Liver X Receptor Activation Potentiates the Lipopolysaccharide Response in Human Macrophages

Coralie Fontaine,* Elena Rigamonti,* Atsushi Nohara, Philippe Gervois, Elisabeth Teissier, Jean-Charles Fruchart, Bart Staels, Giulia Chinetti-Gbaguidi

Abstract—Macrophages play a central role in host defense against pathogen microbes by recognizing bacterial components, resulting in the activation of an arsenal of anti-microbial effectors. Toll-like receptor (TLR)-4 mediates the recognition of lipopolysaccharide, a pathogen-associated molecular pattern from Gram-negative bacteria. Activation of the TLR-4 signaling pathway by lipopolysaccharide increases antibacterial effects by inducing secretion of cytokines that activate an immune inflammatory response and by generating bactericidal reactive oxygen species via the NADPH oxidase system. Liver X Receptors (LXRs) are nuclear receptors controlling cholesterol homeostasis and inflammation in macrophages. In addition, LXRs are critical for macrophage survival and play a role in the innate immune response in the mouse. In this study, we investigated whether LXR activation also regulates host defense mechanisms in human macrophages. In primary human macrophages, oxidized LDL and synthetic LXR ligands increased TLR-4 gene expression. Transient transfection assays, gel shift and chromatin immunoprecipitation analysis indicated that LXRs induce human TLR-4 promoter activity by binding to a DR4-type LXR response element. LXR induction of TLR-4 mRNA was followed by an induction of TLR-4 protein expression. Moreover, although short-term pretreatment with LXR agonists significantly reduced the inflammatory response induced by lipopolysaccharide, pretreatment of macrophages for 48 hours with LXR agonists resulted in an enhanced lipopolysaccharide response. Finally, LXR activation increased reactive oxygen species generation by enhancing the expression of NADPH oxidase subunits. These data provide evidence for an immunomodulatory function of LXRs in human macrophages via mechanisms distinct from those previously identified in mouse macrophages. (Circ Res. 2007;101:40-49.)

Key Words: macrophages ■ nuclear receptors ■ lipopolysaccharide ■ reactive oxygen species

Macrophages participate in the regulation of innate and adaptive immunity. These cells play a central role in the host defense against pathogen microbes by rapidly recognizing bacterial components and activating an arsenal of antimicrobial effectors. The ability of macrophages to recognize bacterial pathogen-associated molecular patterns is conferred in part by the Toll-like receptor (TLR) family of proteins. Ten members of the TLR family have been described that collectively recognize a wide range of microbial components. Lipopolysaccharide (LPS) is an integral component of the outer membrane of Gram-negative bacteria. LPS signaling is mediated by TLR-4, because invalidation of this gene in mice leads to LPS-unresponsiveness. LPS binding to TLR-4 activates the mitogen-activated protein kinases (MAPK) c-Jun N-terminal kinase (Jnk), p38, and extracellular signal-regulated kinase (Erk) cascades and induces the expression of genes involved in innate immunity (e.g., selectins, NADPH oxidase) and inflammatory response (e.g., monocyte chemoattractant protein [MCP]-1 and tumor necrosis factor [TNF][alpha]), which recruit and/or activate neighboring cells to eliminate pathogens. In addition, activation of TLR-4 by LPS triggers NADPH oxidase activation. NADPH oxidase is a complex enzyme that consists of 5 major subunits: a plasma membrane spanning cytochrome b558 composed of 2 subunits, gp91phox and p22phox, linked to flavin and cytosolic components such as Rac-2, p67phox, and the subunit p47phox, the latter requiring phosphorylation to be active. This enzyme produces superoxide anions that can lead to the generation of toxic reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, all of which can directly cause oxidative damage to bacteria. Interestingly, several recent lines of evidence indicate that the TLR-4 signaling pathway is also activated by ROS.

The liver X receptors, LXRalpha and LXRbeta, are nuclear receptors that regulate genes controlling lipid metabolism and inflammation. LXRs are activated by oxysterols as well as by intermediate products of the cholesterol biosynthetic pathway. LXRs bind as heterodimers with the retinoid X...
receptor (RXR) to specific response elements, termed LXR response elements (LXREs), located in promoters of target genes. LXREs usually consist of a (A/G)GGTCA direct repeat motif spaced by 4 nucleotides (DR4). LXR activation in macrophages induces expression of several genes involved in cholesterol trafficking and efflux including genes encoding the Niemann Pick C (NPC)1/NPC2 proteins, the ATP-binding cassette transporters (ABC)A1 and ABCG1/ABCG4 and apolipoprotein (apo)E. In addition to their well-established role as cholesterol sensors, LXRs regulate transcriptional programs involved in the inflammatory response. In murine macrophages, LXR activation has been shown to inhibit TLR-4–mediated LPS response by antagonizing the nuclear factor κB pathway through a mechanism that is not completely understood. Furthermore, TLR-4 activation can inhibit LXR-induced cholesterol efflux from macrophages, indicating a physiological crosstalk between inflammation and lipid metabolism. Although the anti-inflammatory properties of LXRs are well documented in murine macrophages, the role of LXRs in the inflammatory response in human monocyte/macrophages is controversial. In human monocytes, LXR agonists suppress tissue factor and TNFα expression induced by proinflammatory stimuli; however, TNFα and vascular endothelial growth factor are also direct LXR target genes in human monocyte/macrophages. In addition, a role for LXRs in the control of the innate immune response, especially macrophage survival, in mice has also emerged: LXR activation prevents bacteria-induced macrophage apoptosis and LXR-null macrophages undergo accelerated apoptosis when challenged with intracellular bacteria.

Although the crosstalk between the LXR and TLR signaling pathways is well established in murine macrophages, data showing whether LXRs could also play a role in the TLR-4–LPS signaling pathways in human macrophages are lacking. We demonstrate that LXR activation leads to an increase in TLR-4 expression in human but not in murine macrophages. Our results show that this regulation occurs at the transcriptional level via a LXRE in the human TLR4 promoter. Induction of TLR-4 by LXR activation enhances signaling pathways in response to LPS, leading to an increased MCP-1 and TNFα secretion. Moreover, LXR activation increases ROS generation in both resting and LPS-stimulated macrophages by enhancing the expression of the NADPH oxidase subunits. Our results provide evidence for novel mechanisms through which LXRs could modulate the adaptive immune response in human macrophages.

Materials and Methods

Cell Culture

Mononuclear cells were isolated by Ficoll gradient centrifugation from donor blood. Mature monocyte-derived macrophages were used for experiments after 10 days of culture. Murine bone marrow–derived macrophages were prepared from C57BL/6J mice. For experiments, medium was changed to medium without serum (see the online supplement, available at http://circres.ahajournals.org).

RNA Extraction and Analysis

Total cellular RNA from macrophages was extracted using TRIzol (Life Technologies), and reverse transcription was performed using random hexameric primers and Superscript reverse transcriptase (Life Technologies). cDNA were quantified by quantitative PCR on an MX 4000 apparatus (Stratagene) using specific primers (see the online data supplement).

Electrophoretic Mobility Shift Assays

LXRα and RXRα were in vitro transcribed and subsequently translated. For blocking experiments, 1 μg of polyclonal anti-LXRα antibody (P-20; Santa Cruz Biotechnology) was added to the binding reaction. The radiolabeled probes were added, and the binding reaction was incubated at room temperature. Protein complexes were resolved by 6% non-denaturing polyacrylamide gel electrophoresis (see the online data supplement).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) experiments were performed using differentiated THP-1 macrophages. Cell lysate was immunoprecipitated using anti-LXRα (P-20; Santa Cruz Biotechnology) or anti-IgG antibodies (Santa Cruz Biotechnology) as negative control. The same lysate volume was kept without immunoprecipitation for subsequent purification of input genomic DNA. Immunoprecipitated DNA was PCR amplified using primers covering either the −555 to −349 region of the human TLR-4 promoter containing the (−477/−461) TLR-4-LXRE or part of the β-actin gene as negative control (see the online data supplement).

Plasmid Cloning and Transient Transfection Experiments

HuH7 cells were transfected with reporter plasmids and with expression vectors using the cationic lipid RPR120535B. Cells were subsequently incubated in medium containing 2% Ultroser (BioSepra, Cergy Pontoise, France) and T0901317 (1 μmol/L) or GW3965 (1 μmol/L) for 48 hours and luciferase and β-galactosidase assays were performed (see the online data supplement).

Short-Interfering RNA

Short-interfering (si)RNA specific for human LXRα and LXRβ (SMARTpool siRNA) and nonsilencing control siRNA were purchased from Dharmacon. Human macrophages were transfected using the transfection reagent DharmaFECT Reagent 4. Forty-eight hours after transfection, cells were incubated in the presence of T0901317 (1 μmol/L) or vehicle (DMSO) and harvested 24 hours later.

Protein Extraction and Western Blot Analysis

Protein lysate was separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with polyclonal anti-TLR-4 (H-80; Santa Cruz Biotechnology); anti-p47phox and anti-p91phox (kindly provided by Dr A. Shah, GKT School of Medicine, King’s College, London, UK); anti-β-actin (I-19; Santa Cruz Biotechnology); anti-MAPK p38, phospho-p38, Erk, and phospho-Erk (Cell Signaling Technology) antibodies. Immunoreactive bands were quantified using ChemiGenius 2 (Syngene) (see the online data supplement).

Fluorescein Isothiocyanate–Conjugated LPS-Binding Assay

Primary human macrophages treated or not with T0901317 (1 μmol/L) or GW3965 (1 μmol/L) were incubated with fluorescein isothiocyanate (FITC)-conjugated LPS (200 ng/mL). Cellular binding of FITC-LPS was analyzed by flow cytometry.

Cytokine Secretion in Cell Culture Supernatants

MCP-1 and TNFα secreted in cell culture supernatants were quantified using ELISA purchased from Peprotech according to the instructions of the manufacturer.

ROS Quantification

Cells were exposed to T0901317 (1 μmol/L) or GW3965 (1 μmol/L) for 48 hours and then incubated with dichlorofluorescein diacetate.
treatment (Figure 1C). Strikingly, LXR activation by T0901317 (2 μmol/L) or GW3965 (2 μmol/L) had no impact on TLR-4 expression in murine bone marrow–derived macrophages, pointing to a species-specific regulation of TLR-4 by LXR (Figure 1D). ABCA1 gene expression was measured as positive control of LXR activation both in human and murine macrophages (data not shown). Together, these results demonstrate a species-specific increase of TLR-4 mRNA levels after LXR activation.

**LXRs Bind to the Human TLR-4 Promoter In Vitro and In Vivo**

To determine whether TLR-4 is directly responsive to LXRs, the human TLR-4 proximal promoter was examined by bioinformatics analysis to determine the presence of potential LXRE sites. A potential LXRE very similar to the consensus sequence was found between nucleotides −477 and −461 upstream of the transcription initiation site (Figure 2A). Electrophoretic mobility shift assays were performed to test the ability of the human LXRα/RXRα heterodimer to bind to this putative (−477/−461) TLR-4–LXRE. A specific DNA–protein complex was formed when in vitro synthesized LXRα
and RXRα proteins were incubated with the \(^{32}\)P-labeled probe covering the TLR-4–LXRE (Figure 2B, lane 7). RXRα or LXRα alone did not bind to this site, confirming that these receptors cannot bind as homo- or monomers. The binding of LXRα/RXRα to the probe is specific, because the complex was competed by an excess of cold wild-type (lanes 8 to 10) or mutated (lanes 11 to 13) oligonucleotides to the reaction mixture. The specificity of LXRα/RXRα binding was verified by the addition of a polyclonal anti-LXRα antibody, which inhibited the formation of the LXRα/RXRα complex (lane 6). Furthermore, no binding was observed on the mutated (−477/−461) TLR-4–LXRE (Figure 2B, lane 15). Similar results were obtained for the LXRβ/RXRα heterodimer (data not shown). These data demonstrate that LXRα and LXRβ bind as a heterodimer with RXRα to the (−477/−461) TLR-4–LXRE present in

the promoter of the human TLR-4 gene. Alignment of the human and murine TLR-4 5′-promoter region sequence indicated that the LXRE site is not conserved between the human and the murine promoters, probably explaining the species-specific regulation of TLR-4 by LXR (Figure 2A).

To validate the binding of LXRα to the native TLR-4 promoter in vivo, a ChIP assay was performed in human THP-1 macrophages, in which TLR-4 mRNA is also strongly induced by treatment with LXR agonists (see the online data supplement). The genomic DNA region encompassing the LXRE of the TLR-4 gene was immunoprecipitated with a polyclonal LXRα-specific antibody. DNA precipitates were isolated and then subjected to PCR by using primer pairs covering either the −555/−349 TLR-4 gene promoter (top) or a β-actin gene (bottom) region. Control PCRs were performed without DNA (H2O) or with nonimmunoprecipitated genomic DNA (input). The data shown are representative of 2 independent experiments.
LXR/ RXRα Heterodimers Induce TLR-4 Promoter Activity

To test whether LXRα/RXRα activate transcription from the (∼477/−461) TLR-4–LXRE site, 3 copies of this element were cloned in front of the herpes simplex virus thymidine kinase promoter to obtain the (TLR-4–LXRE)3x-Tk-Luc luciferase reporter vector. In HuH7 cells, cotransfection of pCMX-hLXRα and pSG5-hRXRα expression vectors on the (TLR-4–LXRE)3x reporter vector led to significant induction of transcriptional activity compared with empty pCMX and pSG5 vectors. This effect was enhanced by T0901317 (1 μmol/L) or GW3965 (1 μmol/L) (Figure 3A). Induction of transcriptional activity after LXRα/RXRα transfection and activation was abolished when the mutated LXRE site was tested (Figure 3A). To further investigate whether LXRα activates the TLR-4 promoter, transfection assays were performed using luciferase reporter constructs driven by the natural TLR-4 promoter29 of 620 bp or by the TLR-4 promoter fragment that contains 480 bp and lacks the TLR-4–LXRE site. Activity of the 620-bp TLR-4 promoter was induced by LXRα/RXRα cotransfection and enhanced by the presence of T0901317 (1 μmol/L) (Figure 3B). By contrast, the truncated 480-bp TLR-4 promoter was unaffected by either LXRα/RXRα cotransfection or T0901317 treatment. In addition, introduction of specific mutations of the LXRE in the context of the 620-bp TLR-4 promoter abolished induction of the TLR-4 promoter by either LXRα/RXRα cotransfection in the absence or in the presence of T0901317 (1 μmol/L) (Figure 3B).

Similar results on both TLR-4–LXRE site and natural human TLR-4 promoter were obtained when LXRβ and RXRα were transfected under the same experimental conditions (data not shown). These results demonstrate that the human TLR-4 promoter contains a functional LXRE that confers responsiveness to LXRα.

LXR Expression Is Necessary for the Induction of TLR-4 Gene Expression by LXR Agonists

To address whether the stimulatory effect of LXR ligands on TLR-4 gene expression was mediated by LXR, a siRNA approach was used to reduce LXRα and LXRβ expression. Quantitative PCR analysis indicated that transfection of a specific double LXRα/LXRβ siRNA pool significantly suppressed LXRα and LXRβ gene expression by ∼70% and 80%, respectively, in comparison with control siRNA-transfected cells (Figure 4A and 4B). ABCA-1 gene induction, measured as positive controls, was also significantly reduced (Figure 4C). Taken together, these data demonstrate that LXR ligands induce TLR-4 gene expression through a receptor-dependent mechanism.

LXR Activation Increases TLR-4 Protein Expression

To determine whether TLR-4 gene regulation by LXR ligands is followed by an increase at the protein level, total proteins were isolated from primary human macrophages incubated with T0901317 (1 μmol/L) or GW3965 (1 μmol/L) at different time points. TLR-4 protein levels were subsequently measured by Western blot analysis. Treatment with both LXR agonists increased significantly TLR-4 protein levels after 48 hours of treatment (Figure 5A and 5B).
Flow cytometry in primary human macrophages treated with T0901317 (1 μmol/L) or GW3965 (1 μmol/L) for 48 hours. T0901317 (Figure 5C) and GW3965 (Figure 5D) increased the binding of FITC-LPS to primary human macrophages by 22 ± 5% (P < 0.01) and 37 ± 11% (P < 0.01) compared with untreated cells, respectively.

**LXR Activation Regulates the Response of TLR-4 Signaling Pathways to LPS in Primary Human Macrophages**

To determine the functional consequences of TLR-4 upregulation by LXR agonists, MAPK activation was examined by measuring Jnk, Erk, and p38 phosphorylation using specific antibodies. Interestingly, Jnk, Erk, and p38 phosphorylation in response to LPS was enhanced in primary human macrophages pretreated for 48 hours with LXR agonists (Figure 6A and 6B). In addition, secretion of MCP-1 and TNFα in response to LPS was significantly enhanced in macrophages pretreated with the LXR agonists for 48 hours, whereas short-term pretreatment with LXR agonists inhibited the LPS response as previously reported by Walcher et al22 (Figure 6C and 6D). Moreover, 48-hour pretreatment with the LXR agonists strongly enhanced the LPS-induced expression of the MCP-1, TNFα, and COX-2 genes in primary human macrophages as well as in THP-1 differentiated macrophages (see supplemental Figure II).

**LXR Activation Increases ROS Production in Human Macrophages**

LPS is known to induce ROS production in macrophages, most likely by activating membrane associated NADPH oxidase. Therefore, ROS was measured after LPS stimulation of primary human differentiated macrophages incubated with the LXR ligand T0901317 (1 μmol/L) for 48 hours. As

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*Figure 4. LXR ligands induce TLR-4 gene expression through a receptor-dependent mechanism. Human macrophages were transfected with nonsilencing control or silencing LXRα/β siRNA; 48 hours later, cells were treated with or without T0901317 (1 μmol/L) for 24 hours, and mRNA levels of LXRα (A), LXRβ (B), TLR-4 (C), and ABCA1 (D) were analyzed by quantitative PCR. Results were normalized to cyclophilin mRNA and expressed relative to the levels in control siRNA-transfected cells set as 1 (mean ± SD of 2 independent experiments). Statistically significant differences between treatments and control are indicated (t test; *P < 0.05, **P < 0.01, ***P < 0.001).*
reported previously, ROS production was induced by LPS in macrophages. Interestingly, LXR activation increased ROS release in both resting and LPS-stimulated macrophages (Figure 7A), suggesting a direct regulation of ROS production by LXRs. The increase in ROS production in the presence of LXR agonists was associated with the induction of membrane NADPH oxidase activity, because the effect was abolished by diphenyliodonium, a specific flavin inhibitor (Figure 7B). Similar results were obtained using THP-1 differentiated macrophages (see supplemental Figure III).

It was next investigated whether LXRs regulate the expression of genes encoding NADPH oxidase subunits in macrophages. p47phox and gp91phox mRNA levels were increased in human macrophages treated for 24 hours with T0901317 or GW3965, an effect dependent on the expression of LXRα/LXRβ, as demonstrated by the loss of gene induction by T0901317 in cells transfected with an LXRα/LXRβ siRNA (see supplemental Figure IV). By contrast, p22phox and p67phox transcripts were not affected by LXR activation (data not shown). Treatment with LXR agonists for 24 hours also led to an induction of p47phox and gp91phox protein expression in human macrophages (see supplemental Figure IV).

Strikingly, LXR activation had no impact on NADPH oxidase subunit gene expression nor on ROS production in murine bone marrow–derived macrophages, pointing again to a species-specific regulation of NADPH oxidase by LXR agonists (see supplemental Figure V).

**Discussion**

Our results demonstrate that LXR agonists induce the expression of the LPS receptor TLR-4 in human but not in murine macrophages. A LXRE site was identified in the human TLR-4 promoter that mediates the transactivation by LXRs/RXRα. The mouse and human TLR-4 genes are highly conserved. However, notable differences exist with respect to the elements implicated in gene regulation, which may account for species differences in terms of tissue expression and modulation by different stimuli. The LXRE site is not conserved between the human and mouse TLR-4 promoters, likely explaining the species-specific regulation of TLR-4 by LXRs. Increased expression of TLR-4 resulted in an enhanced responsiveness of human macrophages to LPS. Indeed, LXR agonist pretreatment enhanced MAPK activation.
as well as MCP-1 and TNF\(\alpha\) secretion in LPS-stimulated human macrophages. Moreover, we show that LXR activation increases ROS production in LPS-activated human macrophages. This effect was also observed in resting macrophages. Enhanced ROS production in LXR-activated macrophages was associated with an increase in the expression of the NADPH oxidase subunits p47\(^{\text{phox}}\) and gp91\(^{\text{phox}}\).

TLR-4 is critical for the recognition of LPS, a chief pathogen-associated molecular pattern from Gram-negative bacteria. TLR-4 activation by LPS engages multiple mechanisms that control the initiation of the adaptive immune response involving in the surveillance, attack, containment, and clearance of pathogens. Cell migration from peripheral blood into the inflamed tissue involves tightly controlled sequences of events. Activation of TLR-4 results in the induction of cytokine expression, which regulates cell migration and activation to the sites of inflammation. In addition, LPS-activated macrophages produce ROS, which could directly kill bacteria within the phagosome. Because MCP-1 and TNF\(\alpha\) secretion in response to LPS as well as ROS production are induced in LXR-activated macrophages, it appears that LXRs may play a role in the macrophage response against bacteria. Indeed, LXRs may contribute to bacterial elimination through recruitment and/or activation of neighboring cells as well as through the production of antibacterial ROS.

Progression of the innate immune response is also regulated at the level of macrophage survival. Of note, certain bacterial pathogens target TLR-4–initiated antiapoptotic mechanisms to induce the death of activated macrophages and thereby evade detection and destruction by the host immune response. Interestingly, LXR\(\alpha\)-deficient mice are
highly susceptible to infection by the intracellular bacteria *Listeria monocytogenes*. Bone marrow transplant studies pointed to altered macrophage function as the major determinant of this susceptibility. LXR activation antagonized pathogen-induced apoptosis of murine macrophages through upregulation of the antiapoptotic factors AIM and Bcl-xl as well as inhibition of proapoptotic gene expression. However, we were not able to detect AIM and Bcl-xl expression in primary human macrophages under our experimental conditions (data not shown), thus excluding the possibility that this mechanism is operational in this human model. Nevertheless, silencing of LXRs gene expression in human peripheral blood mononuclear cells resulted in significant upregulation of the expression of the proapoptotic c-myc gene. In addition, in the antiapoptotic branch of TLR-4 signaling, nuclear factor κB and p38 MAPK cooperate to induce transcription of 2 antiapoptotic genes, Pai-2 and Bfl-1/A1, the products of which block the concurrent activation of the proapoptotic pathways. Thus, it cannot be excluded that LXR agonists, via induction of TLR-4, also modulate the apoptotic response of human macrophages.

Recently, Walcher et al have identified an anti-inflammatory action of LXRs in human cells. These authors showed that short-term, acute cotreatment of Th-1 primed human monocytes with LXR agonists, together with interferon γ, led to a slight decrease in TNFα secretion. In line with these observations, we observed that pretreatment of primary human macrophages with LXR agonists for 0 to 6 hours before stimulation with LPS resulted in a reduction of LPS-induced MCP-1 and TNFα secretion. However, when macrophages were pretreated for 48 hours with LXR agonists, an increase of the LPS/TLR-4 signaling pathway was observed. Thus, it appears that LXR activation prepares macrophages to allow an enhanced antibacterial response via induction of the TLR-4 signaling pathway, whereas, once the inflammatory stimulus is present, LXRs exert antiinflammatory actions. This combination of chronic and acute effects suggests that the LXR pathway may have evolved to potentiate the role of the macrophage in the response to and resolution of inflammation. Clearly, the impact of LXR signaling on macrophage gene expression is complex and context dependent.

In conclusion, our results identify a novel role for LXRs in the preparation of macrophages to exert antibacterial activities via induction of TLR-4 expression and NADPH oxidase activity.

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### Disclosures

None.

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MATERIALS AND METHODS

Cell culture
Mononuclear cells isolated by Ficoll gradient centrifugation from blood of healthy normolipidemic donors were suspended in RPMI 1640 medium containing gentamycin (40mg/ml), glutamine (0.05%) and 10% pooled human serum. Differentiation of monocytes into macrophages occurs spontaneously by adhesion of cells to the culture dishes. Mature monocyte-derived macrophages were used for experiments after 10 days of culture. Human monocytic THP-1 cells (ATCC, Rockville, Maryland, USA) were maintained in RPMI 1640 medium containing 10% of FCS and differentiated for 72h with 167nmol/L PMA. Murine bone marrow-derived macrophages were prepared from C57BL/6J mice. Bone marrow cell suspensions were isolated by flushing the femurs and tibias with PBS and cells were cultured as previously described. For experiments, medium was changed to medium without serum.

RNA extraction and analysis
Macrophages were treated with oxLDL (50 µg/ml) or the LXR activators T0901317 or GW3965 at the indicated concentrations and time-points as described. Total cellular RNA was extracted using Trizol (Life Technologies, France) and reverse transcription was performed using random hexameric primers and Superscript reverse transcriptase (Life Technologies, France). cDNA were quantified by quantitative real-time PCR on a MX 4000 apparatus (Stratagene) using specific primers indicated in Table1. Amplification was performed in a volume of 20µl containing 100nmol/L of each primer, 4mmol/L MgCl2, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene) and SYBR Green 0.33X (Sigma-Aldrich). The conditions were 95°C for 10min, followed by
40 cycles of 30sec at 95°C, 30sec at 55°C and 30sec at 72°C. mRNA levels were subsequently normalized to cyclophilin mRNA.

**Electrophoretic mobility shift assays (EMSA)**

LXRα and RXRα were in vitro transcribed from pCMX-hLXRα and pSG5-hRXRα plasmids, respectively using T7 polymerase and subsequently translated using the TNT coupled transcription/translation system (Promega, Madison, WI). Proteins were incubated for 10min at room temperature in a total volume of 20µl containing 0.5µg poly(dI-dC) and 0.5µg herring sperm DNA in the following binding buffer: Hepes 30mmol/L, KCl 60 mmol/L, DTT 1 mmol/L, 0.1% Triton X100, 0.5% glycerol. For blocking experiments, 1µg of polyclonal anti-LXRα antibody (Santa Cruz P-20) was added to the binding reaction. The radiolabeled probes (LXREwt: 5’-AAAGAGGTATGTAAGGTAGAATGAGGTCATTATG-3’, LXREmut: 5’-AAAGAGGTATGTAACACAGAATGAGGTCATTATG-3’) were added and the binding reaction was incubated for a further 15min at room temperature. Protein complexes were resolved by 6% non-denaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-EDTA (TBE) at room temperature. For competition experiments, 1, 10 and 100-fold excess of unlabeled oligonucleotides were added to the binding reaction 10min before the labeled probes.

**Chromatin immunoprecipitation (ChIP) assays**

ChIP experiments were performed as described by 3 and modified by Giraud et al. 4. Briefly, differentiated THP-1 macrophages were treated with the LXR ligand T0901317 (1µmol/L) for 2h. Cell lysates were sonicated on ice, 15 times for 15sec, and separated by 45sec. A volume of lysate equivalent to 20 x 10⁶ cells was immunoprecipitated using 4µg of anti-LXRα antibody (Santa Cruz P-20) or of an anti-IgG antibody (Santa Cruz) as a negative control. The
same lysate volume was kept without immunoprecipitation for subsequent purification of input genomic DNA. One-tenth of the immunoprecipitated DNA was PCR amplified twice for 35 cycles (30sec at 95°C, 30sec at 55°C, and 30sec at 72°C) using primers covering either the –555 to –349 region of the human TLR-4 promoter containing the (-477/-461)TLR-4-LXRE: for, 5’-CCTACATATCGAAGTCCTAAC-3’; rev, 5’-CTCACAATTCTAGAGGCTTCCAGT-3’; or part of the β–actin gene: for, 5’-CGAGCCATAAAAGGCAACTTTCG-3’; rev, AGGAAGAGGAGGAGGAGGAGTTT-3’ as a negative control.

**Plasmid cloning and transient transfection experiments**

The hLXREwt and hLXREmut constructs were obtained by inserting 3 copies of the double-strand wild type or mutated LXRE in the pTK-pGL3 plasmid. The hTLR-4pGL3 constructs were obtained by inserting 480 bp or 620 bp fragments of the human TLR-4 promoter (hTLR-4wt) in the multicloning site of the pGL3 vector. Fragments were obtained by PCR amplification using human genomic DNA as template and the following primers: hTLR-4-444For: 5’-CCAACATAACGCGTGTCCTTATAAGAAGGGG-3’, hTLR-4-586For: 5’-GAAGGGACGCAGTCAAATTGTGGAGCTGC-3’ and hTLR-4+38Rev: 5’-GGTGGATCCAAAGCAGTCTCGGGC-3’.

HuH7 cells were cultured in 24-well plates. Cells were transfected with reporter plasmids and with expression vectors (pSG5, pCMX, pSG5-hRXRα or pCMX-hLXRα) using the cationic lipid RPR 120535B as described. Cells were subsequently incubated in medium containing 2% Ultroser (BioSepra, France) and T0901317 (1µmol/L), GW3965 (1µmol/L) or DMSO as vehicle for 48h. At the end of the experiment, cells were lysed and luciferase and β-galactosidase assays were performed.
Protein extraction and western blot analysis

Cells were lysed in buffer (50mmol/L Tris-HCl, 300mmol/L sucrose, 50mmol/L NaCl, proteinase inhibitor cocktail, pH 7.3) and 20µg of protein lysate was separated by SDS-PAGE and transferred to nitrocellulose. Equal loading of the gel was verified by Ponceau red staining. Membranes were subjected to immunodetection with polyclonal anti-TLR-4 (Santa Cruz H-80), polyclonal anti-p47phox and polyclonal anti-p91phox (kindly provided by Dr. A.Shah, London) and anti-β-actin (Santa Cruz I-19) antibodies. Rabbit polyclonal antibodies against MAPK p38, phospho-p38 (Thr180/Tyr182), Erk and phospho-Erk (Tyr204) were purchased from Cell Signaling Technology (St Quentin en Yvelines, France). Immunoreactive bands were revealed using an ECL detection kit (Amersham Pharmacia Biotech) and signals were analyzed by densitometry and quantified using ChemiGenius 2 (Syngene).

FITC-conjugated LPS-binding assay

Primary human macrophages treated or not with T0901317 (1µmol/L) or GW3965 (1µmol/L) were incubated with FITC-conjugated LPS (200ng/ml) in PBS containing 1% human serum for 30min at 4°C. After washing, cellular binding of FITC-LPS was analyzed by flow cytometry.

ROS quantification

Cells were exposed to T0901317 (1µmol/L) or GW3965 (1µmol/L) for 48h and then incubated with dichlorofluoresceine diacetate (DCFH-DA, 10µmol/L) for 30min. At the end of incubation, medium was removed, cells were washed with PBS and lysed in 1mL NaOH 1M. DCF fluorescence was read at λ_{exc}=485 nm and λ_{em}=530 nm. In some experiments, cells were pre-incubated for 30min with diphenyleneiodonium (DPI, 10µmol/L) before LXR
activation and ROS quantification. Cellular proteins were measured by Peterson assay and results were expressed as arbitrary fluorescence units (AFU/µg protein).
REFERENCES


FIGURE LEGENDS OF THE SUPPLEMENTARY DATA

Online Figure 1. LXR activation induces TLR-4 expression in THP-1 differentiated macrophages.

THP-1 macrophages differentiated for 72h with PMA were incubated for 24h with T0901317 or GW3965 at 1µM or with DMSO alone as control. TLR-4 mRNA levels were analyzed by quantitative PCR and normalized to those of cyclophilin. Results are representative of those obtained from 3 independent experiments and are expressed relative to the levels in untreated cells set as 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences between treatments and control are indicated (t test; *p<0.05; **p<0.01).

Online Figure 2. LXR activation enhances LPS-induced MCP-1, TNFα and COX-2 mRNA in primary human as well as in differentiated THP-1 macrophages.

(A) Schematic diagram of the experimental conditions used. (B) Primary human macrophages or (C) PMA differentiated THP-1 cells were treated with T0901317 (1µmol/L), GW3965 (1µmol/L) or vehicle for 48h. Cells were washed and incubated with LPS 100ng/ml for the indicated time periods. MCP-1, TNFα and COX-2 mRNA levels were analyzed using real-time quantitative PCR and normalized to those of cyclophilin. Results are representative of those obtained from 3 independent experiments and are expressed relative to the levels in LPS treated cells set as 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences between treatments and control are indicated (t test; *p<0.05; **p<0.01; ***p<0.001).

Online Figure 3. LXR agonists induce ROS production in differentiated THP-1 cells.

Differentiated THP-1 macrophages pre-incubated or not with NADPH oxidase inhibitor DPI, were treated with T0901317 or GW3965 (1µmol/L) dissolved in DMSO or with DMSO alone as control for 24h. ROS production was measured as described in “Materials and Methods”.
Results are expressed as arbitrary fluorescence units (AFU)/µg protein. Results are representative of those obtained from 3 independent experiments and are expressed relative to the levels in untreated cells set as 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences between treatments and control are indicated (t test; *p<0.05; **p<0.01).

**Online Figure 4. LXR activation induces mRNA and protein levels of p47phox and gp91phox in primary human macrophages.**

p47phox (A) and gp91phox (B) mRNA were analyzed by Q-PCR in human macrophages treated with T0901317 (1µmol/L) or GW3965 (1µmol/L) for 24h. Results are normalized to those of cyclophilin and expressed relative to control cells set as 1. Results are representative of those obtained from 3 independent macrophage preparations and are expressed relative to untreated cells set as 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences between treatments and control are indicated (t test; *p<0.05, **p<0.01). Human macrophages were transfected with nonsilencing control-siRNA or with siRNA against LXRα or LXRβ. 48h after transfection cells were treated or not with T0901317 (1µmol/L) for 24h. mRNA of p47phox (C) and gp91phox (D) were analyzed by Q-PCR. Results are normalized to those of cyclophilin and expressed relative to control-siRNA transfected cells set as 1. Statistically significant differences between treatments and control are indicated (t test; *p<0.05, ***p<0.001). (E) p47phox, (F) gp91phox and β-actin levels were measured by Western Blot and quantified using ChemiGenius2 (Syngene). Results are representative of 3 independent macrophage preparations.

**Online Figure 5. LXR agonists do not induce ROS production in bone marrow-derived macrophages.**

Bone marrow-derived macrophages were treated with T0901317 or GW3965 (2µmol/L) dissolved in DMSO or with DMSO alone as control for 24h. ROS production was measured
as described in “Materials and Methods”. Results are expressed as arbitrary fluorescence units (AFU)/μg protein. Results are representative of those obtained from 3 independent macrophage preparations and are expressed relative to the levels in untreated cells set as 1. Each bar is the mean value ± SD of triplicate determinations.
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Online Figure 1
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Figure 2
Online Figure 3
Online Figure 4
Figure 5