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27-Hydroxycholesterol up-regulates CD14 and predisposes monocytic cells to superproduction of CCL2 in response to lipopolysaccharide

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ABSTRACT

We investigated the possibility that a cholesterol-rich milieu can accelerate response to pathogen-associated molecular patterns in order to elucidate mechanisms underlying aggravation of atherosclerosis after bacterial infection. The consumption of a high-cholesterol diet resulted in enhanced the expression of CD14 in arteries of ApoE$^{-/-}$ mice. 27-Hydroxycholesterol (27OHChol), the most abundant cholesterol oxide in atherosclerotic lesions, induced the significant expression of CD14 by THP-1 monocytic cells, but not by vascular smooth muscle cells or Jurkat T cells. Additions of lipopolysaccharide (LPS) to 27OHChol-treated THP-1 monocytic cells resulted in superinduction in terms of the gene transcription of CCL2 and the secretion of its gene product. In contrast, cholesterol did not cause increased the expression of CD14 in the aforementioned cells, and the addition of LPS to cholesterol-treated monocytic cells did not result in enhanced the expression of CCL2. The conditioned medium isolated from THP-1 cells exposed to 27OHChol plus LPS further induced the migration of monocytic cells in comparison with conditioned media obtained from THP-1 cells treated with 27OHChol or LPS alone. Treatment with 27OHChol also resulted in the enhanced secretion of MMP-9 and soluble CD14 (sCD14), and the secretion of sCD14 was blocked by a selective MMP-9 inhibitor. The inhibition of the ERK pathway resulted in significantly attenuated the secretion of sCD14 via mechanisms that were distinct from those by PI3K inhibition. We propose that 27OHChol can prime monocytes/macrophages by up-regulation of CD14 such that LPS-mediated inflammatory reaction is accelerated, thereby contributing to aggravated development of atherosclerotic lesions by enhancing recruitment of monocytic cells after infection with Gram-negative bacteria.
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1. Introduction

Atherosclerosis is initiated and promoted by an inflammatory process considered to be caused by accumulation and oxidative modification of cholesterol in the artery [1]. Of the oxidative-modified cholesterol derivatives, 27-hydroxycholesterol (27OHChol) is the most abundantly detected form of cholesterol oxide in atherosclerotic plaques from different sites [2,3]. During the early stage of atherosclerosis, circulating leukocytes, largely monocytes and T lymphocytes, bind to cell adhesion molecules expressed on endothelial cells and migrate into the intima, and migrated leukocytes further amplify the inflammatory responses [1,4], which is directed by chemokines. Among chemokines, the activity of chemokine (C-C motif) ligand 2 (CCL2) directs the migration of monocytes to the atherosclerotic lesions [5,6]. Therefore, understanding of the roles of oxidative-modified cholesterol derivatives in CCL2 expression is critical as its activity is closely linked to severity of atherosclerosis.

A large number of different infectious agents, including *Mycoplasma pneumonia*, *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Helicobacter pylori*, *Streptococcus* spp., influenza A virus, hepatitis C virus (HCV), cytomegalovirus (CMV), and herpes simplex virus (HSP), have been found in atherosclerotic plaques [7]. The infectious burden is considered to contribute to the pathogenesis of atherosclerosis. The development of atherosclerotic lesions is accelerated in mice fed with high-cholesterol diet infected with Gram-negative bacteria such as *C. pneumoniae* and *P. gingivalis* or with Gram-positive bacteria like *Streptococcus mutans* [8–10]. Acceleration can be due to direct effects of the bacteria on vascular cells, including promotion of endothelial dysfunction and activation of T lymphocytes and monocytes/macrophages [11]. The direct effects explain the roles of infecting bacteria in inflammation in the vasculature. However, we propose that the underlying mechanisms for development of aggravated atherosclerotic lesions after bacterial infection should be understood in the context of hypercholesterolemia because atherosclerosis requires cholesterol in abundance in its pathogenesis.

Abbreviations: ERK, extracellular-signal-regulated kinase; HAoSMCs, Human aortic smooth muscle cells; 27OHChol, 27-hydroxycholesterol; LPS, lipopolysaccharide; mCD14, membrane-associated CD14; PI3K, phosphoinositide-3-kinase; sCD14, soluble CD14

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Groups of infectious pathogens have molecular motifs conserved within a class of microbes referred to as pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) in infected organisms [12]. Lipopolysaccharide (LPS) is the prototype PAMP derived from Gram-negative bacteria. LPS released after infection is recognized by CD14, a PPR expressed by monocytes/macrophages. CD14 binds to LPS and transfers the bound LPS to Toll-like receptor 4 (TLR4), which initiates inflammatory responses by mediating the expression of chemokines like CCL2 [13]. CD14 plays a pivotal in initiating responses to LPS. Macrophages and animals deficient in CD14 show markedly reduced sensitivity to LPS and no symptoms of septic shock after exposure to LPS or Gram-negative bacteria, respectively [14,15]. Therefore, the increased expression of CD14 in macrophages within atherosclerotic lesions [16] suggests that inflammatory reaction against bacterial PAMPs can be enhanced in atherosclerosis.

In the current study, we determined the probability that cell homeostasis is modified by the lipids present within atherosclerotic lesions such that inflammatory reaction against infecting bacteria or bacterial PAMPs is accelerated in atherosclerosis. We attempted to determine whether cholesterol or its oxidized form present in atherosclerotic lesions can affect the expression of CD14 and inflammatory response to LPS. We demonstrated for the first time that 27OHChol induces the up-regulated expression of CD14 in monocytic cells and predisposes them to superinduction of CCL2 expression in response to LPS. We also sought to elucidate molecular mechanisms for the up-regulated expression of CD14 induced by 27OHChol.

2. Materials and methods

2.1. Animal experiments

Animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines as previously described [17]. Experimental protocols were approved by the Pusan National University Animal Care and Use Committee.

2.2. Cells

THP-1 cells and Jurkat T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells and Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2. Human aortic smooth muscle cells (HAoSMCs) purchased from Cambrex (East Rutherford, NJ, USA) were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 15% FBS and the antibiotics.

2.3. Reagents

Cholesterol and 27OHChol were purchased from Research Plus, Inc. (Barnegat, NJ, USA). Lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA, USA). LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126 was purchased from Cell Signaling Technology (Danvers, MA, USA). Oxidized LDL (oxLDL) was purchased from Biomedical Technologies Inc. (Ward Hill, MA, USA). MMP-9 inhibitor I was purchased from Calbiochem (San Diego, CA, USA).

2.4. Reverse transcription (RT) – real-time polymerase chain reaction (PCR)

Total RNA was reverse-transcribed for 1 h at 42 °C with Moloney murine leukemia virus reverse transcriptase, followed by polymerase chain reaction (PCR) and real-time PCR using primers. The primers were mouse GAPDH: 5′-acacattgggggtaggaaca-3′ (forward) and 5′-aactttggcattgtggaagg-3′ (reverse); mouse CD14: 5′-ctgatctcagccctctgtcc-3′ (forward) and 5′-gtcccagcccagtgaaagac-3′ (reverse); human GAPDH: 5′-gagtcaacggatttggtcct-3′ (forward) and 5′-tgtggtcatgagtccttcca-3′ (reverse); human CD14: 5′-aggcctcaaggtactgagca-3′ (forward) and 5′-ctgttgcagctgagatcgag-3′ (reverse); human MMP-9: 5′-ttcatcttccaaggccaatc-3′ (forward) and 5′-gccattcacgtcgtccttat-3′ (reverse); and human CCL2: 5′-tctgtgcctgctgctcatag-3′ (forward) and 5′-cagatctccttggccacaat-3′ (reverse). Please see the supplementary data for more details.

2.5. Flow cytometric analysis

THP-1 cells were harvested by centrifugation and incubated for 40 min with anti-CD14 antibody conjugated with fluorescent dye (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. After washing twice with PBS, cells were resuspended in 1% paraformaldehyde in phosphate-buffered saline (PBS). Fluorescence was analyzed by flow cytometry.

2.6. Enzyme-linked immunosorbent assay

The levels of CCL2, sCD14, and MMP-9 secreted into the culture media were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.7. Chemotaxis assay

The migration of THP-1 cells was measured using Transwell Permeable Supports (Costar, Cambridge, MA, USA) as previously described [18].

2.8. MMP-9 gelatinolytic activity in cell supernatants

MMP-9 activity in cell supernatants was measured by zymography using 8% polyacrylamide–SDS gels containing 0.2% type A gelatin (Sigma-Aldrich). Please see the supplementary data for more details.

2.9. Western blot analysis

CD14 and TLR4 proteins were detected by Western blot analysis. The intensity of each immunoblot band was determined by using digitizing software Un-Scan-It Gel (Silk Scientific Inc., Orem, UT). Please see the supplementary data for more details.

2.10. Statistical analysis

Statistical analysis by Student's *T* test or one-way ANOVA followed by Dunnett's multiple comparison test was performed using PRISM (version 5.0) (GraphPad Software Inc., San Diego, CA, USA). A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Elevated CD14 expression in the aortas of an animal model of atherosclerosis

Because infection with Gram-negative bacteria resulted in accelerated atherosclerosis in an animal model of atherosclerosis [8,10], we attempted to determine whether the expression of CD14, which initiates response to LPS of bacteria, was changed in atherosclerotic arteries. We compared the transcription of the CD14 gene between $ApoE^{-/-}$ mice and wild-type mice fed a high-cholesterol diet. CD14 transcripts were detected in six aortas out of six wild-type mice, and its transcription was enhanced in $ApoE^{-/-}$ mice (Supplementary Fig. 1). In comparison with C57BL/6 wild-type mice, significantly elevated levels of CD14

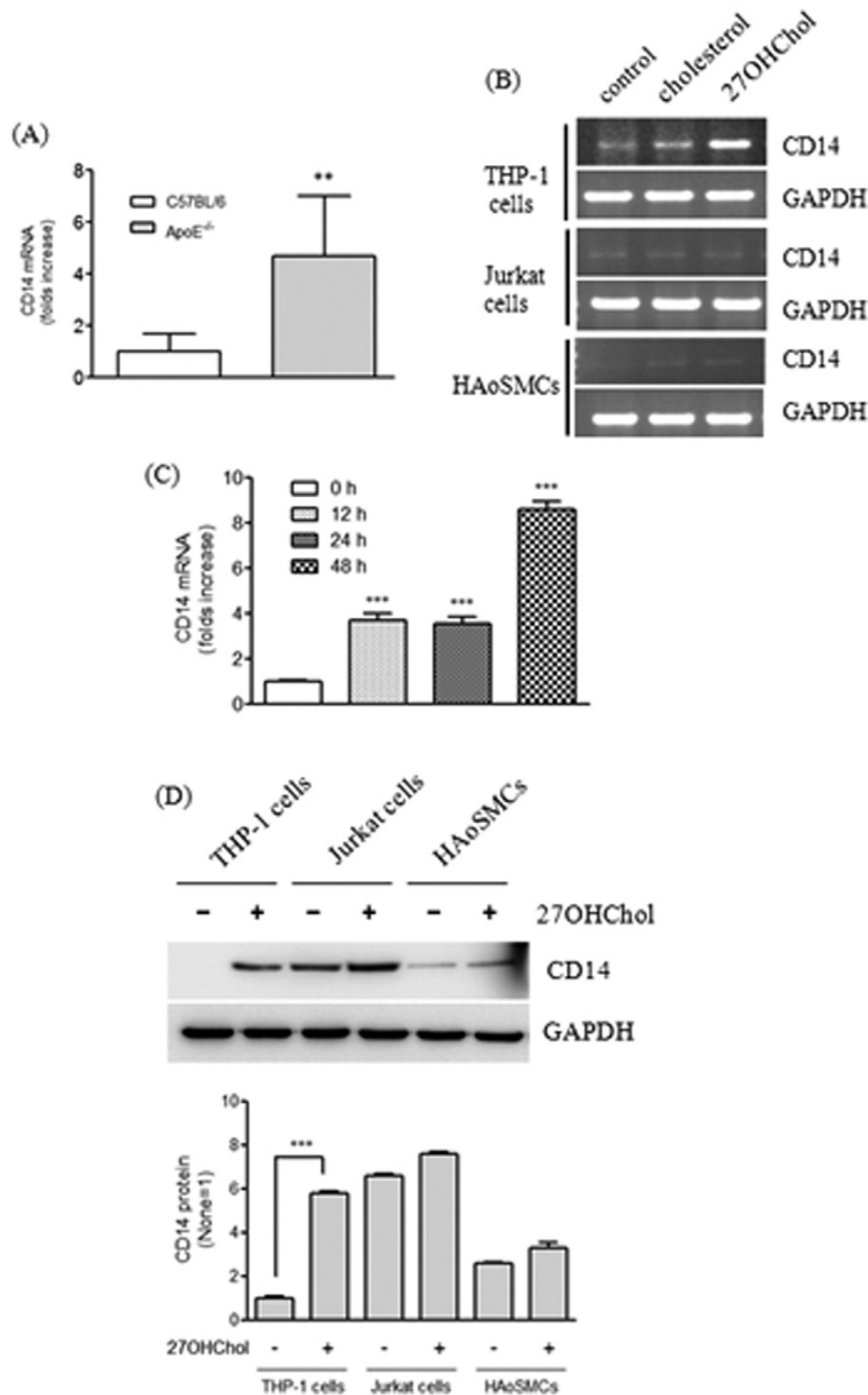

Fig. 1. Increased expression of the CD14 in a cholesterol oxide-rich milieu. (A) The levels of CD14 transcripts were assessed by real-time PCR using total RNA extracted from the aortas of wild-type C57BL/6 mice and ApoE$^{-/-}$ mice after a high-cholesterol diet for 12 weeks. The *y*-axis values represent fold increases of CD14 mRNA levels normalized to GAPDH levels relative to those of the C57BL/6 mice. Data are expressed as mean $\pm$ SE ($n = 6$ individual mice for each group). $^{**}P < 0.01$ vs. C57BL/6. (B) THP-1 cells, Jurkat T cells, and HAoSMCs were serum starved for 24 h and incubated for 48 h in the absence or presence of cholesterol (5 μg/ml) or 27OHChol (2.5 μg/ml). Total RNA was isolated from the cells, and CD14 transcripts were amplified by RT-PCR. (C) THP-1 cells (2.5×10^6 cells/100 mm culture dish) were serum starved for 24 h in 10 ml RPMI 1640 containing 0.1% BSA (endotoxin free), followed by incubation with 27OHChol (2.5 μg/ml) for the indicated time periods. The levels of CD14 transcripts were assessed by real-time PCR. The *y*-axis values represent fold increases of CD14 mRNA levels normalized to GAPDH levels relative to those of THP-1 cells incubated in medium alone (0 h). Data are expressed as mean $\pm$ SD ($n = 3$ replicates for each group). $^{***}P < 0.001$ vs. control. (D) THP-1 cells, Jurkat T cells, and HAoSMCs were serum starved for 24 h and incubated for 48 h in the absence or presence of 27OHChol (2.5 μg/ml). CD14 protein was detected by Western blot analysis.

transcripts were observed in ApoE$^{-/-}$, as determined by real-time PCR (Fig. 1A). In addition, fluorescence immunostaining showed that the immunoreactivity of CD14 was also enhanced in atherosclerotic lesions, as examined using aortic roots of ApoE$^{-/-}$ and wild-type mice (Supplementary Fig. 2). These results indicate up-regulation of CD14 in the arteries of ApoE$^{-/-}$ mice.

3.2. Up-regulated expression of CD14 in monocytic cells by 27OHChol

We attempted to determine the lipids responsible for the expression of CD14 in the atherosclerotic artery. Because cholesterol and 27OHChol are the two most abundant lipids in atherosclerotic lesions, we attempted to determine whether they had an effect on levels

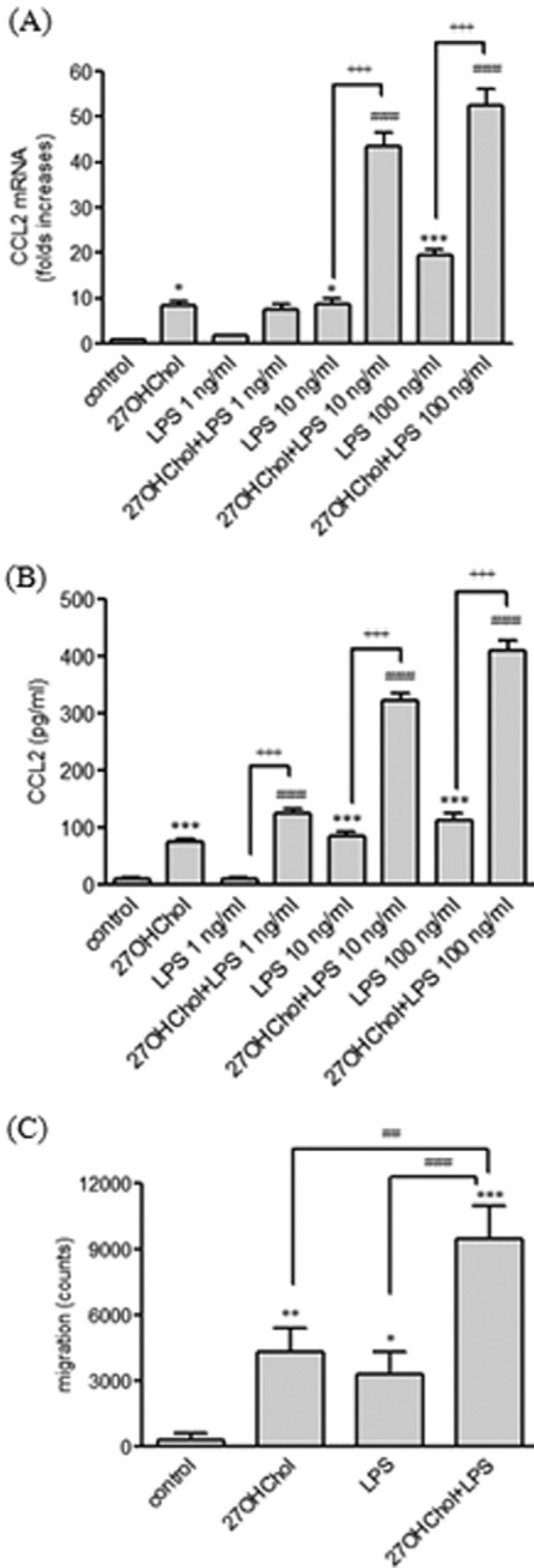

Fig. 2. Enhanced expression of CCL2 in response to LPS in the presence of 27OHChol. (A, B) Serum-starved THP-1 cells were incubated for 24 h in the absence or presence of 27OHChol (2.5 μg/ml) and stimulated for 9 h with or without the indicated amount of LPS. (A) The levels of CCL2 transcripts were assessed by real-time PCR. The *y*-axis values represent fold increases of CCL2 mRNA levels normalized to GAPDH levels relative to those of control THP-1 cells incubated in medium alone. Data are expressed as mean ± SD ($n = 3$ replicates for each group). $^{*}P < 0.05$ vs. control; $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol; $^{+++}P < 0.001$ vs. corresponding LPS. (B) The amount of CCL2 secreted into culture media was measured by ELISA. Data are expressed as mean ± SD ($n = 3$ replicates for each group). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol; $^{+++}P < 0.001$ vs. corresponding LPS. (C) Monocytic cells were exposed to conditioned media isolated from THP-1 cells treated with 27OHChol, LPS, or 27OHChol plus LPS, as described above. Migration of monocytic cells in response to the conditioned media was determined by chemotaxis assays. Data represent the mean ± SD of migrated cells counted from three individual samples for each group. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control; $^{\#\#}P < 0.01$ vs. 27OHChol; $^{\#\#\#}P < 0.001$ vs. LPS.

of CD14 in vascular cells. HAoSMCs, THP-1 monocytic cells, and Jurkat T cells were treated with cholesterol and 27OHChol, prior to examination of CD14 (Fig. 1B). CD14 transcripts were weakly detected from THP-1 cells, HAoSMCs, and Jurkat T cells, and the markedly elevated transcription of CD14 was observed in THP-1 cells in the presence of 27OHChol (Fig. 1C). However, a higher concentration of cholesterol did not affect CD14 expression in the three types of cells (Fig. 1B). We investigated whether 27OHChol influenced the expression of CD14 protein. Treatment with 27OHChol led to increased level of CD14 protein mainly in THP-1 cells in comparison with Jurkat cells or HAoSMCs (Fig. 1D). These results indicate that the oxidized form of cholesterol, rather than cholesterol, induces the expression of CD14 in monocytic cells.

3.3. Superproduction of CCL2 by monocytic cells in response to LPS in the presence of 27OHChol

CD14 binds LPS, and LPS-CD14 complexes elicit production of chemokines by the activation of monocytes and macrophages via TLR4 [19]. Since 27OHChol induced the up-regulated expression of CD14, we attempted to determine whether response to LPS was influenced by the presence of 27OHChol. THP-1 cells were treated with cholesterol or 27OHChol, followed by addition of LPS, and the expression of CCL2 was then analyzed by real-time PCR and ELISA (Fig. 2). LPS induced the transcription of CCL2 in THP-1 cells in a concentration-dependent manner. The levels of CCL2 transcripts increased by 1.7-, 8.7-, and 19.5-fold in the presence of 1, 10, and 100 ng/ml of LPS, respectively, in comparison with control. 27OHChol alone increased the level of CCL2 transcripts by 8.3-fold. The addition of LPS to 27OHChol-treated cells resulted in a further increase in induction of CCL2 transcription; CCL2 transcripts were elevated by 43.5-fold and 52.6-fold with the addition of LPS at a final concentration of 10 and 100 ng/ml to 27OHCHol-treated cells, respectively (Fig. 2A. Please see Supplementary Fig. 3 for RT-PCR). 27OHChol and LPS affected the secretion of CCL2 in a pattern similar to those observed with the transcription of the gene. LPS enhanced CCL2 secretion in a concentration-dependent manner in THP-1 cells. 27OHChol also induced the secretion of CCL2, and the addition of LPS to 27OHChol-treated cells resulted in further enhanced the secretion of CCL2 (Fig. 2B). In contrast, the addition of LPS to THP-1 cells incubated with cholesterol did not enhance induced the expression of CCL2 (Supplementary Fig. 4).

We also investigated whether 27OHChol affected the expression of TLR4 because LPS-CD14 complex induces inflammatory responses via the receptor [19,20]. Treatment with 27OHChol or cholesterol did not influence the expression of TLR4, as determined by RT-PCR and Western blot analysis (Supplementary Fig. 5).

In order to determine whether secreted CCL2 was functional, we performed a chemotaxis assay, as previously described [18]. The significant migration of monocytic cells was induced in response to the supernatant containing CCL2 which was isolated after stimulation of THP-1 cells with 27OHChol or LPS. The migration was further induced in

response to the conditioned media obtained after stimulation of THP-1 cells with LPS in the presence of 27OHChol (Fig. 2C).

3.4. Enhanced secretion of sCD14 in the presence of 27OHChol

CD14 is found in two forms; a membrane-associated form (mCD14) and a soluble form (sCD14) [21]. We attempted to determine whether 27OHChol affected release of CD14. THP-1 cells released a low amount of soluble CD14 and the amount of released soluble CD14 was elevated in proportion to duration of treatment with 27OHChol (Fig. 3A). The amount of sCD14 detected in the media was 29.2 ± 10.5 pg/ml when cells were not stimulated, and it increased to 76.8 ± 19.6 pg/ml, 270.5 ± l31.0 pg/ml, and 1035.4 ± 126.8 pg/ml after treatment with 27OHChol for 12 h, 24 h, and 48 h, respectively. Cell surface CD14 binds LPS and initiates response to LPS [21]. Because LPS response was enhanced in the presence of 27OHChol, we attempted to determine whether 27OHChol also influenced levels of CD14 protein on the surface. Results of flow cytometric analyses showed an increased level of CD14 on the cell surface in the presence of 27OHChol. The percentage of control THP-1 cells that expressed a high level of CD14 was 0.5%, which increased to 13.8% in the presence of 27OHChol (Fig. 3B).

The formation of sCD14 can occur after cleavage of mCD14 by protease like MMP-9 [22]. Therefore, we investigated the question of whether MMP-9 participated in 27OHChol-induced the secretion of sCD14. First, we investigated the effects of 27OHChol on the expression of MMP-9. We measured the expression of MMP-9 via quantitative assays. MMP-9 expression was induced at 12 h after the treatment of monocytic cells with 27OHChol and sustained up to 48 h after the treatment (Supplementary Fig. 6). In accordance with the real-time PCR data, the treatment of monocytic cells with 27OHCHol resulted in significantly increased the secretion of MMP-9, as determined by ELISA, in proportion to the duration of treatment (Fig. 3C). The activity of MMPs secreted by THP-1 cells was analyzed by gelatin zymography using the supernatant of smooth muscle cells as a positive control. Both MMP-2 as well as MMP-9 gelatinolytic activity was detected in the supernatant of smooth muscle cells. The treatment of THP-1 cells with 27OHChol resulted in increased MMP-9 activities in supernatants in a time-dependent manner up to 48 h post-treatment (Fig. 3D). The addition of MMP-9 inhibitor I, a cell permeable, selective, reversible inhibitor of MMP-9, to 27OHCHol-treated monocytic cells resulted in significantly attenuated the secretion of sCD14 (Fig. 3E). The MMP-9 inhibitor I, however, did not influence the expression of tumor necrosis factor-α (TNF-α) or CD88 whose expression was reported to be induced in the presence of 27OHChol [18,23] (Supplementary Fig. 7). These results indicate that MMP-9 is involved in 27OHChol-induced the secretion of sCD14 from monocytic cells.

For the investigation of the effects of oxLDL on CD14 expression, THP-1 cells were treated with oxLDL in parallel with 27OHChol, and the levels of CD14 transcripts and secretion of sCD14 were determined by real-time PCR and ELISA, respectively (Fig. 3F and Supplementary Fig. 8). 27OHChol induced the significant transcription of the CD14 gene as well as release of sCD14, as described above. OxLDL also affected CD14 expression primarily at the protein level. OxLDL induced the slight elevation of CD14 transcription by 1.8-fold but significantly enhanced sCD14 release, although to a lesser extent than 27OHChol, from THP-1 cells.

3.5. Differential regulation of CD14 expression by PI3K and ERK pathways

We previously reported that 27OHChol activated both phosphoinositide-3-kinase (PI3K)/Akt and extracellular-signal-regulated kinase (ERK) pathways [17]. Therefore, we investigated the role of each signaling pathway in the expression of CD14 using pharmacological inhibitors. The inhibition of the ERK pathway using U0126 resulted in the attenuated transcription of CD14 (Fig. 4A) and near abrogation of sCD14 secretion induced by 27OHChol (Fig. 4B). In the mean time, LY294002, an inhibitor of the PI3K pathway, did not influence the transcription of the CD14 gene (Fig. 4A) but significantly inhibited the secretion of sCD14 (Fig. 4B), which indicated that the PI3K pathway regulated the secretion of sCD14. Therefore, we investigated the effects of LY294002 on MMP-9 expression in parallel with U0126. Both LY294002 and U0126 almost completely inhibited the transcription of MMP-9 and secretion of its gene product induced by 27OHChol (Fig. 4C). The inhibition was not due to cytotoxicity of the inhibitors because an addition of LY294002 or U0126 did not reduce viability of 27OHchol-treated THP-1 cells (Supplementary Fig. 9). Collectively, these results showed that LY294002 inhibited the secretion of sCD14 by blocking MMP-9 expression rather than by affecting the expression of the CD14 gene.

4. Discussion

ApoE$^{-/-}$ mice are a popular animal model for examining the *in vivo* influence of cholesterol on gene expression in conjunction with pathogenesis of atherosclerotic lesions. The consumption of a high-cholesterol diet leads to atherosclerosis throughout the arterial tree of ApoE$^{-/-}$ mice with accumulation of cholesterol, which undergoes oxidative modifications [24–26]. Using this animal model, we observed significantly increased the transcription of the CD14 gene in the arteries of ApoE$^{-/-}$ mice in comparison with wild-type mice after a high-cholesterol diet. These results indicate that a high-cholesterol milieu is sufficient for induction of CD14 expression in the arteries.

Atherosclerotic lesions are heterogeneous not only in the cellular composition but also in lipid composition. The most abundant lipid in atherosclerotic lesions is cholesterol, followed by 27OHChol [24]. We found that 27OHChol, but not cholesterol, induced the transcription of CD14. The effect of 27OHChol appears to be cell-type specific because up-regulation of CD14 occurred in macrophages, but not in vascular smooth muscle cells (VSMCs) and T cells. These results are in line with those of the previous study by Waldo et al. [16], who reported that CD14-positive cells were predominantly macrophages within atherosclerotic lesions. Taken together, our results indicate that oxidative-modified cholesterol derivatives, like 27OHChol, will induce the expression of CD14 in macrophages within atherosclerotic lesions.

We observed an exaggerated response of monocytic cells to LPS in the presence of 27OHChol, as determined by CCL2 expression. 27OHChol and LPS appeared to synergistically induce CCL2 expression, as the levels of transcription as well as the secretion of CCL2 induced by treatment with 27OHChol plus LPS were much greater than summation of the levels observed with each treatment alone. These results are in agreement with those of the previous report in that CD14 binds LPS and activates cells by subsequent transfer of bound LPS to TLR4 and that activated cells express various cytokines and chemokines, including CCL2 [20,27–29]. Because CCL2 plays a major roles in recruitment of monocytes into atherosclerotic lesions [6,29], we investigated the chemotactic activity of the secreted CCL2. The migration of THP-1 monocytic cells was also further induced in response to conditioned media containing CCL2 secreted from 27OHChol-treated monocytic cells in response to LPS. Taken together, our results indicate that 27OHChol induces up-regulation of CD14, which will bring about an accelerated inflammatory response to LPS in atherosclerotic lesions.

GPI-anchored mCD14 is released by shedding in response to various stimuli, probably mediated by proteases such as leukocyte elastase and metalloproteinases [22,30]. Surfactant protein D has been reported to increase sCD14 in a process mediated by MMP-9 and MMP-12 in mouse macrophages [22]. We observed a substantial reduction in 27OHChol-induced the secretion of sCD14 by inhibition of MMP-9, indicating that 27OHChol can induce the secretion of sCD14 from monocytic cells via MMP-9-dependent shedding. CD14 can also be secreted as a soluble molecule via different mechanisms [31]. CD14 molecules stored

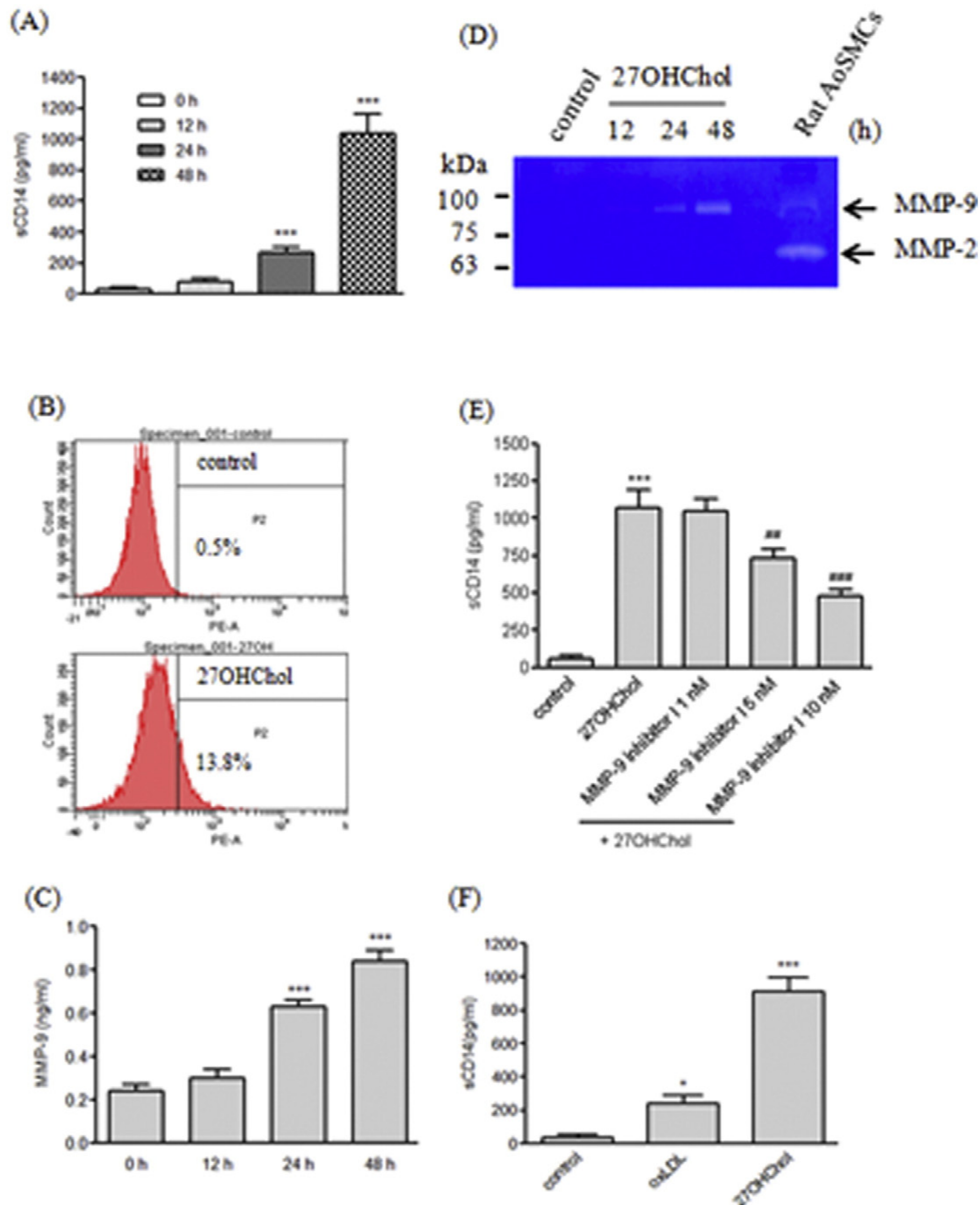

Fig. 3. Effects of 27OHChol and oxLDL on production of sCD14. (A) Serum-starved THP-1 cells were incubated with 27OHChol (2.5 μg/ml) for the indicated time periods, and the amount of sCD14 secreted into culture media was measured by ELISA. Data are expressed as mean ± SD ($n = 3$ replicates for each group). ***$P < 0.001$ vs. control. (B) Serum-starved THP-1 cells were incubated in the absence or presence of 27OHChol, followed by immunostaining of THP-1 cells for cell surface CD14. Flow cytometry was performed for analysis of fluorescence. (C) Serum-starved THP-1 cells were incubated with 27OHChol (2.5 μg/ml) for the indicated time periods, and the amount of total MMP-9 (pro- and active) secreted into culture media was measured by ELISA. Data are expressed as mean ± SD ($n = 3$ replicates for each group). ***$P < 0.001$ vs. control. (D) Serum-starved THP-1 cells were incubated with 27OHChol (2.5 μg/ml) for the indicated time periods. The activity of MMP-9 secreted by the cells was assessed by zymography. Control THP-1 cells were incubated for 48 h in medium alone, and rat AoSMCs were treated with platelet-derived growth factor (5 ng/ml) for 12 h. (E) Serum-starved THP-1 cells were incubated for 24 h in the absence or presence of 27OHChol, followed by incubation for 24 h with or without MMP-9 inhibitor I (10 nM). The amount of sCD14 secreted into culture media was measured by ELISA. Data are expressed as mean ± SD ($n = 3$ replicates for each group). ***$P < 0.001$ vs. control. ##$P < 0.01$ vs. 27OHChol. ###$P < 0.001$ vs. 27OHChol. (F) Serum-starved THP-1 cells were incubated with 27OHChol (2.5 μg/ml) or oxLDL (50 μg/ml) for 48 h, and the amount of sCD14 secreted into culture media was measured by ELISA. Data are expressed as mean ± SD ($n = 3$ replicates for each group). *$P < 0.05$ vs. control. ***$P < 0.001$ vs. control.

intracellularly can escape GPI anchor attachment and can be secreted with the C-terminal leader sequence [31]. Therefore, the possibility that 27OHChol might influence direct secretion from intracellular vesicles should not be excluded.

We attempted to identify signaling pathways involved in CD14 production induced by 27OHChol. PI3K and ERK pathways were selected because involvement of both signaling pathways in action of 27OHChol as well as oxysterol mixture has been reported [17,18,32]. We found that the ERK and PI3K signaling pathways have both common and distinct roles in 27OHChol-induced up-regulation of CD14. The PI3K pathway is required for the secretion of sCD14, and the ERK pathway is required for the transcription of CD14 as well as the secretion of

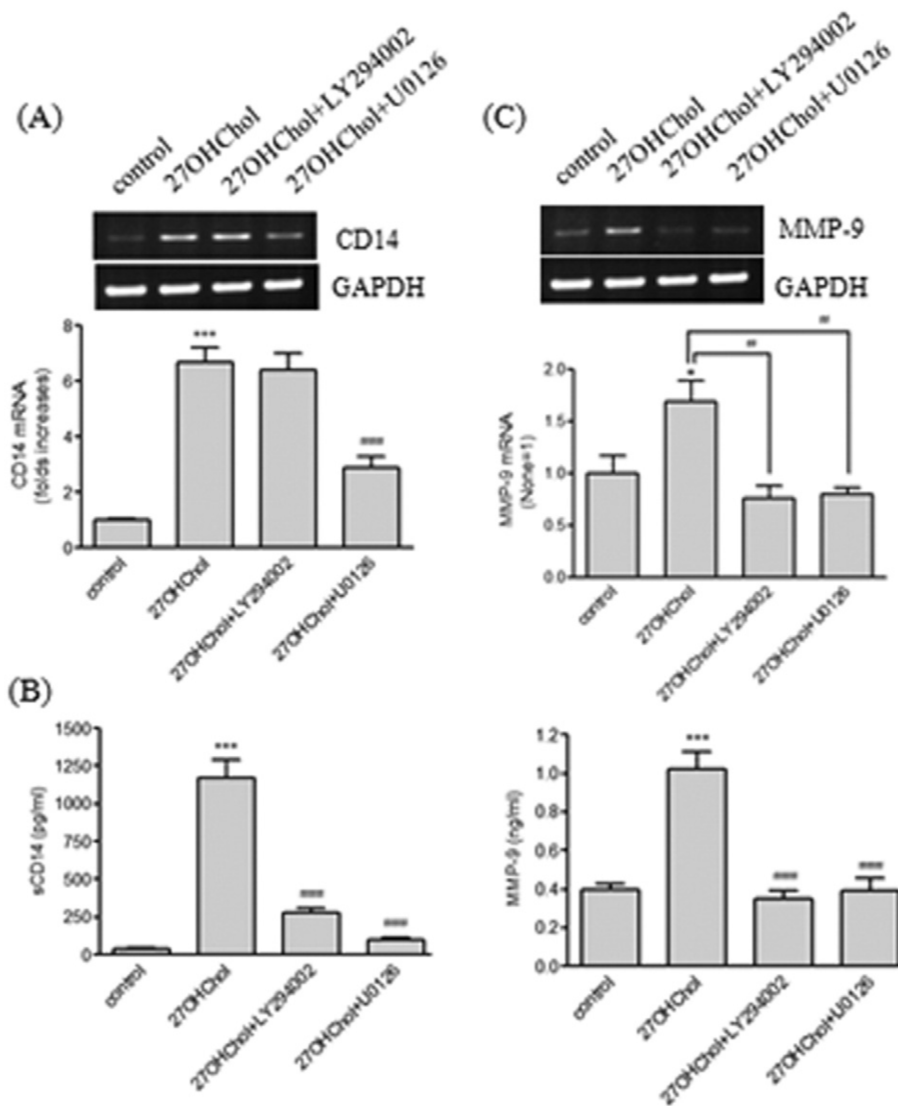

Fig. 4. Effects of inhibitors of the PI3K and ERK pathways on the expression of CD14 and MMP-9. (A–C) Serum-starved THP-1 cells were treated with 27OHChol for 48 h after pre-incubation for 2 h in the absence or presence of the indicated inhibitors (10 μM each). (A) CD14 transcripts were amplified by RT-PCR (upper panel), and the levels of CD14 transcripts were assessed by real-time PCR. The *y*-axis values represent fold increases of CD14 mRNA levels normalized to GAPDH levels relative to those of control THP-1 cells incubated in culture medium alone. (lower panel). Data are expressed as mean ± SD ($n = 3$ replicates for each group). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol. (B) The amount of sCD14 secreted into culture media was measured by ELISA. Data are expressed as mean ± SD ($n = 3$ replicates for each group). $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. 27OHChol. (C) MMP-9 transcripts were amplified by RT-PCR (upper panel), and the bands on agarose gels were quantified by using Un-Scan-It Gel (middle panel). The amount of MMP-9 secreted into medium was measured by ELISA (lower panel). Data are expressed as mean ± SD ($n = 3$ replicates for each group). $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. 27OHChol.

sCD14. These results suggest a major role ERK pathway in enhanced the transcription of CD14 and a complex control of sCD14 secretion in the presence of 27OHChol. The current study, however, did not attempt to determine whether PI3K and ERK acted in an independent or cooperative manner. Additional study is necessary in order to elucidate the types of connections or crosstalk that may be occurring between the two pathways in the presence of 27OHChol.

Plaque oxysterols have been reported to induce the expression of MMP-9 in promonocytic cells [18,32]. However, the underlying mechanisms for the 27OHChol-induced expression of MMP-9 remain unknown. In the current study, we provided molecular mechanisms for induction of MMP-9 expression by 27OHChol in monocytic cells. We found that the ERK pathway was required in 27OHChol-mediated production of MMP-9, in line with previous studies that reported participation of this pathway in MMP-9 expression induced by oxysterol mixture and oxLDL [32,33]. In addition, we found that the extent of inhibition of transcription and the secretion of MMP-9 by treatment with LY294002 was comparable to those observed by U0126. These results indicate that the PI3K pathway is also essential for induction of MMP-9 expression. Collectively, our data indicate that both the ERK and PI3K pathways should be active for induction of MMP-9 expression by 27OHChol, thereby leading to sCD14 secretion, in monocytic cells.

OxLDL, a complex mixture of various components composed of lipid hydroperoxide, aldehyde, and cholesterol oxides [34,35], exhibits multiple effects. OxLDL stimulates the expression of intercellular adhesion molecule-1 (ICAM-1) by human umbilical vein endothelial cells (HUVEC) and increases leukocyte-endothelial cell adhesion [36,37]. Therefore, the oxidation of accumulated LDL has been suggested to promote inflammation. In the current study, we found a new effect of oxLDL on vascular cells; it induced release of sCD14 from monocytic cells. Of the two lipids examined, 27OHChol was more efficient than oxLDL in induction of sCD14 release. We think that this observation may be a result of lipid composition of oxLDL. OxLDL used in this study was Cu^{2+}-oxidized LDL, which predominantly contains sterols oxygenated in the 7-position in the order of 7-ketocholesterol, 7β-hydroxycholesterol, and 7α-hydroxycholesterol, rather than 27OHChol

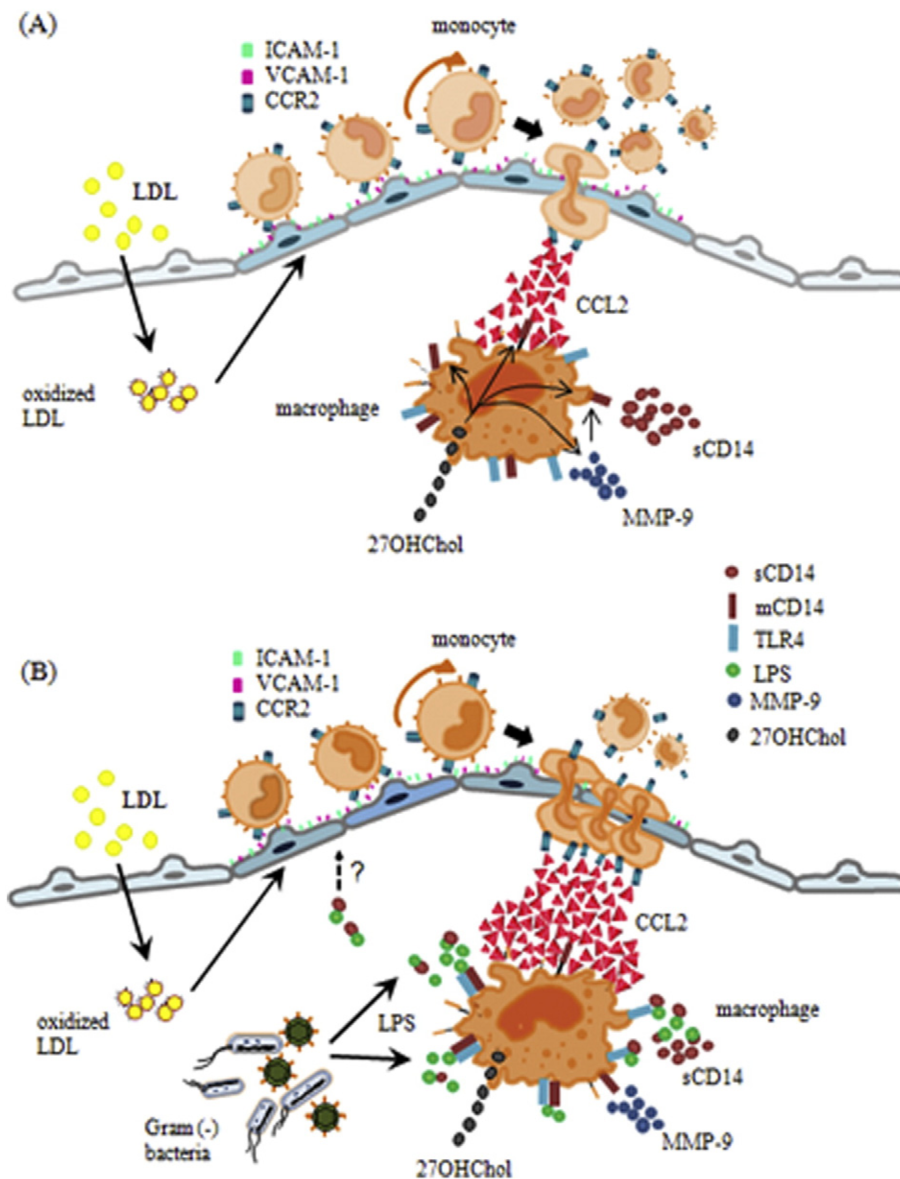

Fig. 5. Enhanced inflammatory response to LPS in the presence of 27OHCHol. (A) 27OHChol promotes the secretion of CCL2 and MMP-9 from macrophages. Secreted CCL2 participates in recruitment of monocytes and MMP-9 contributes to proteolytic shedding of CD14 whose expression was also up-regulated by 27OHChol. Therefore, exposure of macrophages to 27OHChol leads to a condition with elevated levels of mCD14 and sCD14 on and around macrophages, respectively. (B) The elevated levels of CD14 allow formation of more LPS-CD14 complexes and transfer of more LPS to TLR4, which further activates macrophages such that substantially high amounts of CCL2 are secreted, thereby enhancing migration of more monocytes and aggravating inflammation in atherosclerotic lesions.

[34,35]. These findings suggest that these 7-oxygenate cholesterols of oxLDL can also induce the secretion of sCD14. Currently, the question of whether and how the aforementioned 7-oxygenate cholesterols influence the secretion of sCD14 is under investigation.

CD14 is a pattern recognition receptor with broad ligand specificity. In addition to LPS, CD14 recognizes diverse structures from invading microbes, such as lipoteichoic acid (LTA) from Gram-positive bacteria [38], lipoarabinomannan (LAM) from mycobacteria [39], and viral double-stranded (ds) RNA [40]. Therefore, it is possible that the elevated expression of CD14 induced by 27OHChol may influence inflammatory responses to diverse pathogens detected in atherosclerotic lesions. Based on our results and previous findings, we propose a model for accelerated inflammation by 27OHChol and LPS in atherosclerotic lesions (Fig. 5). Macrophages exposed to 27OHChol secrete CCL2 and MMP-9 and show an elevated level of mCD14. CCL2 will direct the migration of monocytes attached on endothelium which express cell adhesion molecules induced by oxLDL, and MMP-9 will enhance the secretion of sCD14 by conversion of mCD14 to sCD14 (Fig. 5A). Elevated levels of mCD14 and sCD14 result in enhanced delivery of LPS to TLR4 after infection with Gram-negative bacteria, resulting in superinduction of CCL2 expression and enhanced migration of monocytes (Fig. 5B). Since sCD14 induces responses of CD14-negative endothelial cells as well as CD14-positive macrophages to LPS [27,41], secreted sCD14 may induce activation of endothelial cells in response to Gram-negative bacteria in the atherosclerotic lesions.

Conflict of interest

Authors do not have any conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.12.003.

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