REGULATION OF TNF-α IN MONOCYTES BY LXR AND RXR

THE ROLE OF LIVER-X-RECEPTOR AND RETINOID-X-RECEPTOR IN THE REGULATION OF TUMOUR NECROSIS FACTOR-α EXPRESSION AND PRODUCTION IN HUMAN MONOCYTES

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ABSTRACT

Liver X receptor (LXR) is a member of the nuclear hormone receptor superfamily that is activated by hydroxylated cholesterol derivatives referred to as oxysterols. It has also been shown to play a crucial role in regulating cholesterol trafficking and lipid metabolism in liver and macrophages. Furthermore, LXR has also been directly implicated in the reduction of atherosclerosis in several murine models of the disease by virtue of its ability to promote reverse cholesterol efflux from intima-resident lipid-loaded macrophages. While roles for LXR in monocyte biology have focused primarily on cholesterol trafficking, evidence for other functions for the receptor outside of its traditional role as a mediator of cholesterol homeostasis is lacking. Presented herein is evidence that LXR also serves as a mediator of cytokine expression. This work has shown that treatment of human peripheral blood monocytes or monocytic THP-1 cells with the LXR ligand 22(R)-hydroxycholesterol (22R-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-alpha (TNF-α). Promoter analysis, inhibitor studies, and order-of-addition experiments demonstrated that TNF-α induction by 22R-HC and 9cRA occurs by a novel two-step process. The initial step involves 22R-HC-dependent induction of TNF-α mRNA, and intracellular accumulation of TNF-alpha 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-alpha protein occurs via a separable RXR-dependent step that requires de novo transcription and protein synthesis.

Furthermore, the RXR-dependent secretory event can be mimicked by agents that induce monocytic differentiation like phorbol esters that culminate in RXR activation by a pathway that does not require exogenous ligand. In this context, RXR was also shown to be a down stream target of the protein kinase C (PKC) signal transduction cascade, that results in the activation of RXR and the induction of secretory factor(s) which facilitate secretion of LXR-derived TNF-α. These studies have provided evidence that should help to expand the currently known role for LXR in monocyte biology and have furthermore identified a new role for RXRs in promoting the secretion of soluble factors like cytokines. Furthermore, in light of reports that show LXR activity promotes a reduction in atherosclerosis, it stands to reason that this regulatory circuit of LXR-dependent production of TNF-α from monocytes would similarly contribute to the attenuation of atherosclerosis *in vivo*.

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ABBREVIATIONS

AA	Amino acids
AF-1, -2	Activation domain-1, -2
Apo E	Apolipoprotein E
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CBP	CREB-binding protein
CMV	Cytomegalovirus
Cys	Cysteine
DBD	DNA-binding domain
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleoside triphosphates
DR	Direct repeat
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electromobility shift assay
ER	Estrogen receptor
FXR	Farnesoid X receptor
GR	Glucocorticoid receptor
GRIP1	Glucocorticoid receptor-interacting protein
HDL	High-density lipoprotein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRE	Hormone response element
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IPTG	Iso-propyl-thiogalactoside
Kb	Kilobase
KDa	Kilodaltons
LBD	Ligand-binding domain
LDL	Low-density lipoprotein
LXR	Liver X receptor
LXRE	LXR response element
MMTV	Mouse mammary tumour virus
MR	Mineralocorticoid receptor
N-CoR	Nuclear receptor corepressor
NP-40	Nonidet P-40
ONPG	*o*-nitrophenyl-β-D-galactopyranoside

PBS	Phosphate-buffered saline
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonylfluoride
PPAR	Peroxisome proliferator activated receptor
PPRE	PPAR response element
22R-HC	22(*R*)-hydroxycholesterol
22S-HC	22(*S*)-hydroxycholesterol
RAR	Retinoic acid receptor
RLU	Relative light units
RNA	Ribonucleic acid
RXR	9-*cis* retinoic acid receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMRT	Silencing mediator for retinoid and thyroid-hormone receptors
SRC-1	Steroid receptor co-activator-1
SREBP	Sterol response element binding protein
SV40	Simian virus 40
TBP	TATA-binding protein
TE	Tris-EDTA
TFIIA,B,D,E,F,H	Transcription factor IIA, IIB, IID, IIE, IIF, IIH
TK	Thymadine kinase
TNF-α	Tumour necrosis factor-α
TR	Thyroid hormone receptor
Tris	Tris(hydroxymethyl)aminoethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1: Introduction

General transcription

The spatial and temporal control of gene expression is an essential process in the maintenance and continuation of life. Differential gene regulation controls much of development, homeostasis, and indeed, helps to determine the point at which apoptosis takes place. The classical mechanism by which most eukaryotic genes that encode messenger ribonucleic acid (mRNA) are transcribed is through the RNA polymerase II (RNAP) machinery. RNAP is a large complex comprised of basal transcription factors (e.g. TATA-binding protein (TBP), transcription factor (TF) IID, TFIIB, and TFIIH) that assemble on core elements in the promoter regions of target genes (i.e. the TATA-box located approximately 20 base pairs upstream of the transcription start site) and initiates the transcription of DNA into RNA (reviewed by Roeder, 1996 and Greenblatt, 1997). Whether or not a mRNA-encoding gene becomes expressed is largely determined by the cellular conditions or metabolic demands of the cell. The control of RNA transcription often occurs by modulating the activity of the RNAP machinery either by modifications of local chromatin structure which in turn affects the assembly of basal transcription factors, the recruitment of these factors, or the stability of the basal transcription machinery, and hence its ability to initiate transcription (reviewed by Roeder, 1996 and Greenblatt, 1997).

In many genes whose expression pattern is tightly controlled either spatially or temporally as opposed to constitutive expression, these genes are often induced in response to a stimulus, either a) chemical, such as in the case of a developmental morphogen, b) environmental, as in the changes that occur in cellular alkalinity, or c) in response to changes in metabolic demand in the cell, such as changes in glucose utilization.

In these specific instances, the stimulus affects gene expression by modulating the activity of another set of transcription factors, referred to generally as activators if they augment gene expression or repressors if they diminish gene expression. Activators or repressors bind to regions in the promoters of target genes either upstream of the transcription start site or downstream, but usually not within the coding sequence of the particular gene. The regions to which these accessory transcription factors bind are thus appropriately referred to as enhancer or repressor regions. The general mechanism of action of this subset of transcription factors involves binding of the *trans*-acting factor to its cognate *cis*-acting DNA elements, and either directly interacting with the RNAP2 machinery or acting as a scaffold upon which other coregulatory proteins bind. This larger complex of proteins in turn interacts with the assembled basal transcription machinery and either facilitates activation or repression of transcription (reviewed by Greenblatt, 1997). See Figure 1 for a generalized model of enhancer-mediated activation of RNAP2.

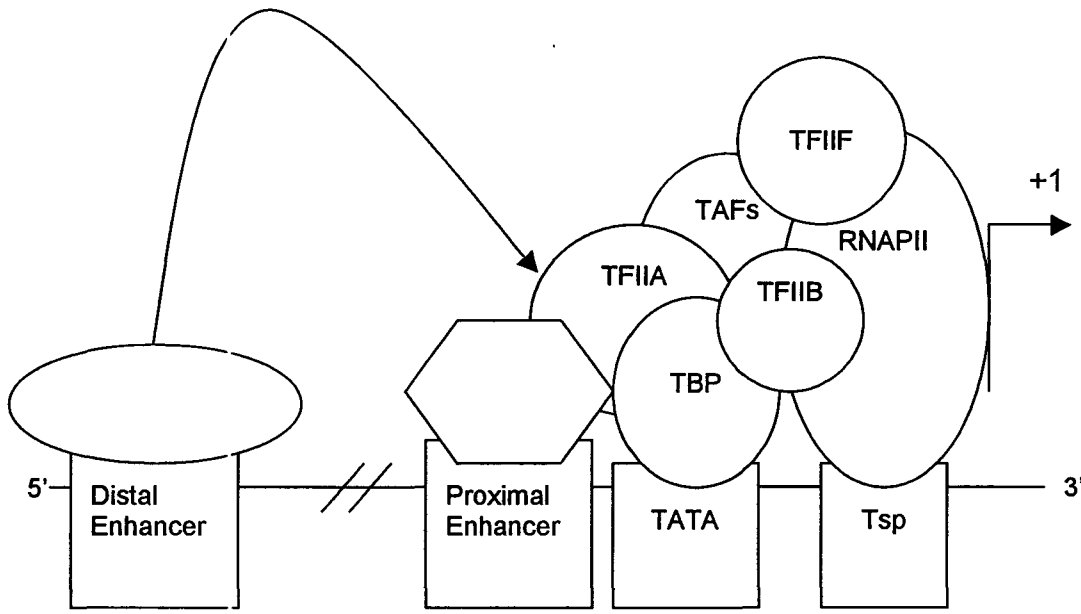

Figure 1. The generalized model of enhancer-mediated activation of RNA polymerase II complex. Adapted from Lemon and Tjian, 2000.

Nuclear Hormone receptors: Introduction

Nuclear hormone receptors are a superfamily of these enhancer region binding transcription factors. Nuclear hormone receptors are ligand-activated transcription factors that upon binding specific ligands become transcriptionally-competent, bind to specific enhancer response elements, and interact with the basal RNA polymerase II apparatus to activate transcription (reviewed by Giguere, 1999). Nuclear hormone receptors are almost exclusively nuclear-localized and are bound to specific hormone receptor response elements (HREs) that upon ligand engagement induce transactivation of target genes. Some receptors such as thyroid hormone receptor (TR) and retinoid X receptor (RXR) can bind as monomers, homodimers, or heterodimers (reviewed by Giguere, 1999 and Mangelsdorf and Evans, 1995). Furthermore, ligands for nuclear hormone receptors tend to be highly lipophilic and as such can pass freely through the plasma membrane and can be easily transported to the nucleus whereupon it binds the hormone receptor and activates it.

The classic nuclear steroid hormone receptors such as glucocorticoid (GR), mineralocorticoid (MR), and estrogen (ER) bind to HREs as monomers, and their ligands are derived from endocrine sources along the hypothalamic-pituitary axis (reviewed by Wilson and Foster, 1992). After these ligands have been generated, they circulate the body and arrive at target tissues, diffuse into them, and bind their cognate receptors with affinities ranging between 0.01 to 10nM (reviewed by Chawla et al., 2001a). The processes these hormones regulate are primarily developmental, including sexual differentiation, reproduction, elements of carbohydrate metabolism, and electrolyte

balance (reviewed by Chawla et al., 2001a). In contrast, the lipophilic ligands for nuclear hormone receptors that heterodimerize with RXR, such as the peroxisome proliferator-activated receptor (PPAR), retinoic acid receptor (RAR), and bile acid receptor (FXR) are derived primarily from dietary sources, and their activity does not appear to be subject to negative feedback inhibition like those receptors that comprise the classic nuclear hormone receptors as described above. The relative binding affinity of these latter receptors for their cognate ligands is comparatively much less ranging from 1 to 10μM (reviewed by Chawla et al., 2001a). Furthermore, the emergent role that these latter receptors play in general physiology is to serve as lipid sensors, and the processes in which they participate are usually positive, feed-forward, metabolic cascades that control genes involved in lipid homeostasis and related processes.

Nuclear Hormone Receptors: Structure and Mechanism of Action

Nuclear hormone receptors have relatively conserved modular domain structures. Each receptor contains well-conserved DNA-binding domains localized towards the amino-terminus and well-conserved carboxy-terminal ligand-binding domains. Both DNA- and ligand-binding specificity between members of the receptor family arise from subtle differences in primary amino acid sequence that serve to recognize and bind enhancer region DNA sequences and the receptor's cognate ligands (see Figure 2 for a schematic representation of the general structure of a nuclear hormone receptor). The amino-terminus of most nuclear hormone receptors bears a transcriptional activation domain, referred to as an autonomous ligand-independent transactivation function (AF-1)

domain, whilst at the extreme carboxy-terminus a second transactivation domain is found (AF-2). These regions participate in activation of the RNAP complex in concert with a gamut of other steroid receptor coactivator proteins (reviewed by Leo and Chen, 2000). In addition, these AF domains have been shown to potentiate transactivation in their isolated states, AF-1 being able to transactivate in a ligand-independent manner while AF-2 transactivates almost exclusively in a ligand-dependent fashion (Schulman et al., 1997 and Willy and Mangelsdorf, 1997). Interestingly, AF-1 and AF-2 can also synergize and robustly induce transactivation. This is thought to occur as a result of ligand-dependent interactions between the N- and C-termini facilitated perhaps by the host of co-factors that become recruited (reviewed by Mangelsdorf and Evans, 1995).

The classic mechanism of nuclear hormone receptor activation is one of a receptor assembled on its HRE in the absence of bound ligand being transcriptionally inactive. This repressive state is maintained by the association of nuclear receptor corepressor proteins such as SMRT/TRAC and NCoR/RIP13 with the receptor (reviewed by Giguere, 1999). Upon ligand engagement, conformational changes in the receptor take place dissociating the corepressor proteins, facilitating tight binding to the cognate HRE and the assembly of coactivator molecules, thus permitting transactivation. These nuclear receptor-specific coactivator molecules, such as steroid receptor cofactor (SRC)-1 and TIF2/GRIP1 (Onate et al., 1995, Smith et al., 1996, and Kamei et al., 1996) interact with hormone receptors in a ligand-dependent manner and serve to form a scaffold of proteins that in turn recruit other coregulatory molecules that associate with RNAP2 like CBP/p300 or PCAF, two factors that contain intrinsic histone acetyltransferase (HAT)

activity. These latter molecules remodel local chromatin structure by direct histone acetylation and facilitate the assembly of the basal RNAP apparatus (Zamir et al., 1997, Torchia et al., 1997, Heinzel et al., 1997, reviewed by Leo and Chen, 2000 and Pazin et al., 1997).

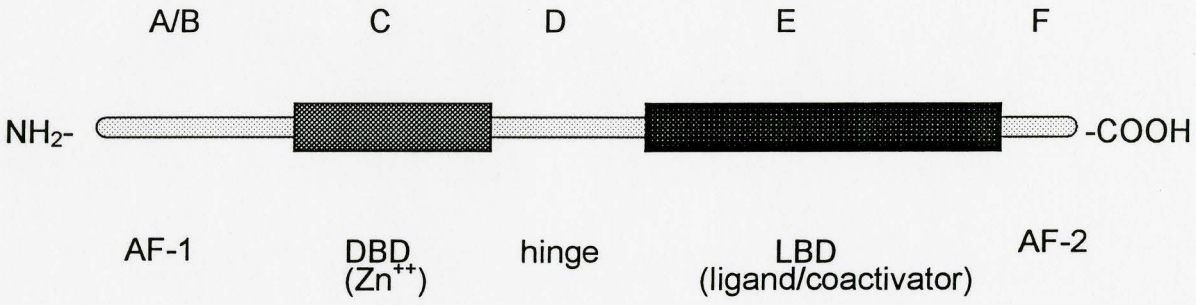

Figure 2. The generalized structure of nuclear hormone receptors.
Letters correspond to domains. A/B-domain is responsible for transactivation and contains the AF-1 domain, C-domain contains the DNA-binding domain (DBD) characterized by zinc-finger DNA-binding motifs, D-domain refers to a hinge region that provides structure, E-domain corresponds to the ligand-binding domain (LBD), and F-domain is also required for transactivation containing the AF-2 domain. (Adapted from Giguere, 1999)

Nuclear Hormone Receptor Response Elements

As mentioned, nuclear hormone receptors modulate gene expression of target genes by binding to hormone response elements (HREs) in the enhancer regions of target genes. The types of DNA sequences nuclear hormone receptors recognize in the promoters of target genes are usually characterized by a hexanucleotide repeat (5'-AGGTCA-3', being the canonical sequence) separated by an intervening sequence of variable length ranging from 1 to 5 nucleotides (reviewed by Mangelsdorf and Evans, 1995). The orientation of these repeats, either direct, inverted, or everted, in addition to the length of intervening sequence, helps to confer receptor-type specificity and the modality of receptor binding (e.g. as monomers, or homo/heterodimers) (reviewed by Mangelsdorf and Evans, 1995, Chawla et al., 2001a, and Desvergne and Wahli, 1999). However, many of the HREs characterized in nuclear hormone receptor target genes thus far do not possess this canonical sequence and are variations of this canonical sequence some with considerable sequence degeneracy (Zhang et al., 2001, Laffitte et al., 2001a, Repa et al., 2000b, Tontonoz et al., 1998, and Chawla et al., 2001b). The location of different HREs within a given promoter region is also variable, ranging from being close to the core TATA-box promoter, such as in the HRE corresponding to LXR:RXR in *abc1* (-70bp), or several kilobases away, as is the situation with the PPAR:RXR HRE in *acyl-coA oxidase* (-2943bp) (Costet et al., 2000 and Zhang et al., 1992, respectively).

The class of nuclear hormone receptors that heterodimerize with RXR bind to response elements that are arranged in a direct repeat configuration (DR) with an intervening sequence ranging between 0 and 5 base pairs. For example, a response

element recognized by the PPARs contains two hexanucleotide half sites separated by 1 base pair of intervening sequence and is thus referred to as a DR1. Other members of the nuclear hormone receptor superfamily bind to other configurations such as DR3, for the VDR:RXR heterodimer, DR4, for the thyroid receptor (TR):RXR heterodimer and the LXR:RXR heterodimer, and DR5 for the RXR:RAR heterodimer (reviewed by Mangelsdorf and Evans, 1995). As described above, the characterization of target genes for these receptors has found that in many cases, the canonical sequence ascribed to the half-sites is not found *in vivo*. For example, the gene that encodes for liver HMG-CoA synthase, a protein involved in ketogenesis, is a target for transactivation by PPAR (Rodriguez et al., 1994). When compared to a consensus DR1 (5'-AGGTCA$(x)_1$AGGTCA-3'), the HMG-CoA PPAR response element is 5'-AGACCTtTGGCCC-3' (Rodriguez et al., 1994). Similarly, the response element to which LXR:RXR bind in the transactivation of the *abc1* gene (Costet et al., 2000) is an imperfect DR4 when compared to the canonical DR4. The characterized LXR response element for ABC-1 is 5'-AGGTTActacCGGTCA-3', a significant departure from the canonical sequence originally defined for nuclear hormone receptors (reviewed by Giguere, 1999).

Physiological Functions of Nuclear Hormone Receptors

The principle physiological role of nuclear hormone receptors is in the expression of genes essential in sexual development and in general intermediary metabolism. Table 1 is a sample of specific nuclear hormone receptors and their associated functions in metabolism. However, some receptors, like PPARγ, have been shown to have essential roles in adipocyte differentiation (Rosen and Spiegelman, 2001, Moore et al., 2001 and Fajas et al., 1999). Other receptors have been shown to play roles in modulating inflammation, specifically inhibiting the inflammatory programme in leukocytes in response to pro-inflammatory stimuli (Ricote et al., 1998, Huang et al., 1999, and Na et al., 1999). In the context of inflammation control, RXR, PPARα, and PPARγ are implicated by virtue of their abilities to interfere with nuclear factor (NF)-κB signaling in response to an inflammatory stimulus (Ricote et al., 1998, Huang et al., 1999, and Na et al., 1999). Indeed, many of the anti-inflammatory effects of hyperlipidemic drugs and glucocorticoids have been attributed to their ability to attenuate NF-κB signaling, since they are agonists for PPARα or require RXR as a heterodimerization partner. Furthermore, the essential roles of receptors such as PPARγ and RXR in development are illustrated by attempts by other groups to generate gene knockout animals of PPARγ and RXR. Disruption of either of these genes that encode these receptors results in embryonic lethality. Consequently, there are many other functions for nuclear hormone receptors that are outside of their classical roles in metabolism.

Table 1. Generalized function of certain nuclear hormone receptors in intermediary metabolism (adapted from Giguere, 1999, Desvergne and Wahli, 1999, and Repa and Mangelsdorf, 2000)

Receptor	Metabolic Function	Reference
Peroxisome Proliferator-Activated Receptor (PPAR)	α-isoform: β-oxidation γ-isoform: glucose transport δ-isoform: placentation, adiposity	Rosen and Spiegelman, 2001, Rosen and Spiegelman, 2000; Spiegelman et al., 2000, and Barak et al., 2002
Farnesoid X Receptor (FXR)	bile acid homeostasis	Sinal et al., 2000
Thyroid hormone receptor (TR)	increased metabolic rate	Yen, 2001
Liver X Receptor (LXR)	cholesterol biosynthesis and traffic	Peet et al., 1998a, Repa et al., 2000a, and Chawla et al., 2001b

Liver X Receptor: Overview, Structure, and Expression Profile

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 (Willy et al., 1995 and Peet et al., 1998a). LXRα was cloned originally by screening a human liver λgt11 cDNA library using the DNA-binding domain of human RARα (Willy et al., 1995). The open reading frame for LXRα gives rise to a translated protein product of 447 amino acids, approximately 49,000 kDa. Human LXRα shares close similarity to the rat nuclear hormone receptor RLD-1 and human NER or UR (Apfel et al., 1994; Shinar et al., 1994, and Song et al., 1994). Bearing 92% sequence homology to RLD-1, LXRα is believed to be the human homologue of RLD-1 (Willy et

al., 1995). By comparison to LXRα, LXRβ is virtually identical, but is however larger having 461 amino acids (Shinar et al., 1994). See Figure 3 for a schematic representation of the structures of LXRα, LXRβ, and RLD-1.

Based on the amino acid sequences for both LXRα and LXRβ, and the predicted functional domains contained therein, LXRs possess the same modular structure described above like other nuclear hormone receptors (Willy et al., 1995, Shinar et al., 1994, and Desvergne and Wahli, 1999). DNA-binding specificity is conferred by folds and structures found in the C-domain (DBD) while ligand-binding specificity is conferred by the carboxy-terminal LBD.

Northern blot analysis has shown that LXR mRNA expression is found predominantly in tissues with high metabolic activity such as kidney, intestine, spleen, adrenals, monocytes, and, as its name suggests, liver (Willy et al., 1995 and Venkateswaran et al., 2000b). In the specific case of monocytes, it has been shown that LXR expression increases over the course of monocytic differentiation into macrophages and in an autoregulatory manner in response to ligand activation (Laffitte et al., 2001b).

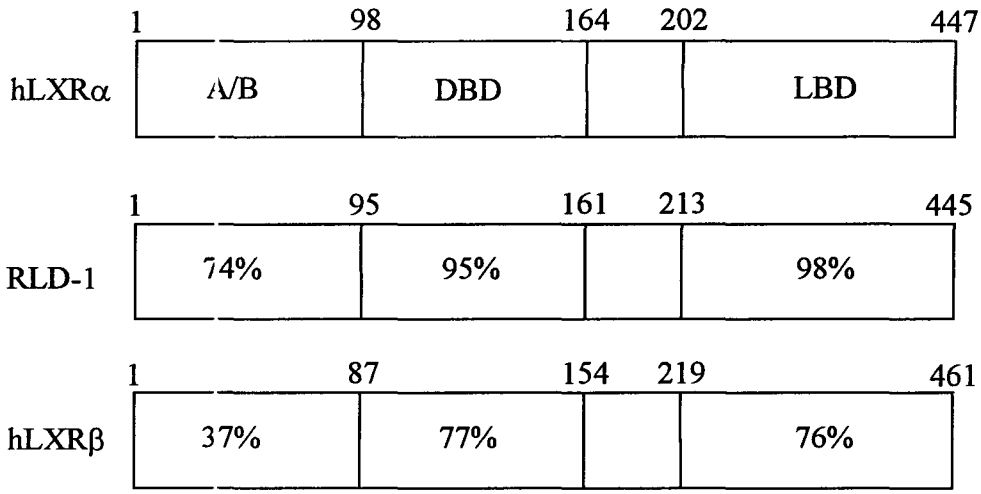

Figure 3: Domain homology comparison between human LXRα and related homologues. Percent sequence identity between modular domains of human LXRα, rat RLD-1, and human LXRβ are provided (Willy et al., 1995, Apfel et al., 1994, and Shinar et al., 1994). Sequence comparisons were carried out using DNAStar Software Package.

LXR/RXR: A "Permissive Heterodimer" and the "Phantom-Ligand" Effect

While ligand-binding is essential to activate nuclear hormone receptors, in some cases however, a nuclear hormone receptor dimer can be activated by a ligand for one of the receptors even though the ligand for the cognate receptor is absent, the so-called "phantom-ligand effect" (Schulman et al., 1997). This effect has been documented exclusively in heterodimers containing RXR, and LXR/RXR heterodimers can be activated in this fashion. Activation of these types of heterodimers by ligands for either RXR or the dimerization partner is thought to have its origin in the nature of the conformational changes induced within the heterodimer that permit transactivation even in the absence of one of the ligands for either partner. The structural basis behind the phantom ligand effect is that upon ligand-binding to RXR, which is positioned 5' in the heterodimer, the conformational changes that occur in RXR induces conformational changes in the AF-1 domain of the 3' dimerization partner, which results in transactivation (Schulman et al., 1997). This characteristic of some RXR heterodimers not only expands the number of potential signals that can induce expression of genes under the control of these particular transcription factors but serves to assist in identifying candidate target genes for different heterodimer combinations if a selective ligand to either of the dimer partners is used, and the other dimerization partner is known. Consider the transactivation of the LXR target gene *abc1*, the gene that encodes the ATP-binding cassette protein (ABC)-1 involved in cholesterol trafficking from monocytes and implicated in Tangier's disease (Chawla et al., 2001a and Lu et al., 2001). Studies have shown that activators for either LXR or RXR are sufficient to induce expression of *abc1*

(Repa et al., 2000a and Venkateswaran et al., 2000a). These types of heterodimers, which can be activated by ligands for either receptor, are collectively referred to as is "permissive" heterodimers. Peroxisome proliferator-activated receptor (PPAR)γ:RXR and farnesoid receptor (FXR):RXR heterodimers are also permissive heterodimers (reviewed by Aranda and Pascual, 2001). Not all RXR heterodimers are permissive however. Some heterodimer combinations cannot be activated when a single ligand for either RXR and the other dimerization partner are present. These dimers are referred to as "non-permissive" heterodimers. PPARα:RXR are examples of these types of dimers, and thus require activators for both PPARα and RXR for transactivation (reviewed by Desvergne and Wahli, 1999). Similarly, retinoic acid receptor (RAR):RXR and vitamin D receptor (VDR):RXR dimers are also non-permissive heterodimers (reviewed by Aranda and Pascual, 2001).

LXR response elements, activators, and target genes

As previously described, LXR transactivates target genes through heterodimerization with its cognate receptor RXR (Willy et al., 1995). Furthermore, LXR/RXR heterodimers facilitate gene expression primarily through binding to response elements in a DR4 arrangement, with 5'-AGGTCA-3' as the half-site consensus sequence (Willy et al., 1995 and Willy and Mangelsdorf, 1997). However, as discussed above, many LXR response elements (LXREs) do not always possess this consensus sequence (Refer to Table 2 for LXREs mapped in target genes). In transactivation, LXR/RXR heterodimers were found to have a specific polarity with RXR occupying the 5' half-site

and LXR occupying the 3' half-site (Willy and Mangelsdorf, 1997). Interestingly, this polarity is shared with VDR, TR, and RAR heterodimers containing RXR (reviewed by Mangelsdorf and Evans, 1995).

Table 2. LXREs characterized in LXR target genes.

Gene	LXRE	Reference
SREBP-1c	GGGTTA (ctgg) CGGTCA	Repa et al., 2000b
ApoE	GAATCA (ctta) AGGTCA	Laffitte et al., 2001a
ABC1	AGGTTA (ctac) CGGTCA	Costet et al., 2000
Cyp7a	TGGTCA (ctca) AGTTCA	Lehmann et al., 1997
LXR	TGACCT (caag) TGATCC	Laffitte et al., 2001b
Fatty acid synthase (FAS)	GGGTTA (ctgc) CGGTCA	Joseph et al., 2002
Lipoprotein lipase (LPL)	GGTTTA (cact) GATTTA	Zhang et al., 2001
CETP	GGGTCA (ttgt) CGGGCA	Luo and Tall, 2000

With the finding that LXR plays a pivotal role in cholesterol homeostasis (Peet et al., 1998a and reviewed by Peet et al., 1998b), the discovery that LXR ligands are in fact cholesterol derivatives was not surprising. Referred to colloquially as "oxysterols", these activators are oxygenated derivatives of cholesterol that are formed through steroid and bile acid synthetic pathways (reviewed by Schroepfer, Jr., 2000 and Wolf, 1999). Ligand-binding studies have shown that the most potent oxysterol activators of LXR are 22(*R*)-hydroxycholesterol (22(*R*)-HC), 24*(S)*,25 epoxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (Janowski et al., 1996, Janowski et al., 1999, and Fu et al., 2001).

Table 3. Oxysterol activators of LXR. (adapted from Janowski et al., 1999) CH=cholesterol, HC=hydroxycholesterol.

Oxysterol	EC_{50} for LXRα (μM)	EC_{50} for LXRβ (μM)
24(S), 25-epoxy CH	4	3
24(S)-HC	4	3
22(R)-HC	5	3
25-HC	7	<10% at 40μM
7(α)-ol,24(*S*),25-EC	8	6

See Figure 4 for a schematic for the structures of oxysterols that can interact with LXRs. These compounds have been found free in serum and in association with atherogenic oxidized low-density lipoprotein (oxLDL) particles (Dixon et al., 1970 and Kandutsch et al., 1978).

Physiological Roles of LXR

Lxr-/- mice and further studies have shown that LXR is primarily a cholesterol sensor that regulates the expression of genes in the liver, which influence cholesterol metabolism and homeostasis in a number of different tissues (Peet et al., 1998a). The first LXR target gene identified was the gene encoding the enzyme cholesterol 7α hydroxylase, *cyp7a* (Lehmann et al., 1997 and Peet et al., 1998a). CYP7A is the enzyme that catalyzes the rate-limiting step in bile acid biosynthesis that results in the conversion of cholesterol into bile acids such as cholic acid and chenodeoxycholic acid, which are subsequently excreted from the body via the large intestine (See Figure 5). Recently, LXRs have been shown to regulate the expression of additional ATP-binding cassette proteins (ABCG5 and ABCG8) in the liver and intestines (Repa et al., 2002). These latest

ABC proteins have been implicated in the pathogenesis of the autosomal recessive disorder sitosterolemia, and the transcriptional regulation of these proteins by LXR further points to the fundamental role of this receptor hepatic cholesterol trafficking. Other LXR target genes to date include fatty acid synthase (FAS), lipoprotein lipase (LPL), and cholesteryl ester transfer protein (CETP), all of which are involved in mediating cholesterol biosynthesis and trafficking illustrating the essential role of LXRs in cholesterol homeostasis and supporting the *in vivo* data collected from *lxr* -/- mice demonstrating that cholesterol metabolism is severly impaired in the absence of LXR expression (Joseph et al., 2002, Zhang et al., 2001, Luo and Tall, 2000, Peet et al., 1998a and Repa et al., 2000a).

The pivotal role of LXR in mediating cholesterol and fatty acid homeostasis comes from the identification that sterol regulatory element-binding protein-1c (SREBP-1c), a key transcription factor that regulates expression of genes essential for fatty acid biosynthesis, is also subject to transcriptional regulation by LXR (Repa et al., 2000b, Yoshikawa et al., 2001, and DeBose-Boyd et al., 2001). SREBP-1c-activated genes include acetyl-CoA synthetase, acetyl-CoA carboxylase, FAS, and glycerol phosphate acyltransferase, genes that are essential to lipid biosynthesis and fatty acid esterification (reviewed by Edwards et al., 2002). Consequently, the regulation of SREBP-1c by LXR demonstrates further the intimate role that this receptor plays in the coordinate regulation of not only cholesterol metabolism but also lipid homeostasis.

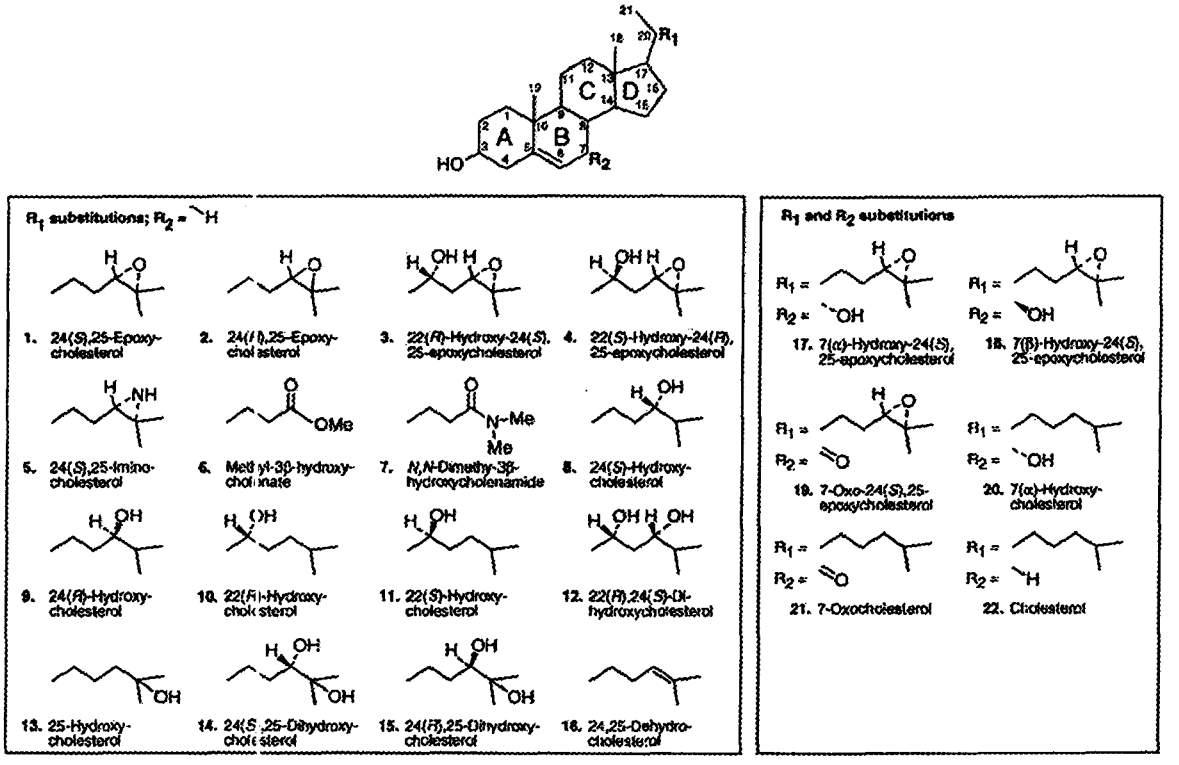

Figure 4. Structures of naturally occurring oxysterols that interact with LXR. (adapted from Janowski et al., 1999)

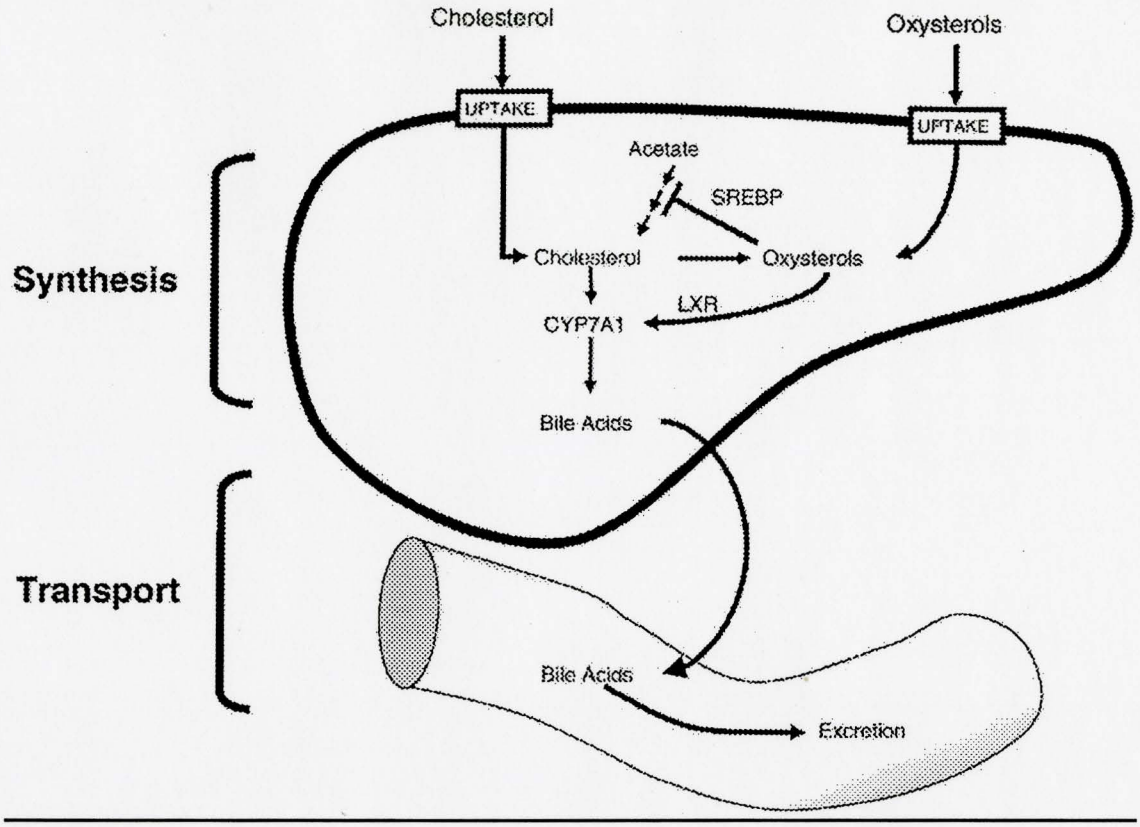

Figure 5. Mechanism for regulation of CYP7A and its relation to cholesterol synthesis and bile acid turnover. Adapted from Bjorkhem and Diczfalusy 2002.

LXR: Role in monocyte biology and atherosclerosis

In addition to having a fundamental role in hepatic and peripheral cholesterol metabolism and transport, intriguing work has shown that LXRs also plays a fundamental role in macrophage biology. LXR has been shown to regulate cholesterol efflux from lipid-loaded macrophages and to regulate the expression of lipoprotein lipases that promote hydrolysis of triglycerides at the plasma membrane (Costet et al., 2000, Venkateswaran et al., 2000c, Venkateswaran et al., 2000b, Laffitte et al., 2001b, Laffitte et al., 2001a, and Zhang et al., 2001). Modulation of cholesterol efflux is achieved by LXR-dependent induction of genes encoding the aforementioned ATP-binding cassette proteins ABC-1 and ABCG1, which encode plasma membrane-associated reverse cholesterol transport proteins that mediate cholesteryl ester and free cholesterol efflux from monocytes and lipid-loaded macrophages (Schwartz et al., 2000, Costet et al., 2000, Venkateswaran et al., 2000c, and Venkateswaran et al., 2000a). Upon removal from the macrophage, this cholesterol is subsequently incorporated into high density lipoprotein (HDL) and into apolipoprotein E (apoE)-containing particles, transported back to the liver where it can be converted to bile acids and excreted (Peet et al., 1998a and Willy et al., 1995). In fact, LXR has been shown to also control the expression of apoE, a 34kD protein, which is also a surface constituent of plasma lipoproteins and a high-affinity ligand for the LDL receptor in the liver (Laffitte et al., 2001a). The role of apoE here is to mediate hepatic uptake of chylomicron remnants, of very low-density lipoprotein (VLDL), and even of HDL in order to clear remnant plasma lipoproteins (reviewed by Weisgraber, 1994).

The pivotal role of LXRα in regulating reverse cholesterol transport in macrophages is particularly relevant since lipid accumulation in these cells is pivotal to the etiology and pathogenesis of atherosclerosis. Atherosclerosis is a condition that affects millions of people worldwide (Plutzky, 1999). This disorder is marked by a progressive infiltration of monocytes into the arterial intimal space of the coronary, carotid, and other Great arteries (reviewed by Lusis, 2000). In these areas, particularly at points where vascular branching occurs, one finds substantial turbulent blood flow, and this facilitates monocyte adhesion to integrins on the vascular endothelium and subsequent monocytic diapedesis. Once resident within arterial intimae, monocytes accumulate oxidized low-density lipoprotein (oxLDL), differentiate into lipid-loaded macrophages, so-called "foam cells", and contribute to the characteristic fatty streak lesion of an early atheroma. These early lesions are clinically silent but provide the foundation upon which more complex lesions develop which can result in clinical manifestations such as plaque rupture, thrombus formation, and even myocardial infarction (reviewed by Lusis, 2000).

The accumulation of oxLDL in these cells is mediated primarily by CD36 and SR-A. CD36 is a plasma membrane protein and a member of the scavenger receptor class B family. It is also the receptor for the hemostatic modulator thrombospondin-1 (reviewed by Nicholson et al., 2000). Interestingly, CD36 has been shown to be a target for PPARγ-dependent transactivation, thereby providing a further link between atherogenesis and nuclear hormone receptors (Tontonoz et al., 1998 and Nagy et al., 1998). By promoting a reduction in intracellular cholesterol stores and lipid accumulation

in macrophages, LXRα is considered to participate in a series of transcriptional cascades that are anti-atherogenic. An intriguing study has provided evidence to this end by demonstrating that upon lipid loading of monocytes mediated by CD36, LXRα is activated and subsequently induces expression of ABC-1 in a coordinated regulatory cascade that involves PPARγ-dependent induction of *lxrα* expression and subsequent LXRα-dependent induction of *abc1*, which correlated with a reduction of atherosclerosis in *ldlR -/-* mice (Chawla et al., 2001b). These findings provided evidence of an exquisite mechanism available to macrophages to remove excess cholesterol and to maintain cholesterol balance that is mediated by nuclear hormone receptors. The hypothesis that LXR activity in monocytes is anti-atherogenic has been lent further support by recent studies showing that selective agonists of RXR and LXR significantly reduce lesion size and the progression of atherosclerosis in *apoE -/-* animals (Claudel et al., 2001 and Joseph et al., 2002). In these reports, the reduction in atherosclerotic lesion size and prevalence was attributed to the induction *abc1* and the direct promotion of reverse cholesterol efflux from intimally resident foam cells.

OxLDL, oxysterols, their effects on cytokine production

Exposure of monocytes and other cell types to oxLDL and its constituent oxysterols and oxidized lipids exert many other effects on gene expression (reviewed by van Reyk and Jessup, 1999). These effects include an alteration of gene expression not necessarily involved with the regulation of cellular lipid content and homeostasis. For instance, studies have shown that the expression of cytokines such as (TNF-α),

interleukin (IL)-1α, IL-1β, IL-6, IL-8, and platelet-derived growth factor (PDGF) are differentially modulated in macrophages in response to oxLDL or various oxysterols (Hamilton et al., 1990, Jovinge et al., 1996, Hsu et al., 2001, and Mikita et al., 2001). Similarly, other studies have demonstrated diverse effects of oxLDL and oxysterols in vascular endothelial cells and smooth muscle cells (Niemann-Jonsson et al., 2000, Takei et al., 2001, and Huang et al., 2001). The cohort of cytokines described above can 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 (reviewed by Lusis, 2000). They can also promote T-cell infiltration and tissue necrosis that can arguably contribute to the late stage necrotic core within complex atherosclerotic lesions (Glass and Witztum, 2001). Nuclear hormone receptors have been implicated in mediating the production of some of these cytokines. PPARα agonists have been shown to induce MCP-1 and IL-8 (Lee et al., 2000). Conversely, PPARγ has been shown to downregulate cytokine production in macrophages in response to an inflammatory stimulus such as bacterial lipopolysaccharide (LPS) (Chawla et al., 2001b).

Tumour Necrosis Factor (TNF)-α

Of these cytokines, TNF-α is perhaps the one cytokine whose effects are most widely studied and understood. TNF-α was initially discovered in 1975 by Carswell as one of the molecules induced upon exposure of macrophages to bacterial endotoxin and named for its ability to induce hemorrhagic tumor atrophy and necrosis in mice (Carswell

et al., 1975). Since the initial discovery of TNF, a substantial body of literature has been devoted to the study of this cytokine and its effects both *in vitro* and *in vivo*.

TNF-α is produced primarily by activated macrophages, though recently studies have shown that it is also produced by adipose, endometrial, and smooth muscle tissue (reviewed by Beutler and Cerami, 1989, Hotamisligil et al., 1993, von Wolff et al., 1999, and Yamakawa et al., 1999, respectively). The gene for TNF-α is found on chromosome 6 with its other TNF ligand family member lymphotoxin-α (LTα) within the locus that contains the major histocompatibility complex (reviewed by Ware et al., 1996). The gene for TNF-α was cloned originally in 1984 (Pennica et al., 1984), and the proximal promoter region identified and subcloned in 1993 (Takashiba et al., 1993). The gene for TNF-α contains an open reading frame that gives rise to translated product of 26kDa, which is subsequently proteolytically cleaved by the metalloprotease TNF-α converting enzyme (TACE) yielding a 17kDa molecule that is secreted from TNF-α-producing cells (Kriegler et al., 1988 and Moss et al., 1997). In solution, the 17kDa monomers assemble to form 56kDa trimers that comprise fully functional TNF-α *in vivo*.

The effects of TNF-α on target tissues are pleiotropic. TNF-α has been shown to be involved in host defense in response to infection, to elicit anti tumour activity, and to modulate metabolic function (reviewed by Beutler, 1999). In the context of inflammation, TNF-α is the first cytokine produced by monocytes upon exposure to bacterial endotoxins, the so-called "acute phase response": a process that results in the subsequent production of IL-1β, and IL-6 from activated monocytes (reviewed by Dinarello, 2000).

The control of TNF-α expression is stringently controlled both transcriptionally and translationally, since one of the potential pitfalls of excessive TNF-α production is the activation of systemic inflammatory responses such as what is observed in sepsis (reviewed by Dinarello, 2000).

Apart from inflammation, TNF-α is also involved in mediating apoptosis. Serving as a paracrine factor in this context, secreted TNF-α has been shown to interact with specific TNF receptors on target cells, of which there are two: TNFR1 (p55) and TNFR2 (p75). The specific engagement of TNF-α with TNFR1 (p55) is primarily associated with the induction of apoptosis since the signal transduction pathways induced by engagement eventually lead to activation of apoptosis-associated caspase-8 and caspase-10, and the subsequent activation of caspase-3 and poly(ADP-ribose) polymerase (PARP) inactivation (reviewed by Szatmary, 1999). TNF signaling through TNFR2 is thought to result in activation of NF-κB through the phosphorylation of the inhibitor of κB (IκB) on serine 32 and serine 36 by IκB kinase (IKK). Phosphorylation of IκB results in its ubiquitination and subsequent proteolysis by the 26S proteosome (Baeuerle and Baltimore, 1988 and reviewed by Szatmary, 1999). See Figure 5 for a schematic of TNF signaling through its cognate receptors.

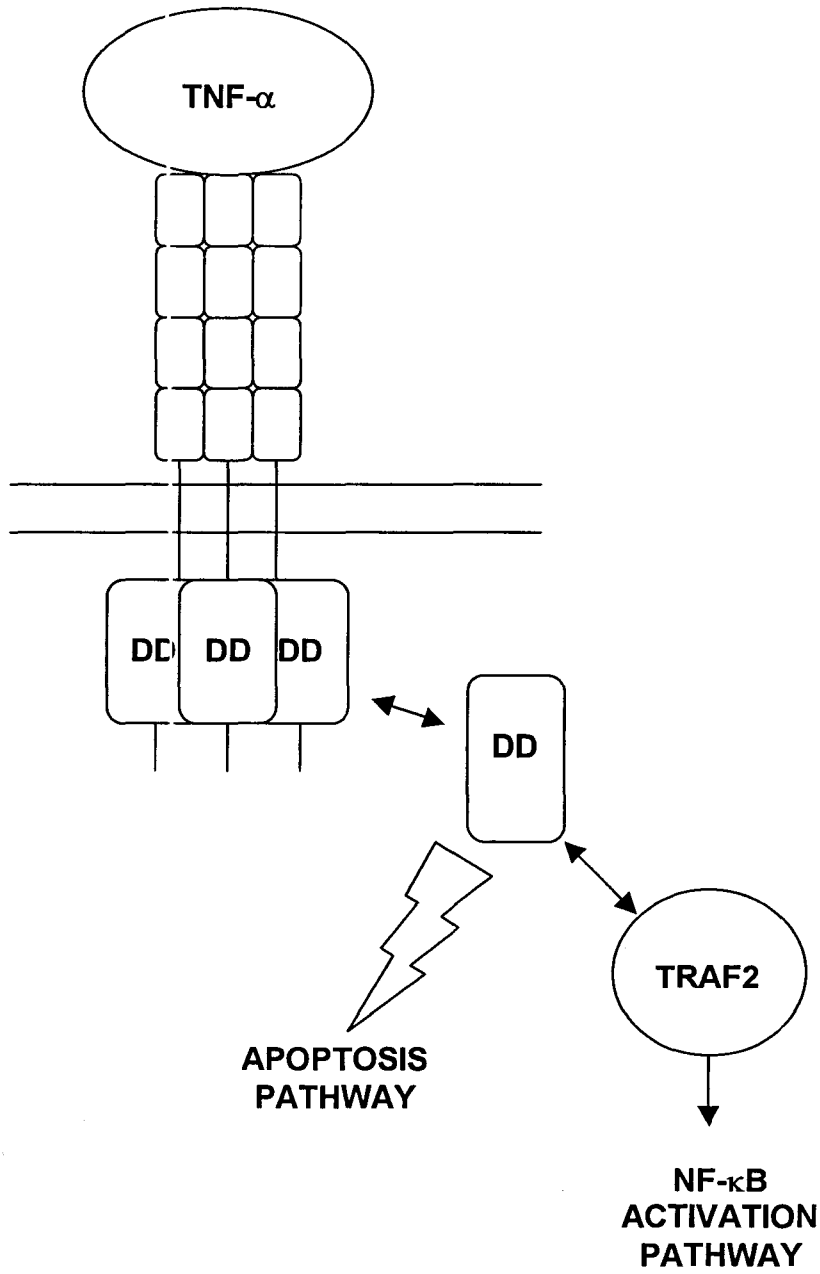

Figure 6. Schematic outline of general signal transduction pathways elicited by TNF-α upon binding to TNF receptors. Adapted from Dinarello, 2000. DD: death-domain.

TNF-α and Atherogenesis

TNF-α immunoreactivity has been detected in human atherosclerotic plaques and has been thought to contribute to the development of atherosclerotic lesions by promoting further inflammation within the existing lesion (Niemann-Jonsson et al., 2000). However, the specific role of TNF-α in atherogenesis is now controversial since a recent report in which the *tnfα* gene was deleted in a murine model of atherosclerosis showed that the extent of atherosclerosis in *tnfα* -/- mice was not significantly different from wild type animals (Schreyer et al., 2002). In light of these data, it is thought that the role of TNF-α in atheromae may be merely a marker of inflammation within lesions or perhaps contributing to the apoptosis of intima-resident cells by signaling through p55 or p75 TNF receptors. This hypothesis is supported by recent evidence demonstrating that smooth muscle cells extracted from atherosclerotic plaques readily undergo apoptosis upon stimulation with TNF-α and signaling through the TNF receptors, suggestive that TNF-α may play a protective role in the control of atherosclerotic lesion progression (Niemann-Jonsson et al., 2001).

Project Rationale

While much work in the field of LXR research has focused on the role of LXR in cholesterol metabolism and homeostasis, a role for LXR in functions outside of that physiological milieu has yet to be determined. While oxLDL provides ligands for LXRα (Brown et al., 1996, Fu et al., 2001) and, as described, also exerts pleiotropic effects on

gene expression that are unrelated to lipid metabolism, such as cytokine and growth factor production, a role for LXR in cytokine production in monocytes/macrophages has not been described This work was designed to investigate such a role. Given the many functions that other nuclear hormone receptors play in other biological processes aside from general lipid homeostasis, such as PPARγ, a receptor that is essential not only in metabolism but development and insulin sensitization, the potential that LXR will have multiple functions is not outside the realm of possibility. To this end, an examination of the cytokine profile of human monocytes/macrophages upon stimulation with oxysterol activators of LXR and activators of RXR was undertaken to investigate the possibility for multiple LXR-dependent functions. Initial results of these studies and the subsequent dissection of the underlying mechanisms involved are presented herein. In short, the body of work presented here provides evidence that should redefine the view that LXR is wholly a master regulator of cholesterol metabolism. Presented here is evidence that both LXR and RXR are involved in the dynamic regulation of TNF-α expression and secretion in human monocytes.

CHAPTER 2: Materials and Methods

Materials

Chemicals and Reagents

The chemicals and reagents to carry out this research and their origins are listed below.

Actinomycin D	Sigma Chemical Company
Agarose (electrophoresis grade)	Life Technologies
Agarose (NuSieve-GTG)	FMC Bioproducts
Agar	Becton Dickinson and Company
Amino acids	Sigma Chemical Company
Ampicillin	Sigma Chemical Company
BioRad protein assay	BioRad Laboratories
Bovine serum albumin	Pharmacia Biotech
Charcoal, Dextran-coated	Sigma Chemical Company
Cycloheximide	Sigma Chemical Company
Deoxynucleotide triphosphates (dNTPs)	Pharmacia Biotech
Dithiothreitol (DTT)	Sigma Chemical Company
Dimethylsulfoxide (DMSO)	Caledon Laboratories
Ethanol	Sigma Chemical Company

Goat IgG-agarose conjugate	Santa Cruz
Hydroxycholesterols:	
25-hydroxycholesterol	Sigma Chemical Company
22(*R*)-hydroxycholesterol	Research Plus
22(*S*)-hydroxycholesterol	Sigma Chemical Company
LG100268	Ligand Pharmaceuticals
LG101208	Ligand Pharmaceuticals
L-glutamine	Life Technologies
Luciferin	Biosynth
E. coli O127 lipopolysaccharide (LPS)	Sigma Chemical Company
MEM sodium pyruvate solution (100mM)	Life Technologies
Molecular weight standards:	
50 bp DNA ladder	Life Technologies
1 Kb Plus DNA ladder	Life Technologies
low-range SDS-PAGE standards	BioRad Laboratories
high-range SDS-PAGE standards	BioRad Laboratories
Nonidet P-40 (NP-40)	BDH Chemicals
Oxidized low-density lipoprotein, human (oxLDL)	Intracel
Penicillin (5000U/mL) / streptomycin (5000μg/mL)	Life Technologies
Phenylmethylsulfonylflouride (PMSF)	Boehringer Mannheim
Protease inhibitor cocktail tablets (EDTA-free)	Boehringer Mannheim
Protein G-sepharose	Boehringer Mannheim

Poly-dIdC	Pharmacia Biotech
5X reporter lysis buffer	Promega
9-*cis* retinoic acid (9cRA)	Sigma Chemical Company
Salmon sperm DNA	Sigma Chemical Company
Sephadex G-50	Pharmacia Biotech
Serum, fetal calf	Life Technologies
Sodium dodecyl sulfate (SDS)	Sigma Chemical Company
TNF-α N-19 (polyclonal goat anti-human TNF-α IgG)	Santa Cruz
Triton X-100	BDH Chemicals

Enzymes

The enzymes used to conduct this research, in addition to their companies of origin, are listed below. All enzymatic reactions were conducted according to the manufacturer's specifications.

Calf intestinal alkaline phosphatase	New England Biolabs
DNA polymerase I (Klenow)	New England Biolabs
Lysozyme	Sigma Chemical Company
Restriction endonucleases	New England Biolabs
RNAse A	Pharmacia Biotech

T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase	New England Biolabs
Vent DNA polymerase	New England Biolabs

Radiochemicals

L-[^{35}S]-methionine (1151Ci/mmol)	NEN Life Science Products
[α-^{32}P]dATP (3000Ci/mmol)	NEN Life Science Products
[γ-^{32}P]dATP (3000Ci/mmol)	NEN Life Science Products
[^{14}C]-labeled molecular weight markers	Life Technologies

Oligonucleotides

Oligonucleotides used for generating double-stranded DNA probes for electromobility shift assays (EMSA) and amplifying DNA using PCR were generated by MOBIX, McMaster University, Hamilton, Ontario. Only the sense strands are given.

Oligonucleotide Number	Oligonucleotide Sequence	Purpose
AB6746	5'-CTTGCGGTTCCCAGGGTTTAAATAAGTTC ATCTA-3'	LXRE-ΔMTV (forward)
AB6747	5'-TAGATGAACTTATTTAAACCCTGGGAACC GCAAG	LXRE-ΔMTV (reverse)
AB26496	5'-ACCTCTGGGGAGATGTGACCACAGCA ATGGGTAGGAGAATGTCCAGGGCTATGG AAGTCGAGTATCGGGGACCCCCCCTTAA-3'	Human TNF-α promoter –932→–851 (forward)

AB26834	5'-ACCTCTGGGGAGATGTGACCACAGCAAT GGG-3'	Human TNF-α promoter –932→–900 (forward)
AB26835	5'-CCATTGCTGTGGTCACATCTCCCCAGA GGT-3'	Human TNF-α promoter –932→–900 (reverse)
AB26836	5'-TGTCCAGGGCTATGGAAGTCGAGTATCG-3'	Human TNF-α promoter –894→–866 (forward)
AB26837	5'-CGATACTCGACTTCCATAGCCCTGGACA-3'	Human TNF-α promoter –894→–866 (reverse)
AB27089	5'-TGTCCATTTCTATGGAAGTCGAGTATCG-3'	Mutant Human TNF-α promoter region –894→–866 (forward) for EMSA
AB27090	5'-CGATACTCGACTTCCAATCAAATGGACA-3'	Mutant Human TNF-α promoter region –894→–866 (reverse) for EMSA
AB27864	5'-GGGTAGGAGAATGTCCATTTCTATGGAA GTCGAGTATCGGGG-3'	Mutant Human TNF-α promoter region –894→–866 (forward) for transfection
AB27865	5'-CCCCGATACTCGACTTCCATAGAAATGG ACATTCTCCTACCC-3'	Mutant Human TNF-α promoter region –894→–866 (reverse) for transfection

Bacterial Strains and Growth Conditions

E. coli DH5α (American Type Culture Collection) were used in the growth and preparation of the plasmid DNA used in these studies. Bacteria were routinely grown at 37°C in 2YT (1.6% bactotryptone, 1% bacti-yeast, and 0.5% NaCl) that was supplemented with 100μg/mL ampicillin.

Mammalian Cells and Cell Lines and culture conditions

The COS-1 cell line are African Green Monkey Kidney cells derived from CV-1 simian cells transformed by an origin-defective mutant of SV40, which encodes for wild-type T antigen (Gluzman, 1981). These cells were obtained from ATCC. COS-1 were maintained at 37°C in Dulbecco's minimal essential media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine.

THP-1 cells are a human acute monocytic leukemia cell line (Tsuchiya et al., 1980) and obtained from ATCC. These cells were maintained at 37°C in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum.

Human peripheral blood mononuclear cells (PBMCs) were collected from healthy donors and monocytes were isolated from buffy-coat preparations using a MACS Monocyte Isolation kit (Milentyi Biotec, USA) 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.

Plasmids

Commercially-available plasmids

pSG5: From Stratagene, pSG5 is an ampicillin-resistant eukaryotic expression vector containing an SV40 early promoter and an SV40 poly-A signal with a T7 promoter upstream of the multiple cloning site (MCS).

pRc/CMV: From Invitrogen, pRc/CMV is an ampicillin-resistant eukaryotic expression vector containing enhancer-promoter sequences from the gene of human cytomegalovirus (CMV) and a poly-A signal from bovine growth hormone (BGH) gene. It also contains a T7 RNA polymerase and SP6 promoter flanking either side of the multiple cloning site.

pGL2*luc*: From Promega, pGL2*luc* is an enhancerless SV40 promoter/luciferase which contains an ampicillin resistance gene.

Plasmids Constructed by Others

pSG5-RXRα: contains full length human RXRα, cloned into the *Eco*RI site of pSG5 (Marcus et al., 1993).

pRC/CMV-LXRα: contains full length human LXRα (Miyata et al., 1996).

TK-LXRE*luc*: contains three copies of the LXRE-ΔMTV DNA response element cloned into the *Hin*DIII site of TK-*luc* in a tandem repeat (Willy et al., 1995).

pXP1-TNF(-1311)*luc*: contains 1.3kB of the human TNF-α promoter/regulatory region linked to the luciferase gene (Rhoades et al., 1992).

Plasmids constructed for Project

pTNF(-914-359)*luc* was generated by a *BstX*I collapse of pTNF(-1311)*luc*.

pTNF(-971-762)*luc* was generated by a *Msc*I collapse of pTNF(-1311)*luc*.

pTNF(-641-493)*luc* was generated by a *Stu*I collapse of pTNF(-1311)*luc*.

pTNF(-987-105)*luc* was generated by a *Sst*I collapse of pTNF(-1311)*luc*.

pTNF(-932/-851)*luc* 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 *Bam*HI of the luciferase expression vector pGL2*luc* (Promega).

pTNF(-894/-866)*luc* contained a single copy of a synthetic double-stranded oligonucleotide corresponding to nucleotides -894 to -866 (annealed AB26836 and AB26837) of the TNF-α promoter was cloned into the *Bam*HI site of expression vector pGL2*luc* (Promega). All plasmid constructions were verified by DNA sequence analysis.

pTNFmut(-894/-866)*luc* contained a single copy of a synthetic double-stranded oligonucleotide corresponding to nucleotides -894 to -866 (annealed AB27089 and AB27090) of the TNF-α promoter was cloned into the *Bam*HI site of expression vector pGL2*luc* (Promega). All plasmid constructions were verified by DNA sequence analysis.

pTNFmut(-1311)*luc* was generated by mutating pTNF(-1311)*luc* using site-directed mutagenesis employing the QuickChange™ Kit (Stratagene) and oligonucleotides AB27864 and AB27865. All plasmid constructions were verified by DNA sequence analysis.

All plasmids constructed herein were verified by DNA sequence analysis (MOBIX, McMaster University)

Methods

Purification of Plasmid DNA

Small-Scale Plasmid DNA Purification

The method used for small-scale (miniprep) preparation of plasmid DNA is lysis-by-boiling method as follows. Plasmid DNA was extracted from 1.5mL of pelleted saturated bacterial culture, grown overnight at 37°C in 5mL of 2YT supplemented with ampicillin (final concentration 100μg/mL). The resulting pellet was resuspended in 350μL of STET buffer (0.1M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, and 5% Triton X-100) and 25μL of fresh lysozyme (10mg/mL in 10mM Tris-HCl pH 8.0). The sample was subsequently incubated in a boiling water bath for 40 seconds followed by a 10-minute centrifugation in order to pellet the cell debris. The resulting supernatant was collected in a fresh tube, and 200μL of ammonium acetate (7.5M) and 700μL of isopropanol were added. The sample was frozen in liquid nitrogen for 5 minutes and centrifuged at 14,000rpm at 4°C for 30 minutes. The resultant nucleic acid pellet was washed with 70% ethanol and resuspended in TE (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) containing RNAase A (100μg/mL).

Large Scale Plasmid DNA purification (Qiagen Method)

The method for large scale (maxiprep) preparation of plasmid DNA, using Qiagen prepared DNA purification columns (anionic silica-gel resin which binds doubled stranded DNA and neither RNA nor cellular debris), is based on an alkaline lysis procedure. Briefly, a 50mL flask of 2YT media containing ampicillin (100μg/mL) was

inoculated with a single colony and was grown overnight in a shaking incubator at 37°C. Subsequently, 10mL of the overnight culture was added to 500mL of 2YT supplemented with ampicillin (100μg/mL) and incubated at 37°C overnight. This culture was harvested the next day, and plasmid DNA was purified using the Qiagen columns according to manufacturer specifications.

DNA Quantification by Fluorometry

Plasmid DNA was quantified fluorometrically using a method designed by Hoefer and using a Hoefer Mini-Fluorometer (TKO 100) according to manufacturer specifications. All DNA quantifications were measured relative to the Calf Thymus DNA standard (1μg/μL).

Transformation of Plasmid DNA into Bacteria By Heat Shock

50μL of competent *E. Coli* DH5α cells (Gibco BRL) were incubated on ice with 5-10ng of plasmid DNA for 30 minutes. The resultant mixture was subsequently incubated at 37°C for 20 seconds and then placed back on ice for 2 minutes. Cells were then incubated at 37°C for 1 hour in 200μL of 2YT so as to permit expression of the transformed plasmid and then streaked on an agar containing plate of 2YT supplemented with ampicillin (100μg/mL) and incubated at 37°C overnight.

In vitro Transcription and Translation

In vitro transcription and translation reactions were carried out using Promega TNT® coupled system. This system allows for simultaneous transcription and translation

of cDNAs within the same reaction mixture. Briefly, the reaction mixture comprised of 25μL of TNT® rabbit reticulocyte lysate, 2μL of TNT® reaction buffer, 1μL of appropriate TNT® RNA polymerase (SP6 or T7), 1μL of amino acid mixture, 1μL of RNAsin ribonuclease inhibitor (40U/μL), 2μg of cDNA-containing plasmid DNA, and sterile, nuclease-free water to a final volume of 50μL. The mixture was incubated at 30°C for 2 hours and translated product analyzed by SDS-PAGE.

Cytokine assays

THP-1 and human monocytes (10^6 cells/ml) were cultured as described above and incubated in the presence of the various compounds, as indicated. Control cells received the equivalent amount of vehicle (dimethylsulphoxide (DMSO), ethanol, or water) as indicated. Following the prescribed period of incubation, supernatants were collected, and 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, USA) according to the manufacturer's instructions. Briefly, 96-well microtitre plates (Nunc, USA) were coated with 100μL capture antibody (mouse anti-cytokine, 1:250) in coating buffer (0.1M sodium carbonate, pH 9.5) overnight at 4°C. The next day, wells were aspirated and wash three times with wash buffer (PBS pH 7.4, 0.05% (v/v) Tween-20) and blocked with 200μL assay diluent (PBS, pH 7.5 and 10% (v/v) fetal bovine serum) and incubated at room temperature for 1 hour. Following this, wells were aspirated and washed three times with wash buffer, and 100μL of sample were added and incubated for 2 hours at room temperature. Wells were washed again

with wash buffer five times following the 2 hour incubation, and detection antibody (biotinylated mouse anti-cytokine, 1:250 in assay diluent) coupled to avidin-horseradish peroxidase conjugate (1:250) was added to the wells and incubated for 1 hour at room temperature. Wells were then washed five times with wash buffer, and 100μL of TMB Substrate Reagent (a 1:1 mixture of Substrate Reagent A (H_2O_2 in solution) and Substrate Reagent B (3, 3',5, 5'-tetramethylbenzidine in organic solvent)) was added to each well and incubated for 30 minutes at room temperature. The reaction was stopped by addition of 100μL of 2N H_2SO_4. Colour intensity was measured using an ELISA plate spectrophotometer at λ=450nm. The calculation of cytokine concentration in supernatants was determined based on results derived from a standard curve for each cytokine studied.

RNA analysis

Total RNA from THP-1 cells (4×10^6 cells/sample) was isolated using the RNeasy Mini isolation kit (Qiagen, USA) and subjected to Northern analysis under standard conditions using random-primed [^{32}P]-radiolabelled probes generated from cDNAs for human TNF-α, and GAPDH, as follows. Briefly, 10μg of RNA was subjected to electrophoresis on a 1% agarose/0.66M formaldehyde gel for 4 hours at 100V. Subsequently, the gel was washed in sterile, nuclease-free water, and a nylon screen (Gene Screen Plus™, DuPont NEN) soaked in sterile, nuclease-free water and 10X SSPE (1.5M NaCl, 0.1M NaH_2PO_4-H_2O, 0.01M EDTA-Na_2) was applied onto the gel, and transfer of RNA from the gel was carried out using 10X SSPE transfer solution overnight at room temperature. The next day, the nylon screen was removed and washed in 2X

SSPE, and RNA was crosslinked using UV light (Stratalinker) and baked to the nylon screen for 1 hour at 80°C under a vacuum.

Generation of a radioactive cDNA probe was carried out as follows. Briefly, 50ng of cDNA to be labeled and random hexamer primer (3μg/μL, MOBIX, McMaster University) were incubated in a total volume of 40μL. This mixture was boiled in order to denature the DNA and cooled on ice for 5 minutes. To this mixture was added, 6μL of 10X Klenow Buffer, 3μL of 1.0mM dGTP, dCTP, dTTP, 50μCi of [α-^{32}P]dATP, and 3μL of Klenow polymerase, and incubated at 30°C for 2 hours. The volume was adjusted to 150μL with sterile water, and passing the mixture over two Sephadex G-50 columns purified the DNA probe. Radioactivity incorporation was measured by scintillation analysis.

Hybridization of the radiolabelled DNA probe to the RNA was carried out as follows. The nylon screen was incubated for 2-4 hours in prehybridization solution (5X SSPE, 50% (w/v) deionized formamide, 5X Denhardt's solution, 1% SDS, 10% dextran sulfate, sodium salt (MW 500,000)) at 42°C in a hybridization canister. Following this incubation, the radiolabelled probe was denatured by boiling and added (5×10^5 cpm/mL) to fresh hybridization solution (as described above), and incubated at 42°C for 16 hours in a hybridization canister. Following incubation, the nylon screen was washed twice successively with 2X SSPE for 15 minutes, followed by 2X SSPE, 2% SDS for 45 minutes at 65°C, and once with 0.1X SSPE at room temperature for 15 minutes. The nylon screen was then exposed to Kodak X-Ray film. Radioactive bands were quantified

by phosphorimager analysis of the nylon screen and normalized to the radioactivity present in the GAPDH signal, which was used as an internal standard.

Transfections and luciferase assay

Transient transfection of COS-1 cells was carried out using lipofectamine (Gibco BRL) according to the manufacturer's instructions. Briefly, COS-1 cells (3×10^5 cells/ 6cm dish) were transfected using 4μL lipofectamine along with 0.5μg of a luciferase reporter gene, 0.5μg of cDNA-containing eukaryotic expression plasmid or empty eukaryotic expression plasmid, and 0.5μg of pCMV*lacZ*, 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 the appropriate empty eukaryotic expression vectors. Cell extracts were prepared 48 hours post-transfection, and luciferase assays and β-galactosidase assays were carried out as previously described (Marcus et al., 1993). Briefly, harvesting of cells consisted of washing them twice with PBS and the addition of 400μL/well of 1X reporter lysis buffer. Each sample was collected into a clean Eppendorf tube, vortexed at high speed for 10 seconds, and then centrifuged for 2 minutes at 12,000 rpm at room temperature. The supernatants were collected and then assayed for luciferase and β-galactosidase activity.

Assessment of luciferase activity involved using a Lumat LB 9507 luminometer, a machine that mixes 20μL of sample with 100μL of luciferase assay buffer (470μM fire-fly luciferin, 270μM coenzyme A, 530μM ATP, 33.3mM DTT, 20mM Tricine, 1.07mM

$(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67mM $MgSO_4$, 0.1mM EDTA). The machine then measures the luciferase activity and expresses this activity in relative light units (RLU).

Assessment of β-galactosidase activity was done as follows. Briefly, 20μL of lysate was added to 400μL β-galactosidase buffer (10mM KCl, 1mM $MgSO_4$, 100mM sodium phosphate, 50mM β-mercaptoethanol (pH 7.5)). To this was added 150μL of ONPG (4mg/mL), and the resultant mixture was incubated for 1-2 hours at 37°C. The reaction was terminated using 200μL of 1M Na_2CO_3 and 50μL isopropanol. The product of the reaction *o*-nitrophenol was detected using a spectrophotometer set to 420nm.

Luciferase activity was normalized to β-galactosidase activity by dividing RLU by OD_{420}. The average and standard deviations were calculated for each experiment by using values generated from three independent transfections for each test condition.

Metabolic labelling and immunoprecipitation

THP-1 cells (4×10^6 cells per sample) were cultured in methionine- and cysteine-free RPMI containing 1% dialyzed fetal bovine serum and 100mCi/mL [^{35}S]-methionine/cysteine (NEN Life Science Products, USA) for 12 hrs at 37°C. Cell extracts were prepared accordingly and precleared using 1μg of goat IgG-agarose conjugate (Santa Cruz, USA) overnight at 4°C on a rocking platform. This mixture was centrifuged for 20 seconds at 12,000 rpm, and the supernatant was transferred to a clean tube. To measure the radioactivity of the proteins present in this supernatant, 5μL of each supernatant was subjected to the trichloroacetic acid method in order to precipitate the protein, and the radioactivity was assessed by scintillation counting. Equivalent amounts

of radioactivity from each sample were loaded, and immunoprecipitation of TNF-α was carried out using 0.5μg polyclonal goat anti-human TNF-α IgG (TNF-α N-19, Santa Cruz, USA) for 1 hour at 4°C on a rocking platform. This immune complex was precipitated by addition of a protein G-sepharose slurry (Boehringer-Ingelheim, Germany) and incubated for 4 hours at 4°C on a rocking platform. Following this, the samples were centrifuged and washed in a buffer containing 50mM Tris-HCl (pH 7.5), 0.1% Nonidet P40, and 0.05% sodium deoxycholate. These complexes were then resolved on 15% SDS-polyacrylamide gel electrophoresis, the gel dried, and exposed to Kodak X-ray.

Electrophoretic mobility shift analysis (EMSA)

EMSA was used to determine the binding of proteins to DNA. Using human LXRα and RXRα synthesized *in vitro* by transcription of their corresponding cDNAs and translation using the Promega TNT® coupled system, EMSA was carried out as described previously (Marcus et al., 1993, Miyata et al., 1996). Briefly, EMSA doubled stranded DNA oligonucleotide probes were generated by annealing the synthetic single stranded oligonucleotides described above. This was achieved by heating the two oligonucleotides at 75°C for 10 minutes and then cooling them to room temperature at a rate of 1°C/minute. Annealed DNA was subjected to electrophoresis on a 4% NuSieve gel (containing 0.5μg/mL ethidium bromide). The DNA was electrophoresed onto NA45 paper and eluted by heating the paper at 65°C for 30 minutes in DEAE elution buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0, 1M NaCl). The paper and buffer was

then centrifuged at 14, 000 rpm for 5 minutes, and the resulting supernatant was collected and to which was added 10μL of 1M $MgCl_2$ and absolute ethanol, flashed frozen in liquid nitrogen, and centrifuged at 14, 000 rpm for 30 minutes at 4°C. The DNA pellet was washed in 70% ethanol and resuspended in sterile water. DNA concentration was calculated by fluorometry, and radiolabelling reactions were carried using DNA concentrations of approximately 20pmol/μL. Radiolabelling of the DNA probe was carried out using 20pmol of DNA probe, 6μL of Klenow buffer, 3μL each of 1.0mM dGTP, dCTP, dTTP, 50μCi of [α-^{32}P]dATP, 3μL of Klenow polymerase, and sterile water. This mixture was incubated at room temperature for 2 hours, and the volume adjusted subsequently to 150μL using sterile water. The probe was subsequently purified by passing the DNA solution over two Sephadex G-50 columns, and 2μL of the resultant probe was analyzed using a scintillation counter for radiolabel incorporation.

Binding reactions were carried out with the radiolabelled synthetic double-stranded oligonucleotide probe and *in vitro* translated non-radioactive proteins. Briefly, 2μL of each *in vitro* translated protein were coincubated with 20pmol of radiolabelled DNA probe, 1μL of polydIdC (4μg/μL), 1μL of bovine serum albumin (BSA; 4μg/μL), 1μl of salmon sperm DNA (4μg/μL), 5μL of Buffer C (20mM HEPES-KOH, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF, 25% glycerol), and sterile water to a final volume of 20μL. This mixture was incubated at 30°C for 30 minutes, and subsequently, 1μL of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in sterile water) was added and mixed. This mixture was

then subjected to electrophoresis at 200 volts at 4°C for 3 hours on a non-denaturing 4% polyacrylamide gel. The gel was then dried and exposed to Kodak X-Ray film.

CHAPTER 3: Results

LXR/RXR activators induce TNF-α protein from THP-1 monocytes and human blood-derived monocytes.

To examine the role of LXR/RXR activators in cytokine expression, THP-1 human monocyte/macrophage cells (ATCC) were used. THP-1 is a monocytic cell-line that has been used previously to investigate the role of nuclear hormone receptors in monocyte/macrophage biology (Tontonoz et al., 1998 and Nagy et al., 1998). They are grown in suspension culture and can be differentiated into macrophages using phorbol esters or ionomycin whereupon the cells will adhere to the culture vessel. These cells express both LXR (α and β isoforms) and its heterodimeric partner RXRα (Chawla et al., 2001b and Chawla et al., 2001c). The expression of both LXR and RXR in THP-1 cells was confirmed by Western Blot (data not shown). For these initial experiments, differentiation of THP-1 monocytes into macrophages was not induced so as to maintain a generalized monocytic phenotype and to best mimic the phenotype monocytes would likely be in *in vivo* when encountering the LXR and RXR activators used, 22(*R*)-HC-hydroxycholesterol (22R-HC) and 9-*cis* retinoic acid (9cRA). In all experiments conducted, the concentrations of ligands used in these experiments and for all experiments described subsequently have been shown to maximally activate both LXR (10μM) and RXR (10μM) (Janowski et al., 1999).

To examine the role of oxysterol activators of LXR and 9cRA on cytokine production, THP-1 cells were incubated for 96 hours in the presence or absence of these ligands and the cytokine production profile was examined. Using enzyme-linked immunosorbent assay (ELISA) to quantify the levels of cytokine present in the supernatants, it was found that a significant level of TNF-α was detected in supernatants from THP-1 cells that were co-incubated with 22R-HC and 9cRA (Figure 7A). The production of IL-1β or IL-6 was not observed under any conditions, except for when THP-1 cells were cultured in the presence of the *E. Coli* O127 endotoxin lipopolysaccharide (LPS). This result with LPS is consistent with previous results by others showing that IL-6, IL-1β, and TNF-α are induced upon stimulation with LPS, the so-called LPS-induced "acute phase response" (Arai et al., 1990). Interestingly, production of TNF-α only occurred when both 22R-HC and 9cRA were present, not individually (Figure 7A). Furthermore, coincubation of THP-1 cells with 22R-HC and the natural RAR-ligand all *trans* retinoic acid (atRA), a compound that does not activate RXR, did not result in any appreciable production of TNF-α. Therefore, these results suggest that the observed effects may be mediated by LXR and RXR and not by RAR.

When these results are compared against data collected from THP-1 cells treated with 22(*S*)-HC-hydroxycholesterol (22S-HC) and 9cRA, they were suggestive that LXR and RXR may be mediating this induction for co-administration of 22S-HC and 9cRA did not result in any TNF-α production. 22S-HC is the enantiomer of 22R-HC and is a compound that binds LXR with comparable affinity as 22R-HC (K_i 22R-HC = 380 ± 50nM; K_i 22S-HC = 150 ± 10nM) but does not activate the receptor (Janowski et al.,

1999). This compound is effective in examining LXR activation, and therefore, in these experiments 22S-HC would fulfill the role of an effective LXR-antagonist and help to assess the LXR dependence of the findings. Based on this effect, these experiments were also repeated using freshly isolated primary human peripheral blood mononuclear cells (PBMCs). As seen with THP-1 cells, under the same culture conditions, TNF-α was only observed when PBMCs were exposed jointly to 22R-HC and 9cRA, and not under any conditions where 22S-HC was used (Figure 7B).

As observed in cells treated with the LPS, secretion of TNF-α was accompanied by the coordinate secretion of other primary pro-inflammatory cytokines, such as IL-1β and IL-6, in a manner consistent with the classic acute phase response (Arai et al., 1990). As stated however, 22R-HC and 9cRA co administration had no effect on the production of IL-1β or IL-6 from THP-1 cells or from primary monocytes implying that 22R-HC/9cRA stimulates a pathway of induction of TNF-α that is cytokine specific and is distinct from other pathways, mediated perhaps by LXR and RXR.

TNF-α mRNA has been shown to be induced by oxidized low-density lipoprotein (oxLDL) (Hsu et al., 2001; Mikita et al., 2001). Since 22R-HC is found in oxLDL (Brown et al., 1996), oxLDL was tested to see if it could stimulate TNF-α from THP-1 cells either alone or in concert with 9cRA, in a manner like that observed with 22R-HC and 9cRA. Therefore repeating the experiments described above but substituting oxLDL for 22R-HC, incubation of oxLDL had little effect on TNF-α production as compared to control untreated cells. However, co-administration of oxLDL with 9cRA led to a robust induction of TNF-α as compared to vehicle-treated cells (Figure 7C). Interestingly, the

extent of TNF-α induction under these conditions was less than when 22R-HC is co-incubated with 9cRA, perhaps reflecting the relative concentration of 22R-HC in oxLDL, an oxysterol that has been detected in oxLDL (Brown et al., 1996). Thus, under these experimental conditions, oxLDL cooperates with 9cRA in the induction of TNF-α protein expression in THP-1 cells, corroborating early reports implicating oxLDL in TNF-α production.

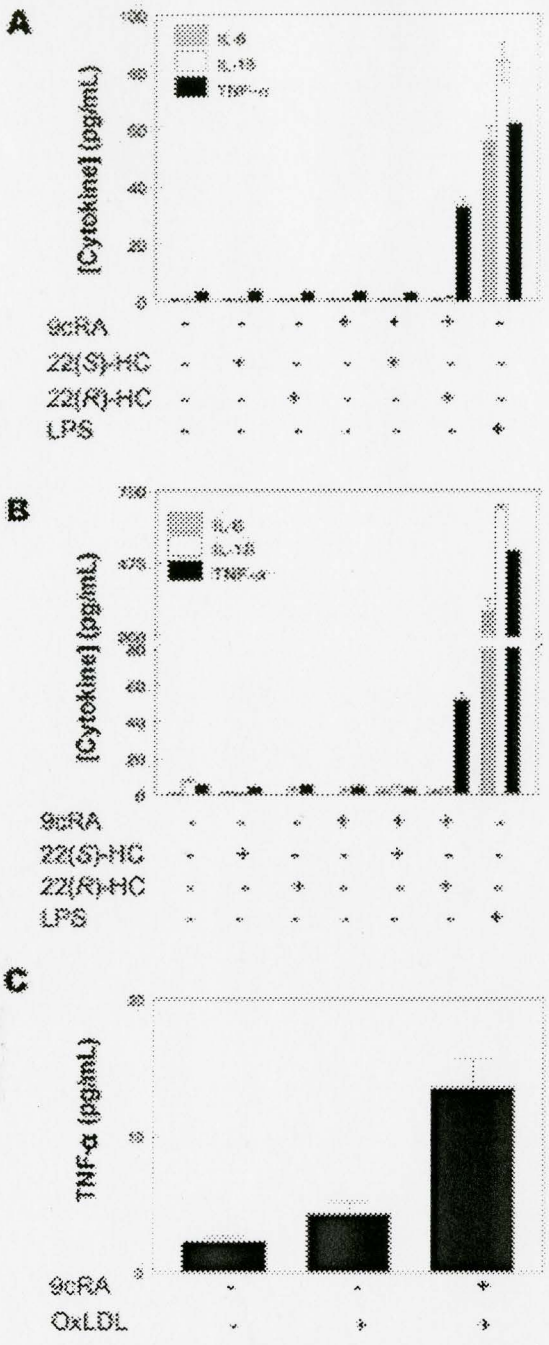

Figure 7. 22(*R*)-hydroxycholesterol, oxidized LDL (oxLDL) and 9-*cis* retinoic acid (9cRA) specifically induce production of TNF-α from THP-1 cells and primary human monocytes. (**A**), THP-1 cells or (**B**), freshly prepared primary human monocytes were incubated for 96 hours in the presence of 22R-HC, 22S-HC, and/or 9cRA (each 10μM), or LPS (10ng/mL), as indicated and the levels of TNF-α, IL-1β, and IL-6 were quantified by ELISA. (**C**) THP-1 cells were incubated for 96 hours in the presence of oxLDL (50μg/mL) and 9cRA (10μM), as indicated and levels of TNF-α were quantified by ELISA. All data presented represents the average (± SD) of three independent experiments.

Several lines of preliminary evidence indicate that 22R-HC/9cRA-dependent induction of TNF-α may be mediated through endogenous LXR. As mentioned earlier, 22S-HC failed to induce TNF-α secretion from THP-1 cells or primary monocytes, either alone or in combination with 9cRA. Furthermore, 22S-HC inhibited 22R-HC/9cRA-mediated induction of TNF-α in a dose-dependent manner (Figure 8). Indeed, a two-fold molar excess of 22S-HC relative to 22R-HC virtually eliminated extracellular TNF-α production. This inhibition was not due to generalized cell toxicity, since final concentrations of oxysterols up to 30 μM had no deleterious effects on cell viability as determined by trypan blue staining (not shown). As such, 22S-HC is believed to be competing with 22R-HC for LXR binding, hence accounting for the concentration dependent reduction in TNF-α production.

The kinetics of 22R-HC/9cRA-dependent TNF-α induction were also investigated. Detectable amounts of TNF-α were present at the earliest time point examined (12 hours) and accumulated over 96 hours (Figure 9). Recently published findings have shown that LXR expression is induced in THP-1 cells in the presence of LXR activators like 22R-HC (Whitney et al., 2001 and Laffitte et al., 2001b). Indeed, these same studies demonstrated that LXR serves to autoregulate its expression in the presence of ligand by driving its own expression via multiple LXREs in the LXR proximal promoter. Thus, in this current system and in the constant presence of LXR activator, LXR expression will be induced, and the expression of TNF-α in the presence of 22R-HC/9cRA can be expected to increase if LXR/RXR are mediating its induction.

The foregoing, along with further evidence described below, is consistent with TNF-α induction being directly controlled by pre-existing, endogenous factors such as LXR and RXR.

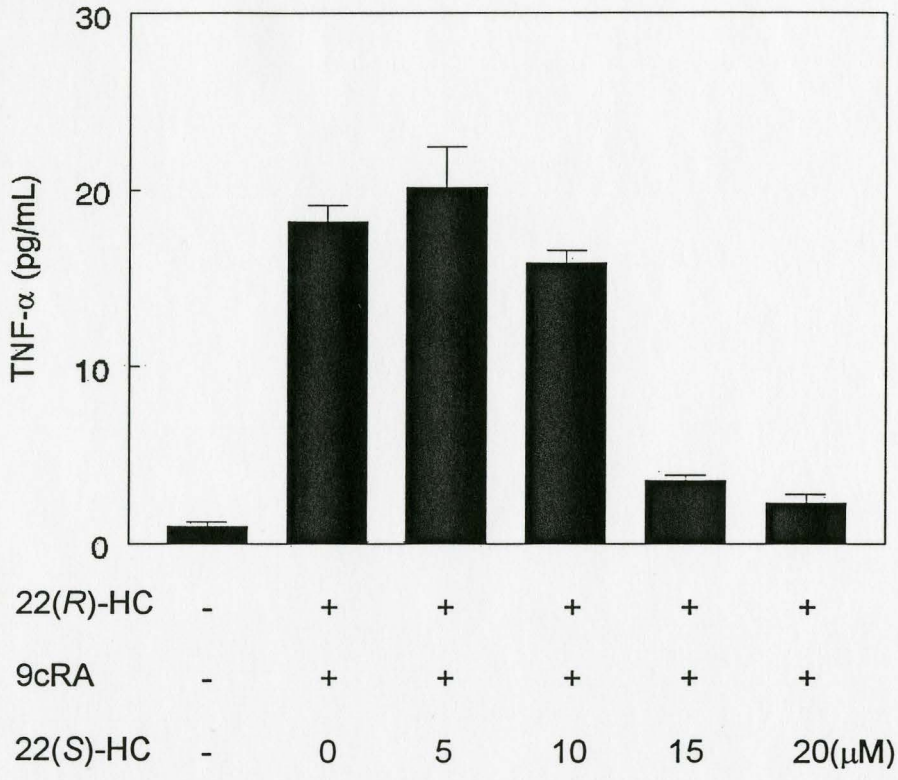

Figure 8. 22S-HC inhibits 22R-HC/9cRA-mediated secretion of TNF-α. THP-1 cells were incubated with 22R-HC and 9cRA (10μM each) for 96 hours in the presence of increasing concentrations of 22S-HC as indicated. Supernatants were collected and assayed for TNF-α as above in Fig. 1. Cell viability remained at or above 95% over the course of the experiment as monitored by trypan blue exclusion. The data presented represent the average (± SD) from three separate experiments.

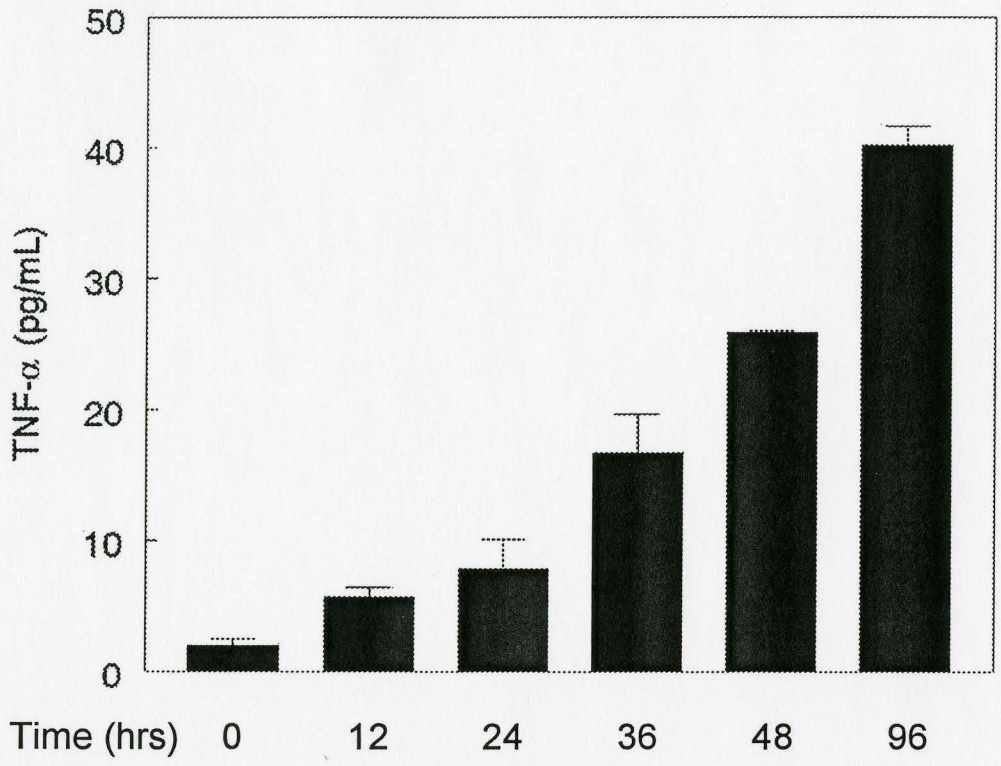

Figure. 9 Time-course of TNF-α production from THP-1 cells in the presence of 22R-HC and 9cRA. THP-1 cells were incubated in the presence of 22R-HC and 9cRA (10μM each) for 96 hours, and supernatants were assayed for TNF-α by ELISA at the times indicated. The values presented represent the average (± SD) from three independent experiments.

LXR/RXR activators induce TNF-α mRNA

To more closely examine if LXR/RXR activators are indeed modulating expression of TNF-α at the level of transcription, the expression of TNF-α mRNA in the presence or absence of 22R-HC and 9cRA was assessed. Based on data presented in Figure 10, 22R-HC treatment of THP-1 cells lead to a robust near 11-fold induction of TNF-α mRNA while co-incubation of 22R-HC/9cRA resulted in 7-fold induction of the same mRNA. Incubation with 22S-HC or 22S-HC/9cRA did not result in increased TNF-α mRNA. Comparatively, incubation with oxLDL resulted in a similar robust induction of TNF-α mRNA, nearly to the same degree as 22R-HC achieved. Interestingly, the addition of 9cRA did not further augment the 22R-HC-mediated increase; indeed, TNF-α mRNA expression was somewhat diminished (from 11-fold to 7-fold) under these circumstances. A detailed time course analysis of TNF-α mRNA induction by 22R-HC showed that TNF-α mRNA is induced by 22R-HC at least as early as 12hrs post-incubation and remain at this steady state level for the duration of the time course (Figure 11). Therefore, the production of TNF-α protein in the presence of 22R-HC/9cRA as early as 12hrs appears to correlate with an increase in TNF-α mRNA expression in the presence of oxysterol activators of LXR.

To provide further evidence that endogenous factors like LXR and RXR may be involved the 22R-HC-dependent induction of TNF-α mRNA, the 22R-HC-dependent induction of TNF-α in the presence or absence of the translation inhibitor cycloheximide

was evaluated. The 22R-HC-mediated increase in TNF-α mRNA was not ablated in the presence of cycloheximide, consistent with induction of mRNA being mediated by pre-existing, endogenous factors such as LXR and RXR (Figure 12). Consequently, oxysterol LXR activators result in the increased expression of TNF-α mRNA but this induction appears to not require 9cRA.

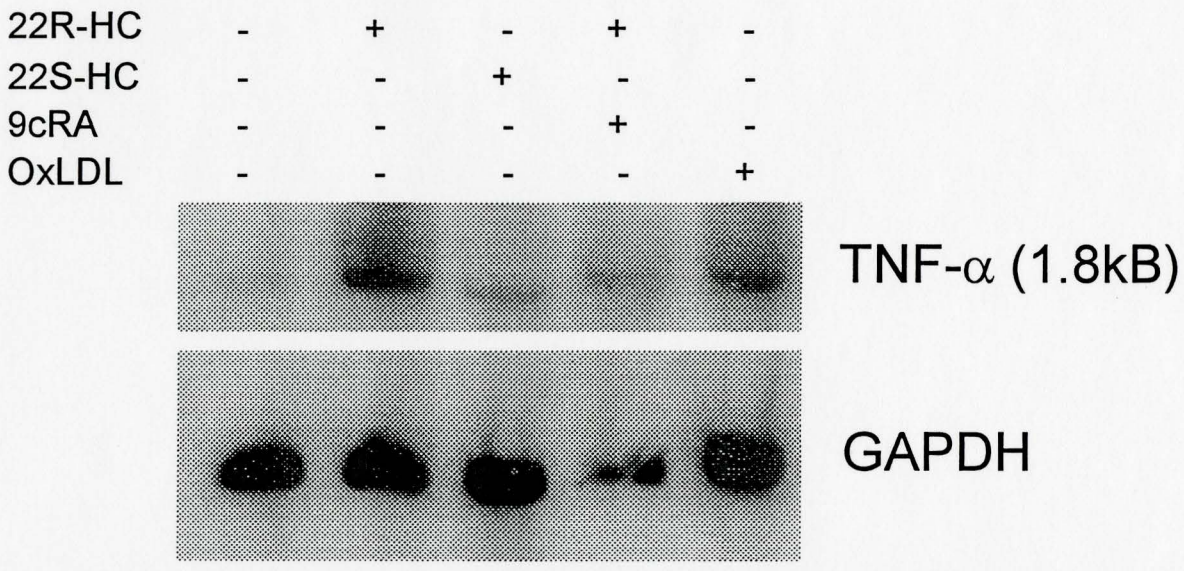

Figure 10. Oxysterol activators of LXR and OxLDL induce the expression of TNF-α mRNA in THP-1 cells. THP-1 cells were incubated for 96 hours with 22R-HC, 22S-HC, and/or 9cRA (10μM each) or oxLDL (50μg/mL), as indicated compounds, and total RNA was isolated from treated cells and subjected to Northern blot analysis using a human TNF-α cDNA probe or a human GAPDH cDNA probe.

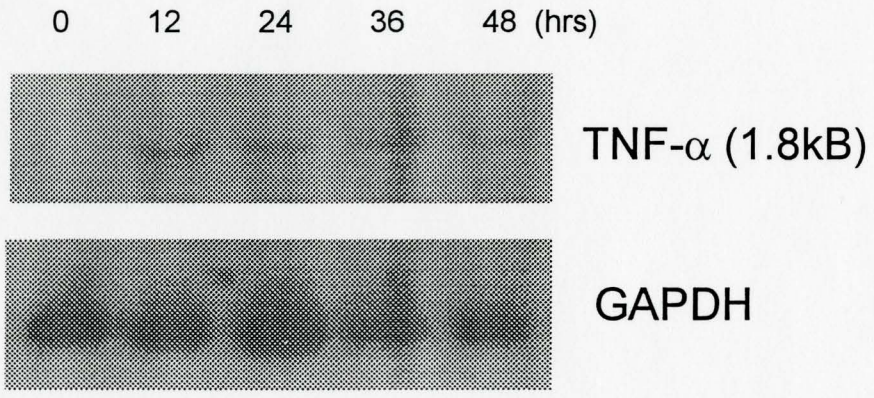

Figure 11. 22R-HC-dependent induction of TNF-α mRNA occurs over time. THP-1 cells were incubated with 22R-HC (10μM) for the times indicated and TNF-α mRNA was subjected to Northern blot analysis as described above.

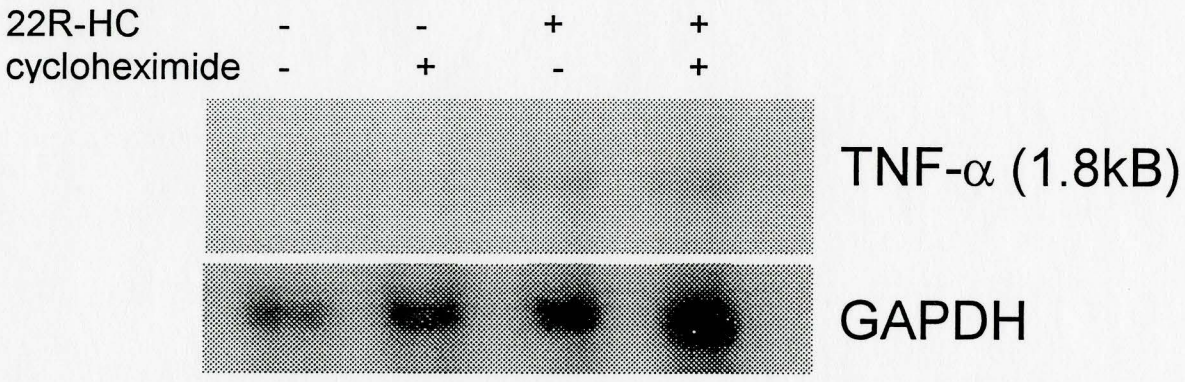

Figure 12. Expression of 22R-mediated TNF-α mRNA does not require *de novo* protein synthesis. THP-1 cells were incubated for 48 hours with 22R-HC (10μM) in the presence or absence of cycloheximide (16mM). Total RNA was subjected to Northern blot analysis as above.

22R-HC-mediated TNF-α mRNA is translated but remains cell-associated.

It was unusual though intriguing to observe induction of TNF-α mRNA by 22R-HC on its own; however, incubation with 22R-HC is insufficient to result in the production of TNF-α protein in the supernatants of THP-1 cells. This was suggestive that perhaps the mRNA produced by either 22R-HC is different from that produced by 22R-HC/9cRA-produced TNF-α mRNA leading to different translational outcomes. Alternatively, perhaps 22R-HC and 9cRA participate in distinct steps along the TNF-α expression pathway, one step involved in transcriptional induction and another involved in translation and secretion. TNF-α is a cytokine whose expression can be controlled either at the level of transcription initiation or translation. In the context of TNF-α translational control, it has been shown that stability of TNF-α mRNA by elements in the 3'-UTR can determine whether or not the mRNA is translated (Piecyk et al., 2000 and Raabe et al., 1998). Knowing this and in light of the previous observations, it was important to determine if the mRNA produced by 22R-HC is translated so as to better understand what role 9cRA may play in facilitating production of TNF-α in the culture supernatants.

To examine if 22R-HC-induced TNF-α mRNA is translated, THP-1 cells were metabolically labeled with ^{35}[S]-methionine/cysteine and immunoprecipitation analysis using antibodies to human TNF-α was carried out. Cell-associated immunoreactive TNF-α, with a mobility consistent with the mature size of the protein (17kDa), was detected in

extracts prepared from cells exposed to 22R-HC for 12 hours, a time chosen based on the time-course experiments previously described. The levels were approximately 3-4 times higher than the control, untreated cells (Figure 13). Consistent with the Northern blot analysis, treatment with 22S-HC or 22S-HC/9cRA had no effect on TNF-α protein levels. Also as expected, significant levels of cell-associated TNF-α protein were not observed in cells co-administered 22R-HC/9cRA arguably because TNF-α protein is secreted under these circumstances. Similarly, very low levels of cell-associated TNF-α protein were detected in LPS-treated cells; again, a consequence of the protein being rapidly secreted following its synthesis. The 26kDa pre-processed TNF "precursor" protein was not detected at all in these experiments even after prolonged exposure of the film. Therefore and intriguingly, TNF-α mRNA induced by 22R-HC is translated, and the protein is processed; however, the protein remains cell-associated.

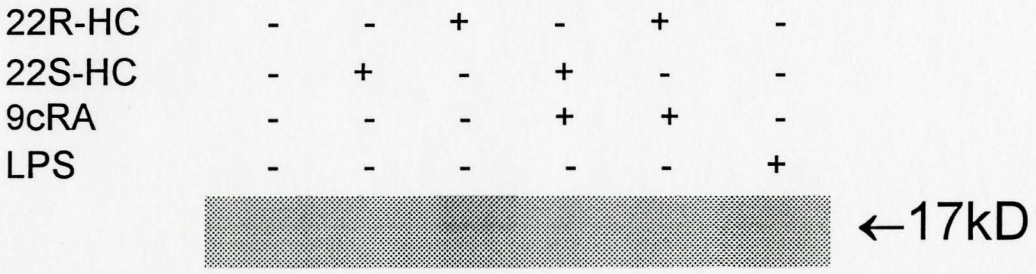

Figure 13. 22R-HC induces TNF-α mRNA and protein expression in THP-1 cells. THP-1 cells were metabolically labelled with ^{35}[S]-methionine/cysteine in the presence of the indicated compounds for 12 hours (10μM 22R-HC and 9cRA, 10ng/mL LPS). Cell extracts were immunoprecipitated with anti-human TNF-α IgG and proteins were resolved by SDS-polyacrylamide gel electrophoresis. Data are representative of 2 independent experiments. The 17 kD band represents the mature form of TNF-α.

LXR/RXR directly transactivate the human TNF-α promoter

The induction of TNF-α mRNA in the presence of oxysterol activators of LXR but not in the presence of the pan-LXR antagonist 22S-HC suggests that the induction of TNF-α is likely mediated through LXR and occurs by activating the TNF-α promoter. In order to clarify and to determine this directly, transient transfection assays were carried out with the assistance of H. Patel using COS-1 cells and a luciferase reporter gene linked to the full 1.3kb proximal promoter/regulatory region of the human TNF-α gene (pXP1-TNF(-1311)*luc*, Rhoades et al., 1992). COS-1 cells (ATCC# CRL-1650) are a transformed African Green monkey kidney cell line derived from the CV-1 cell line but transduced with the viral SV40 gene that encodes T antigen (Gluzman, 1981). 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 (Miyata et al., 1996). Oxysterols had no appreciable effect on the basal activity of the TNF-α reporter gene (Figure 14). Similar findings were also obtained using a specific LXR target reporter gene composed of a canonical DR4 (pTK-DR4*luc*, Willy et al., 1995) demonstrating that COS-1 cells express low levels of endogenous LXR (data not shown). However, in the presence of ectopically expressed human LXRα, 22R-HC led to an approximate 10-fold induction in the TNF-α reporter gene activity compared with vehicle-treated cells (Figure 14). 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, 22R-HC led to a 20-fold increase in promoter activity, demonstrating the RXR is required for the maximal induction of TNF-α expression in this context (Figure 14). 9cRA alone had a marginal effect on the LXRα/RXRα-dependent transactivation of the reporter vector, though it was still greater comparably to cells transfected with LXRα/RXRα and the reporter construct. The explanation for these findings may relate to the fact that LXR/RXR are "permissive" nuclear hormone receptor heterodimers, heterodimers that can be activated to varying degrees of efficacy by ligands of either heterodimer partner. Interestingly however, 9cRA attenuated 22R-HC, LXRα/RXRα-mediated transactivation by approximately 50%, consistent with findings from the Northern analysis of THP-1 cells.

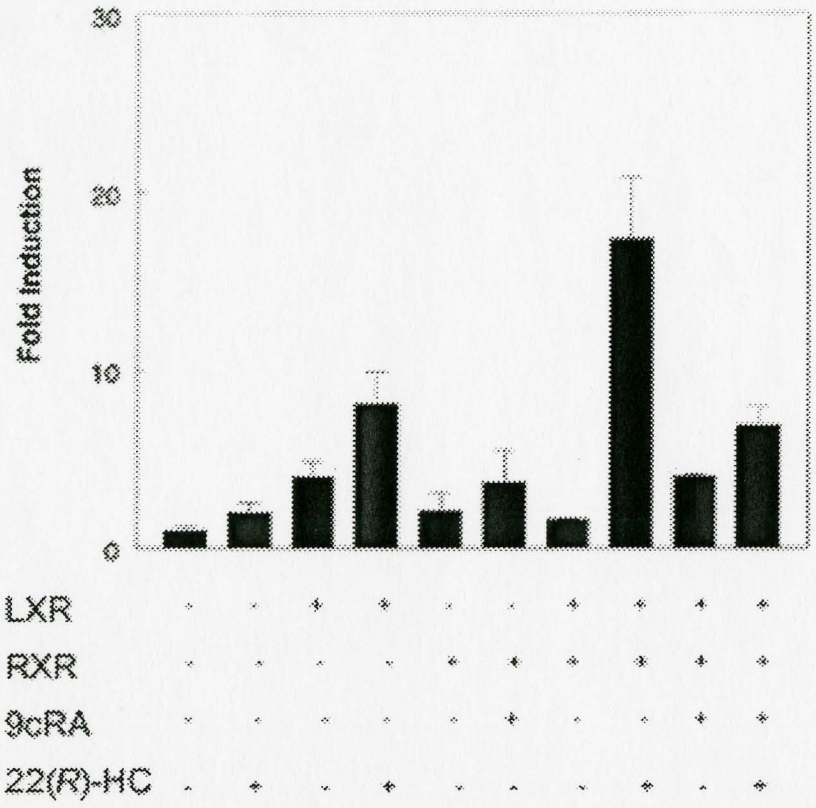

Figure 14. The human TNF-α promoter is a target for transactivation by LXRα and RXRα. COS-1 cells were transfected with a human TNF-α promoter/luciferase reporter gene (TNF-α(-1311)*luc* in the absence or presence of expression vectors for human LXRα and human RXRα along with 22R-HC (10μM) and 9cRA (10μM), as indicated, and luciferase activity was measured. The values presented represent the average (± SD), relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate, and normalized for protein and β-galactosidase expression levels.

LXRα/RXRα heterodimers bind directly to an upstream LXRE in the TNF-α promoter

The finding that the TNF-α gene is a direct target for LXR-dependent transactivation and that in THP-1 cells, induction of TNF-α mRNA occurs in the absence of *de novo* protein synthesis implied that LXR/RXR may bind directly to DNA in the TNF-α proximal promoter. As discussed earlier, 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 AGGTCA separated by 4 nucleotides (DR4) (Willy et al., 1995). The human TNF-α promoter contains numerous transcription factor binding sites, including multiple sites for NF-κB and AP-1, however, a canonical DR4 was not apparent, though several candidate DR4s were identified. To begin to identify promoter elements that mediate LXR responsiveness, a series of deletions in the TNF-α promoter were produced and tested for their activity in transfection assays in the presence of co-expressed LXRα/RXRα. As shown in Figure 15A, the wildtype TNF-α promoter was activated 18-fold by 22R-HC in the presence of co-expressed LXRα/RXRα, whereas promoter derivatives lacking nucleotides spanning -987 to -105 or -914 to -359 were inactive. A derivative where nucleotides -971 to -762 were deleted remained partially active, resulting in a 3-fold induction, while interestingly a reporter gene derivative missing nucleotides -640 to -493 was induced 9-fold (Figure 15A). These findings indicate that the region within the TNF-α promoter that confers maximal responsiveness to LXRα/RXRα lies between -971 to –762.

A cursory examination of the DNA sequence between -971 and -762 region revealed the presence of two degenerate AGGTCA motifs configured in a DR4 arrangement centered at residues -918 (site 1) and –879 (site 2) respectively, that could potentially serve as LXREs (Figure 15B). A synthetic sub fragment spanning residues -932 to -851 that contained these elements was responsive to LXR/RXR and 22R-HC, being activated 5-6-fold above background when appended to a heterologous promoter (pTNF(-932/-851)*luc* (Figure 15A).

To determine if LXR binds directly to this promoter region, a radiolabelled oligonucleotide probe corresponding to this region was tested by EMSA using LXRα and RXRα proteins synthesized *in vitro*, with the assistance of H. Patel. A protein/DNA complex was observed only in the presence of both LXRα and RXRα (Figure 16A). This complex was LXR/RXR-specific since it was competed in a dose-dependent manner by an unlabelled *bona fide* LXRE oligonucleotide (MTV LXRE, Willy et al., 1995). To determine if LXRα/RXRα targets either of the putative LXREs, oligonucleotides corresponding to site 1 and site 2 were used in DNA-binding competition assays. The site 2, but not the site 1 oligonucleotide was able to compete out binding of LXRα/RXRα to the -932/-851 fragment (Figure 16B), indicating that LXRα/RXRα targets site 2. Consistent with this, radiolabelled site 2 probe (Figure 16C), but not site 1 probe, formed a specific protein-DNA complex with LXRα/RXRα. 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 (Figure 16C). Unequivocal confirmation that site 2 constitutes a *bona fide* functional

LXRE is shown in Figure 17. Transfection of COS-1 cells with a reporter construct containing site 2 (pTNF(-894/-866)*luc*) was responsive to LXR/RXR and 22R-HC nearly 40-fold above control transfections. Conversely, the mutant variant (pTNFmut(-894/-866)*luc*) was unresponsive to LXR/RXR in these transfections. Furthermore, when the same site was mutated in the context of the full-length TNF-α (pTNFmut(-1311)*luc*) promoter, the LXR/RXR responsiveness of the promoter was abolished (Figure 18). From these observations, one can conclude that the DR4 arrangement at –879 in the TNF-α is a target for LXR/RXR and the outcome of this binding is activation of the TNF-α promoter *in vivo*. Consequently, it stands to reason that the induction of TNF-α mRNA by 22R-HC in THP-1 cells is a direct result of LXR/RXR binding and transactivation of the TNF-α promoter within these cells.

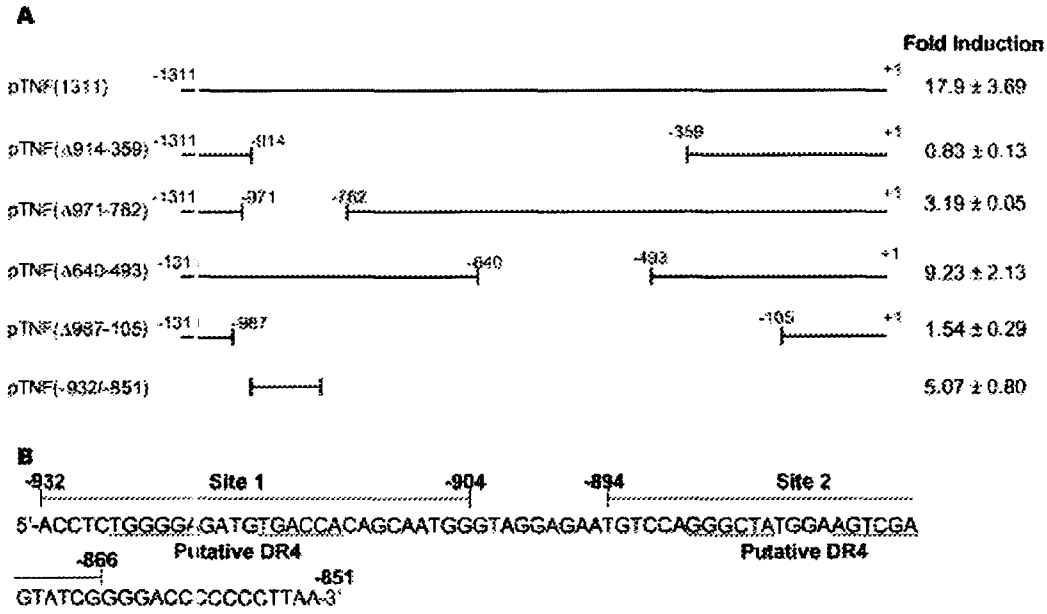

Figure 15. LXRα/RXRα transactivates of the human TNF-α promoter via a distal response element. (**A**) COS-1 cells were transfected as described above with pTNF(-1311)*luc* or various promoter derivatives, as indicated, along with human LXRα and human RXRα expression vectors in the presence of 22R-HC (10μM). The values presented represent the average (± SD) 22R-HC, LXRα/RXRα-mediated fold-induction (relative to corresponding untreated cells which were taken as 1) from three independent transfections carried out in triplicate and normalized for protein and β-galactosidase expression levels. (**B**) Relevant nucleotide sequence of the TNF-α promoter showing the position of two putative LXREs (site 1 and site 2).

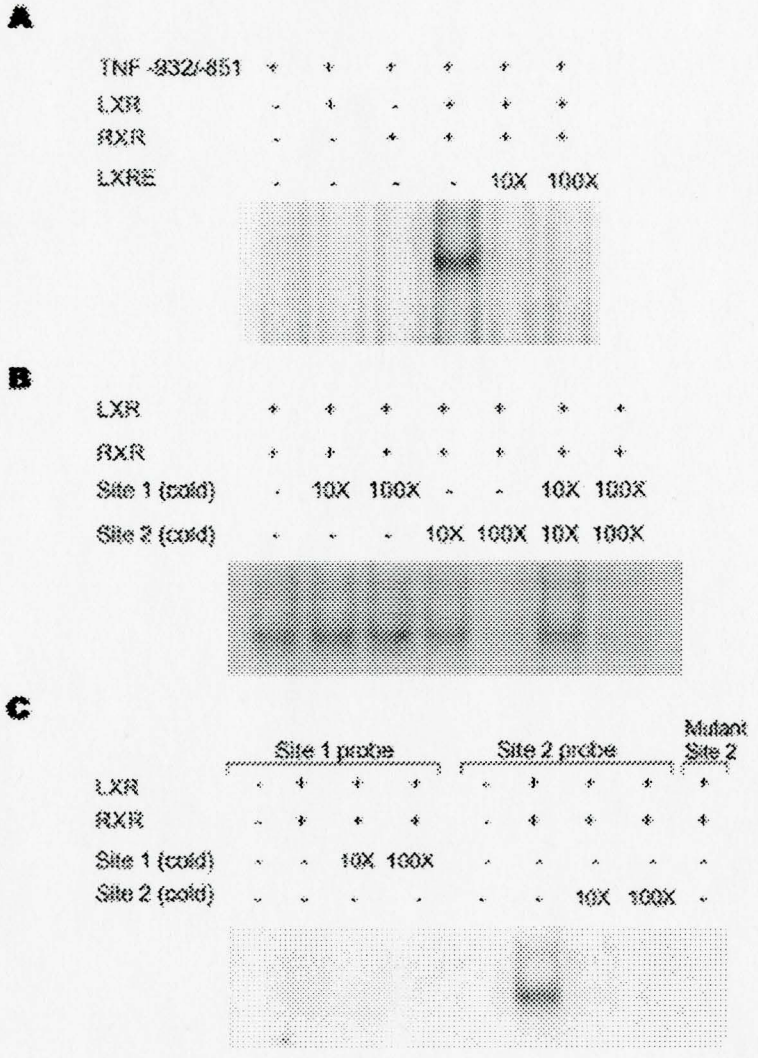

Figure 16. LXR/RXR bind directly and specifically to site 2 distal element in the TNF-α promoter (A) A radiolabelled DNA probe corresponding to nucleotides -932 to -851 of the TNF-α promoter was incubated with *in vitro* synthesized LXRα and/or RXRα, in the presence or absence of excess unlabelled MTV LXRE probe, as indicated, and protein/DNA complexes were resolved by gel electrophoresis. The first lane represents probe incubated with unprogrammed reticulocyte lysate. (B) Site 2 oligonucleotide inhibits protein/DNA complex formation. Labelled -932/-851 probe was incubated with LXRα/RXRα in the absence or the presence of 10 or 100-fold molar excess of unlabelled oligonucleotide corresponding to site 1 and/or site 2, as indicated. (C) LXRα/RXRα binds to site 2. Labelled probes corresponding to site 1, site 2, or the mutated site 2 oligonucleotide were incubated with LXRα/RXRα in the absence or presence of excess unlabelled competitor probe, as indicated, and protein DNA complexes were resolved by gel electrophoresis.

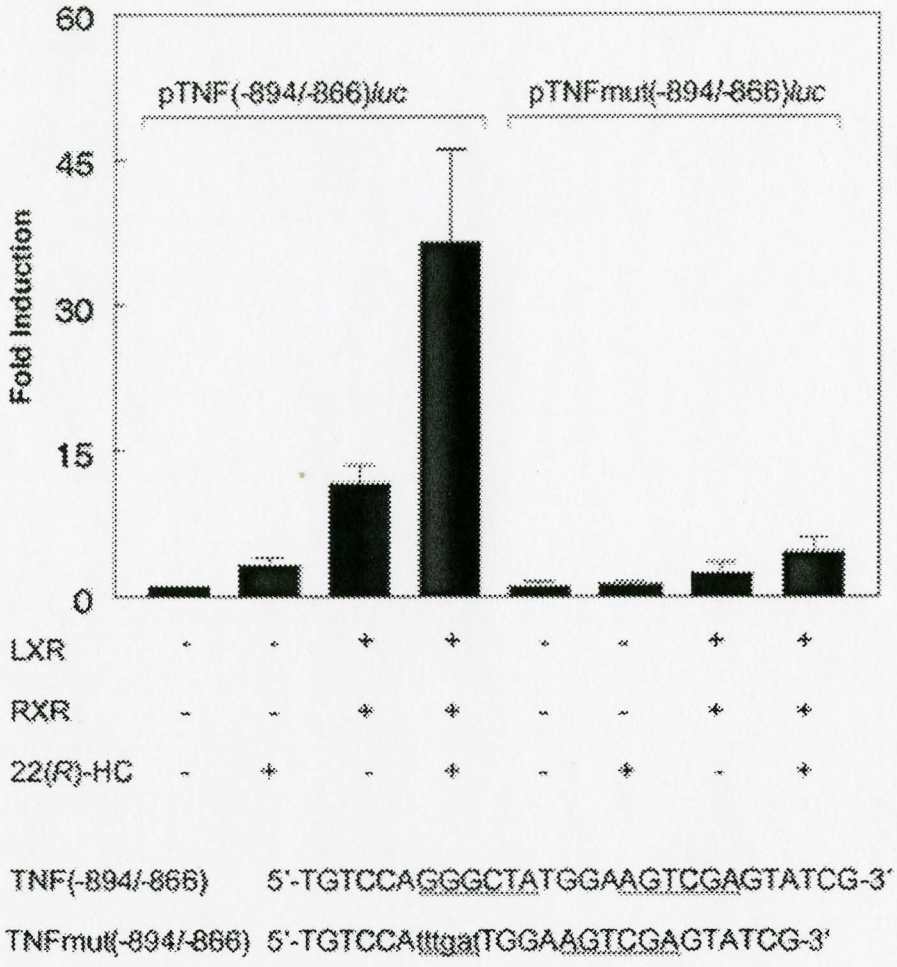

Figure 17. The TNF-α LXRE confers LXR/RXR responsiveness to a heterologous promoter. Luciferase reporter genes containing a single copy of the wild type site 2 oligonucleotide (pTNF(-894/-866)*luc*) or of a mutant derivative (pTNFmut(-894/-866)*luc*) were transfected into COS-1 cells in the presence or absence of 22R-HC along with expression vectors for LXRα and RXRα as indicated, and luciferase activity was measured. The values presented represent the average (± SD), relative to untreated cells (taken as 1) from two independent transfections carried out in triplicate, and normalized for protein and β-galactosidase expression levels. The sequence of the wild type and mutant oligonucleotides are indicated.

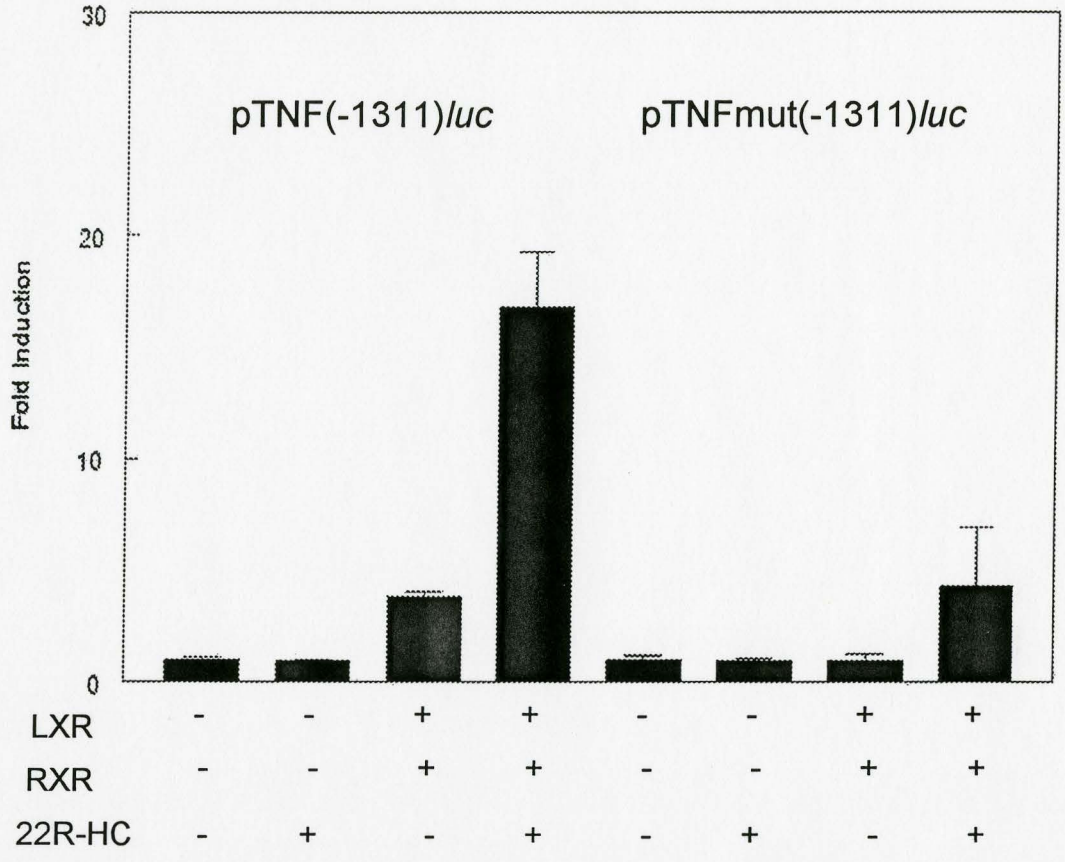

Figure 18. The TNF-α LXRE confers LXR/RXR responsiveness in the wild type TNF-α promoter. Luciferase reporter genes containing the mutated TNF LXRE in the full length TNF-α promoter (pTNFmut(-1311)*luc*) and wild type TNF-α promoter (pTNF(-1311)*luc* were transfected into COS-1 cells in the presence or absence of 22R-HC along with expression vectors for LXRα and RXRα as indicated, and luciferase activity was measured. The values presented represent the average (± SD), relative to untreated cells (taken as 1) from two independent transfections carried out in triplicate, and normalized for protein and β-galactosidase expression levels.

9cRA triggers release of TNF-α from cells via a separate LXR-independent pathway that requires de novo transcription and protein synthesis

As described above, while 22R-HC increased TNF-α mRNA and protein levels in THP-1 cells in an LXR-dependent manner, soluble TNF-α was only detected in the presence of co-administered 9cRA. Since 22R-HC administration results not only in TNF-α mRNA production but also processed (17kDa) TNF-α protein, this suggests that 9cRA, either alone or in conjunction with 22R-HC, participates in a distinct, post-translation step that triggers TNF-α release from cells.

To begin to elucidate this multi-step pathway, order-of-addition experiments with oxysterols and 9cRA in the presence of various inhibitors were carried out. The basic approach involved pre-incubating THP-1 cells with 22R-HC under various conditions for 12 hours to promote TNF-α mRNA and protein accumulation as previously shown. The findings that at 12 hours, TNF-α protein accumulates in THP-1 cells in response to 22R-HC though is not secreted (Figure 13) but is however detected at the same time period when both 22R-HC and 9cRA are present allowed for this systematic addition of compounds to be useful in elucidating what role 9cRA played in the post-translational release of TNF-α from these cells. 9cRA and additional various inhibitors as indicated in Figure 19, were administered after this 12-hour interval and cells were incubated for a further 12 hours, after which extracellular TNF-α was measured by ELISA. In Figure 19 lane d, addition of 22R-HC at t=0 followed by 9cRA at t=12 hours led to a significant induction of extracellular TNF-α protein in comparison to untreated cells or cells treated

with 22R-HC alone (lanes a and b). TNF-α was not detected when 22S-HC was substituted in place of 22R-HC prior to addition of 9cRA (Figure 19, lane i).

Co-incubation of 22R-HC at t=0 with cycloheximide (cyclo), a compound that inhibits protein translation by interfering with peptidyl transferase activity in the 80S ribosomal subunit but does not affect 22R-HC-dependent TNF-α mRNA induction as shown previously ablated soluble TNF-α production. Furthermore, 22S-HC (which inhibits 22R-HC induction as previously shown) followed by 9cRA at 12 hours abolished soluble TNF-α production. This was expected since TNF-α mRNA and/or protein is not induced under these conditions (lane c and h). Inclusion of 22S-HC with 9cRA at 12 hours had no effect on extracellular TNF-α production (Figure 19, compare lanes g and h, respectively). These results suggest that the secretion step triggered by 9cRA is independent of LXRα. However, inclusion of cycloheximide (lane f) or the transcription inhibitor actinomycin D (lane e) along with 9cRA at 12 hours completely abolished TNF-α secretion. Thus, 9cRA appears to be required for the *de novo* synthesis of a factor(s) that, while unnecessary for 22R-HC mediated induction of TNF-α mRNA and protein, is required at a post-translational step for release of cell-associated TNF-α.

Surprisingly, extracellular TNF-α was not detected when cells were first pre-incubated with 9cRA followed by 22R-HC (Figure 19, compare lane d with lane j). This could simply be due to degradation or loss of 9cRA activity during the 12-hour pre-incubation time interval prior to addition of 22R-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, which requires TNF-α for its own positive regulation. This scenario could also provide an explanation for the findings that sequential addition of 22R-HC followed by 9cRA led to a more robust production of TNF-α over a 24 hour interval as compared to when these compounds were co-administered (compare Figure 19 lane d with the 24-hour time point shown in Figure 9). These conditions would thus lead to the accumulation of intracellular TNF-α protein prior to secretion mediated by the subsequent addition of 9cRA.

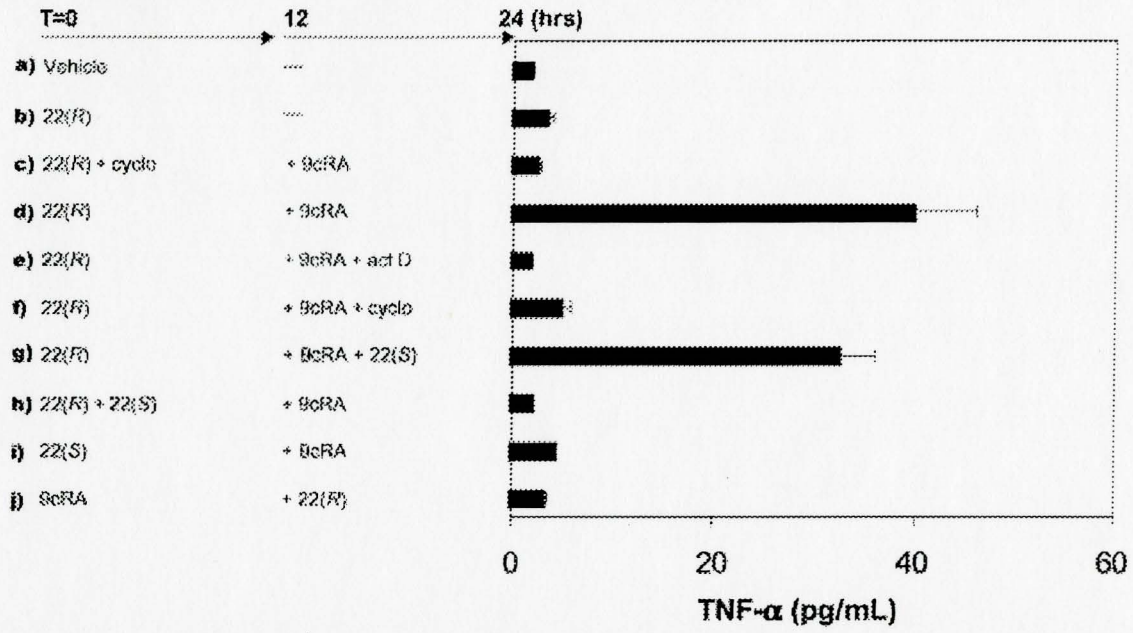

Figure 19. 9cRA triggers release of TNF-α protein in a process that requires *de novo* transcription and protein synthesis but is independent of LXR. THP-1 cells were sequentially incubated at t=0 and t=12 hours with the indicated compounds. Cells were incubated for a further 12 hours and culture supernatants were assayed for TNF-α by ELISA. Final concentrations were as follows; 22R-HC, 22S-HC, and 9cRA 10μM; cycloheximide (cyclo) 16mM; and actinomycin D (act D) 5μg/mL, respectively. The values presented represent the average (± SD) from three independent experiments carried out in triplicate.

The secretory step mediated by 9cRA is dependent upon RXR activation

Identifying that 9cRA mediates the production of a heretofore-uncharacterized protein factor involved with secretory step along the pathway of LXR-dependent TNF-α mRNA and protein expression suggested that endogenous RXR might be mediating this process. As described earlier, 9cRA is the natural ligand for RXR (Mangelsdorf and Evans, 1995, Schulman et al., 1997). While 9cRA is believed to be a specific RXR activator, it also binds to RAR with comparable affinity. 9cRA binds to RXRs with an affinity of 1.4-2.4 nM, and similarly 9cRA binds to RARs with an affinity of 0.2-0.8 nM (Allegretto et al., 1993). However, 9cRA transactivates RXRs much more efficiently than it does RAR even though 9cRA binds RAR with a higher affinity. As such, whether or not 9cRA is activating expression of this secretory factor through RXR/RXR homodimers or RXR/RAR heterodimers is difficult to ascertain using exclusively 9cRA. A novel class of highly selective RXR ligands, so-called "rexinoids", whose EC_{50} are nearly 100-fold lower than 9cRA have been developed by Ligand Pharmaceuticals. These compounds target RXR specifically and do not activate RAR as 9cRA has been shown to do. Vis-à-vis 9cRA, the EC_{50} for the rexinoid LG100268 is 0.05μM (M. Leibowitz, personal communication). These compounds have been extremely useful in elucidating the specific role of RXR in various processes, ranging from adipocyte gene expression (Singh et al., 2001) to the further clarification of ABCA1 in monocytes (Repa et al., 2000a). In order to clarify if RXR is driving the expression of the TNF-α secretion factor, THP-1 cells were incubated in the presence or absence of oxysterol and LG100268. As

shown in Figure 20, coincubation of THP-1 cells with LG100268 and other activators of LXR, 22R-HC, and T0901317, resulted in the production of TNF-α as judged by ELISA analysis. T0901317 is a synthetic compound produced by Tularik Corporation that binds LXR with a very high affinity and selectivity, more so than 22R-HC. It has been extremely useful in determining the LXR-dependency of a variety of processes (Repa et al., 2000a and Joseph et al., 2002). The levels of TNF-α produced using these compounds were comparable to those achieved using 22R-HC/9cRA. However, 22R-HC/LG100268 resulted in significantly more TNF-α, while T0901317/LG100268 resulted in nearly 50% less TNF-α than either 22R-HC/9cRA or 22R-HC/LG100268.

These differential effects on TNF-α production between the above treatments may reflect the degree of TNF-α transactivation achieved by 22R-HC, T0901317, and even LG100268, which can all activate LXR/RXR heterodimers. However, the transactivation potential of T0901317 may be less than that of 22R-HC. This oxysterol may not bind as tightly to LXR as T0901317 but may induce conformational changes in the receptor that are more favorable to maximal transactivation than T0901317. The same may be true for LG100268 given that upon binding RXR, it alters the conformation of the LXR/RXR heterodimer in such a way so as to permit transactivation of the TNF-α promoter, thereby augmenting TNF-α mRNA production. LG100268 may further induce expression of the secretory factor through RXR. This could account for the increased TNF-α protein detected in samples co incubated with 22R-HC and LG100268. This hypothesis is substantiated by Northern blot analysis demonstrating that TNF-α mRNA is induced in

the presence of LG100268 (data not shown). Indeed, TNF-α mRNA has also been shown recently to be induced by LG100268; however the reasons for this induction were not clear (Singh et al., 2001). Given the evidence presented here, the LG100268-mediated induction of TNF-α mRNA by Singh et al., 2001 was likely mediated through LXR/RXR.

Definitive evidence for the RXR dependency in the expression of a factor(s) required for the secretion of LXR-derived TNF-α comes from order of addition experiments based on the methodology described above. Pre-incubation of THP-1 cells with 22R-HC for 12hrs followed by addition of LG100268 and incubation for a further 12hrs resulted in a robust production of TNF-α protein to a level similar to that achieved with 9cRA under similar circumstances (Figure 21). Like earlier experiments where 9cRA was incubated initially followed by 22R-HC supplementation, initial treatment of THP-1 cells with LG100268 for 12 hours followed by 22R-HC supplementation failed to produce a significant amount of TNF-α (Figure 21). This again may be a function of the factor produced via LG100268 being labile and/or requires TNF-α for its sustained expression. Based on the selectivity of the rexinoid in specifically activating RXR and the absence of any TNF-α produced upon co incubation of THP-1 cells with 22R-HC and a RAR-specific activitor, atRA (data not shown), one can conclude that the secretory step involved in the release of TNF-α protein produced via LXR is certainly controlled by either RXR homodimers or another RXR heterodimer that does not include either RAR or LXR.

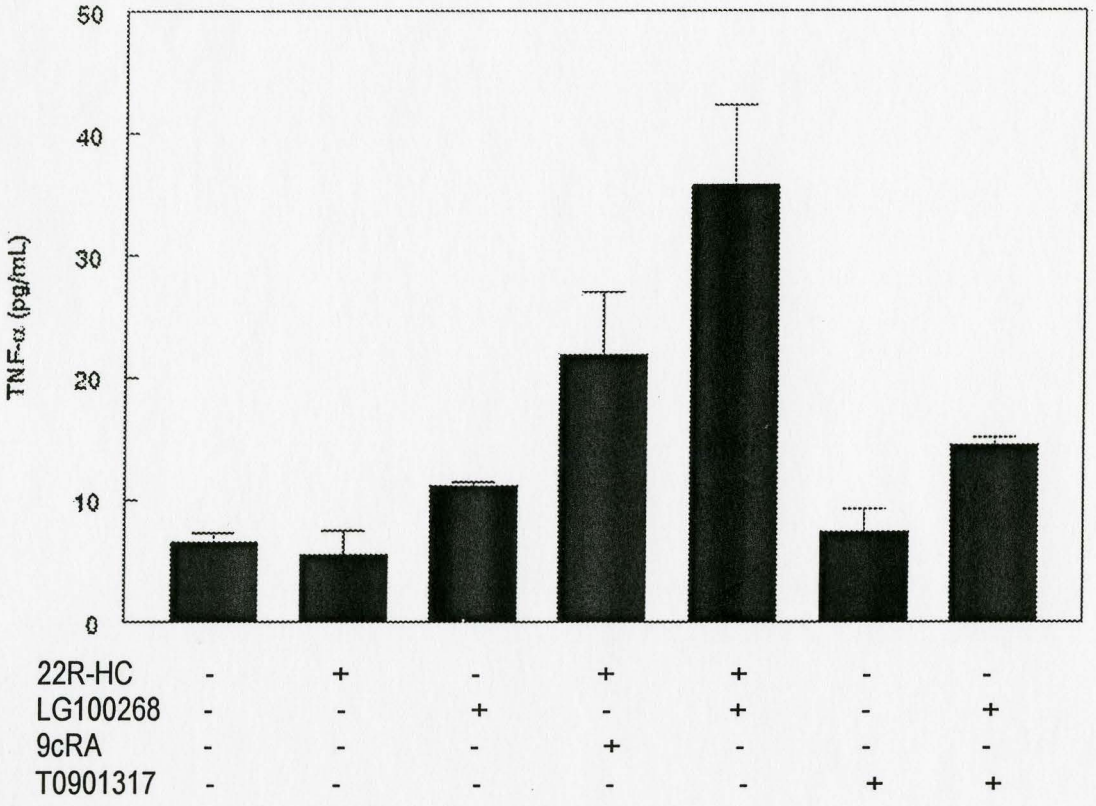

Figure 20. Co-incubation of THP-1 cells with 22R-HC and synthetic LXR activators in addition to LG100268 result in production of TNF-α. THP-1 cells were cultured for 48 hrs in the presence or absence of either 22R-HC (10μM), LG100268 (0.1μM), 9cRA (10μM), or T0901317 (0.1μM) as indicated. The supernatants were collected, and the amount of TNF-α present in the supernatants was determined by ELISA. Experiments were carried out in triplicate

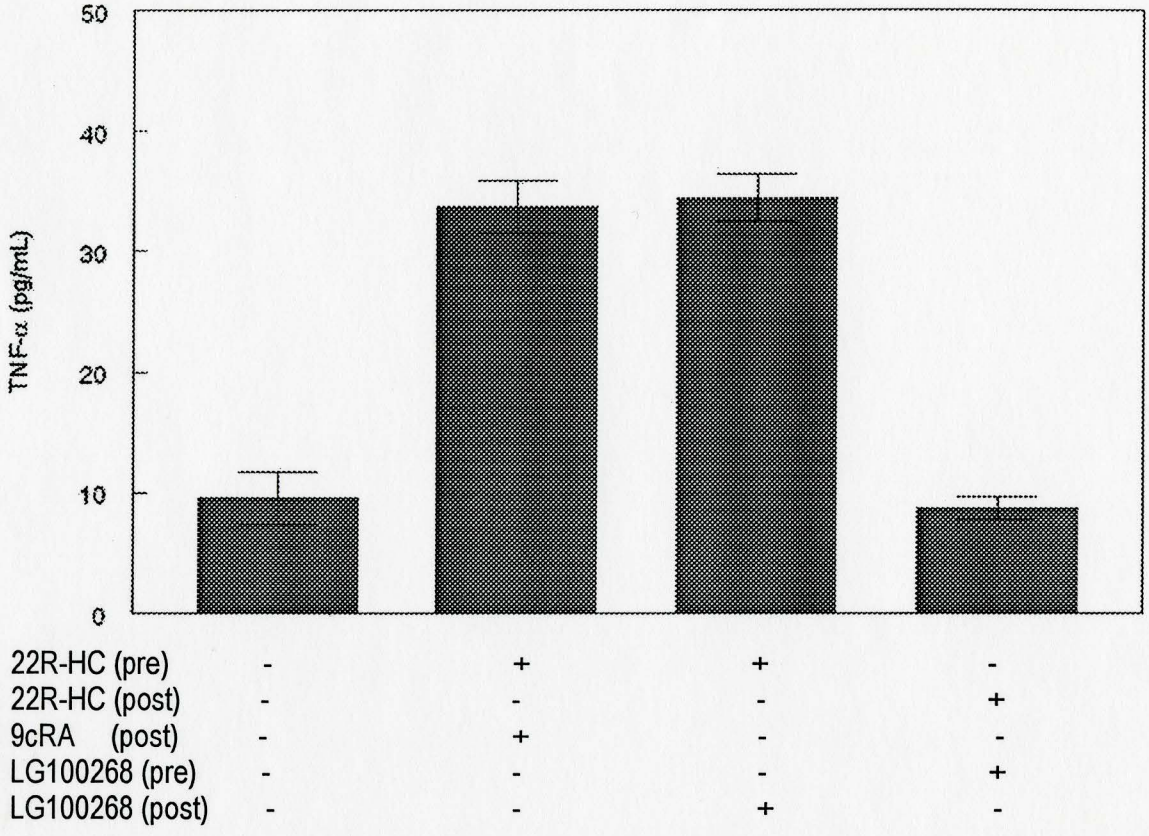

Figure 21. LG100268 triggers the release of TNF-α protein following an initial pre-incubation of THP-1 cells with 22R-HC. THP-1 cells were sequentially incubated with the indicated compounds 12 hrs initially (pre) followed by a further 12 hrs with another compound as indicated (post). 22R-HC (10μM), LG100268 (0.1μM), or 9cRA (10μM). Following the total 24 hr incubation, the supernatants were collected, and the levels of TNF-α present were determined by ELISA. Experiments were carried out in triplicate

Different stimuli cooperate with LXR activators to produce TNF-α from human monocytes

Given the evidence for a LXR/RXR dependent pathway leading to TNF-α expression and production from THP-1 cells, investigations were undertaken to examine the effect of different chemical or cytokine stimuli on the LXR/RXR dependent TNF-α expression pathway. In the context of atherogenesis, monocytes differentiate into macrophages once they become intima-resident. Furthermore, much of the cytokine production associated with atherogenesis is derived from these intima-resident macrophages (Lusis 2000). Consequently, to determine what role this regulatory circuit involving LXR and RXR in the production of TNF-α may play in macrophages, THP-1 cells were differentiated into macrophages using the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (PMA). PMA is a compound that mimics diacylglycerol, thus activates PKC in monocytes. PMA induces monocytes to differentiate into macrophages. Differentiation of THP-1 monocytes into macrophages using PMA followed by treatment with activators of LXR and RXR exerted interesting but differential effects on TNF-α production. A 48-hour pretreatment of THP-1 cells with PMA to differentiate them into macrophages (Mikita et al., 2001), followed by a PBS wash, and incubation for a further 48 hours with fresh media in the presence or absence of LXR and RXR activators resulted in TNF-α production from cells treated with 22R-HC alone (25.626pg/mL TNF-α produced), 22R-HC and LG100268 (65.121pg/mL), and LG100268 alone (11.525pg/mL) (Figure 22). Interestingly, 22R-HC and 9cRA coincubation did not augment the production of TNF-α above the levels achieved with

22R-HC treatments alone (25.919pg/mL vis-à-vis 25.626pg/mL, respectively). Furthermore, the intriguing result of these experiments was that in PMA-differentiated THP-1 cells, 9cRA was no longer required in order to observe TNF-α in the supernatant of cells treated with 22R-HC (Figure 22). It is possible that the differentiation program activated by PMA in THP-1 cells leads to the expression of this secretory factor. Furthermore given the lack of any additional effect with 9cRA, it is possible that expression of this secretory factor achieves a maximum level as a result of PMA-stimulation such that further activation with 9cRA fails to induce additional expression.

In light of the increased production of TNF-α in the presence of 22R-HC and LG100268, it is possible that the larger levels may be due in part to LG100268 augmenting the level of TNF-α mRNA in an additive manner with 22R-HC. This scenario is plausible since LG100268 can activate the permissive LXR/RXR heterodimer (Repa et al., 2000a). This reasoning helps to also explain the production of TNF-α when differentiated THP-1 cells were treated with LG100268 alone. Indeed, it was demonstrated that LG100268 is able to induce TNF-α mRNA expression based on Northern blot data collected previously, which lend support to this hypothesis (data not shown). One can infer that these differing levels of production are not due to a further augmentation of the secretory factor's expression since the presence of 9cRA, no additive effect on TNF-α production was observed, despite 9cRA being able to activate RXR. Therefore, the increased level of TNF-α production using 22R-HC and LG100268 is attributed to augmentation of TNF-α mRNA and not augmentation of the secretory factor(s).

These data suggest that RXR may be activated within the signaling cascades induced by PMA. The possibility that RXR becomes activated in the absence of ligand engagement, as part of a larger generalized signaling cascade is not without precedence. Nuclear hormone receptors can be phosphorylated by the activation of a diverse set of phosphorylation cascades. For example, RXR can be phosphorylated by cyclin B, ERK1, MKK4/SEK1, and PKA, events that in a particular cellular context either activate or repress RXR activity (Adam-Stitah et al., 1999, Harish et al., 2000, Lee et al., 2000, and Dowhan and Muscat, 1996). Furthermore, PPAR signaling can be modulated through PKC-dependent phosphorylation, and even LXRα has been shown to be a target for PKA-mediated phosphorylation, both of which enhance their respective activity (Latruffe et al., 2000 and Tamura et al., 2000, respectively). Based on these findings and in light of evidence showing that upon pretreatment of THP-1 cells with PMA, the need for 9cRA to promote TNF-α secretion in response to LXR-dependent induction of TNF-α is no longer required implies that these stimuli may be activating a series of signaling cascades that lead to the phosphorylation of a factor(s) that either activates RXR indirectly through a heretofore unknown protein mediator that cooperates with RXR, or directly by phosphorylation of the receptor itself thereby activating it.

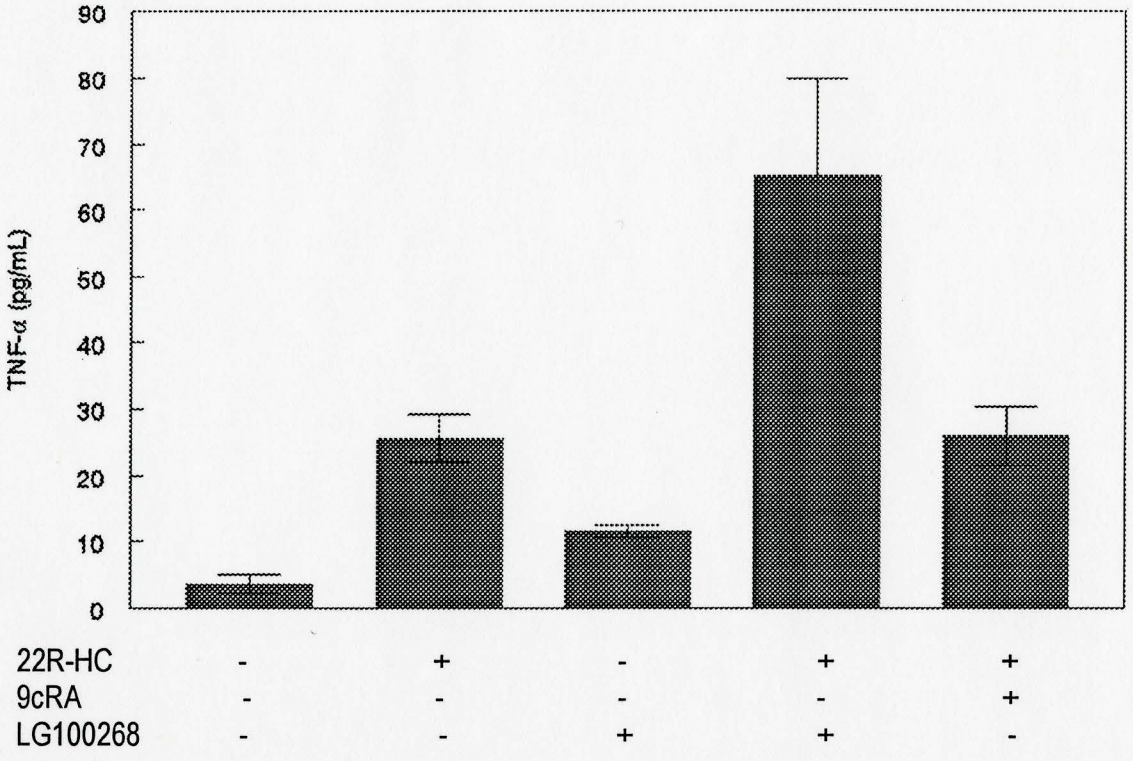

Figure 22. Effects of oxysterols and RXR activators on TNF-α production in PMA-differentiated THP-1 macrophages. THP-1 cells were differentiated into macrophages by 48 hr treatment with PMA (50ng/mL). Following 48 hr incubation, cells were washed twice with PBS, and fresh media was added supplemented with either 22R-HC (10μM), 9cRA (10μM), or LG100268 (0.1μM), as indicated. Cells were incubated for a further 48 hrs, after which the supernatants were collected, and the levels of TNF-α were determined by ELISA. Experiments were carried out triplicate.

The secretory factor(s) is a component of the PMA-dependent signaling cascade

The data collected thus far suggest that the RXR-dependent step involved in the secretion of LXR-derived TNF-α protein becomes activated upon PMA stimulation and furthermore does not require exogenous ligand activation. It was important to know if RXR is a direct downstream target of the signal transduction cascades activated by PMA. To begin to elucidate the signaling pathway that leads to RXR activation and if RXR is indeed a direct target for these signaling cascades a highly selective RXR antagonist (LG101208) was used. Preincubation of THP-1 cells with the antagonist in the presence of PMA for 48 hrs followed by washing and supplementation with LXR or RXR agonists showed that TNF-α production was reduced by nearly 80% in the presence of the RXR antagonist (Figure 23). This reduction was not due to the inhibition of TNF-α mRNA production through LXR/RXR since northern analysis of TNF-α mRNA produced in the presence of 22R-HC and the antagonist resulted in an induction of mRNA on par with that achieved by 22R-HC alone (Figure 24). Consequently, these experiments demonstrated that the inhibition of TNF-α production from these cells in the presence of LG101208 is due thus to an inhibition of the second secretory step and not the expression of TNF-α mRNA by LXR/RXR. Furthermore, when both 22R-HC and LG100268 were added following the initial 48 hr incubation with PMA and LG101208, TNF-α protein secretion was restored, albeit not to levels observed when 22R-HC and LG100268 are added following only PMA incubation. These data showed that LG100268 relieves the

inhibition of the secretory factor's expression by LG101208, arguably by competing with LG101208 for RXR and thus activating the receptor. Based on these results, the RXR dependency of the second step is further supported but additionally a connection between the signaling cascade initiated by PMA and the activation of RXR and subsequently the secretory step, which facilitates secretion of TNF-α has been made.

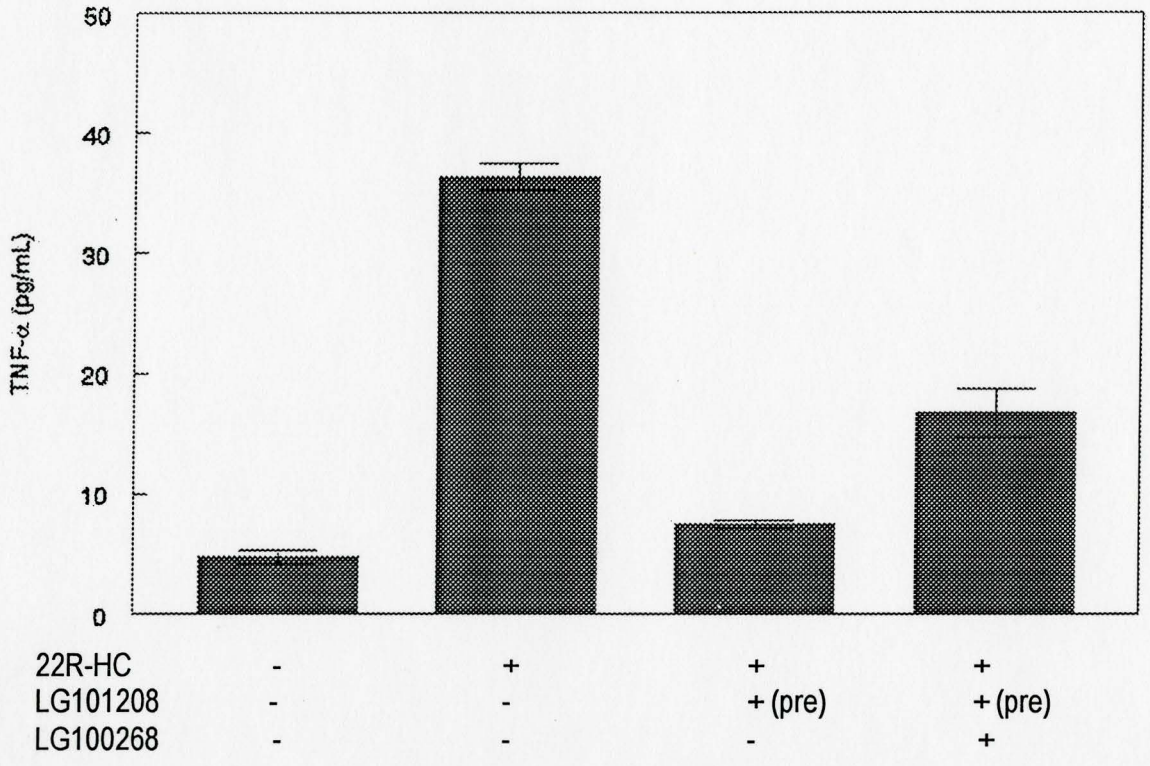

Figure 23. The effect of pre-incubation of THP-1 cells with PMA and an RXR antagonist on TNF-α secretion followed by addition of LXR/RXR activators. THP-1 cells were differentiated with PMA (50ng/mL) for 48 hrs, in the presence or absence of an RXR antagonist, LG101208, (0.1μM) (pre). Following 48 hrs, cells were washed twice with PBS, and fresh media was added supplemented with either 22R-HC (10μM) and/or LG100268 (0.1μM), as indicated. These cells were then incubated for a further 48 hrs, and the levels of TNF-α in the supernatants were measured by ELISA. Experiments were carried out in triplicate.

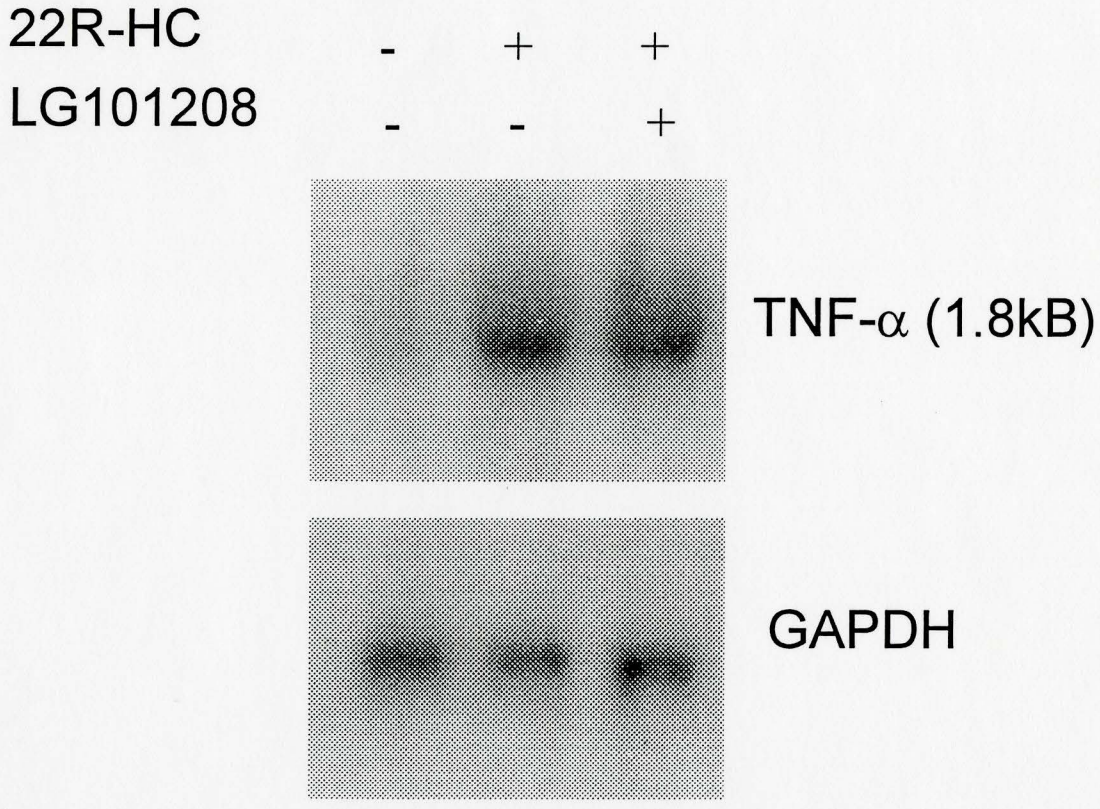

Figure 24. Rexinoid antagonist LG101208 does not inhibit 22R-HC-dependent induction of TNF-α mRNA. THP-1 cells were incubated for 48 hrs in the presence of 22R-HC (10μM) and the rexinoid antagonist LG101208 (0.1μM), as indicated. Following this RNA was isolated and subjected to Northern analysis as described above.

The secretory step initiated by PMA is sensitive to inhibition of protein kinase C (PKC).

Since PMA is an analogue of diacylglycerol (DAG), which activates PKC, and based on evidence collected that implicates PMA-dependent signaling in the activation of the RXR-dependent secretory step, it is possible that the secretory pathway activated by PMA is dependent on PKC. To examine this potential link, a broad-spectrum inhibitor of PKC, calphostin C, was used. Calphostin C competes with DAG for the DAG-binding site in PKC (Kobayashi et al., 1989). When THP-1 cells were coincubated with PMA and calphostin C for 48 hrs, followed by washing and incubation with 22R-HC for a further 48 hrs, the production of TNF-α was reduced by nearly 75% in the presence of calphostin C relative to cells treated initially with only PMA followed by 22R-HC supplementation (Figure 25). When THP-1 cells were supplemented with both 22R-HC and LG100268, the production of TNF-α in the supernatants was restored to levels near those achieved when 22R-HC is added following PMA-stimulation (Figure 25). Interestingly, when LG100268 is added initially with calphostin C and PMA, and 22R-HC is subsequently added, robust production of TNF-α is detected, again at levels that approach those achieved by 22R-HC following PMA-stimulation (Figure 25). These data show that the secretory step induced by LG100268 and RXR is indeed an end point in the PKC-dependent signaling cascade, and this further shows that activation of RXR is a component of the signaling cascades elicited by PMA.

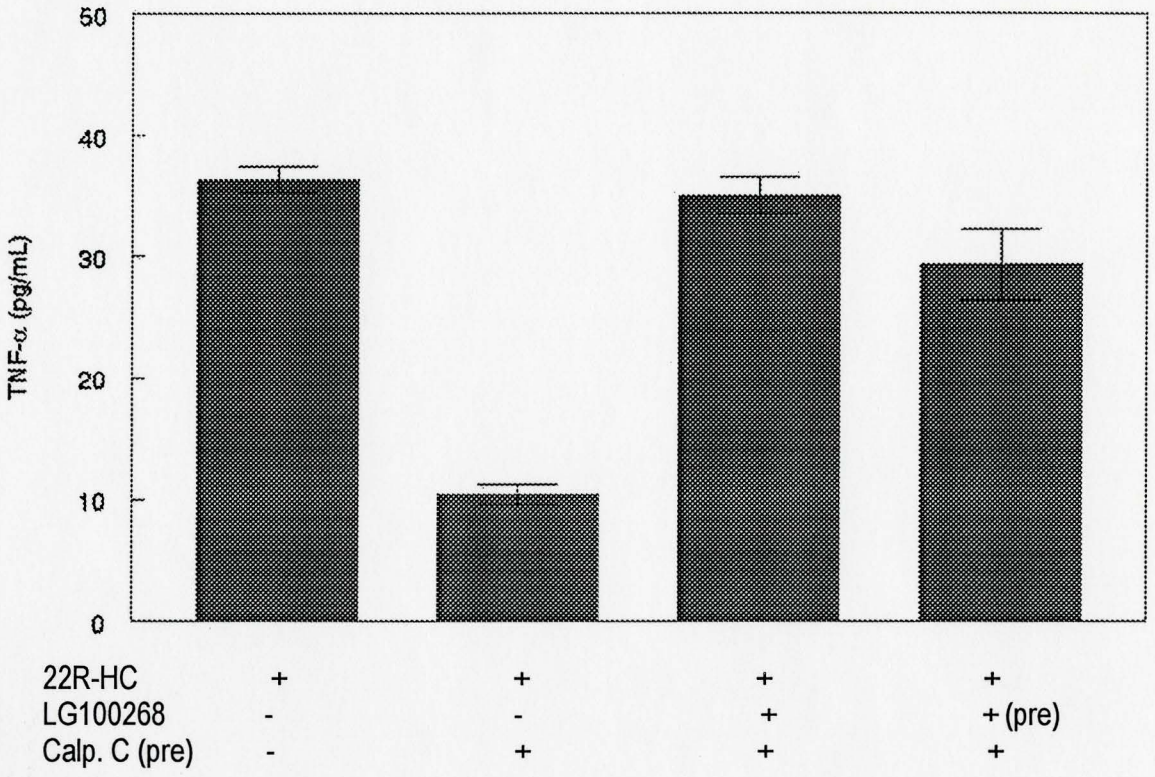

Figure 25. The effect of pre-incubation of THP-1 cells with PMA and calphostin C on TNF-α secretion followed by addition of LXR/RXR activators. THP-1 cells were incubated for 48 hrs with PMA (50ng/mL) and in the presence or absence of calphostin C (calp. C) (1μM) and LG100268 (0.1μM) (pre), as indicated. Following 48 hrs, cells were washed twice with PBS, and fresh media was added supplemented with 22R-HC (10μM) and LG100268 (0.1μM), as indicated. Cells were incubated for a further 48 hrs, and the levels of TNF-α in the supernatants were measured by ELISA. Experiments were carried in triplicate.

CHAPTER 4: Discussion

Major Findings of Work

The emergent role of LXR pathways that control rates of lipid accumulation and efflux and in coordinating cellular responses to lipid loading in monocytes and macrophages, and the relationship between monocytes, lipid accumulation, and atherogenesis has made LXR the subject of extensive and intense study (Claudel et al., 2001, Chawla et al., 2001c, Chawla et al., 2001b, and Tontonoz et al., 1998). The studies described herein have expanded the role for LXR in monocyte biology to now include cytokine expression and furthermore to expand the role of nuclear hormone receptors, specifically RXR, in mediating the secretion of soluble factors. Initially, it was shown that LXR and its oxysterol ligands specifically stimulate TNF-α mRNA synthesis. This is achieved in part through the binding of LXR/RXR directly to an LXRE within the proximal promoter of the TNF-α gene. This is the first demonstration that TNF-α or any cytokine is a direct target for LXR-dependent transactivation. Intriguingly, LXR-dependent of TNF-α mRNA was shown to be translated; however, protein remains cell-associated. Secretion of TNF-α is mediated by the induction of a gene(s) that is under the control of RXR. However, this secretory step is independent of LXR and is indeed part of a generalized differentiation program within monocytes that culminates in the activation of RXR resulting in the expression of these "secretory" factors.

Production of TNF-α from human monocytes by LXR/RXR activators

Oxidized LDL and its constituent oxysterols and oxidized lipids exert pleiotropic effects on gene expression and cellular function in macrophages that have been directly implicated in the development and pathogenesis of atherogenesis and atherosclerosis (Lusis, 2000). OxLDL provides ligands for both PPARγ and LXRα, and recent studies have shown that these receptors cooperate in interconnected pathways that modulate the uptake and removal of cholesterol in monocytes following lipid loading (Chawla et al., 2001b).

The intriguing result from the initial treatments of THP-1 and peripheral blood monocytes with LXR and RXR activators was that production of TNF-α did not require monocyte differentiation. Unstimulated monocytes do not normally secrete TNF-α unless activated and differentiated into macrophages (reviewed by Arai et al., 1990). Consequently, it was hypothesized that these compounds were affecting cellular function via a pathway that is independent of monocyte activation or differentiation or perhaps is mimicking a process involved with either of these events. Furthermore, coexpression of other proinflammatory cytokines such as IL-1β or IL-6 was not detected, which in the context of TNF-α is almost always observed. These data implied that the production of TNF-α in response to these compounds was the product of a novel and a unique pathway that was TNF-α-specific.

The LXR-selectivity in this process came from experiments showing that the enantiomer of 22R-HC, 22S-HC, failed to exert any comparable effect on TNF-α secretion either alone or in concert with 9cRA or LG100268 (Figures 7 and 20 respectively). As described earlier, 22S-HC is an effective inhibitor of LXR, since it binds the receptor with comparable affinity as 22R-HC yet fails to induce transactivation. Consequently, when increasing concentrations of 22S-HC were added to cells under 22R-HC and 9cRA stimulation and the levels of TNF-α production decreased, it followed that this was due to 22S-HC competing with 22R-HC for LXR binding, if indeed LXR/RXR were mediating the response. Furthermore, the time course experiments of TNF-α production demonstrated that a gradual accumulation of TNF-α in cellular supernatants occurred when cells were stimulated with 22R-HC and 9cRA, suggesting that the production of TNF-α in THP-1 cells may be due to transcriptional induction and may indeed involve LXR/RXR.

This hypothesis was confirmed by northern analysis and transfection studies that demonstrated the TNF-α promoter is a direct target for transactivation by LXR/RXR heterodimers. Northern blot analysis showed that oxysterol activators of LXR induced the expression of TNF-α mRNA, while 22S-HC failed to do so (Figure 10). This result alluded to an LXR-dependence in this process as did northern analysis of RNA from cells treated with 22R-HC and the translation inhibitor cycloheximide showing that TNF-α mRNA was still induced by 22R-HC even in the presence of cycloheximide (Figure 12). This result showed that *de novo* translation of other proteins induced by 22R-HC are not required for the induction of TNF-α mRNA suggestive that pre-existing factors like

LXR/RXR are involved. These results and those collected from northern analysis of RNA from cells treated with 22R-HC over a defined time course all suggested that LXR is likely involved directly in the transcriptional induction of TNF-α mRNA in THP-1 cells (Figure 11).

The subsequent mapping of a functional LXRE to –879 in the TNF-α promoter, which is manifest by the direct binding of LXRα/RXRα to a DR4 arrangement definitively showed that LXR/RXR activates the *tnf-α* gene (Figure 16). Intriguingly, promoter derivatives that retained the LXRE failed to transactivate the *luciferase* gene to the same level that the wildtype, full-length TNF-α promoter achieved (Figure 15A). These results suggested that other regions in the TNF-α promoter likely cooperate with the LXRE to produce maximal transactivation in our system. The TNF-α promoter contains many different binding sites for other transcription factors, such as AP-1, NF-κB, and Oct-1, all of which can contribute to the transcriptional induction of the *tnf-α* gene (Takashiba et al., 1993 and reviewed by Ware et al., 1996). It is possible that *in vivo* such transcription factors or others may be involved and cooperate with LXR/RXR directly, or through the battery of cofactors that become recruited to the heterodimer upon ligand engagement.

This hypothesis is plausible given studies showing that the transcription factors *c-jun/c-fos* (AP-1) and the p50 subunit of NF-κB interact with SRC-1 (Lee et al., 1998 and Na et al., 1998). These interactions were shown to potentiate both AP-1- and NF-κB-mediated transactivations through a further cooperation with CBP-p300 integrators of the basal transcription machinery. Consequently, since LXR/RXR dependent transactivations

involve the recruitment of SRC-1 (Spencer et al., 2001 and Gan et al., 2001) and given the role that transcription factors such as AP-1 and NF-κB play in TNF-α gene expression, it is conceivable that cross talk may occur between LXR/RXR and these transcription factors *in vivo*. It will be interesting to elucidate any cross talk between these transcription factors and LXR/RXR. Certainly, the interplay with other transcription factors will provide insights into the mechanisms of transcriptional activation and the potential convergence with these other signaling pathways that are known to be important for TNF-α gene expression.

RXR-dependency of the secretory step involved in LXR-dependent TNF-α production

TNF-α is an essential cytokine mediator of inflammation and apoptosis whose production, if not stringently controlled, can result in severe pathologic conditions such as septic shock. Therefore, the expression of TNF-α is under strict control at both the level of transcription and translation. These mechanisms regulate the expression, abundance, modification, processing, stability, subcellular localization and even the secretion of TNF-α mRNA and protein (Piecyk et al., 2000, Dean et al., 2001, Raabe et al., 1998, and Shurety et al., 2000). The findings that TNF-α mRNA induced by LXR/RXR is translated, and the TNF-α protein produced is processed from the 27kDa TNF-α precursor protein to the bioactive 17kDa version suggested that a secretory control step is required to facilitate the release of this processed TNF-α from the monocyte (Figure 13). Inhibitor studies and order-of-addition experiments indicated that

9cRA and the RXR-selective compound LG100268 induce the expression of a factor(s) that is secondary to 22R-HC/LXR-mediated induction of TNF-α mRNA and protein synthesis. Instead, it is part of a secretory step that permits the release of TNF-α from these cells. This post-translational step is independent of LXR since 9cRA-mediated TNF-α release was not ablated in the presence of the LXR antagonist 22S-HC. This step requires, however, *de novo* transcription and protein synthesis, since it was sensitive to inhibition by actinomycin D and cycloheximde, respectively (Figures 19 and 21). Since LG100268 is selective for RXR, these data suggested that the secretory control step involves RXR.

While a role for RXR has been definitively shown by the use of highly selective RXR ligands, it remains uncertain as to whether or not this secretory pathway activated by RXR is due to transactivation of target genes through RXR homodimers or a yet-to-be identified RXR heterodimer. One can eliminate the possibility that LXR or RAR are potential heterodimer partners for RXR in this process since it was shown that 22S-HC fails to inhibit the secretory step in the order-of-addition experiments, and similarly the RAR selective ligand all *trans* retinoic acid (atRA) when co-incubated with 22R-HC does not result in the production of TNF-α. It will be important to clarify the exact RXR combination that is involved in mediating this secretory step, and with the availability of highly selective experimental compounds for different hormone receptors, identifying if another nuclear hormone receptor cooperates with RXR in the activation of this secretory step should be relatively easy to discover.

While a generalized secretory mechanism for cytokines is yet to be described in macrophages that is controlled by RXR, previous reports have shown that 9cRA and RXR can promote the secretion of insulin from glucose-stimulated pancreatic islet cells (Chertow et al., 1997 and Blumentrath et al., 2001). Thus there is precedence that RXR can mediate the secretion of other soluble factors. The finding that processed (17kDa) TNF-α is retained in THP-1 cells following a 22R-HC stimulation implied that the secretory factor induced by RXR activation is producing a series of factors that must be involved in the post-Golgi trafficking of TNF-α to the plasma membrane, and not the machinery involved in processing TNF-α such as the TNF-α converting enzyme (TACE). 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 (Shurety et al., 2000). Scant evidence is available to begin to speculate what this particular factor(s) may be; however, based on the results collected from differentiated THP-1 cells, these secretory factors are also induced within the monocytic differentiation programme (Figures 22).

Indeed, the use of effective and selective inhibitors of RXR demonstrated that RXR becomes activated in a PKC-dependent manner within the differentiation programme initiated by PMA, since inhibition of RXR activity by LG101208 ablated the secretion of TNF-α in response to 22R-HC, but was restored in spite of PKC inhibition by activation of RXR using LG100268 (Figures 23 - 25). These results directly implicated PKC signaling in the activation of RXR. Future studies should be conducted to determine if other signal transduction pathways activated by PKC, for example PI3K

and MEK/MAPK, are involved in the activation of this secretory step. To carry out these experiments, a similar protocol should be followed as the experiments executed using calphostin C; however, Wortmannin, which inhibits PI3K, and PD-98059, a potent and selective inhibitor of MAP kinase kinase (MEK), should be substituted for calphostin C.

The convergence of phosphorylation signal transduction cascades and nuclear hormone receptor signaling is not novel. Indeed, others have shown that several nuclear hormone receptors can be phosphorylated and their transcriptional activity modulated in the absence of ligand engagement (Latruffe et al., 2000 and Tamura et al., 2000). Insofar as RXR phosphorylation is concerned, RXR can be phosphorylated by cyclin B, ERK1, MKK4/SEK1, and PKA. In many instances however, phosphorylation of RXRα and RXRβ results in a repression of its transactivation potential (Adam-Stitah et al., 1999, Harish et al., 2000, and Lee et al., 2000). However, PKA-dependent phosphorylation of RXRγ enhances its transactivation potential (Dowhan and Muscat, 1996). In the cellular pathways elucidated here that culminate in RXR activation, it remains to be seen whether or not RXR is actually phosphorylated as a result of PKC activation. If RXR is directly phosphorylated, it will be important to resolve if this is mediated directly by PKC, or perhaps one of the downstream effectors of PKC such as PI3K, MEK/ERK, or PKA. If RXR is not directly phosphorylated, it raises the possibility of whether or not a coactivator molecule that cooperates with RXR is phosphorylated and in turn facilitates expression of this secretory factor(s). Studies have shown that indeed coactivators such as SRC-1 can be phosphorylated by both PKA and ERK1/2 to enhance ligand independent transactivation of nuclear hormone receptors (Rowan et al., 2000a, Rowan et al., 2000b,

and Tremblay et al., 1999). Furthermore, a transcriptional integrator, p300/CBP, can be phosphorylated by PKC and ERK1 (Yuan and Gambee, 2000 and Gusterson et al., 2002, respectively). p300/CBP has been shown to interact with SRC-1(Yao et al., 1996) and to potentiate nuclear hormone receptor signaling. Consequently, it is possible that either of these accessory proteins could be phosphorylated in response to the signal transduction cascades activated by PMA if indeed RXR is not shown to be phosphorylated.

Another plausible scenario that could result in the activation of RXR within the monocytic differentiation programme is that differentiation results in the production of an endogenous RXR ligand. This endogenous ligand would bind to RXR and activate expression of the gene(s) involved in the secretory programme facilitating release of LXR-derived TNF-α. To date, 9-*cis* retinoic acid is the only identified endogenous ligand that binds to and activates RXR (Mangelsdorf et al., 1992 and Heyman et al., 1992). 9cRA is produced *in vivo* by the concerted effort of retinol dehydrogenases (RDH) using 9-*cis* retinal, which is derived from extracellular sources, and converting it to 9-*cis* retinal. The generation of 9-*cis* retinoic acid is thought to arise from the oxidation of 9-*cis* retinal by the activity of retinal dehydrogenase (reviewed by Duester, 1996). It is conceivable that within the monocytic differentiation programme, this pathway could become activated, produce ligands for RXR, and thus induce the gene(s) involved for TNF-α secretion. In the absence of direct RXR phosphorylation or direct coactivator molecule phosphorylation, this scenario would be the next logical explanation for the activation of RXR in response to monocytic differentiation. See figure 26 for a detailed

schematic of the complete LXR/RXR-dependent pathway leading to TNF-α production in human monocytic cells.

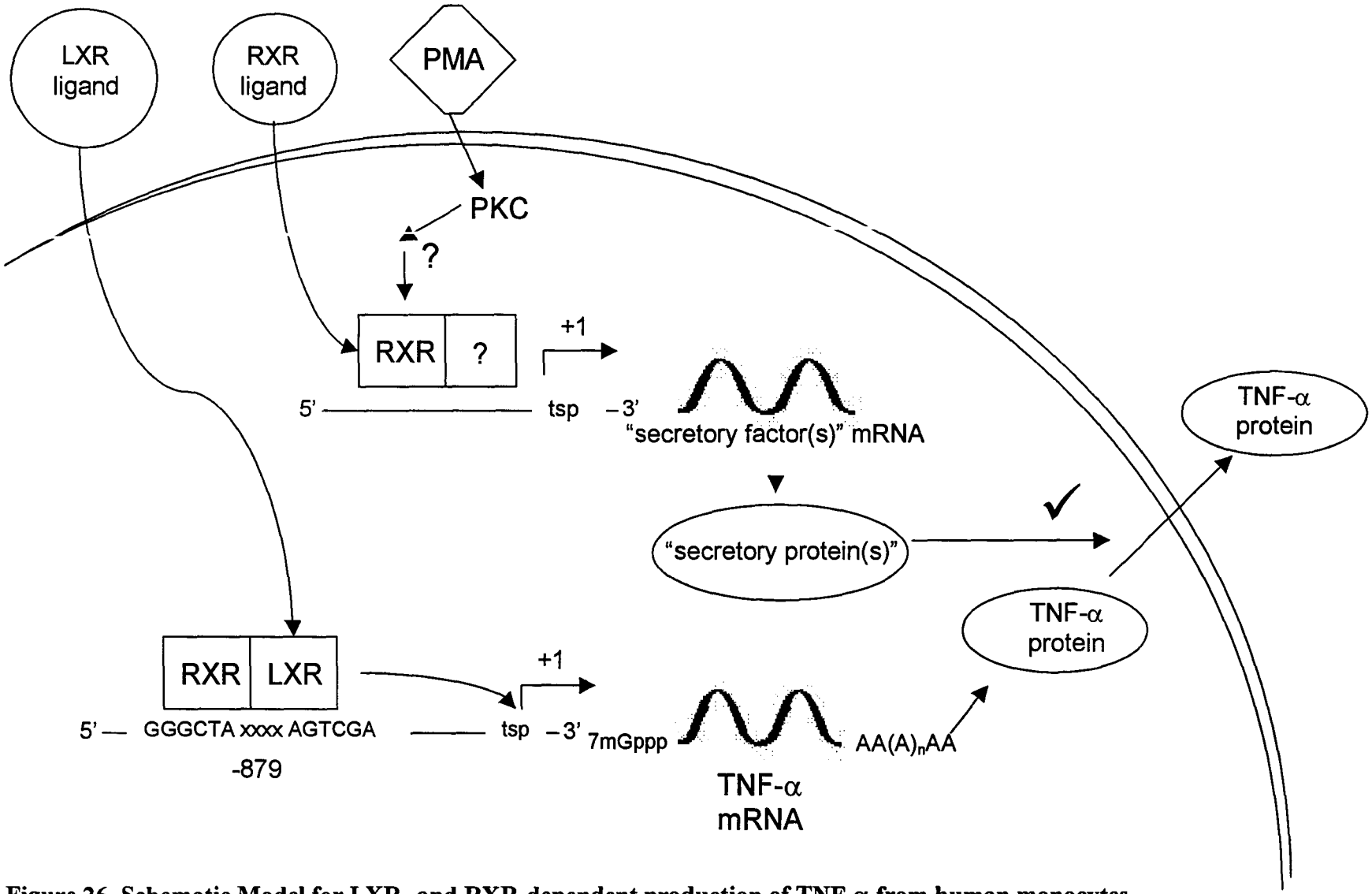

Figure 26. Schematic Model for LXR- and RXR-dependent production of TNF-α from human monocytes

Potential *in vivo* relevance of findings

The identification that TNF-α is subject to transcriptional control by LXR and whose secretion is further controlled by RXR is novel from mechanistic standpoint. The majority of LXR target genes identified to date are those involved primarily in cholesterol biosynthesis, trafficking, and storage (reviewed by Edwards et al., 2002). The discovery that a cytokine is also under the control of LXR raises intriguing questions about the potential *in vivo* consequences of such a regulatory circuit.

The infiltration of monocytes into the arterial intimal space and their subsequent differentiation into lipid-loaded foam cells by receptor-mediated uptake of oxLDL by the scavenger receptors CD36 and SR-A are the earliest steps in atherogenesis (Lusis, 2000, and Glass and Witztum, 2001). It is believed that by promoting reverse cholesterol transport through stimulation of ABC-1 and ApoE1 expression, LXRα is thought to participate in a series of cascades that are anti-atherogenic. Indeed, recent findings have shown that LXR and RXR agonists reduce lesion size and atherogenesis in apoE -/- animals (Repa et al., 2000a, Claudel et al., 2001, and Joseph et al., 2002) providing supportive evidence that LXR activity is anti-atherogenic. Comparatively, the expression of cytokines such as macrophage-colony stimulating factor (M-CSF), monocyte chemotactic protein (MCP)-1, and TNF-α are considered to be pro-atherogenic since these agents can promote monocyte differentiation, further monocyte recruitment, smooth muscle cell proliferation, and necrosis within existing atheromatic lesions (Lusis, 2000, Glass and Witztum, 2001, and Plutzky, 1999). Cytokines like TNF-α are usually pro-inflammatory, when co-expressed with other cytokines and chemokines. However when

expressed in isolation, it is also pro-apoptotic. It is tempting to speculate that in the absence of co-expression of other pro-inflammatory cytokines such as IL-1β and IL-6, this LXR-specific pathway resulting in TNF-α expression and production, may contribute to a series of pro-apoptotic events within the atheroma. Indeed, reports have shown that certain oxysterols induce apoptosis (Brown et al., 1996), and that atherosclerotic lesions contain significant numbers of apoptotic cells as well as immunoreactive TNF-α (Niemann-Jonsson et al., 2001 and Niemann-Jonsson et al., 2000). Furthermore, in light of reports showing that the predominant oxysterol found in atherosclerotic lesions is 27-hydroxycholesterol (Garcia-Cruset et al., 1999 and Garcia-Cruset et al., 2001), and that 27-hydroxycholesterol is an endogenous ligand for LXR in lipid-loaded macrophages (Fu et al., 2001), it is conceivable that the TNF-α detected in atherosclerotic plaques is produced, at least in part, by the LXR-directed expression of the cytokine in intimally resident macrophages. This TNF-α may furthermore behave as a paracrine factor that promotes localized apoptosis within an existing lesion.

The specific LXR-dependent stimulation of TNF-α expression in monocytes/macrophages resident within the intima, coupled with LXR-mediated cholesterol efflux, could contribute to a diminution of the existing lesion size by inducing apoptosis of proliferating smooth muscle cells, foam cells, or infiltrating T cells. However, a deleterious effect of TNF-α expression cannot be completely excluded since TNF-α could also serve to worsen the existing state by stimulating inflammatory cytokine production from infiltrating T cells.

The hypothesis that TNF-α may serve to exacerbate atherogenesis has been questioned recently by a report showing that in a murine model of atherosclerosis where the gene for TNF-α was deleted, the development of atherosclerosis was not inhibited, rather it was worsened (Schreyer et al., 2002). This report suggested that TNF-α likely does not behave as a mediator of inflammatory events within the lesion. Consequently, the hypothesis that LXR-derived TNF-α may cooperate with cholesterol efflux by contributing to the apoptosis of locally resident cells within the lesion is plausible in light of the *in vivo* data regarding the role of TNF-α in atherosclerosis. Further, a recent report using another murine model of atherosclerosis that showed how administration of a synthetic LXR ligand inhibits the development of atherosclerosis (Joseph et al., 2002), it is highly unlikely that the production of TNF-α through this LXR-dependent pathway exacerbates atherosclerosis. Given its role in apoptosis, it is likely that the TNF-α produced through LXR contributes to the reduction of lesion size in concert with the activation of reverse cholesterol efflux via the ABC transporter proteins.

While the regulatory signaling cascades elucidated here remain to be clarified and indeed the exact identity of the secretory factor(s) induced by RXR remain to be discovered, this body of work has certainly contributed to redefining the currently held notion that LXR and RXR are transcription factors that mediate events involved primarily in lipid homeostasis. Certainly in the context of monocyte biology, this has work has added a function for LXR/RXR heterodimers beyond those involving cholesterol trafficking and furthermore expands the role of nuclear hormone receptors within monocytes (See figure 27). This work has provided a foundation for further studies to not

only evaluate the *in vivo* relevance of this regulatory circuit but to furthermore explore the mechanistic components and nuances that cooperate with LXR/RXR in regulating TNF-α expression in monocytes.

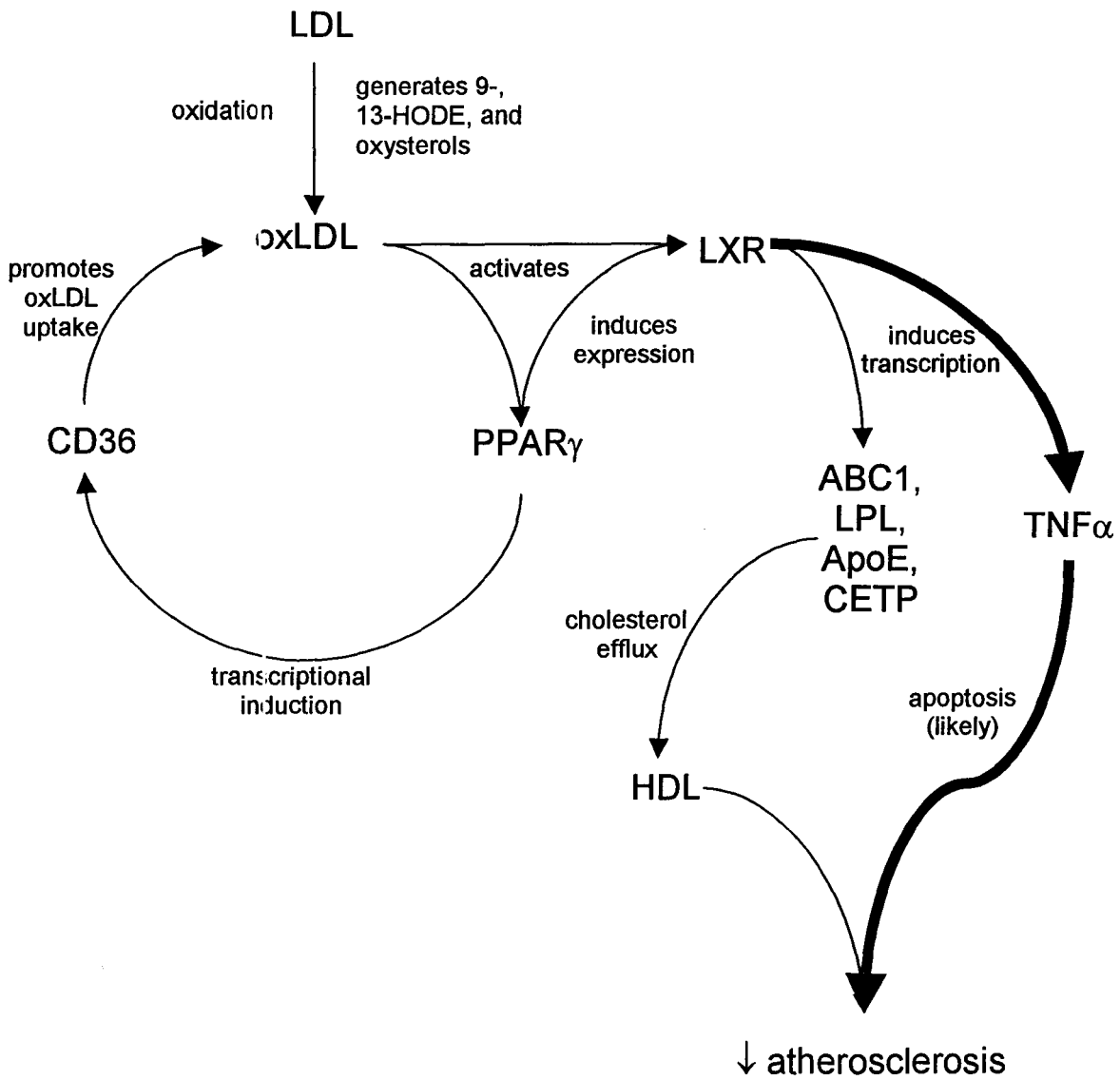

Figure 27. Integration of known nuclear hormone receptor signaling in monocytes. The sequence of events that occur to LDL upon entry into the vascular intima and the molecular events that take place in monocytes following uptake of oxLDL. Pathway elucidated and presented herein in bold and physiological outcomes based on Chawla et al., 2001, Claudel et al., 2001, and Joseph et al., 2002.

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