

Original Article

27-Hydroxycholesterol induces macrophage gene expression via LXR-dependent and -independent mechanisms

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ABSTRACT 27-Hydroxycholesterol (27OHChol) exhibits agonistic activity for liver X receptors (LXRs). To determine roles of the LXR agonistic activity in macrophage gene expression, we investigated the effects of LXR inhibition on the 27OHChol-induced genes. Treatment of human THP-1 cells with GSK 2033, a potent cell-active LXR antagonist, results in complete inhibition in the transcription of LXR target genes (such as LXR α and ABCA1) induced by 27OHChol or a synthetic LXR ligand TO 901317. Whereas expression of CCL2 and CCL4 remains unaffected by GSK 2033, TNF- α expression is further induced and 27OHChol-induced CCL3 and CXCL8 genes are suppressed at both the transcriptional and protein translation levels in the presence of GSK 2033. This LXR antagonist downregulates transcript levels and surface expression of CD163 and CD206 and suppresses the transcription of CD14, CD80, and CD86 genes without downregulating their surface levels. GSK 2033 alone had no effect on the basal expression levels of the aforementioned genes. Collectively, these results indicate that LXR inhibition leads to differential regulation of 27-hydroxycholesterol-induced genes in macrophages. We propose that 27OHChol induces gene expression and modulates macrophage functions via LXR-dependent and -independent mechanisms.

INTRODUCTION

Oxysterols are oxygenated derivatives of cholesterol. As compared to cholesterol, they contain an additional hydroxy, epoxide or ketone group in the sterol nucleus, and/or a hydroxyl group in the side chain [1]. 27-Hydroxycholesterol (27OHChol) is a side-chain oxysterol oxygenated at the 27th carbon atom of cholesterol. This oxysterol is produced via oxidation by sterol 27-hydroxylase (CYP27A1), and metabolized via 7 α -hydroxylation for bile acid synthesis in the liver [1,2]. 27OHChol can also be formed in peripheral tissues either via enzymatic or non-enzymatic mechanisms [2,3]. The production of 27OHChol is enhanced in atherosclerotic lesions of the artery, and increasing amounts detected in

lesions are associated with increasing macrophages [4,5]. The biological function of 27OHChol in peripheral tissues differs from that in the liver. 27OHChol enhances inflammation and triggers an immune response by activating monocytes/macrophages. Following activation, they express chemokines, including chemokine (C-C motif) ligand (CCL) 2, CCL3 and chemokine (C-X-C motif) ligand (CXCL) 8, and pattern recognition receptors that respond to the bacterial pathogen-associated molecular patterns [6-8]. 27OHChol also induces molecules involved in T cell activation on the surface of monocytic cells [9]. Vascular smooth muscle cells and T cells, however, do not express such molecules in response to 27OHChol [10,11]. These findings indicate that macrophages are the major cell type whose gene expression is influenced by



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Author contributions: B-Y.K. and Y.S. performed all experiments. H-r.C. and K.K. designed the study. B-Y.K., D.L., and S-K.E. analyzed and interpreted data. B-Y. K., D.L., H-r.C., S-K.E., and K.K. drafted the manuscript. All authors review the manuscript.

27OHChol.

27OHChol is a functional liver X receptors (LXR) agonist [12]. LXR α (NR1H3) and LXR β (NR1H2) are two isoforms of LXRs expressed with overlapping, but a distinctive pattern. LXR α is dominant in the liver and expressed primarily in the intestine, adipose tissue, and macrophages, whereas LXR β is widely expressed [13-15]. Since LXRs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, they exert their biological effects by controlling the expression of target genes [15]. Activation of LXRs with 27OHChol increases the transcription of LXR-responsive genes involved in cholesterol efflux, such as ABCA1 and ABCG1 in macrophages [12,14]. These findings indicate that 27OHChol induces gene expression via LXRs. However, it is unknown whether the LXR agonistic activity of 27OHChol is involved in the induction of genes other than for cholesterol metabolism.

This study was therefore undertaken to determine the effects of GSK 2033, a potent cell-active LXR antagonist, on the transcription of 27OHChol-induced genes, including inflammatory and cell surface molecules as well as LXR target genes. Our results demonstrate that GSK 2033 differentially regulates the 27OHChol-induced genes in macrophages.

METHODS

Cells and reagents

The human THP-1 monocyte/macrophage cell line (ATCC, #TIB-202) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. Penicillin (50 U/ml) and streptomycin (50 µg/ml) were added to prevent bacterial contamination. 27OHChol and antibodies against LXR α / β , CD14, CD80, and CD86 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GSK 2033 and TO 901317 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-ABCA1 antibody was purchased from Invitrogen (Eugene, OR, USA). Anti-CD163 antibody conjugated with FITC and anti-CD206 antibody conjugated with PE were purchased from BioLegend (San Diego, CA, USA).

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analysis

Total RNA isolated were reverse-transcribed for 1 h at 42°C with 100 U Moloney murine leukemia virus reverse transcriptase in a 10 µl reaction volume, containing 50 mM Tris-HCl (pH 8.3 at 25°C), 55 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 µg oligo (dT) 15 primers, 0.125 mM each dNTP, and 40 U RNase inhibitor. Subsequent qPCR was performed in triplicate using a LightCycler

96 Real-Time PCR System (Roche, Mannheim, Germany), as previously described [8]. Each 20 µl reaction mixture consisted of 10 µl SYBR Green Master Mix, 2 µl forward and reverse primers (10 pM each) of the gene to be quantified, and cDNA template. The thermal cycling conditions consisted of 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 50°C for 10 sec, and 72°C for 10 sec. The relative expression of each gene was calculated as the ratio to the GAPDH gene using the LightCycler 96 software (Version 1.1.0.1320; Roche). The primers used were as follows in Table 1.

Western blot analysis

Cell lysates were separated by 10% SDS-PAGE, and resolved proteins were transferred 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 antibodies against LXR α / β , ABCA1 or β -actin at 4°C overnight. Membranes were washed three times with 0.05% Tween 20/TBS for 10 min each and incubated for 1 h with HRP conjugated secondary Abs (1:5,000 dilution) at room temperature. After 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 680 (GE Health-

Table 1. List of primers used in this study

Gene	Sequence
<i>LXRα</i>	Forward 5'-AAG CCC TGC ATG CCT ACG T-3'
	Reverse 5'-TGC AGA CGC AGT GCA AAC A-3'
<i>ABCA1</i>	Forward 5'-TGT CCA GTC CAG TAA TGG TTC TGT-3'
	Reverse 5'-CGA GAT ATG GTC CGG ATT GC-3'
<i>CCL2</i>	Forward 5'-CAG CCA GAT GCA ATC AAT GCC-3'
	Reverse 5'-TGG AAT CCT GAA CCC ACT TCT-3'
<i>CCL3</i>	Forward 5'-AGT TCT CTG CAT CAC TTG CTG-3'
	Reverse 5'-CGG CTT CGC TTG GTT AGG AA-3'
<i>CCL4</i>	Forward 5'-CTG GGT CCA GGA GTA CGT GT-3'
	Reverse 5'-GCG GAG AGG AGT CCT GAG TA-3'
<i>CXCL8</i>	Forward 5'-TCT GCA GCT CTG TGT GAA GG-3'
	Reverse 5'-AAT TTC TGT GTT GGC GCA GT-3'
<i>TNF-α</i>	Forward 5'-CCC AGG GAC CTC TCT CTA ATC-3'
	Reverse 5'-ATG GGC TAC AGG CTT GTC ACT-3'
<i>CD14</i>	Forward 5'-ACG CCA GAA CCT TGT GAG C-3'
	Reverse 5'-GCA TGG ATC TCC ACC TCT ACT G-3' ;
<i>CD80</i>	Forward 5'-GCA GGG AAC ATC ACC ATC CA-3'
	Reverse 5'-TCA CGT GGA TAA CAC CTG AAC A-3'
<i>CD86</i>	Forward 5'-GGA CTA GCA CAG ACA CAC GGA-3'
	Reverse 5'-CTT CAG AGG AGC AGC ACC AGA-3'
<i>CD163</i>	Forward 5'-AAA AAG CCA CAA CAG GTC GC-3'
	Reverse 5'-CTT GAG GAA ACT GCA AGC CG-3'
<i>CD206</i>	Forward 5'-TGA ATT GTA CTG GTC TGT CCT-3'
	Reverse 5'-CTG TGG TGC TGT GCA TTT ATC T-3'
<i>GAPDH</i>	Forward 5'-GAA GGT GAA GGT CGG AGT-3'
	Reverse 5'-GAA GAT GGT GAT GGG ATT TC-3'

care Life Sciences, Pittsburgh, PA, USA).

Flow cytometric analysis

THP-1 cells were harvested by centrifugation at $200 \times g$ for 5 min at room temperature and incubated for 2 h with antibodies against CD14, CD80 or CD86 (1:100 dilution) in the FACS buffer containing 2 mM EDTA and 0.2% BSA in PBS. After washing with cold-PBS, cells were incubated for 1 h with Alexa Fluor 488-conjugated secondary antibodies (1:200 dilution) at 4°C. Or the harvested cells were incubated with the fluorescent dye-conjugated antibodies (1:100 dilution) against CD163 or CD206 for 1 h at 4°C. Cells were washed with cold-PBS, resuspended in 1% paraformaldehyde, and analyzed by flow cytometry.

ELISA

The amounts of CCL2, CCL3, CCL4, CXCL8, and TNF- α secreted in culture media were quantified using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed via one-way analysis of variance, followed by Dunnett's multiple comparison test, using PRISM (version 5.0) (GraphPad Software Inc., San Diego, CA, USA). A p-value less than 0.05 ($p < 0.05$) is considered to indicate a statistically significant difference.

RESULTS

GSK 2033 inhibits the transcription of LXR target genes

The human LXR α and ABCA1/ABCG1 are targets for regulation by LXR, and ligands for the receptor induce their expression in macrophages [12]. We investigated whether 27OHChol affects expression of the genes along with TO 901317, a synthetic LXR ligand. We observed levels of LXR α transcripts were elevated to 2.25- and 3.49-fold following stimulation with 27OHChol and TO 901317, respectively (Fig. 1A). Assessing the transcript levels of ABCA1 revealed increases up to 1.31- and 8.04-fold by stimulation with 27OHChol and TO 901317, respectively (Fig. 1B). ABCG1 transcripts increased 4.33- and 28.5-fold after stimulation with 27OHChol and TO 901317, respectively (Supplementary Fig. 1). Investigating further whether expressions of LXR α and ABCA1/ABCG1 genes were influenced by GSK 2033, a potent cell-active LXR antagonist [16], revealed that TO 901317- or 27OHChol-induced gene transcription of LXR α and ABCA1/ABCG1 was blocked in the presence of GSK 2033. In line with

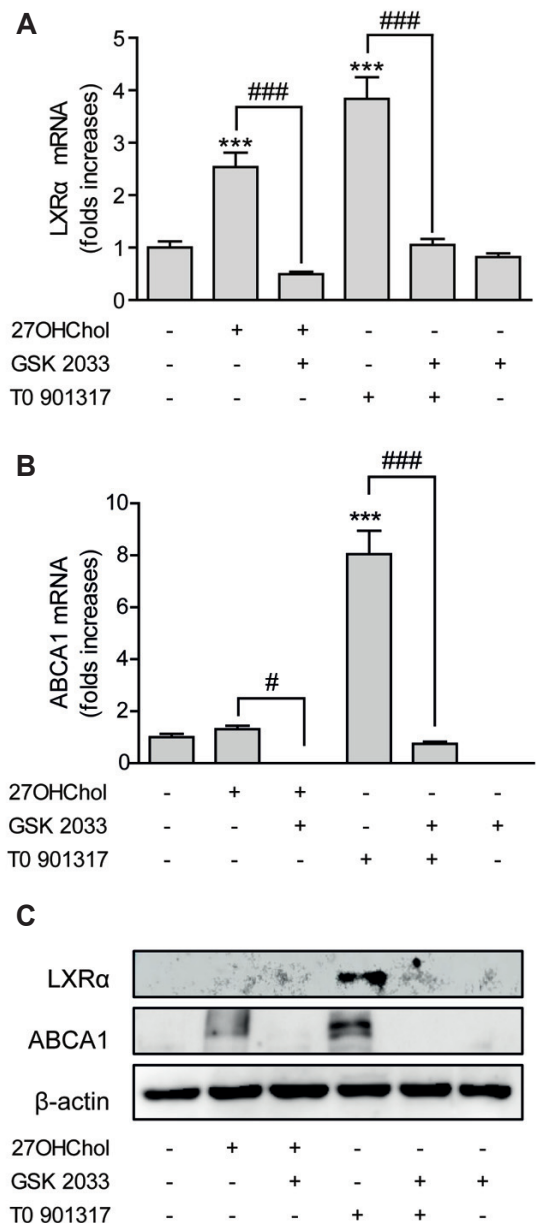


Fig. 1. Inhibitory effects of GSK 2033 on the transcription and protein translation of the LXR α and ABCA1 genes. THP-1 cells were serum-starved overnight and treated with GSK 2033 (1 μ M) for 2 h. Cells were further stimulated with 27OHChol (2.5 μ g/ml) or TO 901317 (1 μ M) for 48 h in RPMI media supplemented with 10% FBS. The levels of LXR α (A) or ABCA1 (B) gene transcripts were assessed by real-time PCR. Data are expressed as the means \pm standard deviation ($n = 3$ replicates for each group). LXRs, liver X receptors; FBS, fetal bovine serum. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. 27OHChol or TO 901317; # $p < 0.05$ vs. 27OHChol or TO 901317. (C) LXR α and ABCA1 proteins were detected by Western blot analysis. Data represent a representative experiment (from 3 independent experiments).

the results of real-time PCR, 27OHChol and TO 901317 elevated protein levels of LXR α or ABCA1, which was completely inhibited by GSK 2033 (Fig. 1C). These results indicate that although less effective than the synthetic LXR agonist, 27OHChol induces

expression of the LXR target genes, and the induction is impaired by GSK 2033.

GSK 2033 suppresses 27OHChol-induced CCL3 gene

27OHChol induces expression of C-C chemokines, including CCL2, CCL3, and CCL4 [11]. We undertook to determine effects of GSK 2033 on the transcription of chemokines to understand the function of LXR inhibition in the induction of C-C chemokines. 27OHChol exposure elevated the levels of CCL2, CCL3, and CCL4 gene transcripts and their gene products (Fig. 2A and B). Although GSK 2033 did not significantly reduce expression of the CCL2 and CCL4 genes, the levels of CCL3 transcripts and protein were profoundly decreased in the presence of GSK 2033.

The antagonist alone did not alter the transcript levels and secretion of the C-C chemokines. These results suggest that of the three C-C chemokines, GSK 2033 suppresses expression of the CCL3 gene alone.

GSK 2033 displays opposite effects on 27OHChol-induced CXCL8 and TNF- α

27OHChol as well as LXR agonists induces the CXCL8 and TNF- α genes which are involved in acute-phase inflammatory responses [8,17,18]. We investigated the roles of LXR agonistic activity of 27OHChol in the expression of CXCL8 and TNF- α . Treatment with GSK 2033 resulted in suppressing the transcription of CXCL8 gene, whereas the transcription of TNF- α further

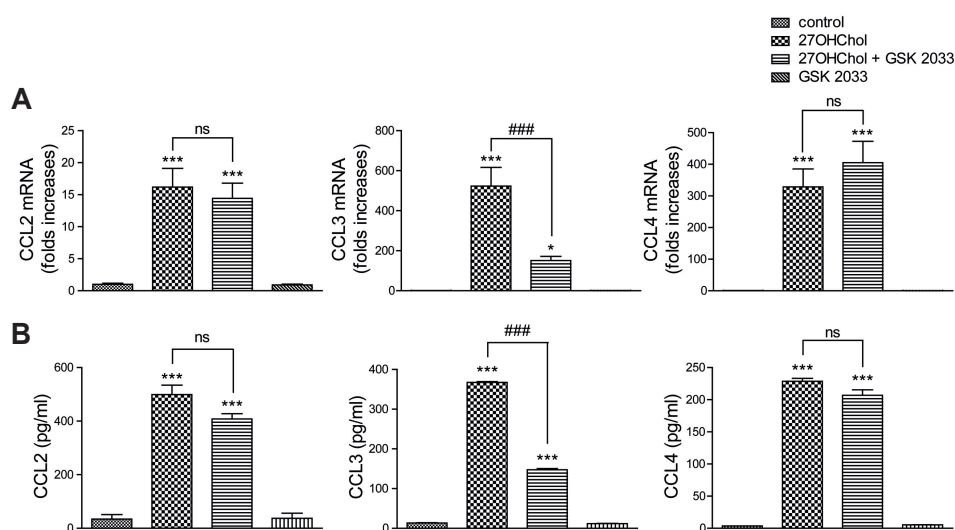


Fig. 2. Differential effects of GSK 2033 on 27OHChol-induced expression of C-C chemokines. After serum-starvation overnight, THP-1 cells were treated with GSK 2033 (1 μ M) for 2 h and stimulated with 27OHChol (2.5 μ g/ml) for 48 h in RPMI media supplemented with 10% FBS. (A) Levels of CCL2, CCL3, and CCL4 gene transcripts were assessed by real-time PCR. (B) The amounts of CCL2, CCL3, and CCL4 proteins secreted in culture media were quantified by ELISA. Data are expressed as the means \pm standard deviation ($n = 3$ replicates for each group). The results are representative of 3 independent experiments. FBS, fetal bovine serum. *** $p < 0.001$ vs. control; * $p < 0.05$ vs. control; ### $p < 0.001$ vs. 27OHChol.

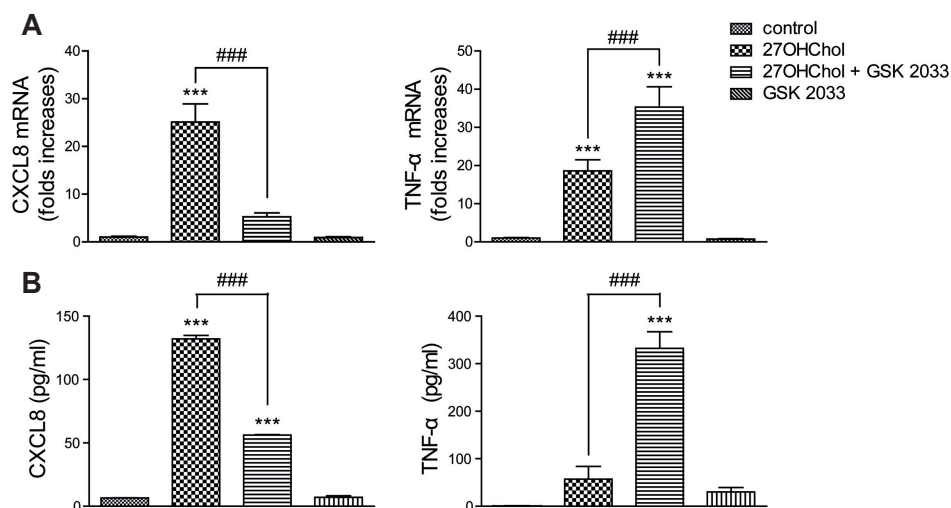


Fig. 3. Opposite effects of GSK 2033 on CXCL8 and TNF- α expression. Serum-starved THP-1 cells were treated with GSK 2033 (1 μ M) for 2 h and stimulated for 48 h with 27OHChol (2.5 μ g/ml). (A) Transcript levels of the CXCL8 and TNF- α genes were assessed by real-time PCR. (B) The amounts of CXCL8 and TNF- α proteins secreted in culture media were quantified by ELISA. Data are expressed as the means \pm standard deviation ($n = 3$ replicates for each group). The results are representative of 3 independent experiments. The results are representative of 3 independent experiments. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. 27OHChol.

enhanced in the presence of GSK 2033 (Fig. 3A). We examined effects of GSK 2033 on CXCL8 and TNF- α secretion. GSK 2033 impaired CXCL8 production, but promoted secretion of TNF- α (Fig. 3B). These results suggest that LXR-agonistic activity of 27OHChol is associated with a repressive effect on TNF- α expression.

GSK 2033 downregulates the transcription of M1/M2 surface markers

27OHChol affect levels of both M1 and M2 markers of macrophages [8]. We used GSK 2033 to estimate involvement of LXR agonistic activity in the induction of M1/M2 markers. 27OHChol increased levels of transcripts and surface expression of M2 markers of CD163 and CD206. Exposure to GSK 2033 resulted in suppressing the transcription of *CD163* and *CD206* genes and downregulating their surface levels (Fig. 4A and B). We also

investigated effects of GSK 2033 on M1 markers of *CD80* and *CD86*, along with *CD14*. The transcription of three genes elevated by 27OHChol was suppressed in the presence of GSK 2033. The LXR antagonist, however, did not downregulate surface expression of CD14, CD80, and CD86 (Fig. 5A and B). These results suggest that LXR activation is required for 27OHChol to induce transcription of the M1/M2 markers and that they are differentially regulated at protein level after LXR inhibition.

DISCUSSION

The LXRs are members of the nuclear receptor superfamily that modulate metabolism, development, proliferation and inflammation through positive and negative regulation of gene expression [13]. LXRs have crucial functions in the regulation of immune responses. Activation of LXR with agonists downregu-

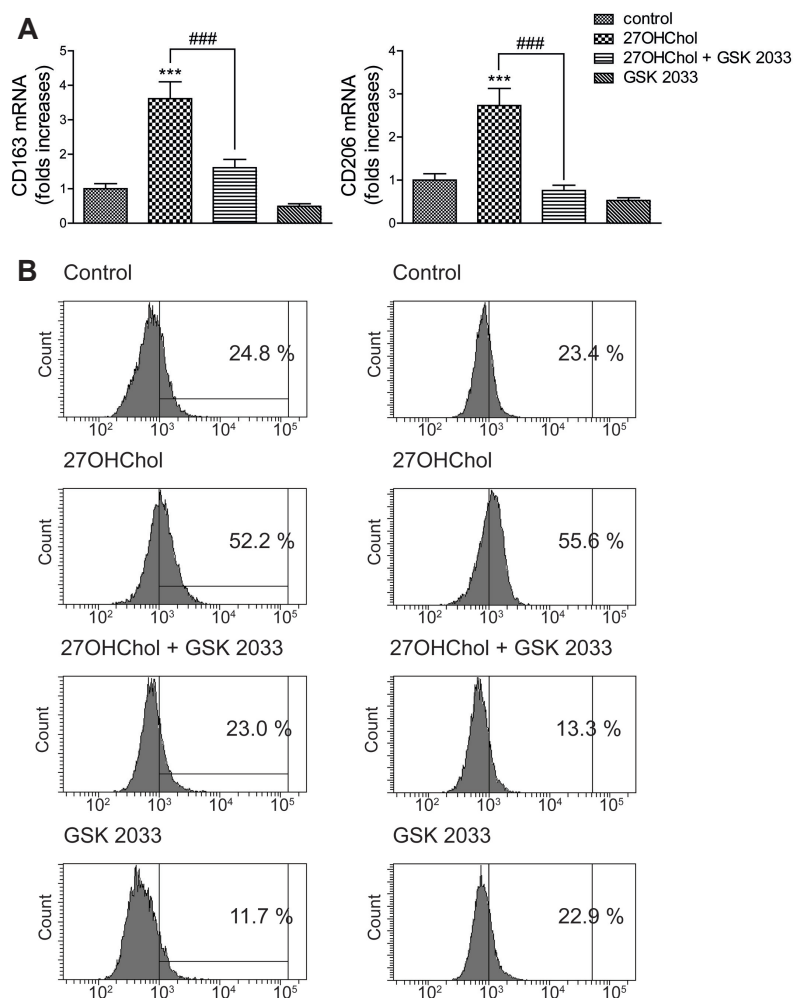


Fig. 4. Suppressive effects of GSK 2033 on M2 surface makers. Following serum-starvation, THP-1 cells were treated with GSK 2033 (1 μ M) for 2 h and stimulated with 27OHChol (2.5 μ g/ml) for 48 h. (A) Transcript levels of the DC163 and CD206 genes were assessed by real-time PCR. Data are expressed as the means \pm standard deviation (n = 3 replicates for each group). (B) After immunostaining of CD163 and CD206 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.

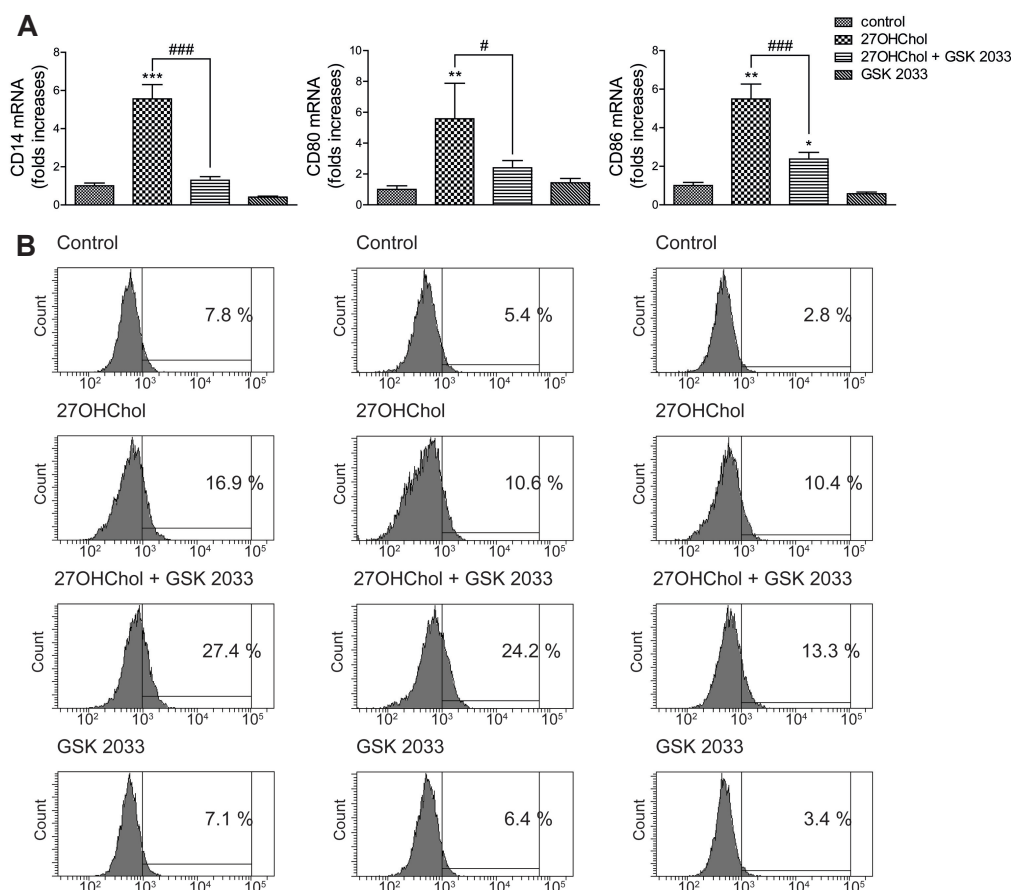


Fig. 5. Differential effects of GSK 2033 on expression of M1 surface markers. Serum-starved THP-1 cells were treated with GSK 2033 (1 μ M) for 2 h, followed by stimulation with 27OHChol (2.5 μ g/ml) for 48 h. (A) Transcript levels of the CD14, CD80, and CD86 genes were assessed by real-time PCR. Data are expressed as the means \pm standard deviation (n = 3 replicates for each group). (B) After immunostaining of CD14, CD80, and CD86 with fluorophore-conjugated Abs, cells were analyzed by flow cytometry. The results are representative of 3 independent experiments. ***p < 0.001 vs. control; **p < 0.01 vs. control; *p < 0.05 vs. control; ###p < 0.001 vs. 27OHChol; #p < 0.05 vs. 27OHChol.

lates the expression of inflammatory genes through a process known as transrepression, and several studies have demonstrated the anti-inflammatory activities of synthetic LXR agonists using different mouse models of inflammatory diseases [19-23]. Endogenous oxysterols, like 27OHChol, are known to activate LXRs [12]. It is evident that this oxysterol is a pro-inflammatory molecule activating macrophages. Activated cells increase cytokine production and upregulate cell surface molecules involved in the immune response [7,9,10]. The current study investigated whether LXR-agonistic activity of 27OHChol affects the cytokine production and upregulates surface molecules after exposure to cell-active GSK 2033 [16], and thereby explored the biological functions of LXRs in macrophage activation.

27OHChol induces expression of diverse genes involved in lipid metabolism, inflammation, and cell differentiation [9,10,12]. Consistent with previous reports [12,14], we found that 27OHChol induces the LXR target genes of *LXR α* and *ABCA1/ABCG1* via LXR-dependent mechanisms. Besides, we have demonstrated that the LXR agonistic activity is involved in expression of *CCL3*, *CXCL8*, *CD14*, *CD80*, *CD86*, *CD163* and *CD206*, since their gene

transcription is inhibited by GSK 2033. The significantly reduced expression of pattern recognition receptors, such as *CD14* and *CD206*, following LXR inhibition, agrees with previous study that has demonstrated that LXR activation impacts the functioning of immune cells to establish an immune response against pathogens [24-26]. The downregulation of CD80 and CD86 is in line with a previous study by Torocsik *et al.* that reported increased expression of these molecules in monocyte-derived dendritic cells after administration of LXR agonists [27]. Taken together, our results suggest that the LXR-agonistic activity of 27OHChol is involved in the complex effects of this oxysterol on immune cells.

27OHChol elevates transcripts of the *LXR α* gene without a noticeable increase in its gene product. TO 901317, however, enhances its expression at both mRNA and protein levels, as shown in Fig. 1. The differences can be caused by the nature of LXR agonistic activity. 27OHChol is a natural LXR agonist, whereas TO 901317 is a synthetic one. TO 901317 displays a strong LXR agonistic activity and fully activates LXR signaling pathways [23]. In contrast, 27OHChol has a weak partial LXR agonistic activity [12]. Even though 27OHChol increases the level of *LXR α* transcripts,

it seems that the activation of additional factors is required for translation of the mRNA into protein.

Cell surface molecules such as CD14, CD80, CD86, CD163, and CD206 are specific markers whose levels are enhanced by 27OHChol on monocytic cells [8]. Among them, CD163 and CD206 belong to M2 polarization markers, and CD80, and CD86 to M1 markers [28]. The results of Figs. 4B and 5B imply that expression of CD163 and CD206 protein is positively regulated by the LXR pathway while CD14, CD80, and CD86 are negatively regulated at protein level in the presence of 27OHChol. Collectively, these results suggest that LXR activity of 27OHChol is also involved in M2 polarization, which agrees with a previous study that reported that 27OHChol drives M2 polarization of human macrophages [29].

In summary, LXR inhibition with GSK 2033 leads to inhibited expression of CCL3 and CXCL8, but not of CCL2 and CCL4, enhanced TNF- α expression, and differential expression of M1/M2 markers on cell surface. We believe that the diverse effects of GSK 2033 are likely to be specific. GSK 2033 alone did not affect cell viability (Supplementary Fig. 2) and had no effect on the basal expression of the aforementioned genes. These results indicate that 27OHChol activates macrophages and affects immune cells via LXR-dependent and -independent mechanisms. Since 27OHChol induces inflammation and modifies immune responses by activating primarily monocytic cells, the molecular mechanisms underlying the cell type-specific effects of this oxysterol are still subject to further studies.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at <https://doi.org/10.4196/kjpp.2021.25.2.111>.

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