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Original Article

Glucocorticoids Impair the 7α-Hydroxycholesterol-Enhanced Innate Immune Response

Yonghae Son [1], **Bo-Young Kim** [1], **Miran Kim** [1], **Jaesung Kim** [1], **Ryuk Jun Kwon** [2,*], **Koanhoi Kim** [1,*]

[1]Department of Pharmacology, School of Medicine, Pusan National University, Yangsan 50612, Korea
[2]Family Medicine Clinic and Research Institute of Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital, Yangsan 50612, Korea

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***Correspondence to**
Koanhoi Kim
Department of Pharmacology, School of Medicine, Pusan National University, 49 Busandaehak-ro, Mulgeum-eup, Yangsan 50612, Korea.
Email: koanhoi@pusan.ac.kr

Ryuk Jun Kwon
Family Medicine Clinic and Research Institute of Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital, 20 Geumo-ro, Mulgeum-eup, Yangsan 50612, Korea.
Email: brain6@hanmail.net

ORCID iDs
Yonghae Son
https://orcid.org/0000-0003-3256-8509
Koanhoi Kim
https://orcid.org/0000-0003-0632-4017

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

ABSTRACT

Glucocorticoids suppress the vascular inflammation that occurs under hypercholesterolemia, as demonstrated in an animal model fed a high-cholesterol diet. However, the molecular mechanisms underlying these beneficial effects remain poorly understood. Because cholesterol is oxidized to form cholesterol oxides (oxysterols) that are capable of inducing inflammation, we investigated whether glucocorticoids affect the immune responses evoked by 7α-hydroxycholesterol (7αOHChol). The treatment of human THP-1 monocytic cells with dexamethasone (Dex) and prednisolone (Pdn) downregulated the expression of pattern recognition receptors (PRRs), such as TLR6 and CD14, and diminished 7αOHChol-enhanced response to FSL-1, a TLR2/6 ligand, and lipopolysaccharide, which interacts with CD14 to initiate immune responses, as determined by the reduced secretion of IL-23 and CCL2, respectively. Glucocorticoids weakened the 7αOHChol-induced production of CCL2 and CCR5 ligands, which was accompanied by decreased migration of monocytic cells and CCR5-expressing Jurkat T cells. Treatment with Dex or Pdn also reduced the phosphorylation of the Akt-1 Src, ERK1/2, and p65 subunits. These results indicate that both Dex and Pdn impair the expression of PRRs and their downstream products, chemokine production, and phosphorylation of signaling molecules. Collectively, glucocorticoids suppress the innate immune response and activation of monocytic cells to an inflammatory phenotype enhanced or induced by 7αOHChol, which may contribute to the anti-inflammatory effects in hypercholesterolemic conditions.

Keywords: 7α-Hydroxycholesterol; Inflammation; GlucocorticoidsMonocytes/macrophages; Pattern recognition receptors

INTRODUCTION

Glucocorticoids are steroid hormones widely used for the treatment of many inflammatory and autoimmune conditions (1). Administration of glucocorticoid drugs produces pharmacological effects in hypercholesterolemic environments (2). Dexamethasone (Dex) decreases the number of immune cells, such as macrophages and T lymphocytes, in the intima of cholesterol-fed rabbits (3,4). Prednisolone (Pdn) inhibited development of inflammatory lesions in the aortas of rabbits fed a high-cholesterol diet (2). Site-specific

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Abbreviations

7αOHChol, 7α-hydroxycholesterol; ATCC, American Type Culture Collection; BCA, bicinchoninic acid; CCR, C-C chemokine receptor; DAMP, damage-associated molecular pattern; Dex, dexamethasone; m-, membrane-bound; PAMP, pathogen-associated molecular pattern; Pdn, prednisolone; PRR, pattern recognition receptor; qRT-PCR, qantitative real-time PCR; s-, soluble; TBS, Tris-buffered saline; TBS-T, TBS containing 0.05% Tween-20.

Author Contributions

Data curation: Son Y, Kim BY, Kwon RJ; Formal analysis: Kim BY, Kim M; Funding acquisition: Kwon RJ, Kim K; Investigation: Son Y, Kim BY, Kim M, Kim J, Kwon RJ; Methodology: Son Y, Kim K; Supervision: Kim K; Writing - original draft: Son Y, Kim BY, Kwon RJ, Kim K; Writing - review & editing: Kim K.

targeting of Pdn nanoparticles reduces inflammation in a high-cholesterol diet rabbit model (5). Glucocorticoids modulate immune responses via gene regulation (6). They inhibit the transcription of inflammation-associated molecules, such as cytokines, chemokines, and receptor molecules, and enhance the release of anti-inflammatory proteins (7). However, the mechanism by which glucocorticoids produce such beneficial effects in animal models of hypercholesterolemia is yet to be determined.

Cholesterol, which is susceptible to oxidation, is oxidized via enzymes and/or auto-oxidation, and the oxygenated derivatives of cholesterol, oxysterols, are abundant in atherosclerotic arteries (8). The 7-oxysterols, which include 7α-hydroxycholesterol (7αOHChol), 7β-hydroxycholesterol, and 7-ketocholesterol, are the second most abundant derivatives next to 27-hydroxycholesterol. These molecules comprise 75%–85% of oxysterols detected in atherosclerotic plaques from different sites (8,9). The 7-oxysterols are the main oxysterols in oxidized low-density lipoproteins and participate in atherosclerosis (10). Among the 7-oxysterols, 7αOHChol is physiologically functional and promotes inflammation by increasing the levels of pro-inflammatory molecules. This oxysterol upregulates the expression of TLR6, a pattern recognition receptor (PRR) involved in innate immunity (11), and activates monocytic cells to produce chemokines, thereby enhancing the migration of immune cells (12,13). 7αOHChol also enhances multiple signaling pathways that affect gene expression (13). These findings suggest that 7αOHChol is a bioactive molecule that modulates the innate immune response.

Monocytic cells, including monocytes and macrophages, are key effector cells that play critical roles in the initiation, maintenance, and resolution of the innate immune response (14). Macrophages are highly heterogeneous plastic cells capable of changing their phenotype and are activated and deactivated during the inflammatory process in response to local microenvironmental stimuli (15). Anti-inflammatory macrophages scan for the danger signals and act to restrict inflammatory responses, whereas pro-inflammatory macrophages initiate and amplify the inflammatory response by upregulating surface expression of receptors and releasing cytokines and chemokines (14,15). Phenotypic changes in macrophages lead to altered disease progression. Thus, controlling this phenotype can serve as a therapeutic approach for inflammatory diseases (16).

We focused our attention on the molecular mechanisms underlying the anti-inflammatory effects of glucocorticoids in a cholesterol-rich environment. Additionally, the question was asked about what effect glucocorticoids have on signal transduction transmitted by 7αOHChol. To better understand this, we conducted an inquiry into the potential impact of Dex and Pdn on the responses mediated by PRRs and their influence on the transition to an inflammatory phenotype subsequent to stimulation with 7αOHChol, using THP-1 human monocytes/macrophages. We then investigated the impact of glucocorticoids on intracellular signaling transduced by 7αOHChol by examining protein phosphorylation.

MATERIALS AND METHODS

Reagents and Abs

7αOHChol was purchased from Research Plus, Inc. (Barnegat, NJ, USA). Dex and Pdn were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. LPS-EK from *Escherichia coli* K12 and FSL-1 (Pam2CGDPKHPKSF)

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were purchased from InvivoGen (San Diego, CA, USA). Abs against TLR6, CD14, p65, pp65, ERK, pERK, and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Abs against Akt, pAkt^{S473}, Src, and pSrc^{T416} were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and treatment

THP-1 monocytic cell line was 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 (50 units/ml) and streptomycin (50 μg/ml). Jurkat T cells that stably express C-C chemokine receptor 5 (CCR5) were maintained in RPMI 1640 medium supplemented with 10% FBS in the presence of G418 (17). THP-1 cells between passages 8 and 10 were used in the experiments. Before treatment with 7αOHChol and glucocorticoids, 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% endotoxin-free BSA as previously described (18). The cells were centrifuged after incubation for the indicated time periods. Supernatants were collected in fresh tubes, stored in a −80°C freezer, and thawed prior to use in chemotaxis assay and ELISA.

ELISA

The secreted levels of CCL2, CCL3, CCL4, soluble CD14 (sCD14), and IL-23 were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The recombinant standards and supernatant samples were added to each well and incubated for 2 h at room temperature of between 20°C and 25°C. After washing the wells, polyclonal Abs against proteins conjugated to HRP with preservatives were added to each well and incubated for 1 h. Following three washes, the substrate provided in the kit was used for color development. After incubation for 30 min in the dark, the reaction was stopped by adding stop solution (2N sulfuric acid). Color intensity was measured at 450 nm using a Sunrise microplate reader (Tecan Austria GMBH, Grödig, Austria). The amount of protein in each sample was evaluated using a standard curve.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed in triplicate using a LightCycler® 96 Real-Time PCR System (Roche Holdings AG, Basel, Switzerland). Each reaction (20 μl) contained 4 μl of complementary DNA template, 10 μl of SYBR Green Master Mix, and 2 μl of 10-pM forward and reverse primers of the target gene, which was amplified under the following cycling conditions: 95°C for 10 min, 45 cycles at 95°C for 10 s, 50°C for 10 s, and 72°C for 10 s. The transcript levels of the target genes were normalized to those of the housekeeping gene GAPDH using the $2^{-\Delta\Delta Ct}$ method (19). Gene expression was calculated relative to that of GAPDH using LightCycler® 96 software (version 1.1.0.1320; Roche Holdings AG) using the following forward (F) and reverse (R) (5′→3′) primers:

CCL2: F, CAGCCAGATGCAATCAATGCC, and R, TGGAATCCTGAACCCACTTCT
CCL3: F, AGTTCTCTGCATCACTTGCTG, and R, CGGCTTCGCTTGGTTAGGAA
CCL4: F, CTGGGTCCAGGAGTACGTGT, and R, GCGGAGAGGAGTCCTGAGTA
CD14: F, ACGCCAGAACCTTGTGAGC, and R, GCATGGATCTCCACCTCTACTG
TLR6: F, AGGGCTGGCCTGATTCTTAT, and R, TGGCACACCATCCTGAGATA
GAPDH: F, GAAGGTGAAGGTCGGAGT, and R, GAAGATGGTGATGGGATTTC

Western blot analysis

After solubilization of the proteins with cell lysis buffer, the protein concentration of the cell lysates was determined using the bicinchoninic acid (BCA) assay. Proteins were separated by

using 10% SDS-PAGE and transferred onto nitrocellulose membranes. Following blocking of nonspecific protein binding for 1 h with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) and 1% skim milk, membranes were incubated with primary Abs diluted in the blocking solution at 4 °C overnight. The membranes were washed three times with TBS-T and incubated for 1 h with HRP-conjugated secondary Abs diluted 1:8,000 in a blocking solution at temperature. After washing three times for 10 min each, the bands were detected using chemiluminescent reagents. Chemiluminescent images were captured using Amersham Imager 600 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Flow cytometry

Following treatment with 7αOHChol with or without glucocorticoids, THP-1 cells were harvested, washed with PBS containing 0.5% BSA (washing buffer), and incubated with 3% BSA in PBS. The cells were then exposed to anti-TLR6 and anti-CD14 Abs diluted 1:100 in washing buffer for 1 h at room temperature. Subsequently, the cells were washed twice with washing buffer and incubated for 40 min with an Alexa Fluor dye-conjugated secondary Ab diluted 1:200 in washing buffer at room temperature in the dark. After washing twice, cells were resuspended in 1% paraformaldehyde/PBS solution. Fluorescence was analyzed using a FACSCanto™ II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Chemotaxis assay

Chemotaxis assays were performed using Transwell Permeable Supports, as previously described (Costar, Cambridge, MA, USA) (20). THP-1 monocytic cells and Jurkat T cells expressing CCR5 (5×10^5 cells each in 100 μl of 0.1% BSA in PBS) were loaded onto the top chamber of 5-μm pore polycarbonate Transwell inserts. Transwell chambers were placed in wells filled with supernatant isolated from cells stimulated with 7αOHChol in the presence or absence of glucocorticoids. Following incubation for 2–3 h at 37 °C in a humidified incubator with 5% CO_2, the number of the cells that migrated to the bottom chamber was counted using a Vi-Cell Counter (Beckman Coulter, Inc., Brea, CA, USA).

Statistical analysis

Data were statistically analyzed using one-way analysis of variance, followed by Dunnett's multiple comparison tests using PRISM (version 5.0; GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p<0.05$.

RESULTS

Glucocorticoids downregulate PRR signaling

We investigated whether glucocorticoids affect TLR6 expression in monocytic cells. The TLR6 transcript levels increased following stimulation with 7αOHChol; however, this increase was attenuated in the presence of Dex and Pdn (**Fig. 1A**). The effect of each glucocorticoid on surface TLR6 was determined using flow cytometry (**Fig. 1B**). Stimulation of THP-1 cell with OHchol resulted in elvelated percentage of TLR6 positive cells from 5.9% to 25.6%, while OHchole-stimulated THP-1 cells in the presence of Dex and Pdn showed TLR6-positive cells with 6.4% and 11.4%, respectively. To evaluate whether glucocorticoids affect the TLR6-mediated response, IL-23 production was determined following treatment with FSL, a synthetic diacylated lipoprotein TLR6 agonist (**Fig. 1C**). THP-1 cells secreted IL-23 with basally low levels, and stimulation with 7αOHChol or FSL-1 alone did not promote secretion. The secretion of IL-23 was enhanced when FSL-1 was added to 7αOHChol-stimulated cells.

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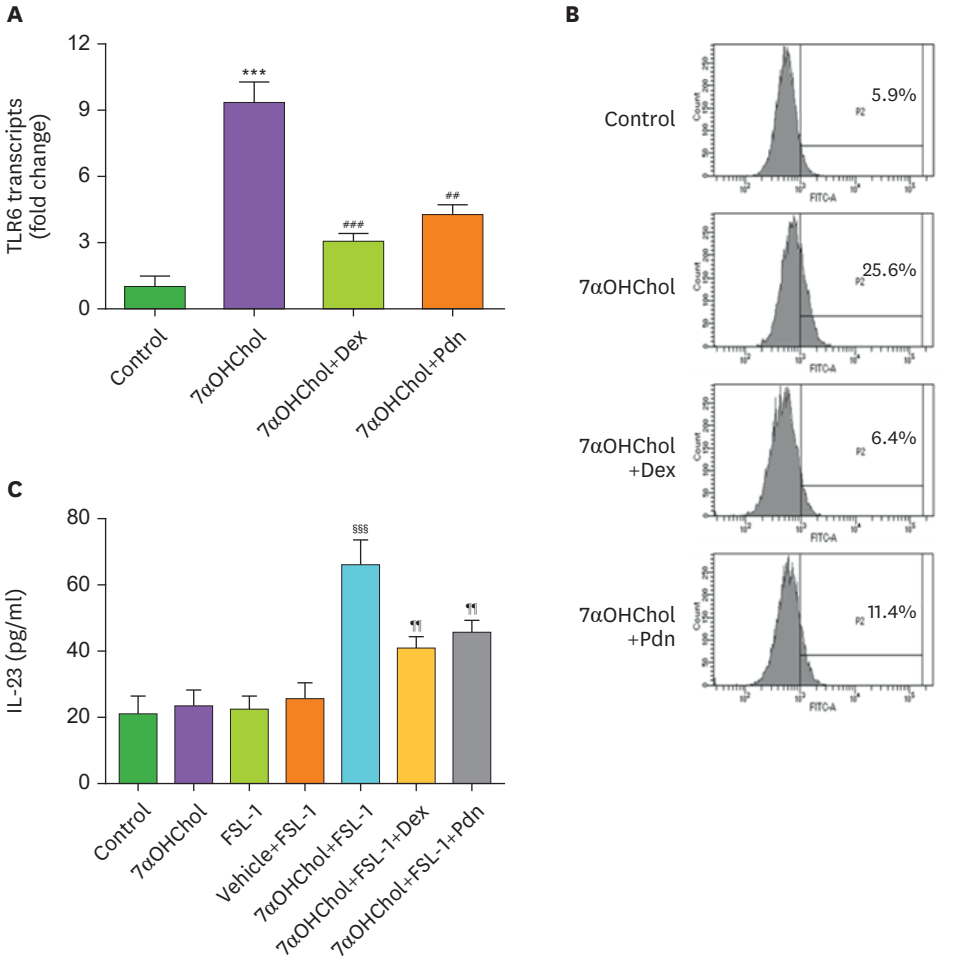

Figure 1. Downregulation of TLR6 and attenuated response to FSL-1.
(A) THP-1 cells are serum-starved for 24 h and treated for 48 h with 7αOHChol (5 μg/ml each) in the absence or presence of the indicated glucocorticoids (1 μM each). Total RNA isolated from the cells is reverse-transcribed, and quantitative real-time polymerase chain reaction is performed to determine the relative levels of TLR6 transcripts. The Y-axis values represent fold increases in the TLR6 messenger RNA levels normalized to the glyceraldehyde-3-phosphate dehydrogenase levels compared with those of control cells. (B) After treatment of serum-starved THP-1 cells with 7αOHChol in the absence or presence Dex or Pdn, cell surface TLR6 is immunostained, and fluorescence is analyzed by flow cytometry. (C) Serum-starved THP-1 cells are treated with 7αOHChol for 24 h and incubated for another 24 h after addition of FSL-1 (100 ng/ml) with or without the indicated glucocorticoids. The amount of IL-23 secreted into culture media is measured by enzyme-linked immunosorbent assay.
Data are expressed as the means ± SDs (n=3 replicates/group).
$^{***}p<0.001$ vs. control; $^{\#\#}p<0.01$ vs. 7αOHChol; $^{\#\#\#}p<0.001$ vs. 7αOHChol; $^{§§§}p<0.001$ vs. control or 7αOHChol or FSL-1; $^{¶¶}p<0.01$ vs. 7αOHChol+FSL-1.

However, secretion was weakened in the presence of Dex and Pdn. These results indicated that glucocorticoids downregulated TLR6 and weakened TLR6-mediated responses.

The effect of glucocorticoids on CD14 expression was also examined. CD14 transcript levels increased after stimulation with 7αOHChol, compared with those in untreated control cells; however, this increase was impaired in the presence of Dex and Pdn (**Fig. 2A**). Stimulation with 7αOHChol increased the percentage of monocytic cells expressing membrane-bound CD14 (mCD14), as determined by flow cytometry; however, Dex and Pdn decreased the percentage of mCD14-positive cells (**Fig. 2B**). CD14 also exists in soluble form. Similarly, 7αOHChol promoted secretion of sCD14 from monocytic cells, which was attenuated in the

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presence of Dex and Pdn (**Fig. 2C**). Because CD14 interacts with bacterial LPS, we investigated whether glucocorticoids affect the LPS response by determining CCL2 production (**Fig. 2D**). Stimulation of THP-1 cells with 7αOHChol resulted in increased secretion of CCL2, which was further enhanced when LPS was added to 7αOHChol-stimulated cells. However, the LPS-enhanced CCL2 secretion diminished in the presence of Dex and Pdn. These results indicated that glucocorticoids downregulated CD14 expression and attenuated the LPS response. Overall, these results suggest that Dex and Pdn inhibit 7αOHChol-enhanced signaling via PRRs, thereby repressing the innate immune response.

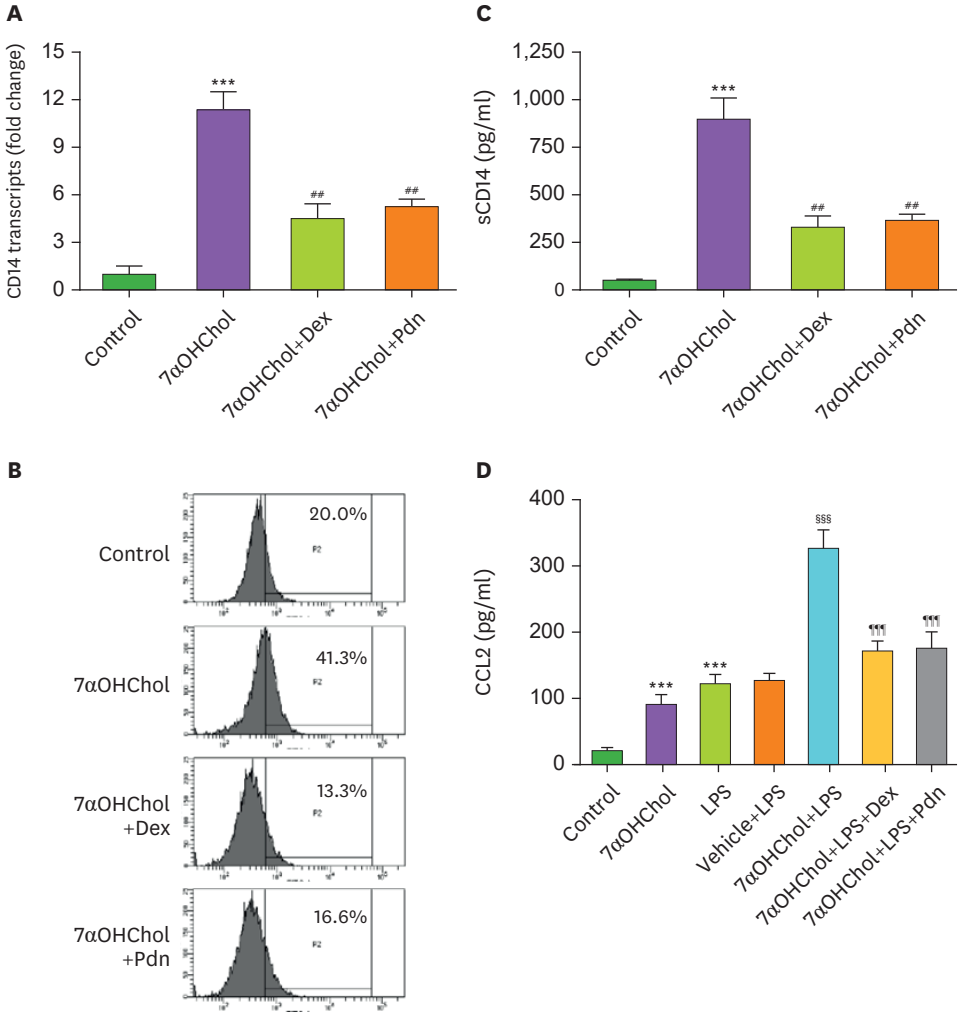

Figure 2. Downregulation of CD14 and weakened LPS response.
(A, B) Serum-starved THP-1 cells are treated for 48 h with 7αOHChol (5 μg/ml) in the absence or presence of the indicated glucocorticoids (1 μM each). (A) Quantitative real-time polymerase chain reaction is performed to determine the relative levels of CD14 transcripts. (B) The mCD14 levels are measured by flow cytometry after immunostaining of surface CD14. (C) Following treatment of serum-starved THP-1 cells for 48 h with 7αOHChol in the absence or presence of Dex or Pdn, released sCD14 is measured by ELISA. (D) Serum-starved THP-1 cells are incubated for 24 h with or without 7αOHChol and then stimulated for 9 h with LPS (100 ng/ml) from *Escherichia coli* K12 in the absence or presence of Dex or Pdn. The amount of CCL2 in the culture media is determined by ELISA. Data are expressed as means ± SDs (n=3 replicates/group).
7αOHChol, 7α-hydroxycholesterol; Dex, dexamethasone; Pdn, prednisolone; LPS, lipopolysaccharide.
***p<0.001 vs. control; **p<0.01 vs. 7αOHChol; §§§p<0.001 vs. 7αOHChol or LPS; ¶¶¶p< 0.001 vs. 7αOHChol+LPS.

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Glucocorticoids inhibit chemokine production and immune cell migration

We investigated whether glucocorticoids affect monocytic cell activation by examining chemokine expression. Transcription of CCL2 increased after stimulation with 7αOHChol, which was attenuated in the presence of Dex and Pdn (**Fig. 3A**). In accordance with the qRT-PCR results, 7αOHChol enhanced the production of CCL2 protein, as determined by ELISA; however, this enhancement diminished in the presence of glucocorticoids (**Fig. 3B**). A chemotaxis assay was performed to examine monocytic cell migration (**Fig. 3C**). Cell migration increased in response to the supernatant isolated from cells stimulated with 7αOHChol, compared with the control. However, a notable reduction in cell migration was observed when utilizing the supernatants of cells stimulated with 7αOHchol in the presence of Dex and Pdn. These results indicate that glucocorticoids regulate CCL2 production and alter monocytic cell migration.

The effect of glucocorticoids on the expression of CCR5 ligands was also examined. The transcript levels of CCL3 and CCL4 increased following stimulation with 7αOHChol; however, this increase was attenuated in the presence of Dex and Pdn (**Fig. 4A**). Stimulation with

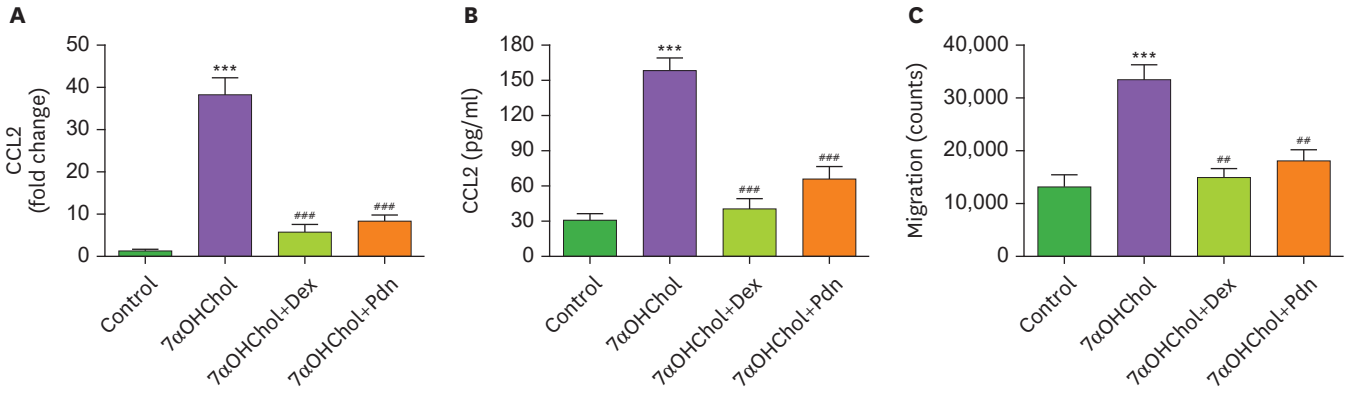

Figure 3. Inhibition of CCL2 expression and monocytic cell migration.
Serum-starved THP-1 cells are treated for 48 h with 7αOHChol (5 μg/ml) in the absence or presence of the indicated glucocorticoids (1 μM each), (A) after which the levels of CCL2 transcripts are assessed by qRT-PCR and (B) the amount of secreted CCL2 protein in the culture media is measured by ELISA. (C) Monocytic cells are exposed to the conditioned media isolated from THP-1 cells stimulated with 7αOHChol (5 μg/ml) with or without the indicated glucocorticoids, and the numbers of migrated monocytic cells are counted. Data are expressed as means ± SDs (n=3 replicates for each group).
***p<0.001 vs. control; ##p<0.01 vs. 7αOHChol; ###p<0.001 vs. 7αOHChol.

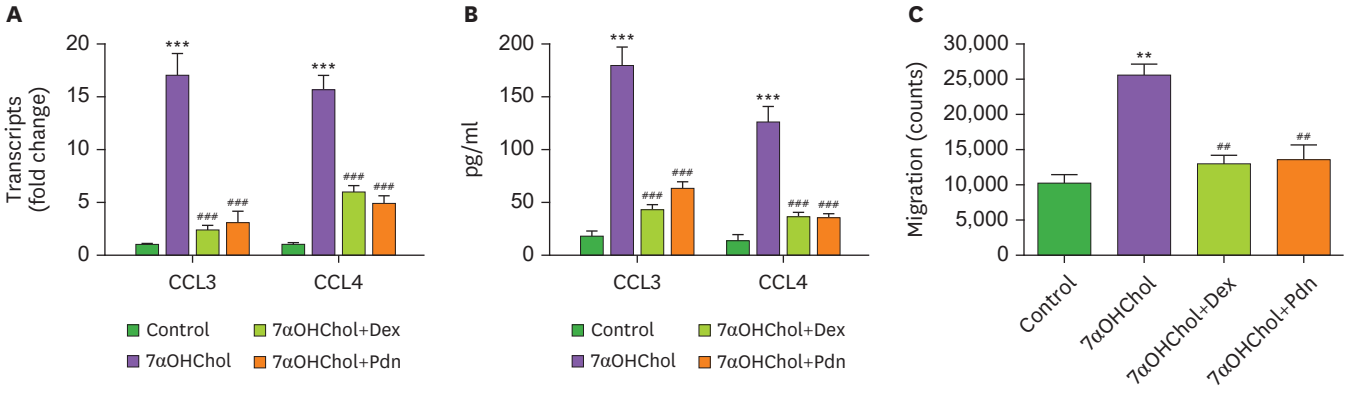

Figure 4. Decreased production of CCR5 ligands and migration of CCR5-expressing T cells.
Following serum starvation, THP-1 cells are cultured for 48 h with 7αOHChol (5 μg/ml) in the presence or absence of the indicated glucocorticoids (1 μM each). (A) The transcript levels of CCL3 and CCL4 genes are assessed by qRT-PCR. (B) The amounts of CCL3 and CCL4 released from THP-1 cells are determined by ELISA. (C) CCR5-positive Jurkat T cells are exposed to conditioned media isolated from THP-1 cells stimulated with 7αOHChol with or without dexamethasone or prednisolone, and their ability to migrate is investigated by a chemotaxis assay. Data are expressed as means ± SDs (n=3 replicates for each group).
**p<0.01 vs. control; ***p<0.001 vs. control; ##p<0.01 vs. 7αOHChol; ###p<0.001 vs. 7αOHChol.

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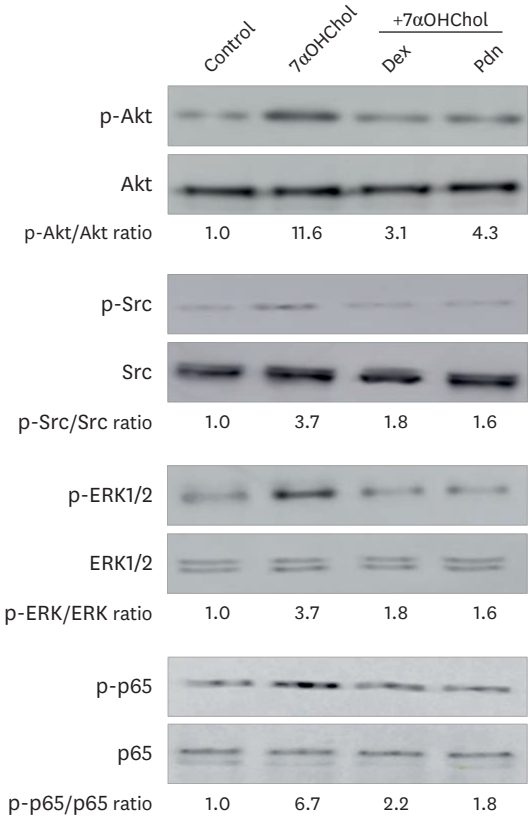

Figure 5. Attenuated phosphorylation of multiple signaling molecules.
Cell lysates are obtained after exposure of THP-1 cells for 6 h to 7αOHChol (5 μg/ml) with dexamethasone or prednisolone (1 μM each). Following the determination of protein concentration, equal amounts of protein are analyzed by western blotting using the indicated Abs.

7αOHChol increased the production of CCL3 and CCL4 proteins, which were impaired by treatment with Dex and Pdn (**Fig. 4B**). Migration assays were performed using the supernatants containing CCR5 ligands. Because Jurkat T cells do not express CCR5, cells expressing CCR5 were used for the chemotaxis assay (**Fig. 4C**). The supernatant isolated from cells stimulated with 7αOHChol promoted migration of the CCR5-expressing T cells. However, cell migration was reduced when the supernatants were isolated in the presence of Dex and Pdn. These results indicated that Dex and Pdn repressed the expression of CCR5 ligands and the migration of CCR5-positive T cells. Collectively, these results suggest that Dex and Pdn suppress 7αOHChol-induced monocytic cell activation to an inflammatory phenotype.

Glucocorticoids attenuate phosphorylation enhanced by 7αOHChol

We attempted to understand the molecular mechanisms underlying the inhibitory effects of glucocorticoids. The results of the current study indicate the suppression of Akt, Src, and ERK because their phosphorylation, which was enhanced by 7αOHChol, was weakened in the presence of Dex and Pdn (**Fig. 5**). Because NF-κB participates in chemokine production, the phosphorylation of the p65 subunit was also determined. Glucocorticoids reduced p65 phosphorylation (**Fig. 5**). These results suggest that glucocorticoids impair the cell signaling transduced by 7αOHChol.

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DISCUSSION

This study elucidates a novel pharmacological function of glucocorticoids, specifically their capacity to attenuate the innate immune responses elicited by 7αOHchol (**Fig. 6**). 7αOHChol upregulates the expression of PRRs such as TLR6 and CD14 on the surface of monocytes/macrophages. Therefore, more pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by receptors, which further activate cells, such that substantially high levels of cytokines and chemokines are secreted, thereby aggravating the immune response. 7αOHChol also induces chemokine production and promotes immune cell migration. Glucocorticoids inhibit the upregulation of receptors and cytokine/chemokine production induced or enhanced by 7αOHChol. These inhibitory effects may slow or suppress inflammation during hypercholesterolemia.

PRRs are proteins mainly expressed by innate immune cells such as macrophages, monocytes, and neutrophils (21). They are capable of recognizing two classes of molecules: PAMPs, such as LPS, mannose, bacterial or viral nucleic acids, peptidoglycans, fungal glucans, and chitin, and DAMPs, such as uric acid, extracellular adenosine triphosphate, and endogenic components released from damaged or dead cells (22,23). Upon binding to PRRs, PAMPs and DAMPs activate monocytes/macrophages, thereby playing a key role in innate immunity and Ag-specific adaptive immune responses (21,24). Because activation through PRRs is crucial for the initiation of the inflammatory response, we investigated the effects of glucocorticoids on PRRs and demonstrated the downregulation of TLR6 and CD14 expression along with impaired production of effector molecules in response to

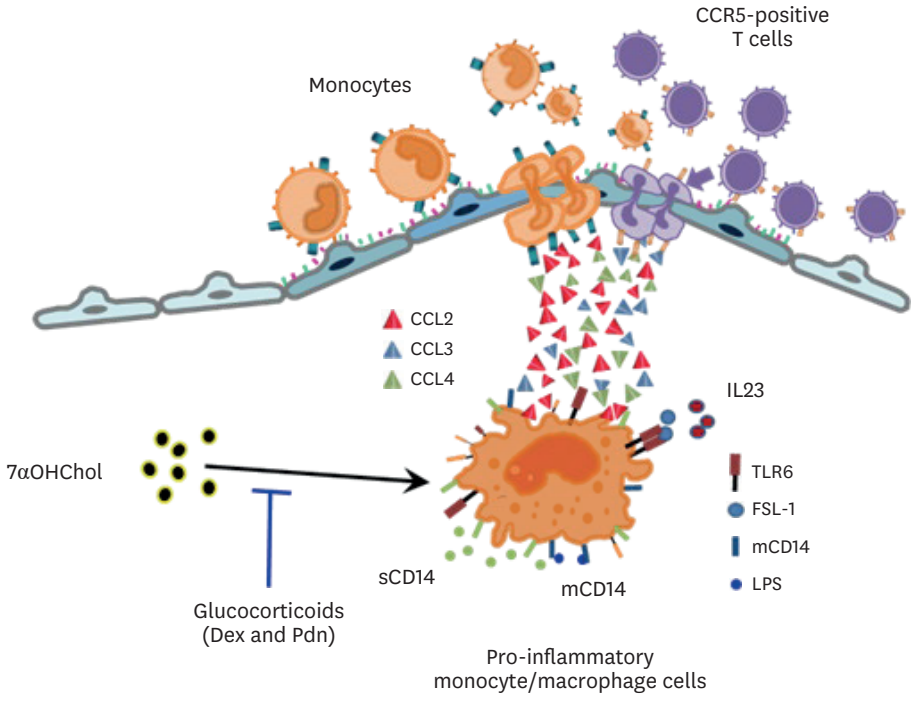

Figure 6. Pharmacological action of glucocorticoids in a milieu rich in 7αOHChol.
7αOHChol enhances the innate immune response by upregulating the surface expression of TLR6 and CD14, leading to increased cytokine and chemokine production. 7αOHChol also promotes the migration of monocytes and specific subtypes of $CD4^+$ effector T cells. Glucocorticoids impair the effects of 7αOHChol on receptor expression, cytokine and chemokine production, and cell migration.

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specific PAMPs of individual receptors. TLR6, also known as CD286, is a transmembrane protein that forms a heterodimer with TLR2. After dimerization with TLR2, the NF-κB intracellular signaling pathway is activated, leading to pro-inflammatory cytokine production and activation of the innate immune response (25). CD14 functions as a co-receptor with the TLR4 for LPS and initiates the LPS response (26). CD14 also recognizes other PAMPs and transmits signals from DAMPs (27,28); sCD14 acts as a DAMP in macrophages (29). However, overactivation of the innate immune system induces septic shock or autoimmunity (30). Therefore, PRRs are important targets for controlling inflammatory and autoimmune disorders (31). Together with these previous findings, the results of this study suggest that glucocorticoids suppress overactivation of the innate immune response by downregulating PRR signaling, which can be enhanced by oxysterols under hypercholesterolemic conditions.

Because CCR5 constitutes a pivotal molecule for the primary migration of $CD4^+$ Th1 cells (32), our results indicate that glucocorticoids regulate the recruitment of Th1 cells along with monocytic cells. The interplay between monocytes/macrophages and Th1 cells plays an important role in the inflammatory immune response via activation of each cell type (33). Monocytes/macrophages produce inflammatory cytokines and chemokines that induce acute phase reactions, recruit leukocytes and lymphocytes, and boost immune cell functions (34,35). Mononuclear cells could affect the polarization of $CD4^+$ T cells toward Th1/Th17 subsets (35). Th1 cells secrete cytokines typically associated with inflammation (36). Th1 cells are involved in classical microphage activation by secreting interferon gamma and induce cell-mediated immune response (36). Interaction between these 2 cell types is likely to occur in atherosclerosis. Atherosclerotic lesions have increased expression of Th1 cell markers and Th1 type cellular immune response with increased inflammatory monocytes and macrophages (37,38), If unregulated, Th1 effector cells contribute to the pathogenesis of organ specific inflammation and autoimmune diseases (39). Therefore, the results of cell migration suggest that glucocorticoids suppress autoimmune responses specific to atherosclerosis by repressing pro-inflammatory Th1 dominance.

Akt, a serine/threonine protein kinase, is activated by growth factor and cytokines, and once activated, phosphorylates downstream targets, including NF-κB, that regulate physiological processes (40). ERK is preferentially activated in response to growth factors and phorbol esters and affects cellular processes by controlling the proteins involved in translation (41). Therefore, the activation of Akt and ERK regulates a variety of cellular processes, including migration, glucose metabolism, cell survival, and protein synthesis (40,41). Oxygenated cholesterol molecules activate kinase pathways and induce cell differentiation, inflammation, and protein synthesis (13,42,43). We observed diminished phosphorylation of Akt and ERK, which was enhanced by 7αOHChol. These results agree with previous findings that glucocorticoids inhibit the Akt and ERK pathways (44,45), indicating the impairment of both pathways by treatment with Dex and Pdn. However, the exact mechanism by which glucocorticoids inhibit both pathways is not yet understood. Further studies are necessary to understand the molecular mechanisms underlying glucocorticoid-mediated regulation of Akt and ERK phosphorylation.

Oxysterol, which is increased in the blood of patients with hypercholesterolemia, has been reported to activate inflammatory immune responses and cause inflammation in various organs, including blood vessels. It has also been reported that it damages ZO-1, a protein in the tight junction of blood vessels, causing blood leakage from blood vessels, tissue leakage from tissues, and vision loss due to leakage of ocular fluid (46). Together with previous

findings, our results suggest that glucocorticoids may be beneficial in the treatment of inflammatory and autoimmune diseases that occur in the setting of hypercholesterolemia, such as atherosclerosis, due to their potent anti-inflammatory and immunosuppressive effects (47-49). Taken together, these results show that glucocorticoids, a widely used clinical drug, effectively suppress the inflammatory immune response triggered by increased oxysterols in the body. However, clinical and laboratory studies have revealed limitations to their therapeutic use. One therapeutic limitation of glucocorticoids in atherosclerosis is their potential to exacerbate insulin resistance and glucose intolerance. This is particularly relevant in patients with preexisting diabetes or metabolic syndromes (50). Long-term glucocorticoid use is associated with an increased risk of hypertension (51). Glucocorticoids also have adverse effects on endothelial function (52). Another important limitation is the potential worsening of dyslipidemia. Glucocorticoids have been shown to increase triglyceride levels and decrease high-density lipoprotein cholesterol levels (53). Therefore, the therapeutic use of glucocorticoids should be carefully considered individually considering the potential risks and benefits of targeted delivery.

The pharmacological action and mechanism of glucocorticoids shown in this paper suggest important directions for future clinical application in patients with hypercholesterolemia and other related diseases. And when it comes to the application of glucocorticoids and other inducing drugs, their point of action can be clearly guessed.

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