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25-Hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol upregulate interleukin-8 expression independently of Toll-like receptor 1, 2, 4 or 6 signalling in human macrophages

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Abstract

Recent studies have shown that Toll-like receptor (TLR)- signalling contributes significantly to the inflammatory events of atherosclerosis. As products of cholesterol oxidation (oxysterols) accumulate within atherosclerotic plaque and have been proposed to contribute to inflammatory signalling in the diseased artery, we investigated the potential of 7-ketocholesterol (7-KC), 7β-hydroxycholesterol (7β-HC) and 25-hydroxycholesterol (25-HC) to stimulate inflammatory signalling via the lipid-recognising TLRs 1, 2, 4 and 6. Each oxysterol stimulated secretion of the inflammatory chemokine interleukin-8 (IL-8), but not IκBα degradation or tumour necrosis factor-α release from monocytic THP-1 cells. Transfection of TLR-deficient HEK-293 cells with TLRs 1, 2, 4 or 6 did not increase sensitivity to the tested oxysterols. Moreover, blockade of TLR2 or TLR4 with specific inhibitors did not reduce 25-hydroxycholesterol (25-HC) induced IL-8 release from THP-1 cells. We conclude that although the oxysterols examined in this study may contribute to increased expression of certain inflammatory genes, this occurs by mechanisms independent of TLR signalling.

Keywords: *Oxysterols, inflammation, atherosclerosis, Toll-like receptors*

Introduction

Atherosclerosis is a chronic inflammatory disease of the arteries, characterised by the recruitment of circulating monocytes into the vessel wall and the resultant formation of cholesterol (Chol)-laden foam cells [1,2]. The identification of the agents and receptors responsible for the initiation and perpetuation of this chronic inflammatory state remains a major focus of current studies. Recent progress in addressing this issue has emerged following the discovery that a family of innate immune system receptors, the Toll-like receptors (TLRs), plays a key role in the inflammatory processes of atherosclerosis [3–5].

The TLRs are a family of type 1 transmembrane pattern-recognition receptors that serve to initiate inflammatory signalling by the detection of conserved pathogen associated molecular patterns (PAMPs) or products of host tissue damage [6]. Recent genetic studies in atherosclerosis-prone Apolipoprotein-E deficient mice have revealed a major role for TLR signalling in the development of atherosclerosis [3–5]. Genetic deletion of a signalling adaptor shared by all of the 10 human TLRs, myeloid-differentiation factor 88, results in a reduction in plaque burden of around 60% [3,4], while deletion of TLR2 or TLR4 alone also leads to a significant reduction in plaque formation [4,5].

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There is also evidence that TLR-signalling may contribute to inflammatory processes in human atherosclerosis [7,8]. Immunohistochemical studies have revealed that while healthy human artery expresses only TLR4, TLRs 1, 2, 4 and 6 are highly expressed in human atheroma [9]. Accordingly, certain markers of TLR-signalling are observed in human atheroma but not healthy artery, such as secretion of the cytokine tumour necrosis factor (TNF)-α [10] and activation of the pro-inflammatory transcription factor NF-κB [11]. Notably, expression of the activated form of NF-κB in plaque appears to co-localise with TLR-expressing cells, leading to the suggestion that these cells may be responding to bound ligand [9].

The ligands responsible for TLR stimulation during atherogenesis remain to be identified, though it is interesting to note that the TLRs expressed in the diseased artery, types 1, 2, 4 and 6, are those responsible for the detection of foreign lipids. Thus, it has been proposed recently that the ligands involved are likely to be of lipid origin and that oxidatively modified lipids, which are present in atheroma [12–14] and which can induce expression of certain inflammatory gene products [15–18] may represent the most likely contributors to the stimulation of TLRs observed in atherosclerosis [17,19,20].

Among potential lipid-ligands that may play a role in these processes, oxidatively modified forms of cholesterol (oxysterols) have gained consideration recently in terms of their ability to stimulate inflammatory signalling [15,16,21–23]. Oxysterols accumulate within atherosclerotic plaque [13] and may be formed enzymatically, such as 24- 25- or 27-HC which perform natural roles as regulators of signalling and Chol metabolism [24], or as a result of auto-oxidation of Chol [25], such as the formation of 7β-hydroxycholesterol (7β-HC) and 7-ketocholesterol (7-KC) during the oxidation of the low-density lipoprotein (LDL) particle [26]. While certain oxysterols are understood to induce expression of inflammatory genes, such as interleukin 8 (IL-8) [15,16] and monocyte chemoattractant protein 1 (MCP-1) [21], little is known of the signalling pathways employed by oxysterols to induce expression of these chemokines.

We therefore investigated the possibility that oxysterols may induce inflammatory signalling via stimulation of the lipid-specific TLR-subtypes 1, 2, 4 and 6, which are involved in the IL-8 response induced by bacterial lipids and lipopeptides. We chose to focus on the responses of macrophages, which are key regulators of inflammatory events within the plaque [1], to 7β-HC and 7-KC, since these are two of the most abundant oxysterols derived from Chol auto-oxidation in the diseased vessel wall [13] and also to 25-hydroxycholesterol (25-HC), as this has been shown to be the most potent oxysterol inducer of inflammatory gene expression in previous studies [15,16,23].

Materials and methods

Reagents

Cholesterol and the oxysterols 7-KC, 7-βHC and 25-HC were purchased from Sigma (Poole, UK), resuspended at 5 mg/ml in ethanol and stored at −20°C prior to use. Phorbol myristic acetate (PMA) and *E. coli* LPS were also from Sigma. *E. coli* LPS was further repurified after purchase to remove TLR2-stimulating lipopeptide based contaminants by phenol-water re-extraction as described previously [27]. The synthetic bacterial lipopeptide Pam_3CSK_4 was from Invivogen (San Diego, USA). Limulus amoebocyte lysate assays confirmed that non-LPS reagents contained less than 30 pg/ml endotoxin as used.

THP-1 cell culture and challenge

Human monocytic THP-1 cells were maintained in RPMI-1640 (Sigma) supplemented with 10% foetal calf serum (FCS), and plated at 5×10^4 cells per well in 96-well plates or 4×10^5 cells per well in 12-well plates in the presence of 0.1 μM dihydroxyvitamin-D3 (Sigma) for 72 h for differentiation to macrophage form prior to challenge. Cells were challenged in triplicate with medium alone, 10 μg/ml of Chol or each oxysterol, 1 ng/ml of *E. coli* LPS, 10 ng/ml of Pam_3CSK_4 or 50 ng/ml PMA. Supernatant TNF-α and IL-8 released from stimulated THP-1 cells was measured at 4 h or 18 h respectively, by ELISA (R&D, Abingdon, UK).

Western blot for determination of IκBα degradation

Dihydroxyvitamin-D3 differentiated THP-1 cells plated in 12-well plates were challenged for 20 min with medium alone, 10 μg/ml of Chol, 7β-HC, 7-KC, or 25-HC, 1 ng/ml LPS or 10 ng/ml of Pam_3CSK_4. Cells were then lysed on ice and 10 μg of protein from each condition was separated by SDS-PAGE and blotted to nitrocellulose at 100 V for 1 h in the presence of 20% methanol. Blots were then blocked with 2% bovine serum albumin for 1 h, probed with antibody to IκBα (Santa-Cruz, Heidelberg, Germany, sc371, 1:5000 dilution) or glyceraldehyde-phosphate-dehydrogenase (Santa-Cruz, sc25778, 1:10,000 dilution) and developed with anti-rabbit secondary conjugated to horse-radish peroxidase (Santa-Cruz, sc2030, 1:10,000 dilution) and enhanced chemiluminescence kit (Pierce, Rockford, USA).

Transfection experiments

Human embryonic kidney (HEK)-293 cells were maintained in DMEM (Sigma)/10% FCS and plated at 2×10^4 cells per well of 96-well plates for transfection experiments. Cells were then transfected with 10 ng of NF-κB sensitive promoter (pELAM) or

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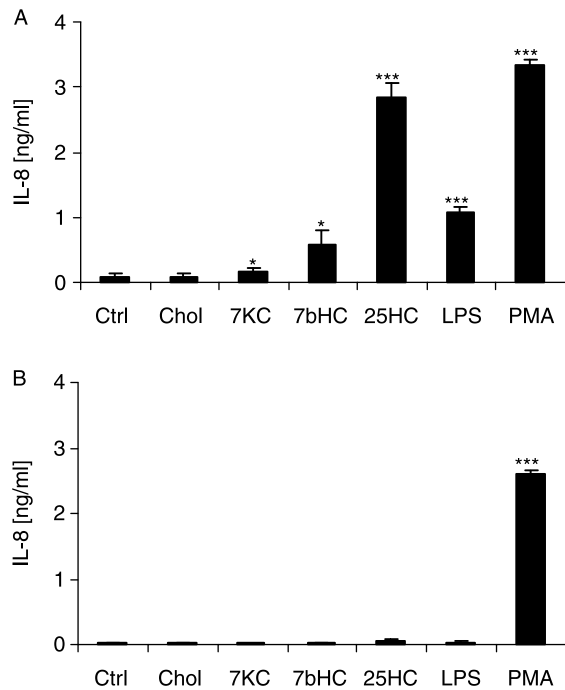

Figure 1. Oxysterols upregulate IL-8 secretion from THP-1 but not HEK-293 cells. Human monocytic THP-1 cells were differentiated for 72 h using 0.1 μM di-hydroxy vitamin D3. Medium was then replaced with fresh medium alone (Ctrl), 10 μg/ml of Chol or each of the oxysterols indicated (7KC; 7β-HC; 25HC), 1 ng/ml LPS or 50 ng/ml PMA. Supernatant IL-8 was measured by ELISA at 18 h (A). HEK-293 cells, which lack TLR-expression, were challenged in the same manner (B). Results shown are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. cells cultured in medium alone.

full-length IL-8 promoter (pIL8) driven reporter constructs, 20 ng of a thymidine-kinase promoter driven renilla expression vector included as an internal transfection efficiency control, 30 ng of plasmid expressing CD14 (kind gift of Professor C. Gregory, University of Edinburgh) and 30 ng each of plasmids expressing TLRs 1, 2, 6, or a plasmid expressing both TLR4 and MD2 (Invivogen) using Genejuice (Merck, Darmstadt, Germany) according to manufacturers instructions. 48 h after transfection, medium was replaced with fresh medium alone (DMEM/10% FCS), or medium containing 10 μg/ml of Chol, 7β-HC, 7-KC or 25-HC, 1 ng/ml LPS or 10 ng/ml Pam_3CSK_4 for a further 18 h. Cells were then lysed and reporter gene expression measured using a Dual-Glo reporter measurement kit (Promega, Southampton, UK). Induction of pELAM or pIL8 was normalised to expression of internal transfection efficiency control pRL-TK and results are presented as fold induction of promoter relative to cells cultured in medium alone.

Inhibition experiments

The synthetic tetra-acyl lipid-A precursor lipid-IVa was purchased from Peptides International (Louisville, USA). The monoclonal anibody to TLR2 clone TL2.5 was from Hycult Biotechnology (Uden, Netherlands). Differentiated THP-1 cells were challenged in triplicate in medium alone, 100 ng/ml of lipid-IVa or 50 μg/ml of anti-TLR2 antibody for 30 min prior to challenge by supplementation of medium with a final concentration of 10 μg/ml of 25-HC or 0.4 ng/ml LPS or 1 ng/ml Pam_3CSK_4. IL-8 was measured at 18 h by ELISA.

Statistical analysis

Results are presented as mean ± SD of triplicate measurements and are representative of at least three similar experiments. Differences were analysed by unpaired two-tailed Student's *t*-test and deemed significant at $p < 0.05$.

Results

Oxysterols induce IL-8 release from macrophages but not epithelial HEK-293 cells

THP-1 cell derived macrophages were challenged with 10 μg/ml of each oxysterol as this concentration was shown in previous studies to induce maximal IL-8 production from macrophages [15,16]. We confirmed that the examined oxysterols increase expression of IL-8 from THP-1 derived macrophages (Figure 1(A)) [15,16]. Consistent with these previous studies, 25-HC resulted in the greatest IL-8 release, while 7-KC consistently, although only very modestly, induced a significant increase in IL-8 production over cells cultured in medium alone (~2–3×). However we found that epithelial HEK-293 cells, which do not express TLRs [28], were much less sensitive to oxysterols, as well as to LPS, in terms of IL-8 production when compared with macrophages (Figure 1(B)). For this reason, and since IL-8 can be upregulated by TLR-signalling [6], we investigated whether TLR-signalling may be involved in the oxysterol mediated upregulation of IL-8 expression.

Transfection of HEK-293 cells with TLRs 1, 2, 4 or 6 does not confer sensitivity to oxysterols

In order to establish whether expression of TLRs could confer responsiveness to oxysterols in HEK-293 cells, these cells were transfected with the TLR-subtypes responsible for the recognition of lipids, TLR4/MD2, or the combination of TLRs 1, 2 and 6, which are thought to heterodimerise to assist in the recognition of smaller bacterial lipids [29]. Using an NF-κB sensitive reporter, we found no evidence that oxysterols induced classical TLR-signalling in these cells, while responses to the established ligands of TLR2 (Pam_3CSK_4) and TLR4 (LPS) were absolutely dependent on expression of their respective TLRs

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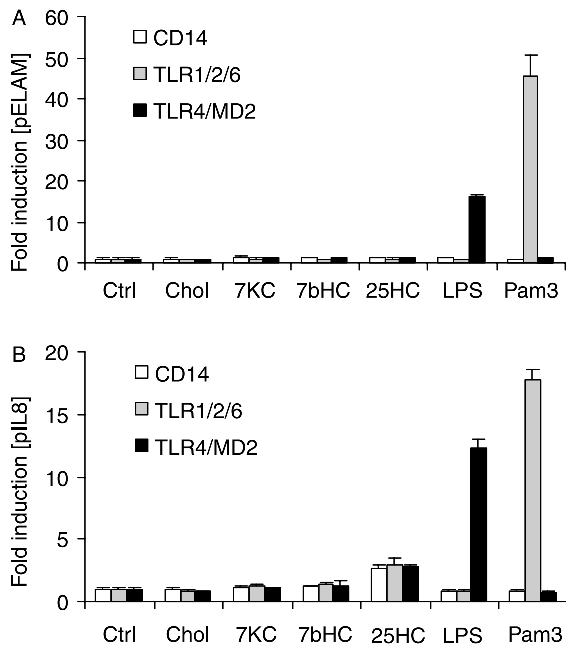

Figure 2. Neither TLR1/2/6 nor TLR4/MD2 transfection confers increased sensitivity to oxysterols. TLR-deficient HEK-293 cells were transfected with CD14 alone, or with additional TLR4/MD2 or the combination of TLRs 1, 2 and 6 and reporter constructs as described in Methods. 48 h post-transfection, medium was replaced with fresh medium alone (Ctrl), 10 μg/ml of Chol or each of the oxysterols indicated (7KC; 7β-HC; 25HC), 1 ng/ml LPS or 10 ng/ml of the synthetic bacterial lipopeptide Pam_3CSK_4 (Pam3). After 18 h, cells were lysed and expression of an NF-κB dependent reporter consruct (pELAM) was measured following normalisation to internal renilla transfection efficiency control. Results are expressed as fold induction vs. cells cultured in medium alone ± SD of triplicate cultures (A). As in (A), except activation of the full-length pIL8 was measured (B). Results shown are representative of three separate experiments.

(Figure 2(A)). Using a full-length IL-8 promoter in the same assay, we found that the oxysterol 25-HC, but not 7-KC or 7-βHC, increased the transcriptional activity of this promoter in the absence of TLR-expression. Moreover, expression of TLRs 1, 2 and 6 or of TLR4/MD2 did not increase responsiveness to any of the oxysterols examined (Figure 2(B)).

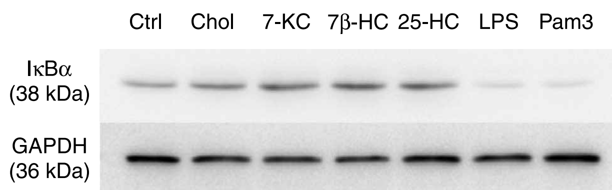

Figure 3. Oxysterols do not induce IκBα degradation in THP-1 cells. Human monocytic THP-1 cells were differentiated for 72 h using 0.1 μM di-hydroxy vitamin D3 then challenged with medium alone (Ctrl), 10 μg/ml of Chol or each of the oxysterols indicated, 1 ng/ml LPS or 10 ng/ml of the synthetic bacterial lipopeptide Pam_3CSK_4 (Pam3). Cells were lysed after 20 min and IκBα degradation was monitored by Western blot. As a loading control, the same lysates were probed with antibody to GAPDH.

Oxysterols do not induce established markers of TLR-signalling in macrophages

A universal feature of signal transduction from all TLRs is activation of the pro-inflammatory transcription factor NF-κB, resulting from degradation of IκBα [6]. However, while established ligands of TLR2 and TLR4 induced IκBα degradation in macrophages, oxysterol treatment of the same cells did not (Figure 3). Similarly, treatment of macrophages with TLR ligands also stimulates secretion of the pro-inflammatory cytokine TNF-α. Again, while established ligands of TLR2 and TLR4 stimulated clear upregulation of TNF-α expression, treatment of the same cells with oxysterols did not (Figure 4).

Inhibition of TLR2 or TLR4 does not block 25-HC induction of IL-8 in macrophages

In order to confirm that oxysterols do not induce IL-8 expression via TLR-signalling in macrophages, further experiments focussed on the effects of the most potent oxysterol inducer of IL-8, 25-HC (Figure 1(A)) [15]. Treatment of THP-1 cells with lipid-IVa, a specific inhibitor of TLR4, blocked LPS induced release of IL-8 completely, though responses to 25-HC remained unchanged. Similarly, while the monoclonal antibody to TLR2 clone TL2.5 blunted significantly IL-8 release in response to the TLR2 ligand Pam_3CSK_4, it did not block responses to 25-HC (Figure 5).

Discussion

Increasing evidence suggests that inflammatory chemokine expression contributes to atherosclerosis [25,30–32]. In particular, increased expression of IL-8 has been implicated, since it is abundantly expressed in foam cells of human plaque [15] and increased plasma IL-8 levels predict future coronary artery disease in human association studies [31]. The chemokine KC, a murine homologue of IL-8, has also been shown to be a principal mediator of monocyte arrest in early carotid artery lesions [25] and replacement of bone marrow of atherosclerosis prone LDL-receptor deficient mice with that from CXCR2 deficient mice, which are unresponsive to KC, results in reduced atherosclerosis in these animals [32].

The agents responsible for upregulating chemokine expression in macrophages within the diseased artery wall remain to be clearly elucidated, though oxysterols have recently been proposed as potential contributors [15,16,21–23]. In particular, oxysterols that have been shown to be present in plaque [13] can upregulate expression of IL-8 [15,16] and MCP-1 [21] from macrophages, both of which are considered to be pro-atherogenic [31–33]. It has also been discovered recently that signalling of the lipid-specific

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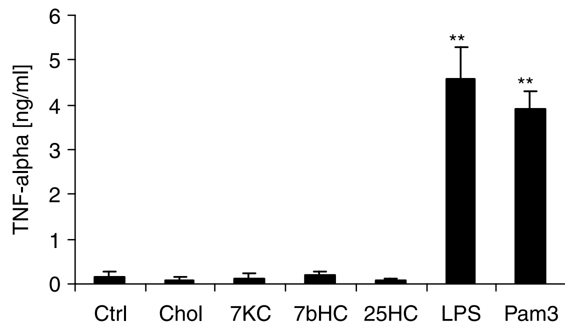

Figure 4. Oxysterols do not induce TNF-α secretion from THP-1 cells. Human monocytic THP-1 cells were differentiated for 72 h using 0.1 μM di-hydroxy vitamin D3 then challenged with medium alone (Ctrl), 10 μg/ml of Chol or each of the oxysterols indicated (7KC; 7β-HC; 25HC), 1 ng/ml LPS or 10 ng/ml of the synthetic bacterial lipopeptide Pam_3CSK_4 (Pam3). TNF-α was then measured by ELISA at 4 h. $^{**}p < 0.01$ vs. cells cultured in medium alone. Results shown are representative of three separate experiments.

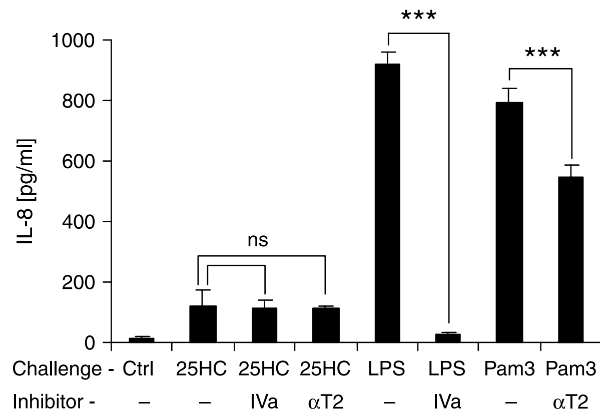

Figure 5. Inhibition of TLR2 or TLR4 does not inhibit 25-HC induced IL-8 release. Human monocytic THP-1 cells were differentiated for 72 h using 0.1 μM di-hydroxy vitamin D3 then pre-incubated for 30 min with medium alone or the specific inhibitors of TLR2 and TLR4 as indicated (IVa, TLR4 inhibitor lipid-IVa; αT2, monoclonal antibody to TLR2 clone TL2.5). Medium was then supplemented with 10 μg/ml of 25-HC, 0.4 ng/ml LPS or 1 ng/ml Pam_3CSK_4 (Pam3) for a further 18 h. Supernatant IL-8 was measured at 18 h. $^{**}p < 0.01$, $^{***}p < 0.001$. Results shown are representative of three separate experiments.

TLRs TLR2 and TLR4 contributes substantially to the progression of atherosclerosis [3–5] and the suggestion has been made that modified endogenous lipids may serve as activating ligands for these TLRs in the diseased artery wall [17–20].

As the main focus of our work is to identify potential ligands responsible for TLR-signalling during athero-sclerosis, we investigated whether TLR-signalling could contribute to the inflammatory properties of certain oxysterols. We chose to examine responses to 25-HC, 7β-HC and 7-KC in particular, since the former has been shown to be a potent inducer of IL-8 in previous studies [15,16] and the other two are among the most abundant oxysterols derived from Chol auto-oxidation in the diseased vessel wall [13]. However, we found no evidence that the examined oxysterols stimulate TLR-signalling in human macro-phages. Two universal features of TLR-signalling, induced by ligands of all of the TLRs, are production of TNF-α from stimulated macrophages and degra-dation of IκBα [6]. Established ligands of TLRs 2 and 4 increased TNF-α release from THP-1 cells, while the oxysterols did not (Figure 4). An earlier study has shown that the oxysterol 25-HC can increase macrophage expression of TNF-α, although only when cells were co-challenged with 9-*cis*-retinoic acid [22]. This was found to be due not to TLR-signalling, but to heterodimerisation of the liver X receptor with its obligate partner the retinoid X receptor, which binds 9-*cis*-retinoic acid. It is possible that similar processes also explain the potential of 25-HC to increase IL-1β release from human macrophages, though no investigation of the mechanism for this process has yet been made [23].

Furthermore, unlike established TLR-ligands, the examined oxysterols did not induce IκBα degradation (Figure 3). A recent study has shown that a biologically relevant mixture of oxysterols, modeled on the concentrations of various oxysterols found in plaque, potentiates the release of another atherogenic chemo-kine, MCP-1, from human monocytic U937 cells [21]. These investigators reported stimulation of ERK-1/2 phosphorylation and also increased nuclear binding of NF-κB, as measured by electrophoretic mobility shift assay [21]. Although we did not use an oxysterol mixture, our results suggest that the activation of NF-κB observed by this group is likely to occur via pathways that are independent of IκBα degradation or TLR-signalling and that other receptors and signalling mechanisms are likely to potentiate this response.

To validate further the notion that oxysterols do not stimulate signalling of the TLRs present in human plaque (TLRs 1, 2, 4 and 6), a HEK-293 cell transfection system was employed. This system has several advantages over the other most widely used method for assessing the capacity of compounds to stimulate TLR-signalling, using knockout mice. Particularly with respect to human TLR2 and TLR4, murine equivalent TLRs can display very different specificities to ligands. For example, certain agents which are agonists of murine TLR4, such as lipid-IVa or penta-acyl *Pseudomonas aeruginosa* LPS, are potent inhibitors of human TLR4 [34,35]. Notably, our studies employed human cells and human TLR and accessory molecule constructs throughout, thereby avoiding such species-specific disparities. This system reliably detected control TLR2 and TLR4 ligands in the picomolar range (not shown), yet overexpression of TLRs 1, 2, 4 or 6 did not confer increased sensitivity to any of the oxysterols examined (Figure 2).

Further studies will be required to elucidate the signalling pathways utilised by oxysterols to enhance

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inflammatory chemokine expression. In particular, the mechanisms for increased expression of IL-8 induced by certain oxysterols requires explanation. Previous studies have shown that oxysterol induction of IL-8 is time and dose-dependent and that cycloheximide inhibits IL-8 release completely, indicating that *de novo* protein synthesis is required [15]. Beyond this, the receptors and signalling pathways involved in oxysterol-mediated IL-8 regulation remain to be fully characterised, though our results demonstrate that TLR-signalling is not involved in responses to 7β-HC, 7-KC or 25-HC. In conclusion, our results and those of previous studies examining the properties of oxysterols suggest that the TLR-stimulating compounds thought to be of relevance to atherosclerosis are not oxysterols.

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