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7α-Hydroxycholesterol Elicits TLR6-Mediated Expression of IL-23 in Monocytic Cells

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Abstract

We investigated the question of whether 7-oxygenated cholesterol derivatives could affect inflammatory and/or immune responses in atherosclerosis by examining their effects on expression of IL-23 in monocytic cells. 7α-Hydroxycholesterol (7αOHChol) induced transcription of the TLR6 gene and elevated the level of cell surface TLR6 protein in THP-1 monocytic cells. Addition of an agonist of TLR6, FSL-1, to TLR6-expressing cells by treatment with 7αOHChol resulted in enhanced production of IL-23 and transcription of genes encoding the IL-23 subunit α (p19) and the IL-12 subunit β (p40). However, treatment with 7-ketocholesterol (7K) and 7β-hydroxycholesterol (7βOHChol) did not affect TLR6 expression, and addition of FSL-1 to cells treated with either 7K or 7βOHChol did not influence transcription of the genes. Pharmacological inhibition of ERK, Akt, or PI3K resulted in attenuated transcription of TLR6 induced by 7αOHChol as well as secretion of IL-23 enhanced by 7αOHChol plus FSL-1. Inhibition of p38 MAPK or JNK resulted in attenuated secretion of IL-23. These results indicate that a certain type of 7-oxygenated cholesterol like 7αOHChol can elicit TLR6-mediated expression of IL-23 by monocytic cells via PI3K/Akt and MAPKs pathways.

Key Words: 7alpha-hydroxycholesterol, IL-23, Macrophages, Toll-like receptor 6

INTRODUCTION

Interleukin (IL)-23 is a heterodimeric cytokine consisting of the IL-23 subunit α (p19) and the IL-12 subunit β (p40) (Oppmann *et al*., 2000). IL-23 is produced mainly by activated macrophages and antigen-presenting cells (APC) including dendritic cells (Oppmann *et al*., 2000) and induces differentiation of naïve CD4+ T cells into IL-17-producing T cells (Th17 cells) (Aggarwal *et al*., 2003), which, in turn, produce a proinflammatory cytokine, IL-17, which enhances T cell priming and stimulates production of other inflammatory mediators (Iwakura and Ishigame, 2006; Korn *et al*., 2009). IL-23 also acts on dendritic cells and macrophages in an autocrine/paracrine manner to stimulate generation of inflammatory cytokines (Iwakura and Ishigame, 2006; Korn *et al*., 2009). Therefore, IL-23-Th17 immune axis contributes to the pathogenesis of chronic inflammatory and autoimmune disease (Langrish *et al*., 2005).

A link between IL-23-IL-17 axis and atherosclerosis, whose pathogenesis is associated with cholesterol, has been proposed. Expression of IL-23 and IL-17 is significantly up-regulated in atherosclerotic lesions of symptomatic patients (Erbel *et al*., 2011) and functional blockade of IL-17A results in markedly reduced development of atherosclerotic lesions and plaque vulnerability in ApoE-deficient mice (Erbel *et al*., 2009). Development of more and significantly larger atherosclerotic lesions occurs in IL-18-deficient $ApoE^{-/-}$ mice compared with $ApoE^{-/-}$ mice, which is correlated with increased expression of IL-23 by smooth muscle cells and macrophages in the lesions (Pejnovic *et al*., 2009). Collectively, these results indicate involvement of IL-23 in a mechanism that promotes development of atherosclerosis. However, it is not known whether cholesterol is involved in increased expression of IL-23 in atherosclerotic lesions.

Atherosclerotic lesions are characterized by accumulation of extracellular lipids among which cholesterol comprises the majority of components (Guyton and Klemp, 1994). The accumulated cholesterol undergoes oxidative modification to cholesterol oxides, oxysterols, non-enzymatically via vivo oxidation or enzymatically during cholesterol catabolism (Schroepfer,

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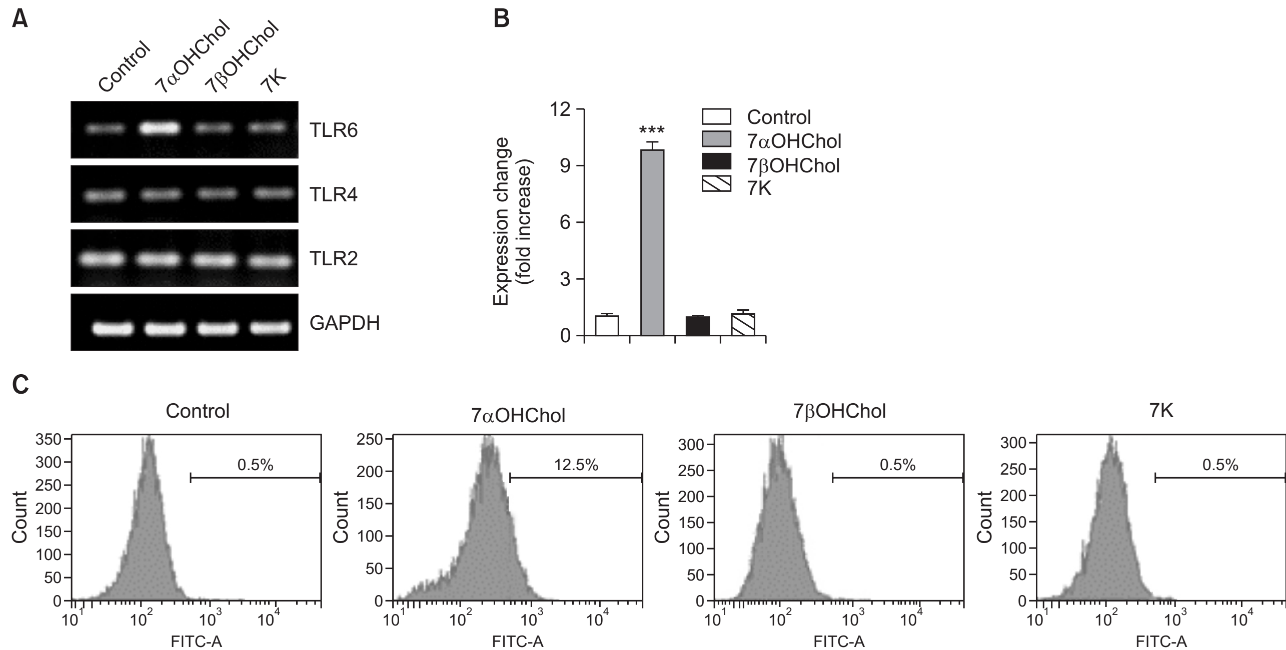

Fig. 1. Expression of TLR6 in THP-1 cells in response to 7-oxygenated cholesterol derivatives. (A) THP-1 cells (1×10^6 cells/60 mm culture dish) were serum starved in 0.1% BSA (endotoxin free) in RPMI 1640 for 24 h and treated with 7αOHChol, 7βOHChol, or 7K (5 μg/ml each) for 48 h. Total RNA was isolated from the cells, and transcripts of indicated TLRs were amplified by RT-PCR. (B) THP-1 cells were serum starved in 0.1% BSA in RPMI 1640 for 24 h and treated with 7αOHChol, 7βOHChol, or 7K (5 μg/ml each) for 48 h. Total RNA isolated from the cells was reverse-transcribed and real-time PCR was performed for determination of the relative levels of TLR6 transcripts. (C) Serum-starved THP-1 cells were treated with 7αOHChol, 7βOHChol, or 7K for 48 h. After immunostaining for cell surface TLR6, flow cytometry was performed for analysis of fluorescence.

2000). 27-Hydroxycholesterol (27OHChol) is the most abundant oxysterol, followed by 7-oxygenated cholesterol catabolites, including 7-ketocholesterol (7K), 7β-hydroxycholesterol (7βOHChol), and 7α-hydroxycholesterol (7αOHChol). These oxysterols comprise 75-85% of oxysterols detected in atherosclerotic plaques from different sites (Carpenter *et al*., 1995; Brown and Jessup, 1999). 7-Oxygenated cholesterols, the main type of oxysterol in oxidized low density lipoprotein (oxLDL) (Brown *et al*., 1997), participate in atherosclerosis by promoting inflammation via up-regulation of chemokines and cytokines (Dje N'Guessan *et al*., 2009). In addition, 7-oxygenated cholesterols are believed to be responsible for the atherogenic effects of oxLDL as 7K and 7βOHChol enhance expression of CXCL8 in human macrophages (Lemaire-Ewing *et al*., 2005; Erridge *et al*., 2007). However, involvement of 7-oxygenated cholesterols in terms of expression of IL-23 is unknown.

We attempted to determine whether 7-oxygenated cholesterol derivatives can induce expression of IL-23. We demonstrated that 7αOHChol can enhance production of IL-23 via TLR6 and sought to identify cellular signaling molecules involved in production of IL-23 in order to understand molecular mechanisms underlying proinflammatory roles of TLR6.

MATERIALS AND METHODS

Cells and reagents

THP-1 cells were purchased from and maintained as recommended by the American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells in passages between 7 and 10 were used for experiments. 7α-Hydroxycholesterol (7αOHChol) and 7β-hydroxycholesterol (7βOHChol) were purchased from Research Plus, Inc. (Barnegat, NJ, USA). 7-Ketocholesterol (7K), LY294002, and SP600125 were purchased from Sigma-Aldrich (St. Louis, MO, USA). FSL-1 was purchased from Invivogen (San Diego, CA, USA). U0126, SB202190, and Akt inhibitor IV (Akti IV) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNAs were isolated using TRIzol reagent and reverse-transcribed to complementary DNA (cDNA) for 1 h at 42°C with Moloney Murine Leukemia Virus reverse transcriptase using the oligod(T)$_{15}$ primer (Promega, Madison, WI, USA), followed by non-quantitative and quantitative real-time PCR. For non-quantitative PCR, transcripts of genes of interest were amplified using Hot Start Taq Polymerase (Promega). The cDNA was denatured at 90°C for 5 min followed by 25 cycles of PCR (95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec) in the presence of forward and reverse primers of the genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Real-time quantitative PCR was performed in triplicate using the LightCycler® 96 Real-Time PCR System (Roche, Germany); each 20-μl reaction consisted of 10 μl of SYBR Green Master Mix, 2 μl of forward and reverse primers (10 pM each) of genes to be analyzed, and cDNA template. Thermal cycling conditions were as follow: 95°C for 10 min, and 45 cycles at 95°C for 10 sec, 50°C for 10 sec, and an elongation period for 10 sec at 72°C.

The relative expression of each gene was then calculated as a ratio to GAPDH using the LightCycler® 96 software (Version 1.1.0.1320). The primers were TLR6: 5'-agggctggcctgattct-tat-3' (forward) and 5'-tggcacaccatcctgagata-3' (reverse); IL23 p19: 5'-gttccccatatccagtgtgg-3' (forward) and 5'-gaggcttg-gaatctgctgag-3' (reverse); IL-12β (p40): 5'-aaggaggcgaggttc-taagc-3' (forward) and 5'-tccttgttgtcccctctgac-3' (reverse); and GAPDH: 5'-gagtcaacggatttggtcct-3' (forward) and 5'-tgtggtcat-gagtccttcca-3' (reverse).

Flow cytometric analysis

THP-1 cells were harvested, washed with PBS, and immunolabelled with anti-TLR6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS (1:50) for 1 h at 4°C. Subsequently, cells were washed twice with cold PBS and incubated for 40 min with FITC-conjugated secondary antibody diluted in PBS (1:100) at room temperature. After washing twice with PBS, cells were resuspended in 1% paraformaldehyde/PBS solution. Fluorescence was analyzed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

The amount of IL-23 secreted from THP-1 cells was measured using a commercially available IL-23 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Recombinant standards provided in the kit and isolated culture media were added to wells of the plate provided in the kit. After incubation for 2 h, wells were washed and incubated with the conjugate at room temperature for 2 h. After several washes, the substrate solution was added, and the color intensity was measured. A standard curve was used for determination of the amount of IL-23 present in the samples. Data are expressed as average ± standard deviation of triplicate experiments.

Statistical analysis

Statistical analyses by ANOVA, followed by Dunnett's multiple comparison tests, were performed using PRISM (version 5.0) (GraphPad Software Inc., San Diego, CA, USA). A p-value less than 0.05 was considered statistically significant.

RESULTS

Up-regulation of TLR-6 expression in the presence of 7αOHChol

We investigated the effects of 7-oxygenated cholesterols on expression of TLR6. THP-1 cells were treated with 7αOHChol, 7βOHChol, and 7K, and transcription of TLR6 was determined. Transcripts of TLR6, which were detected in THP-1 cells using RT-PCR, were induced in the presence of 7αOHChol alone. Transcription of TLR4 and TLR2 was not influenced by 7αOHChol, 7βOHChol, or 7K (Fig. 1A). In determination by realtime PCR, the level of TLR6 transcripts increased by 9.8-fold in the presence of 7αOHChol in comparison with unstimulated (control) cells (Fig. 1B). We also performed flow cytometry in order to examine the question of whether 7-oxygenated cholesterols affected levels of surface TLR6 protein. The percentage of control THP-1 cells that expressed a high level of TLR6 was 0.5%, which increased to 12.5% in the presence of 7αOHChol. However, treatment with 7K or 7βOHChol did not result in an increase in the number of TLR6-positive cells (Fig. 1C). These results indicated that treatment of monocytic cells with 7αOHChol resulted in increased transcription of TLR6 and enhanced levels of its gene

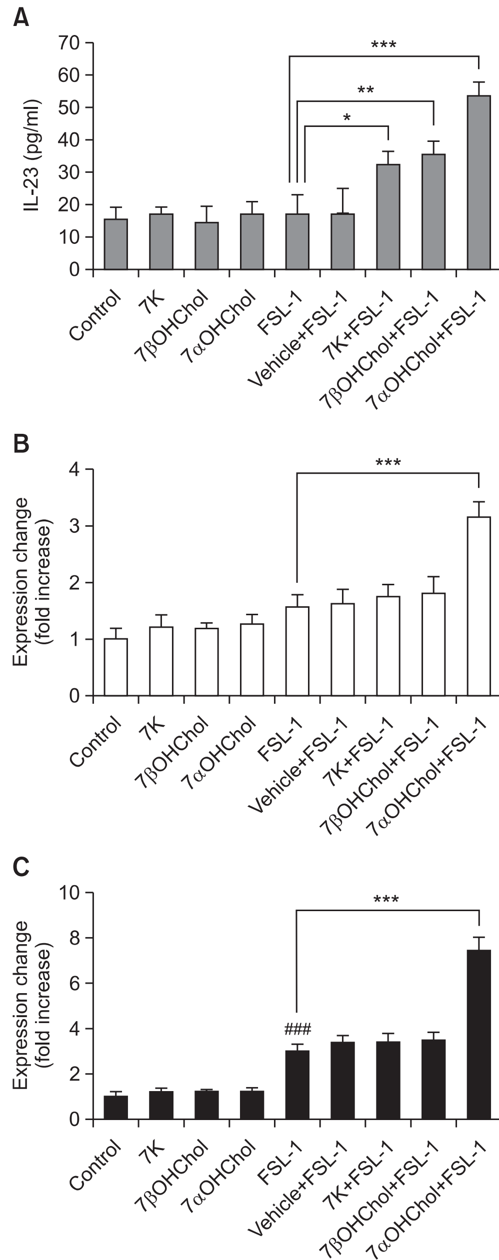

Fig. 2. Effects of 7-oxygenated cholesterol derivatives on TLR6-mediated expression of IL-23. (A) Serum-starved THP-1 cells were treated with 7αOHChol, 7βOHChol, or 7K (5 μg/ml each) for 24 h and incubated after addition of FSL-1 (100 ng/ml) for 24 h. The amount of IL-23 secreted into culture media was measured by ELISA. *p<0.05 vs. FSL-1; **p<0.01 vs. FSL-1; ***p<0.001 vs. FSL-1. (B, C) Serum-starved THP-1 cells were treated with 7αOHChol, 7βOHChol, or 7K (5 μg/ml each) for 24 h and incubated after addition of FSL-1 (100 ng/ml) for 24 h. Total RNA isolated from the cells was reverse-transcribed and real-time PCR was performed for determination of the relative levels of transcripts of IL-23A (B) and the IL-12B (C) genes, respectively. ***p<0.001 vs. FSL-1; $^{###}p$<0.001 vs. control.

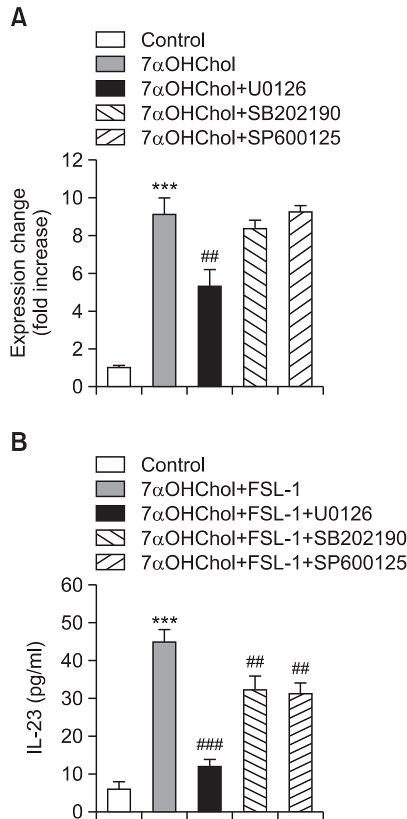

Fig. 3. Effects of inhibitors of MAPKs on expression of TLR6 and IL-23 enhanced in the presence of 7αOHChol. (A) Serum-starved THP-1 cells were treated with or without 7αOHChol (5 μg/ml) for 48 h after pre-incubation for 2 h in the absence or presence of the indicated inhibitors (10 μM each). Total RNA isolated from the cells was reverse-transcribed and real-time PCR was performed for determination of the relative levels of TLR6 transcripts. ***p<0.001 vs. control; ##p<0.01 vs. 7αOHChol. (B) Serum-starved THP-1 cells were treated with or without 7αOHChol (5 μg/ml) for 24 h after pre-incubation for 2 h in the absence or presence of the indicated inhibitors (10 μM each), and incubated after addition of FSL-1 (100 ng/ml) for 24 h. The amount of IL-23 secreted into culture media was measured by ELISA. ***p<0.001 vs. control; ###p<0.001 vs. 7αOHChol +FSL-1; ##p<0.01 vs. 7αOHChol +FSL-1.

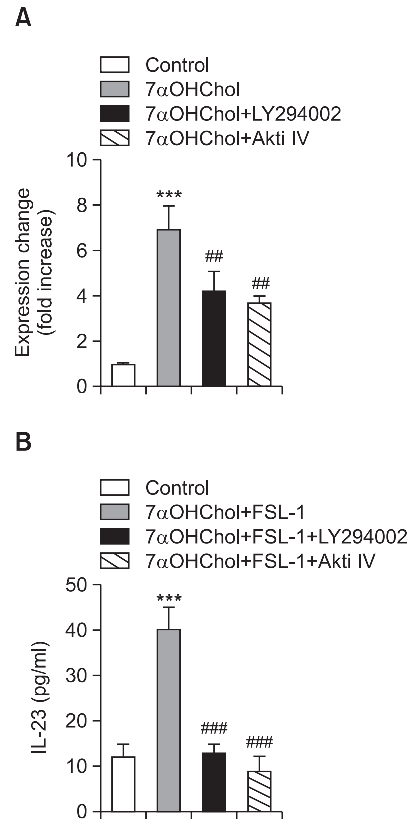

Fig. 4. Effects of inhibitors of PI3K/Akt on expression of TLR6 and IL-23 enhanced in the presence of 7αOHChol. (A) Serum-starved THP-1 cells were treated with or without 7αOHChol (5 μg/ml) for 48 h after pre-incubation for 2 h in the absence or presence of the indicated inhibitors (10 μM each). Total RNA isolated from the cells was reverse-transcribed and real-time PCR was performed for determination of the relative levels of TLR6 transcripts. ***p<0.001 vs. control; ##p<0.01 vs. 7αOHChol. (B) Serum-starved THP-1 cells were treated with or without 7αOHChol (5 μg/ml) for 24 h after pre-incubation for 2 h in the absence or presence of the indicated inhibitors (10 μM each), and incubated after addition of FSL-1 (100 ng/ml) for 24 h. The amount of IL-23 secreted into culture media was measured by ELISA. ***p<0.001 vs. control; ###p<0.001 vs. 7αOHChol +FSL-1.

product on the cell surface.

Augmented expression of IL-23 by a TLR6 agonist in the presence of 7αOHChol

We attempted to determine whether TLR6-mediated signaling was affected in the presence of 7-oxygenated cholesterols. THP-1 cells were treated with the indicated oxysterols, followed by addition of FSL-1, a synthetic TLR6 agonist, and production of IL-23 was then determined. THP-1 cells secreted a low amount of IL-23, and treatment with 7K, 7βOHChol, 7αOHChol, or FSL-1 alone did not result in enhanced secretion of IL-23. Exposure of THP-1 cells to FSL-1 in the presence of 7K, 7βOHChol, or 7αOHChol resulted in increased secretion of IL-23 (Fig. 2A). Among 7-oxygenated cholesterol derivatives used in the experiment, secretion of IL-23 was synergistically increased in the presence of 7αOHChol.

Because IL-23 is a heterodimeric cytokine formed by the IL-23 subunit α (p19) and the IL-12 subunit β (p40) (Oppmann *et al*., 2000), we investigated the question of whether transcription of these subunits was changed in the presence of 7-oxygenated cholesterols using real-time PCR. 7K, 7βOHChol, 7αOHChol, or FSL-1 did not induce transcription of IL-12 subunit β, and transcription of the subunit was induced by addition of FSL-1 to THP-1 cells treated with 7αOHChol. Transcription of IL-23 subunit α was induced by FSL-1, but not by 7K, 7βOHChol or 7αOHChol, and addition of FSL-1 to 7αOHChol-treated cells resulted in enhanced transcription of the subunit (Fig. 2B, C). However, an addition of FSL-1 to 7αOHChol-treated THP-1 cells did not augment transcription of interleukin (IL)-1β and tumor necrosis factor (TNF)-α (data not shown).

Roles of the ERK1/2 pathway in up-regulation of TLR6 and TLR6-mediated expression of IL-23

Because 7αOHChol activated extracellular signal-regulated kinase (ERK) (Kim *et al*., 2014), we investigated the ques-

tion of whether ERK played a role in the action of 7αOHChol-induced expression of TLR6 using U0126 (an ERK kinase inhibitor) in parallel with SB202190 (a p38 MAPK inhibitor) and SP600125 (a JNK inhibitor). Treatment with U0126 resulted in a significant reduction in the level of TLR6 transcripts induced by 7αOHChol, whereas SB202190 and SP600125 did not change transcription of TLR6 (Fig. 3A). MAPKs including ERK, p38 MAPK, and JNK are also activated in response to FSL-1 (Won *et al*., 2012). Therefore, involvement of the kinases in IL-23 expression was also examined. Secretion of IL-23 enhanced by treatment with 7αOHChol plus FSL-1 was blocked by U0126 and significantly attenuated by SB202190 or SP600125 (Fig. 3B). These results indicated that activity of ERK as well as p38MAPK and JNK was required for maximal expression of IL-23 via TLR6 in the presence of 7αOHChol.

Roles of PI3K/Akt in up-regulation of TLR6 and TLR6-mediated expression of IL-23

We reported that treatment of monocytic cells with 7αOHChol resulted in activation of Akt (Kim *et al*., 2014). Therefore, we investigated involvement of PI3K/Akt in 7αOHChol-induced expression of TLR6 using inhibitors of LY294002 and Akti IV. In examination of the effects of the inhibitors on transcription, transcription of TLR6 was significantly attenuated by treatment with both LY294002 and Akti IV. The level of TLR6 transcripts increased by 6.9-fold after stimulation with 7αOHChol in comparison with control, and it was reduced to 4.2-and 3.7-fold in the presence of LY294002 and Akti IV, respectively (Fig. 4A). In addition, the possible involvement of the PI3K/Akt pathway in IL-23 expression was determined. Secretion of IL-23 induced by 7αOHChol plus FSL-1 was almost completely blocked in the presence of LY294002 or Akti IV (Fig. 4B). Collectively, these results indicate involvement of PI3K/Akt in molecular mechanisms of action of TLR6.

DISCUSSION

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) recognizing molecules that are broadly shared by pathogens, i.e., pathogen-associated molecular patterns (PAMPs), but distinguishable from host molecules (Kawai and Akira, 2011). TLRs initiate host defense after recognition of PAMPs from infectious pathogens (Kawai and Akira, 2011). However, activation of TLRs does not always seem to be beneficial to disease and it can be involved in deterioration of disease under certain circumstances. For example, administration of TLR6 synthetic agonists mimicking bacterial PAMPs resulted in enhanced formation of local lesions in low density lipoprotein (LDL) receptor deficient ($LDLR^{-/-}$) mice fed a high fat diet via TLR6 dependent mechanisms (Curtiss *et al*., 2012). These results indicate that TLR6 is necessary for enhancement of atherogenesis when the receptor is activated by bacterial PAMPs. However, the underlying mechanisms linking TLR6 and the enhanced development of atherosclerosis are unknown.

Inflammation plays essential roles in development of atherosclerosis (Libby, 2002). We examined the question of whether TLR6 is activated and involved in expression of pro-inflammatory cytokine in a milieu rich in cholesterol or oxysterols (i.e., cholesterol oxides). Treatment of monocytic cells with 7αOHChol resulted in up-regulated expression of TLR6. For investigation of involvement of the receptor in inflammation, we used a synthetic TLR6 agonist FSL-1 (Pam2CGDP-KHPKSF). Ligation of TLR6 by addition of FSL-1 to monocytic cells treated with 7αOHChol resulted in enhanced production of IL-23 as well as transcription of genes encoding IL-23 subunits. The activation of TLR6 can be said to be specific because treatment with other types of 7-oxygenated cholesterol derivatives like 7K and 7βOHChol did not affect expression of TLR6 and addition of FSL-1 to THP-1 cells treated with 7K or 7βOHChol did not induce transcription of IL-23 subunits. Because 7αOHChol is detected from atherosclerotic lesions (Carpenter *et al*., 1995; Garcia-Cruset *et al*., 2001), there is a possibility that the TLR6 signaling pathway can be activated and may contribute to enhanced expression of IL-23 in the lesions, which provides a mechanism through which expression of IL-23 is enhanced in atherosclerotic lesions.

We attempted to identify cellular factors involved in expression of TLR6. Because MAPKs are serine/threonine-specific protein kinases that mediate gene expression in response to various extracellular stimuli (Kaminska, 2005; Chi *et al*., 2006), their roles in TLR6 expression were investigated. Based on two observations, it seemed that ERK pathway is required for 7αOHChol-mediated activation of TLR6. First, a significant reduction in transcription of the TLR6 gene was observed by inhibition of the ERK pathway, but not of p38 MAPK and JNK, indicating a major role of the ERK pathway in 7αOHChol-induced expression of TLR6. Second, activation of TLR6 with its agonist resulted in enhanced production of IL-23, and inhibition of the ERK pathway resulted in the greatest reduction in IL-23 production. The results of the current study are consistent with those of previous publications in that MAPKs can mediate inflammation in response to activation of TLRs (Kawai and Akira, 2006; Thobe *et al*., 2007). We consider that down-regulation of TLR6 by inhibition of the ERK pathway would contribute to reduced production of IL-23.

PI3K/Akt pathway regulates TLR signaling and outcome of the regulation can be either positive or negative effects on signaling, depending on cell type and stimulus (Hazeki *et al*., 2007; Sandig and Bulfone-Paus, 2012). Treatment with 7αOHChol resulted in enhanced phosphorylation of Akt, indicating activation of the PI3K/Akt pathway in response to 7αOHChol (Kim *et al*., 2014). We observed up-regulated expression of TLR6 in the presence of 7αOHChol. Therefore, we investigated the effects of inhibition of PI3K/Akt on TLR6 signaling. The observations in this study indicate that regulation of the PI3K/Akt pathway will have negative effects on TLR6 signaling; inhibition of PI3K or Akt resulted in down-regulated expression of the TLR6 gene and reduced production of IL-23 via TLR6. These results also indicate that the PI3K/Akt pathway participates in 7αOHChol-induced up-regulation of TLR6 and mediates production of IL-23 in response to ligation of TLR6 in the presence of 7αOHChol. In addition, we observed that treatment of THP-1 cells with 7αOHChol led to increased nuclear translocation of phosphorylated p65, the subunit of nuclear factor κB (NF-κB), indicating activation of the transcription factor (unpublished). It is under investigation whether NF-κB is involved in expression of IL-23.

We demonstrated that treatment of monocytic cells with 7αOHChol resulted in TLR6-mediated expression of pro-atherogenic cytokine IL-23 via up-regulation of the receptor and that inhibition of the ERK or PI3K/Akt pathway leads to reduced expression of IL-23 induced by TLR6 agonist. However,

the current study did not determine whether the ERK pathway and Akt act in an independent or cooperative manner; therefore, in order to understand molecular mechanisms underlying expression of IL-23 induced in combination with 7αOHChol and TLR6 agonist, elucidation of the types of connections or crosstalk that may occur between two pathways is needed.

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