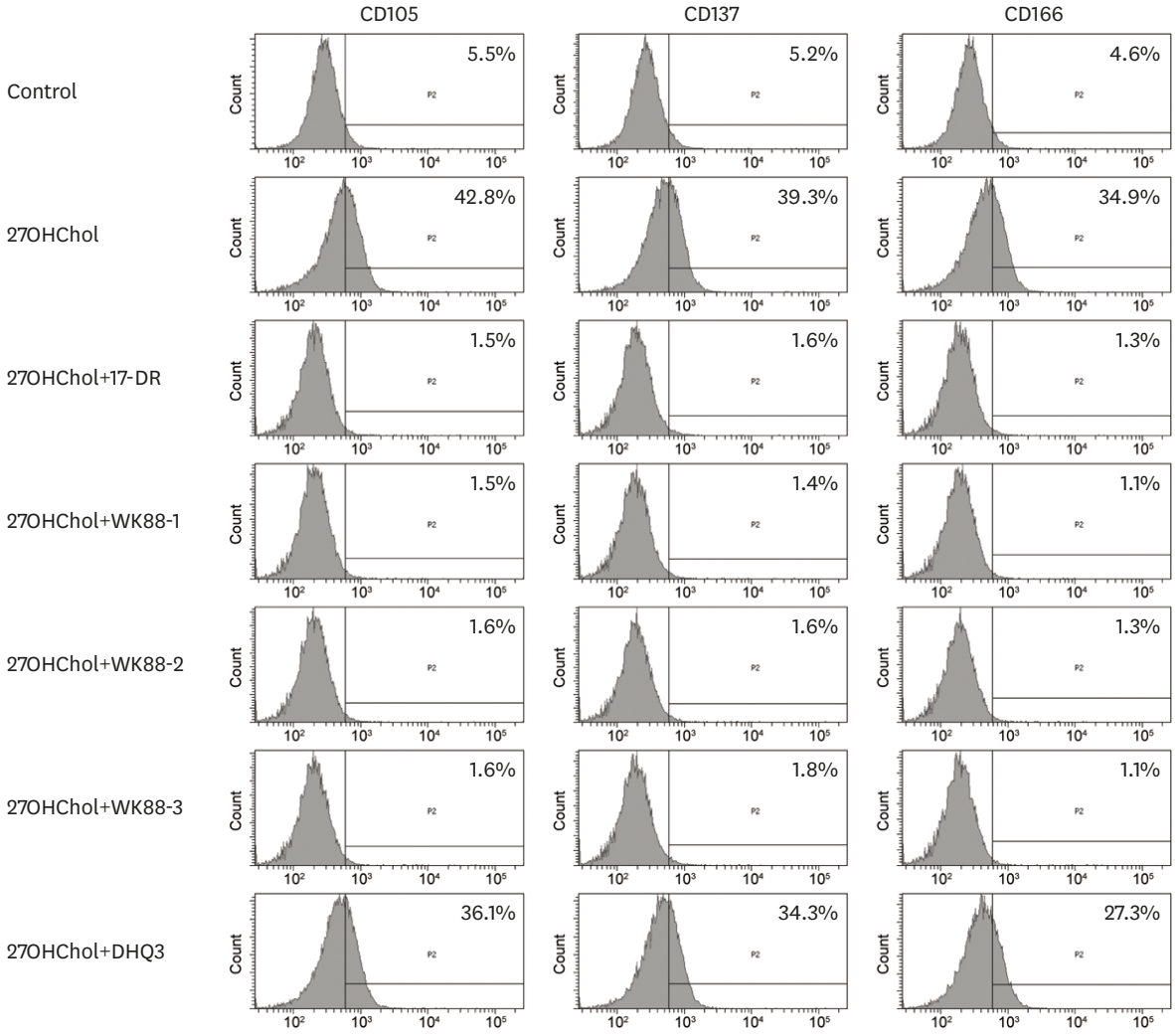

Figure 10. Down-regulation of CD molecules associated with atherosclerosis. Serum-starved THP-1 cells were cultured for 48 h with 27OHChol (2 μg/ml) in the absence or presence of the indicated reblastatins (1 μg/ml each). Cells were immunostained for CD105, CD137 and CD166 with fluorophore-conjugated Abs, and fluorescence was analyzed by flow cytometry. Data represent a representative experiment (from 3 independent experiments).

27OHChol. The increases were completely blocked following treatment with 17-DR, WK88-1, WK88-2, and WK88-3, but not with DHQ3.

DISCUSSION

27OHChol is an endogenous molecule involved in inflammation or immune responses via cell activation and differentiation. Hence, drugs that affect the biological action of 27OHChol are likely to have anti-inflammatory or immunosuppressive effects. In agreement with the idea, diclofenac, a non-steroidal anti-inflammatory drug, and dexamethasone and cyclosporine A, both immunosuppressive drugs, are reported to suppress inflammation or cell differentiation induced by 27OHChol (25-27). Furthermore, 4-*O'*-methylalpinumisoflavone also impairs the immunostimulatory activity of 27OHChol (21). However, it is unknown whether metabolites from *Streptomyces* spp. affect the immunological effects of 27OHChol. In the current study, using THP-1 monocytes/macrophages, we investigated whether reblastatins, including 17-DR, DHQ3,

WK88-1, WK88-2, and WK88-3, influence 27OHChol-mediated responses at the cellular and molecular levels. We believe that the effects of reblastatins demonstrated in the current study are not due to cytotoxicity, because 27OHChol and reblastatins did not cause cell death at the concentrations and duration of treatment used in this study (**Supplementary Fig. 3**).

A common structural feature of the reblastatin derivatives is the 17-methoxyreblastatin structure in the benzene ring (4,6). Among the reblastatins, DHQ3 is the only structure in which the hydrogen connected to the 15th carbon is modified to the hydroxyl group (4,8). Results of this study reveal that, of the reblastatin derivatives used in this study, DHQ3 alone did not influence the expression of CCL2, CXCL10, CXCL11, TNF-α, sCD14 as well as mDC markers, cell migration and endocytic function, suggesting a very weak inhibitory effect of DHQ3 on 27OHChol-induced inflammation and cell differentiation. These findings suggest that modification of the 15th carbon in reblastatin structure is likely to be crucial for exerting inhibition of 27OHChol-induced responses.

Monocytic cells that differentiate in the presence of 27OHChol exhibit high expression of mDC-specific markers, including CD83, CD88, and MHC class II molecules, as well as loss of endocytic function (14). The strong inhibitory effects of reblastatin derivatives on the expression levels of these markers suggest that they effectively suppress cell differentiation into mDC type. The mDC-specific markers activate T lymphocytes via distinct mechanisms. As professional Ag presenting cells, DCs take up soluble Ags, break them into peptides, and present the peptides in conjunction with class II molecules on the cell surface. The recognition of the Ag fragment attached to MHC II molecule results in the T cell-mediated immune response (28). CD83 has a co-stimulatory activity on T lymphocytes and contributes to the development of CD4 positive phenotype (29), whereas CD88 is a C5a receptor and plays an important role in the Th2 immune response (30). Therefore, the results of the current study suggest that reblastatins impair the T cell activation via downregulation of stimulatory signals and Ag presentation in an environment rich in 27OHChol.

Monocytic cells activated by 27OHChol produce molecules that stimulate inflammation and immune responses, including CCL2, CCR5 ligands, M1 chemokines and CD14. CCL2 plays a crucial role in pathogeneses of diseases such as atherosclerosis and rheumatoid arthritis, characterized by monocytic infiltrates, because the chemokine recruits monocytes to the site of inflammation (31). CCR5 ligands preferentially recruit Th1 T lymphocytes that express CCR5 as well as monocytes and thereby promote inflammatory responses (31,32). CD14 binds to LPS and transfers the bound LPS to TLR4, which initiates inflammatory responses by mediating the expression of chemokines including CCL2 (33), and 27OHChol predisposes monocytic cells to superiproduction of CCL2 in response to LPS by up-regulating CD14 on the cell surface (18). CXCL10 is chemotactic for T cells, dendritic cells and monocytes/macrophages and promote T cells adhesion to endothelial cells, whereas CXCL11 is chemotactic for activated T cells (32). Therefore, concurrent inhibition of expression of the molecules aforementioned by reblastatins suggests that they suppress conversion of monocytic cell to an immunostimulatory phenotype in a milieu rich in 27OHChol.

The expression of CD14 is increased in macrophages within atherosclerotic lesions and a chronic infection mimicked by LPS injections provokes a more severe atherosclerosis in apoE$^{-/-}$ mice, suggesting that inflammatory reaction against bacterial PAMPs is likely to be enhanced in atherosclerosis (34,35). We demonstrated that the reblastatins down-regulate CD14 and reduce sCD14 secretion, and they also inhibit the LPS-induced overexpression of

CCL2. In addition, we observed down-regulation of specific markers involved in pathogenesis of atherosclerosis, like CD137, CD105, and CD166. CD137 promotes inflammation of atherosclerotic plaque as monocyte and T cell stimulating factor (36), and CD105 positively affects both plaque inflammation and hemorrhage (37), and CD166, which is overexpressed in atherosclerosis tissues, is chemotactic to monocytes (38). Collectively, these findings imply that reblastatins affect progression of atherosclerosis by impairing inflammation and LPS response.

We attempted to understand the underlying molecular mechanisms contributing to the effects of reblastatins. Results of the current study suggest suppression of Akt activity by reblastatins, which agrees with a previous study that reported involvement of the PI3 kinase/Akt pathway in differentiation and inflammation induced by 27OHChol (16,39). Since NF-κB participates in chemokine production, we further investigated effects of reblastatins on phosphorylation of the p65 subunit (39). Reblastatins exhibit differential effects on phosphorylation of p65; WK88-1 and WK88-3 weakened the response, whereas 17-DR, WK88-2, and DHQ3 did not influence it (**Supplementary Fig. 4**). Signaling molecules that are involved in the inhibitory effects of reblastatins need to be determined through further studies to elucidate their mechanisms of action.

In this study, we report a new pharmacological activity of reblastatins, i.e., the inhibition of 27OHChol-mediated monocytic cell responses. Reblastatins inhibit the differentiation into mDCs and the activation to immuno-stimulatory phenotype that enhance inflammation (**Fig. 11**). Because 27OHChol-induced phenotypes are crucially involved in the onset and progression of chronic diseases, results of the current study indicate the potential of reblastatins to modify pathophysiology of diseases in which oxidized cholesterol molecules are involved.

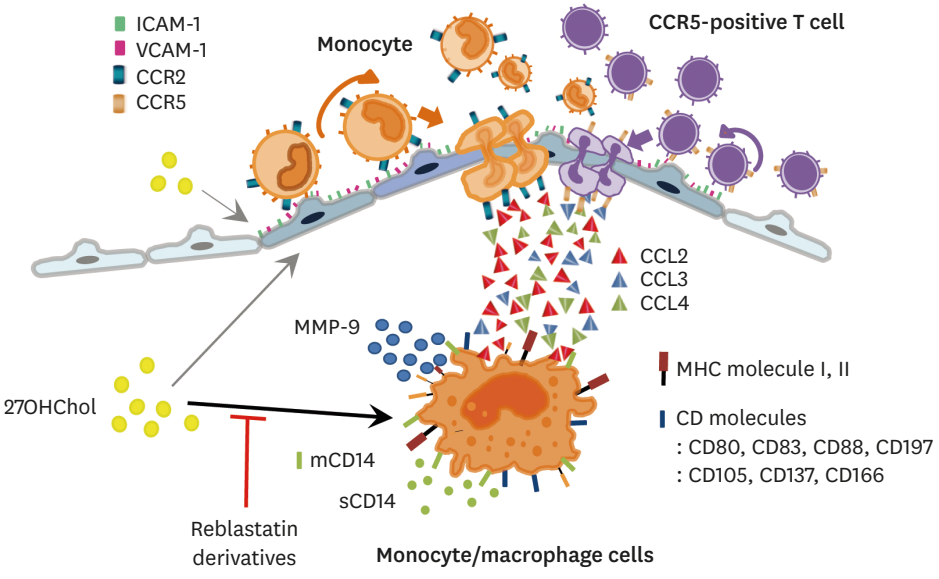

Figure 11. Proposed pharmacological action of reblastatins in a milieu rich in 27OHChol. 27OHChol activates monocytic cells and causes them to express multiple pro-inflammatory molecules which enhance migration of monocytic cells and Th1 T cells. Reblastatins inhibit secretion of chemokines and expression of inflammatory mediators and thereby reduce inflammation induced by 27OHChol. Reblastatins also inhibit differentiation of monocytic cells into mDCs and expression of pro-atherogenic molecules. These inhibitory effects may slow or prevent inflammation and immune responses involved in atherosclerosis.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Concentration effects of reblastatins on CCL2 expression induced by 27OHChol. (A) THP-1 cells (2.5×10^5 cells/ml) were serum-starved, followed by treatment for 48 h with the indicated amounts of reblastatin derivatives in the presence of the 27OHChol (2 μg/ml). CCL2 transcripts were amplified by RT-PCR. (B) Serum-starved THP-1 cells were treated with WK88-3 (from 0.001 to 1 μg/ml) for 48 h in the presence of 27OHChol. The amounts of secreted CCL2 were measured by ELISA. The ED50 was calculated with the GraphPad Prism 5. Data are representative of 3 independent experiments. Values are expressed in mean±SD (n=4).

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Supplementary Figure 2

Time course effects of reblastatin derivatives on the level of surface CD14 on THP-1 cells. Serum-starved THP-1 cells were treated for indicated time periods with reblastatin derivatives (1 μg/ml each) in the presence of the 27OHChol (2 μg/ml). After immunostaining of surface CD14, the levels of fluorescence were assessed by flow cytometry. Data represent a representative experiment (from 3 independent experiments).

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Supplementary Figure 3

Effects of reblastatin derivatives on viability of THP-1 cells. Serum-starved THP-1 cells were treated for 48 h with indicated reblastatin derivatives (1 μg/ml each) in the presence of the 27OHChol (2 μg/ml). Cell viability was determined by using a Vi-Cell XR cell counter (Beckman Coulter, Indianapolis, IN, USA). The viability of cells cultured in medium alone was considered 100%. Data are expressed as the means±SD (n=3 replicates for each group).

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Supplementary Figure 4

Effects of reblastatin derivatives on phosphorylation p65 of NF-κB. After serum-starvation, THP-1 cells were exposed for 4 h to indicated reblastatin derivatives (1 μg/ml each) in the presence of the 27OHChol (2 μg/ml). The levels of p65 and phosphorylated p65 were analyzed by western blotting. Data represent a representative experiment (from 3 independent experiments).

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