Oxysterol Activators of Liver X Receptor and 9-cis-Retinoic Acid Promote Sequential Steps in the Synthesis and Secretion of Tumor Necrosis Factor-α from Human Monocytes*

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Liver X receptor α (LXRα), is a nuclear hormone receptor that is activated by oxysterols and plays a crucial role in regulating cholesterol and lipid metabolism in liver and cholesterol efflux from lipid-loaded macrophages. Here we show that treatment of human peripheral blood monocytes or monocytic THP-1 cells with the LXR ligand 22(R)-hydroxycholesterol (22(R)-HC), in combination with 9-cis-retinoic acid (9cRA), a ligand for the LXR heterodimerization partner retinoid X receptor (RXR), results in the specific induction of the potent pro-apoptotic and pro-inflammatory cytokine tumor necrosis factor-α (TNF-α). Promoter analysis, inhibitor studies, and order-of-addition experiments demonstrated that TNF-α induction by 22(R)-HC and 9cRA occurs by a novel two-step process. The initial step involves 22(R)-HC-dependent induction of TNF-α mRNA, and intracellular accumulation of TNF-α protein, mediated by binding of LXRα/RXRα to an LXR response element at position –879 of the TNF-α promoter. Subsequent cell release of TNF-α protein occurs via a separable 9cRA-dependent, LXRα-independent step that requires de novo transcription and protein synthesis. Our findings reveal a potentially new dimension of the physiological role of LXRα and identify a unique multistep pathway of TNF-α production that may be of consequence to the normal function of LXR in monocyte/macrophages and in disease conditions such as atherosclerosis.

Liver X receptors (LXRα [NR1H3] and LXRβ [NR1H2]) are recently described members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are important in the regulation of genes that govern cholesterol homeostasis in the liver and cholesterol efflux from peripheral tissues. LXRαs are bound by and activated by specific cholesterol metabolites, including oxysterols, and regulate the expression of target genes by binding to specific promoter response elements (LXREs) in association with the obligate heterodimerization partner, retinoid X receptor (RXR), the receptor for 9-cis-retinoic acid (9cRA) (1). Natural ligands for LXR include 22(R)-hydroxycholesterol (22(R)-HC), 24(S),25-epoxycholesterol, and 25-hydroxycholesterol (2–4), compounds that have been found free in serum and in association with atherogenic oxidized low density lipoprotein (oxLDL) particles (5–7). In the liver, LXRα serves as a sterol sensor and regulates the expression of genes that influence cholesterol metabolism and homeostasis, including the genes encoding cholesterol 7α-hydroxylase, which controls the cholesterol/bile acid synthetic pathway, and sterol regulatory element-binding protein-1c, a key transcription factor that regulates expression of genes important in fatty acid biosynthesis (6, 8, 9–11). Studies using lxr null mice have shown that LXRα is essential for normal cholesterol homeostasis and secretion of excess cholesterol in vivo (9).

Recent work has shown that LXRα plays a fundamental role in macrophage biology by regulating cholesterol efflux from lipid-loaded cells. This is manifested by LXRα-mediated induction of genes encoding the ATP-binding cassette proteins ABC-1 and ABCG1, which encode plasma membrane-associated reverse cholesterol transport proteins that mediate cholesterol ester and free cholesterol efflux from monocytes and lipid-loaded macrophages (12–15). Effluxed cholesterol is subsequently delivered to extracellular acceptors, especially high density lipoprotein/ApoE, and transported back to the liver where it is converted to bile acids and excreted (1). The key role of LXRα as a master regulator in the overall process governing cholesterol efflux and reducing intracellular cholesterol levels is underscored by the findings that the genes encoding ApoE, which is necessary for high density lipoprotein formation is also a transcriptional target of LXRα (16). The pivotal role of LXRα in regulating reverse cholesterol transport in macrophages is of particular disease relevance, because lipid accumulation in these cells, through the uptake of oxLDL, is of fundamental importance to the etiology and pathogenesis of atherosclerosis and other chronic inflammatory diseases. oxLDL particles accumulate in macrophages that have infiltrated the arterial intimal space, subsequently developing into lipid-loaded foam cells that comprise the characteristic fatty streak of early atherosclerotic lesions (17). In this context, LXRα, by reducing intracellular cholesterol and lipid accumulation, is considered to be anti-atherogenic. Consistent with this, recent studies have shown that selective agonists of RXR, which activate LXR/RXR heterodimers, significantly reduce lesion size and the progression of atherosclerosis in apoE−/− animals (18).

Exposure of monocytes and other cell types to oxLDL and some of its constituent oxysterols and oxidized lipids exert
pleiotropic effects on gene expression and elicit numerous cellular changes that are associated with normal cell function and molecular processes linked to pathological states (17). These include alteration in the expression of genes that are not necessarily involved in regulating cellular lipid content and homeostasis. For instance, studies have shown that the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, IL-8, and platelet-derived growth factor are differentially modulated in macrophages in response to oxLDL or various oxysterols (19–22). Similarly, other studies have demonstrated diverse effects of oxLDL and oxysterols in vascular endothelial cells and smooth muscle cells (23–25). The cytokines described above provoke local inflammatory responses and can induce apoptosis of macrophages resident within the arterial intimal space and smooth muscle cells in the medial layer of the arterial wall (17). They can also promote T-cell infiltration and tissue necrosis that contribute to the late stage necrotic core within complex atherosclerotic lesions (26).

Although oxLDL provides ligands for LXRα, and macrophages may generate endogenous oxysterols that can activate LXRα (6), a role for LXR and LXR agonists in cytokine production in monocytes/macrophages has not been described. Here, we demonstrate that administration of the LXR-specific ligand 22(R)-HC to peripheral blood-derived human monocytes and to monotypic THP-1 cells, in the presence of 9cRA, results in the specific and selective production of bioactive TNF-α, a key pro-inflammatory and pro-apoptotic cytokine. We further show that TNF-α production occurs via a novel two-step process that involves an initial oxysterol-dependent induction of TNF-α mRNA and intracellular protein accumulation mediated by LXRα/RXRα binding to an LXRE in the TNF-α promoter, and subsequent cell release of TNF-α protein via a separable 9cRA-dependent, LXR-independent step, which requires de novo transcription and protein synthesis. These observations establish a novel role for LXR and oxysterols in monocyte function and identify a unique pathway of cytokine production in these cells.

**EXPERIMENTAL PROCEDURES**

**Reagents—**22(R)-Hydroxycholesterol (22(R)-HC) was obtained from Research Plus Ltd.; 22(S)-hydroxycholesterol (22(S)-HC), 25-hydroxycholesterol, 9-cis-retinoic acid (9cRA), and Escherichia coli 011: B5 lipopolysaccharide (LPS) were obtained from Sigma. Antimycin D and cycloheximide were obtained from Sigma. Oxidized low density lipoprotein (oxLDL) was obtained from Intralcel Perimmune (Rockville, MD).

**Plasmids—**Human LXRα and RXRα mammalian expression vectors pRC/CMV-LXRα and pSG5-RXRα have been previously described (27, 28). Luciferase reporter plasmids used were pXP1-TNF-α (1311/1uc), which contains 1.3 kb of the human TNF-α promoter/regulatory region linked to the luciferase gene (29). Plasmids used in promoter mapping experiments were constructed from pXP1-TNF-α (1311/1uc) as follows: pTNF-α(-914/-359) was generated by a BstXI collapse of pTNF-α (−1311/1uc), pTNF-α(-971/-762)uc was generated by a MscI collapse of pTNF-α(−1311/1uc), pTNF-α(-641/-493)uc was generated by a StuI collapse of pTNF-α (−1311/1uc), and pTNF-α(-897/-1053)uc was generated by a SstI collapse of pTNF-α (−1311/1uc). pTNF-α(-922/−851)uc was generated by cloning a single copy of a synthetic double-stranded oligonucleotide corresponding to nucleotides −932 to −851 of the TNF-α promoter into the BamHI site of the luciferase expression vector pGL2uc (Promega). pTNF-α(-894/−866)uc and pTNF-αmut(-894/−866)uc contained a single copy of a synthetic double-stranded oligonucleotide corresponding to nucleotides −894 to −866 of the TNF-α promoter, and a mutant variant thereof, respectively (sequences provided below), into the BamHI site of expression vector pGL2uc (Promega). All plasmid constructions were verified by DNA sequence analysis.

**Cell Culture—**THP-1 human monocyte cells (obtained from American Type Tissue Collection) were cultured in RPMI 1640 media supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, and 1% v/v L-glutamine. Human peripheral blood mononuclear cells were collected from healthy donors, and monocytes were isolated fromuffy coat preparations using a MACS Monocyte Isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. Cells were cultured in RPMI 1640 supplemented with 10% autologous serum, 1% v/v penicillin/streptomycin, and 1% v/v L-glutamine. Experiments were initiated on the day blood was collected. COS-1 cells (ATCC) were cultured in Dulbecco’s minimum essential medium supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, and 1% v/v L-glutamine.

**Cytokine Assays—**THP-1 and human monocytes (10⁵ cells/ml) were cultured as above and incubated in the presence of the various compounds as indicated in the figure legends. Control cells received the equivalent amount of vehicle (MeSO₂, ethanol, or water) as indicated. The levels of human TNF-α, IL-1β, or IL-6 present in the culture media were measured, as specified, by enzyme-linked immunosorbent assays (ELISA) using OptEIA kits (BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions.

**RNA Analysis—**Total RNA from THP-1 cells (4 × 10⁵ cells/sample) was isolated using the RNeasy Mini isolation kit (Qiagen, Chatsworth, CA) and subjected to Northern analysis under standard conditions using random-primed ³²P-radiolabeled probes generated from cDNAs for human TNF-α, and glyceraldehyde-3-phosphate dehydrogenase as described (30). Radioactive bands were quantified by phosphorimaging analysis of the dried gel and normalized to the radioactivity present in the glyceraldehyde-3-phosphate dehydrogenase signal, which was used as an internal standard. RNA analysis was also performed by reverse transcriptase-PCR using a commercially available kit (MBI Fermentas, Burlington, Ontario, Canada).

**Transfections and Luciferase Assay—**Transfection of COS-1 cells was carried out using LipofectAMINE (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. Briefly, cells (3 × 10⁵ cells/well in 6-well plates) were transfected using 4 μl of LipofectAMINE along with 0.5 μg of a luciferase reporter gene, 0.5 μg of pRC/CMV-LXRα, and 0.5 μg pSG5-RXRα (28), as indicated. Transfections included 0.5 μg of pCMVlacZ, which encodes the gene for β-galactosidase, to serve as an internal control for transfection efficiency. Total DNA and promoter dosage were kept constant with pRC/CMV and pSG5 empty vectors (Invitrogen), as appropriate. RRX and/or LXR ligands were dissolved in ethanol or MeSO₂ and added to a final concentration of 10 μM each. Control cells received the equivalent amount of vehicle. Cell extracts were prepared 48 h post-transfection, and luciferase assays and β-galactosidase assays were carried out as previously described (28).

**Metabolic Labeling and Immunoprecipitation—**THP-1 cells (4 × 10⁵ cells/sample) were cultured in methionine- and cysteine-free RPMI containing 1% dialyzed fetal bovine serum and 100 μM [³⁵S]methionine/cysteine (PerkinElmer Life Sciences) for 12 h at 37 °C in the presence of LXR and RRX ligands (10 μM) or vehicle, as described in the figure legend. Cell extracts were prepared as described (30) and pre-cleared using goat IgG-agarose conjugate (Santa Cruz Biotehnologies, Santa Cruz, CA). Equivalent amounts of radioactivity from each sample were immunoprecipitated with polyclonal goat anti-human TNF-α IgG (TNF-α N-19, Santa Cruz Biotechnology) followed by protein G-Sepharose (Boehringer-Ingelheim, Germany). Immune complexes were resolved by 15% SDS-polyacrylamide gel electrophoresis, and radioactivity in dried gels was quantified by phosphorimaging analysis.

**Electrophoretic Mobility Shift Assay—**Electrophoretic mobility shift assays, using human LXRα and RRXα synthesized in vitro by transcription of their corresponding cDNAs and translation using commercially available systems (Promega), were carried out as described previously (27, 28). Binding reactions were carried out with a radiolabeled synthetic double-stranded oligonucleotide probe corresponding to nucleotides −932 to −851 of the human TNF-α promoter (see Fig. 6B) and probes corresponding to putative LXREs between residues −932/−922, −922/−912, and −912/−904. N-acetyltryptophanamide (N-AcTCTTTGCGGTTCCCAGGGTTTAAATAAGTTCATCTA-3') and residues −894/−866 (site 2, 5'-TGTCACCAGGGTCATGAAGTTCGAG-TATCG3'), respectively (putative DR4 half-sites are underlined). A mutant variant of the site 2 −894/−866 oligonucleotide was also used (5'-TGTCACCTAGTGAGAAGCTATCG3'), the mutated residues are in lowercase. Competitor DNAs included the oligonucleotide 5'-CTTGGGGTCTCCAGGGTAAATTTAAAGTTCTATCG3', corresponding to the LXRE from the MTV promoter (8) and non-radiolabeled versions of the oligonucleotides described above. Protein concentration in binding reactions was kept constant with unprogrammed lysate as appropriate.
RESULTS

Oxysterols and 9cRA Cooperatively Induce the Production of TNF-α from Unstimulated THP-1 Cells and Primary Human Monocytes—To determine if natural oxysterol activators of LXR can stimulate the production of pro-inflammatory and pro-apoptotic cytokines from unstimulated human monocytes, we used THP-1 cells, a human monocyte/macrophage cell line that expresses both LXR (α and β isoforms) and its heterodimeric partner RXRα (31, 32). Treatment of THP-1 cells with either the LXR-specific ligand 22(R)-HC or the RXR-selective ligand 9cRA had no effect on the secretion of the primary cytokines TNF-α, IL-1β, or IL-6, as measured by enzyme-linked immunosorbent assays (ELISA) (Fig. 1A). However, co-administration of both compounds resulted in a potent and specific increase in extracellular TNF-α protein. Similar findings were observed with the LXR activator 25-hydroxycholesterol when used in combination with 9cRA (data not presented). TNF-α induction was observed at physiological concentrations of 22(R)-HC and at levels shown to activate both the α and β isoforms of LXR (EC50 5 μM and EC50 3 μM for LXRα and LXRβ, respectively) (2–4). The amount of extracellular TNF-α produced by co-administering 22(R)-HC and 9cRA was ~50% of the levels observed from cells stimulated with the potent endotoxin lipopolysaccharide (LPS). A similar level of TNF-α production was also observed in primary human peripheral blood monocytes treated with 22(R)-HC and 9cRA (Fig. 1B; 50 and 35 pg/ml for primary cells and THP-1 cells, respectively), indicating that the response to these compounds is not peculiar to THP-1 cells.

As we observed in cells treated with LPS (Fig. 1, A and B), secretion of TNF-α by stimulated macrophages is normally accompanied by the coordinate secretion of several other primary pro-inflammatory cytokines, such as IL-1β and IL-6, in a manner consistent with the acute phase response (33). 22(R)-HC/9cRA, however, had no effect on the production of IL-1β or IL-6 from THP-1 cells or from primary monocytes (Fig. 1, A and B), suggesting that 22(R)-HC/9cRA stimulates a pathway of induction of TNF-α that is selective for this cytokine and distinct from other pathways.

Several reports have shown that oxLDL inhibits LPS-mediated TNF-α expression in activated macrophages, whereas other studies have shown that oxLDL can induce TNF-α in resting macrophages (19–23). However, the findings have been inconsistent and can be dependent upon cell type, species, and/or the concentration, composition, and degree of modification of oxLDL. Given that 22(R)-HC is reportedly present in oxLDL (6), we tested whether oxLDL could also stimulate TNF-α production in resting THP-1 cells. As shown in Fig. 1C, treatment of cells with oxLDL had minimal effect on TNF-α production as compared with control untreated cells. However, co-administration of oxLDL with 9cRA led to an 8-fold induction. Thus, under our experimental conditions, oxLDL cooperates with 9cRA in the induction of TNF-α protein expression in THP-1 cells.

Several lines of evidence indicate that 22(R)-HC/9cRA-dependent induction of TNF-α is specific and is mediated through endogenous LXR. For instance, 22(S)-hydroxycholesterol (22(S)-HC), a stereoisomer of 22(R)-HC that binds to LXR with similar affinity as does 22(R)-HC but does not activate the receptor (4), failed to induce TNF-α secretion from THP-1 cells or primary monocytes, either alone or in combination with 9cRA (Fig. 1, A and B). Importantly, 22(S)-HC inhibited 22(R)-HC/9cRA-mediated induction of TNF-α in a dose-dependent manner. As shown in Fig. 2, a 2-fold molar excess of 22(S)-HC vis à vis 22(R)-HC almost completely eliminated extracellular TNF-α induction. This inhibition was not due to generalized cell toxicity, because final concentrations of oxysterols up to 30 μM had no deleterious effects on cell viability as determined by trypan blue staining (data not presented). Finally, all-trans-retinoic acid, a natural activator of the retinoic acid receptor, could not substitute for 9cRA and failed to induce TNF-α when administered alone or with 22(R)-HC (data not presented). We also examined kinetics of TNF-α induction. As shown in Fig. 3, significant amounts of TNF-α were generated at the earliest time point examined (12 h) and continued to accumulate over 96 h. The foregoing, along with further evidence described below, is consistent with induction being directly mediated by...
Oxysterols Induce TNF-α mRNA and Protein Expression in THP-1 Cells but Subsequent Protein Secretion Requires 9cRA—LXR/RXR belongs to a subclass of nuclear receptor heterodimers that can be activated, under certain circumstances, by ligands for either heterodimer partner (1, 8, 34). The finding that co-administration of 22(R)-HC and 9cRA was necessary to detect extracellular TNF-α implies that both LXR and RXR need to be activated by their cognate ligands to mediate TNF-α induction. However, the simultaneous requirement of both ligands for LXRX/RXR to activate target gene transcription is unusual. An alternative explanation is that 22(R)-HC and 9cRA participate in separate and distinct steps in TNF-α production, a hypothesis tested and discussed further below.

To determine if 22(R)-HC increased TNF-α mRNA levels, we performed Northern blot analysis on mRNA from THP-1 cells. Exposure of cells to 22(R)-HC led to an 11-fold induction in the steady-state level of TNF-α mRNA in comparison to untreated cells (Fig. 4A). oxLDL also resulted in induction of TNF-α mRNA, albeit more modestly than with 22(R)-HC. In contrast, 22(S)-HC did not affect TNF-α mRNA levels. Interestingly, the addition of 9cRA did not further augment the 22(R)-HC-mediated increase; indeed, TNF-α mRNA expression was somewhat diminished (from 11- to 7-fold) under these circumstances. Similar results were obtained when RNA was analyzed by reverse transcriptase-PCR (data not presented). Fig. 4B shows a time course analysis of TNF-α mRNA induction in the presence of 22(R)-HC. As shown in the figure, TNF-α mRNA was detectable at the 12-h time point and remained at this steady-state level. Thus, TNF-α protein secretion from THP-1 cells, as shown in Fig. 3, correlates with induction of TNF-α mRNA. The 22(R)-HC-mediated increase in TNF-α mRNA was not ablated in the presence of cycloheximide, consistent with induction being mediated by pre-existing, endogenous factors (Fig. 4C). Thus, 22(R)-HC increased the steady-state levels of TNF-α mRNA and did so independently of exogenously added 9cRA.

TNF-α mRNA expression is induced by diverse factors in monocytes, however, in the absence of cell activation, the mRNA is normally translationally silent. Translation of TNF-α mRNA is tightly regulated, both by factors that mediate nuclear-cytoplasmic transport and by mRNA silencing and stability factors that recognize determinants in the 3′-untranslated re-
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FIG. 5. The human TNF-α promoter is a target for transactivation by LXRα and RXRα. COS-1 cells were transfected with a human TNF-α promoter/luciferase reporter gene (TNF-α (−1311)/luc) in the absence or presence of expression vectors for human LXRα and human RXRα along with 22(R)-HC (10 μM) and 9cRA (10 μM), as indicated, and luciferase activity was measured. The values presented represent the average (± S.D.), relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate, and normalized for protein and β-galactosidase expression levels.

Fig. 5.

LXR
RXR
9cRA
22(R)-HC

Field Induction

0
10
20
30

LXRα/RXRα Heterodimers Transactivate the TNF-α Promoter—The above findings suggest that the human TNF-α gene is a target for LXR/RXR-mediated transactivation. To determine this directly, we carried out transient transfection assays in COS-1 cells using a luciferase reporter gene linked to a 1.3-kb promoter/regulatory region of the human TNF-α gene (29). COS-1 cells express modest amounts of endogenous RXR but very low levels of LXR, and, therefore, transactivation of LXR target genes in these cells is dependent upon ectopic expression of LXR (27). As shown in Fig. 5, 22(R)-HC had no effect on the activity of the TNF-α reporter gene. Similar findings were obtained with a control LXR target reporter gene pTK-DR4/luc, confirming that these cells express low levels of endogenous LXR (data not presented). However, in the presence of ectopically expressed human LXRα, 22(R)-HC led to an 8- to 10-fold induction in reporter gene activity compared with untreated cells (Fig. 5). Transfection of an expression vector for human RXRα had no significant effect on reporter gene activity, either in the absence or presence of 9cRA. However, in the presence of expression vectors for both LXRα and RXRα, 22(R)-HC led to a 20-fold increase in promoter activity, indicating that full induction required the presence of sufficient amounts of both LXRα and RXRα. 9cRA did not affect LXRα/RXRα activity, indicating that ligand occupancy of RXR was insufficient to activate the LXRα/RXRα heterodimer in this promoter context. Interestingly, however, 9cRA inhibited 22(R)-HC, LXRα/RXRα-mediated transactivation by ~50%, consistent with findings from the Northern analysis of THP-1 cells (see Fig. 3A). The reasons for this reduction are not clear at present.

LXRα/RXRα Heterodimers Bind Directly to an Upstream LXRE in the TNF-α Promoter—LXR/RXR heterodimers activate target gene transcription by binding to LXR response elements (LXRE) that consist of a hexanucleotide direct repeat element related to the consensus half-site TGACCT separated by four nucleotides (DR4) (8). The human TNF-α promoter contains numerous transcription factor binding, including multiple sites for NF-κB and AP-1, however, an obvious LXRE is not apparent. To begin to identify promoter elements that mediate LXR responsiveness, we generated a series of deletions in the TNF-α promoter and tested their activity in transfection assays in the presence of co-expressed LXRα/RXRα. As demonstrated in Fig. 6A, the full-length promoter was induced 18-fold by 22(R)-HC in the presence of co-expressed LXRα/RXRα, whereas deletion derivatives lacking nucleotides spanning −987 to −105 or −914 to −359 (relative to the transcription start site) were inactive. A derivative missing nucleotides −971 to −762 was partially active, resulting in a 3-fold induction, whereas a reporter gene derivative missing nucleotides −640 to −493 was induced 9-fold. These findings indicate that multiple regions in the TNF-α promoter participate in full responsiveness to 22(R)-HC and LXRα/RXRα and that sequences between −971 to −762 are of particular importance.

Examination of the −971 to −762 region revealed the presence of two degenerate TGACCT motifs configured in a DR4 arrangement centered at residues −918 (site 1) and −879 (site 2), respectively, that could potentially serve as LXREs (see Fig. 6B). Consistent with this, a synthetic subfragment spanning residues −932 to −851, which contained these putative elements, was sufficient to confer responsiveness to 22(R)-HC and LXRα/RXRα (5- to 6-fold induction) when appended to a heterologous promoter (pTNF−1987 to −105/luc, Fig. 6A). To determine if LXR binds directly to this promoter region, a radiolabeled oligonucleotide probe corresponding to this region was tested by electrophoretic mobility shift assay using LXRα and RXRα proteins synthesized in vitro. As shown in Fig. 7A, a protein-DNA complex was observed only in the presence of both LXRα and RXRα (lane d). This complex was specific, because it was competed by an unlabeled bona fide LXR oligonucleotide (MTV LXRE; compare lanes d with lanes e and f) but not by nonspecific DNA. To determine if LXRα/RXRα targets either of the putative LXREs, we synthesized oligonucleotides spanning site 1 and site 2 and used these in DNA-binding competition assays. As shown in Fig. 7B, the site 2 but not the site 1 oligonucleotide was able to compete out binding of LXRα/RXRα to the −932 to −851 fragment (compare lanes d and e with lane c), indicating that LXRα/RXRα targets site 2. Consistent with this, radiolabeled site 2 probe (Fig. 7C, lane f), but not site 1 probe (lane e), formed a specific protein-DNA complex with LXRα/RXRα (lanes g and h). Finally, a derivative of site 2 in which the 5′-half site was mutated did not generate a protein-DNA complex with LXRα/RXRα, confirming that LXRα/RXRα targets the DR4 direct repeat element (lane i). Unequivocal confirmation that site 2 constitutes a bona fide LXRE is shown in Fig. 8. Thus, the site 2 oligonucleotide, but not the mutant derivative, conferred LXR/RXR responsiveness onto a heterologous promoter in vivo.
The foregoing establishes the human TNF-α gene as a novel and direct target for LXRα/RXRα binding and transactivation and suggests that the increase in TNF-α mRNA observed in monocytes treated with 22(R)-HC occurs, at least in part, at the level of transcription.

9cRA Triggers Release of TNF-α from Cells via a Separate LXR-independent Pathway That Requires de Novo Transcription and Protein Synthesis—As shown above, although 22(R)-HC increased TNF-α mRNA and protein levels in THP-1 cells, soluble TNF-α was only detected in the presence of co-administered 9cRA. This suggests that 9cRA, either alone or in conjunction with 22(R)-HC, participates in a distinct, post-translation step that triggers TNF-α release from cells. The block to TNF-α secretion is apparently not related to pro-TNF-α protein processing, because the immunoreactive TNF-α protein synthesized in 22(R)-HC-treated cells migrated with an apparent molecular mass of 17 kDa, consistent with the mature form of the protein (see Fig. 4D). Indeed, the 26-kDa pro-TNF-α precursor was not detected.

To begin to unravel this multistep pathway, we carried out
order-of-addition experiments with oxysterols and 9cRA in the presence of various inhibitors. As outlined in Fig. 9, the basic approach involved preincubating THP-1 cells with 22(R)-HC under various conditions for 12 h, a time interval that we previously determined to be sufficient to detect TNF-α mRNA and protein after 22(R)-HC treatment, and extracellular TNF-α after co-administration of 22(R)-HC/9cRA (see Figs. 3 and 4). 9cRA, and additional treatments as indicated in Fig. 9, was administered after this 12-h interval, and cells were incubated for a further 12 h, after which extracellular TNF-α was measured by ELISA. As shown in Fig. 9, lane d, addition of 22(R)-HC at t = 0 followed by 9cRA at t = 12 h led to a 40-fold induction of extracellular TNF-α protein in comparison to untreated cells or cells treated with 22(R)-HC alone (lanes a and b). Thus, significant production of TNF-α was observed over a 24-h time period even when the addition of 22(R)-HC and 9cRA was separated by a period of 12 h. As expected, TNF-α was not detected when 22(S)-HC was substituted in place of 22(R)-HC prior to addition of 9cRA (Fig. 9, lane i).

Co-incubation of 22(R)-HC at t = 0 with the protein synthesis inhibitor cycloheximide (which does not affect TNF-α mRNA induction as shown previously in Fig. 4C) or 22(S)-HC (which inhibits 22(R)-HC induction as shown in Fig. 2) followed by 9cRA at 12 h ablated soluble TNF-α production as expected, because TNF-α mRNA and/or protein is not induced under these conditions (lanes c and h). In contrast, inclusion of 22(S)-HC with 9cRA at 12 h had no effect on extracellular TNF-α production (Fig. 9, compare lanes g and h, respectively). These results suggest that the secretion step triggered by 9cRA is independent of LXRs. However, inclusion of cycloheximide (lane f) or the transcription inhibitor actinomycin D (lane e) along with 9cRA at 12 h completely abolished TNF-α secretion. Thus, 9cRA appears to be required for the de novo synthesis of one or more factors that, whereas not necessary for 22(R)-HC-mediated induction of TNF-α mRNA and protein, are required at a post-translational step for release of cell-associated TNF-α.

In this context it is noteworthy that extracellular TNF-α was not detected when cells were first preincubated with 9cRA followed by 22(R)-HC (Fig. 9, compare lane d with lane j). This could simply be due to degradation or loss of 9cRA activity during the 12-h preincubation time interval prior to addition of 22(R)-HC. However, the finding is also consistent with the possibility that a putative 9cRA-induced factor needed for TNF-α release is labile and/or only transiently available soon after 9cRA treatment. This scenario could also provide an explanation of our findings that sequential addition of 22(R)-HC followed by 9cRA led to a reproducibly more robust production of TNF-α over a 24-h interval as compared with when these compounds were co-administered (40- versus 10-fold, respectively; compare Fig. 9, lane d, with the 24-h time point shown in Fig. 3). Thus, the former conditions would be expected to lead to the accumulation of intracellular TNF-α protein prior to secretion mediated by the subsequent addition of 9cRA. In contrast, simultaneous addition of 22(R)-HC and 9cRA might be expected to result in the presence of less extracellular TNF-α at the 24-h time point because of the prior requirement for TNF-α mRNA synthesis and translation, and the postulated labile nature of a 9cRA-induced release factor.

**DISCUSSION**

The emergent role of LXRs in interconnected pathways that control rates of lipid accumulation and efflux and in coordinating cellular responses to lipid-loading in monocytes and macrophages, and its linkage to pathological conditions such as atherosclerosis has placed this transcription factor under intense scrutiny (18, 31, 32, 38). The findings reported here identify a new and potentially significant role for LXRs and oxysterols in monocytes and macrophages by demonstrating that LXRs and its oxysterol ligands specifically stimulate TNF-α mRNA synthesis. Moreover, we show that cell release of TNF-α protein made in response to LXR activation is mediated via an independent and separable mechanism that is stimulated by the retinoid 9cRA but that does not require LXR. Our findings are unusual in that secretion of TNF-α was specific to this cytokine, and the typical co-expression of other pro-inflammatory cytokines such as IL-1β and IL-6 associated with the inflammatory response, and seen in cells exposed to endotoxins such as LPS, was not observed. The foregoing suggests that oxysterol activators of LXR and 9cRA cooperate in a novel and selective pathway of cytokine induction that may be specific to TNF-α.

Transfection analysis and inhibitor studies demonstrated that the TNF-α promoter is a direct target for transactivation by LXR/RXR heterodimers. This manifests principally through direct binding of LXRs/RXRs to a bona fide LXRE present at position –879 of the TNF-α promoter. In addition to this LXRE, there appear to be other downstream regions of the promoter that are required for full LXR responsiveness. Thus, a promoter derivative that was missing the upstream LXRE (pTNFΔ971–762) was still activated by LXR/RXR, albeit to a much lesser degree than derivatives that retained the identified LXRE. This suggests that full activity requires the presence of a second response element and/or cooperativity with other transcription factors. Ongoing studies to further delin-
ate LXRα protein-DNA and protein-protein interactions, and interplay with other transcription factors will provide insights into the mechanisms of transcriptional activation and potential convergence with other signaling pathways that are known to be important for TNF-α gene expression.

TNF-α plays a predominant and pleiotropic role in inflammation and host defense and, if left unregulated, can cause chronic inflammation and septic shock. Consequently, production of bioactive TNF-α is stringently regulated by multiple transcriptional and post-transcriptional mechanisms that serve to modulate expression, abundance, modification, processing, stability, subcellular localization, and secretion of TNF-α mRNA and/or protein (35, 36, 39). Our findings identify a novel post-translational pathway of control that is stimulated by 9cRA. Inhibitor studies and order-of-addition experiments indicated that 9cRA acts as a step subsequent to 22(S)-HC/LXR-mediated induction of TNF-α mRNA and protein synthesis. This post-translational step is independent of LXR, because 9cRA-mediated TNF-α release was not ablated in the presence of the LXR antagonist 22(S)-HC. This step, however, requires de novo transcription and protein synthesis because it was sensitive to inhibition by actinomycin D and cycloheximide, respectively. The foregoing suggests that 9cRA, potentially through activation of endogenous RXR, stimulates the synthesis of one or more cellular factors required for cellular release of mature TNF-α protein. Although there is no precedent for such a mechanism in macrophages, a previous report suggests that 9cRA and RXR can promote the secretion of insulin from glucose-stimulated pancreatic islet cells (40). It has been reported that biologically active, mature TNF-α in activated macrophages is retained in the Golgi complex and is subsequently translocated from this intracellular pool by various stimulants (39), perhaps mediated by mitogen-activated protein kinase signaling cascades (41). It will be of interest to unravel the mechanisms and pathways involved in 9cRA-mediated release of TNF-α and whether this process is specific to TNF-α made in response to 22(R)-HC/LXR and involves other nuclear hormone receptors such as RXR.

The penetration of monocytes into the vascular intima and their differentiation into lipid-load cells through influx of oxLDL by scavenger receptors such as CD36 and SR-A is one of the earliest steps in atherogenesis (17, 42). In this context, LXRα, by promoting reverse cholesterol transport through stimulation of ABC-1 and ApoE1 expression, is considered to be anti-atherogenic. Recent findings that LXR and RXR agonists reduce lesion size and the progression of atherosclerosis in apoE−/− animals (18) are consistent with this view. In contrast, co-expression of pro-inflammatory cytokines, including TNF-α, are considered pro-atherogenic, because these agents can promote inflammation, apoptosis, and necrosis in atherosclerotic lesions, leading to late-stage lesion calcification and clinical syndrome (17). Indeed, several reports have shown that certain oxysterols induce apoptosis (43) and that atherosclerotic lesions contain significant numbers of apoptotic cells as well as immunoreactive TNF-α (44, 45). However, the selective expression of TNF-α by oxysterol/LXR in the absence of the other primary cytokines normally associated with inflammation suggests that LXR activation does not elicit a generalized pro-inflammatory response. In this context, specific oxysterol/LXR-dependent stimulation of TNF-α expression in monocytes/macrophages resident within the intima, coupled with LXR-mediated cholesterol efflux, could lead to diminishment of lesion size by inducing apoptosis of proliferating smooth muscle cells, foam cells, and/or infiltrating T cells. However, a deleterious effect of TNF-α expression cannot be excluded a priori, because TNF-α could also serve to worsen the existing state by stimulating inflammatory cytokine production from infiltrating T cells. TNF-α elicits pleiotropic and complex physiopathological effects, and the biological relevance of our findings may bear on diverse cellular functions and physiological circumstances that are unrelated to any of the scenarios discussed above. In this context, it is noteworthy that any pathophysiologically relevant consequences of oxysterol-mediated TNF-α induction from monocytes/macrophages would presumably be evident only under specific physiological circumstances where a second signal, mediated by compounds such as 9cRA, is available to stimulate cell release of TNF-α.

In summary, we demonstrate that the human TNF-α gene is a direct target for LXRα transactivation in monocytes and identify a unique and multistep pathway of TNF-α production in these cells. Our findings reveal a potentially important new dimension to the physiological role of LXRα. Further investigations will help to elucidate the biological consequence of these findings to normal cellular function and pathological states.

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