

# Regulation of Sphingomyelin Phosphodiesterase Acid-Like 3A Gene (*SMPDL3A*) by Liver X Receptors<sup>[S]</sup>

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## ABSTRACT

Liver X receptor (LXR)  $\alpha$  and LXR $\beta$  function as physiological sensors of cholesterol metabolites (oxysterols), regulating key genes involved in cholesterol and lipid metabolism. LXRs have been extensively studied in both human and rodent cell systems, revealing their potential therapeutic value in the contexts of atherosclerosis and inflammatory diseases. The LXR genome landscape has been investigated in murine macrophages but not in human THP-1 cells, which represent one of the frequently used monocyte/macrophage cell systems to study immune responses. We used a whole-genome screen to detect direct LXR target genes in THP-1 cells treated with two widely used LXR ligands [*N*-(2,2,2-trifluoroethyl)-*N*-(4-

[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)-ethyl]phenyl]-benzenesulfonamide (T0901317) and 3-[3-[*N*-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy]phenylacetic acid hydrochloride (GW3965)]. This screen identified the sphingomyelin phosphodiesterase acid-like 3A (*SMPDL3A*) gene as a novel LXR-regulated gene, with an LXR response element within its promoter. We investigated the regulation of *SMPDL3A* gene expression by LXRs across several human and mouse cell types. These studies indicate that the induction of *SMPDL3A* is LXR-dependent and is restricted to human blood cells with no induction observed in mouse cellular systems.

## Introduction

Liver X receptors (LXRs) are nuclear hormone receptors that act as oxysterol sensors, regulating genes involved in cholesterol and lipid metabolism (Janowski et al., 1999). Elevated cholesterol levels can lead to enhanced oxysterol production and the activation of LXRs, which increase the gene expression (*transactivation*) of a number of target genes. The capacity of LXRs to promote reverse cholesterol transport via direct gene up-regulation of several ATP-binding cassette (ABC) transporters in macrophages and intestine (e.g., ABCA1/G1/G5/G8) makes them an attractive therapeutic target for the treatment of atherosclerosis (Calkin and Ton-

tonoz, 2010). Activation of LXRs in liver also leads to induction of genes directly involved in lipid synthesis, such as sterol regulatory element-binding protein-1c (SREBP1c), fatty acid synthase, and stearoyl CoA desaturase (Repa et al., 2000). Chronic LXR activation in liver can cause hypertriglyceridemia and hepatosteatosis. LXRs have also been shown to exert anti-inflammatory properties by suppressing genes involved in inflammation, such as tumor necrosis factor- $\alpha$ , interleukins (IL-1 $\beta$  and IL-6), cyclooxygenase 2, inducible nitric-oxide synthase, and nuclear factor- $\kappa$ B in murine macrophages (Joseph et al., 2003). The immunomodulatory effects of LXRs rely on the association of LXRs with corepressor complexes bound to transcription factors, such as nuclear factor- $\kappa$ B, which modulate the expression of inflammatory genes (*transrepression*) (Ghisletti et al., 2007). An additional therapeutic indication for LXRs is in Alzheimer's disease. LXR activation has been shown to increase the levels of the

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**ABBREVIATIONS:** LXR, liver X receptor; ABC, ATP-binding cassette; SREBP1c, sterol regulatory element-binding protein-1c; IL, interleukin; LPS, lipopolysaccharide; LXRE, liver X receptor response element; DR-4, direct repeats separated by any four nucleotides; *SMPDL3A*, sphingomyelin phosphodiesterase acid-like 3A; PMA, phorbol 12-myristate 13-acetate; HEK, human embryonic kidney; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GW3965, 3-[3-[*N*-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy]phenylacetic acid hydrochloride; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-(4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)-ethyl]phenyl]-benzenesulfonamide; LG100268, 2-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)cyclopropyl]pyridine-5-carboxylic acid; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; RT, real time; HRP, horseradish peroxidase; RXR, retinoid X receptor; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; CLL, chemokine (C-C motif) ligand.

apolipoprotein E in murine and human macrophages (Mak et al., 2002) and in rat brain, in which increased apolipoprotein E and its higher lipidation as a result of induction of ABCA1 had been positively associated with amyloid A $\beta$  clearance (Suon et al., 2010).

Although many metabolic pathways are conserved across species, LXRs can regulate their target genes in a species- and isoform-specific fashion. For instance, the rate-limiting enzyme in bile acid synthesis, CYP7A1, is activated by LXRs in mouse but not in human (Goodwin et al., 2003). In addition, in human macrophages, LXR $\alpha$ , but not LXR $\beta$ , has been shown to be involved in an autoregulatory loop upon activation with known LXR ligands. Such an effect has not been observed in murine macrophages (Li et al., 2002). Furthermore, Toll-like receptor 4 is up-regulated by LXR agonists in human but not mouse macrophages (Fontaine et al., 2007).

Gene regulation by LXRs has been extensively studied in murine systems but has not been fully investigated in human THP-1 macrophages. THP-1 cells represent one of the most frequently used cell systems for studying macrophage biology in vitro. In this study we investigated LXR gene regulation at the genome-wide level in THP-1-derived macrophages with and without stimulation of immune response by bacterial lipopolysaccharide (LPS). Analysis of differential gene expression led to the identification of a novel LXR-regulated gene and a DR-4 LXR response element (LXRE) within its promoter. LXRs activate the expression of the *SMPDL3A* gene, either in the presence or the absence of LPS. This study focuses on the regulation of *SMPDL3A* by LXRs across various cell types and tissues in human and rodent species. The *SMPDL3A* gene was originally identified on the basis of its sequence similarity with acid sphingomyelinase, but its function has not been characterized so far other than its increased expression and association with deleted in bladder cancer chromosome region 1 in bladder cancer (Wright et al., 2002). Given the biological importance of acid sphingomyelinases in activated macrophages (Truman et al., 2011), we decided to further investigate the *SMPDL3A* gene regulation by LXRs.

## Materials and Methods

**Cell Culture and Transfections.** THP-1 cells were maintained in RPMI 1640 medium with GlutaMAX (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 50  $\mu$ M  $\beta$ -mercaptoethanol, and antibiotics [penicillin (50 U/ml)-streptomycin (50  $\mu$ g/ml); Invitrogen] at 37°C under 5% CO<sub>2</sub>. Twenty-four hours before treatment, THP-1 cells were plated in 96-well plates at a density of  $5 \times 10^4$  cells/well in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO). Cells were then incubated with LXR ligands in RPMI 1640 medium with GlutaMAX supplemented with 10% delipidated fetal bovine serum (HyClone Laboratories) and antibiotics. HepG2 cells were maintained and routinely propagated in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% CO<sub>2</sub>. CCD 1112 foreskin fibroblasts were maintained and routinely propagated in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% CO<sub>2</sub>. H4, HEK293, and RAW264.7 cells were maintained and routinely propagated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% CO<sub>2</sub>. H4, HEK293, RAW264, CCD 1112, and HepG2 cells were plated in

96-well plates at a density of  $4 \times 10^4$  cells/well and incubated for 24 h with LXR ligands in their respective medium supplemented with 10% delipidated fetal bovine serum and antibiotics.

For siRNA studies, 24 h before transfection, THP-1 cells were plated in 96-well plates at a density of  $5 \times 10^4$  cells/well and differentiated with PMA, as described above. Each transfection was performed with either 30 nM scrambled siRNA (Invitrogen) or 30 nM LXR-specific siRNA (LXR $\alpha$  and LXR $\beta$ ; Invitrogen) using 0.4  $\mu$ l of Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM Reduced Serum Medium (Invitrogen). Twenty-four hours after transfection, cells were washed once with Dulbecco's PBS and treated with the LXR agonist in RPMI 1640 medium with GlutaMAX supplemented with 10% delipidated fetal bovine serum and antibiotics.

The LXR ligands 3-[3-[N-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propoxy]phenylacetic acid hydrochloride (GW3965) and N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)-ethyl]phenyl]-benzenesulfonamide (T0901317) were purchased from Tocris Bioscience (Ellisville, MO). The RXR ligand 2-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)cyclopropyl]pyridine-5-carboxylic acid (LG100268) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada).

**Gene Expression Microarray Analysis.** THP-1 cells were differentiated as described above and plated in 35-mm dishes at  $5 \times 10^6$  cells/dish. Twenty-four hours later, cells were pretreated for 1 h with either DMSO or 1  $\mu$ M T0901317 in delipidated fetal bovine serum-containing media and then incubated with either plain media or LPS (Sigma-Aldrich)-containing media (100 ng/ml) for an additional 8 h.

Total RNA was isolated and purified using RNeasy columns (QIAGEN, Valencia, CA). Reverse transcription and hybridization on two Agilent Human GE 4x44K v2 Microarrays were performed by MOgene LC (St. Louis, MO).

**Analysis of *SMPDL3A* Expression in Cells and Tissues.** For all cells treated in the 96-well format, RNA was isolated and purified using an ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). cDNA was synthesized and subjected to real-time PCR using One-Step RT-PCR reagents (Applied Biosystems). Gene expression analysis was performed according to the method described by Bookout and Mangelsdorf, 2003.

cDNA from Human MTC Panels I and II were purchased from Clontech (Mountain View, CA) and subjected to real-time PCR. Whole blood from human donors was purchased from AllCells, LLC (Emeryville, CA). Human peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood collected into EDTA-containing tubes using standard Ficoll-Paque gradient centrifugation. In brief, 15 ml of blood was transferred to 50-ml tubes, diluted with 15 ml of Dulbecco's PBS and underlaid with 10 ml of lymphocyte separation medium (9.4% sodium diatrizoate and 6.2% Ficoll; MP Biomedicals, Solon, OH). The tubes were centrifuged for 60 min at 800g, with no brake. The cell interface layer was harvested carefully, and the cells were washed three times with PBS (sedimented for 10 min at 800g) and resuspended in RPMI 1640 medium with GlutaMAX supplemented with 10% fetal bovine serum and antibiotics before counting. Cells were plated on six-well plates at a density of  $3 \times 10^6$  cells/well and treated with the LXR ligand for 24 h. The relative gene expression level was determined by RT-PCR as described above. All primer probe sets for the human genes were purchased from Applied Biosystems.

**Animal Studies.** Animal studies were performed in our testing facility in Costa Mesa, California. Male C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) of approximately 11 to 13 weeks of age were given a single daily dose, administered by oral gavage, of either vehicle (1% Polysorbate 80 and 0.5% Natrosol) or T0901317 at 30 mg/kg for 4 consecutive days. Four animals per group were used. Four hours after the last treatment (day 4), blood was collected and preserved in RNAlater (QIAGEN), and animals were sacrificed by cervical dislocation. Liver and intestine tissues were collected and frozen in liquid nitrogen. RNA was isolated from the whole blood

using a RiboPure Blood Kit (Invitrogen). RNA from the other tissues was isolated by lysis with QIAzol reagent and RNeasy columns (QIAGEN). Total RNA was subjected to real-time PCR as described above. Primer probe sets for the rodent genes were purchased from Applied Biosystems.

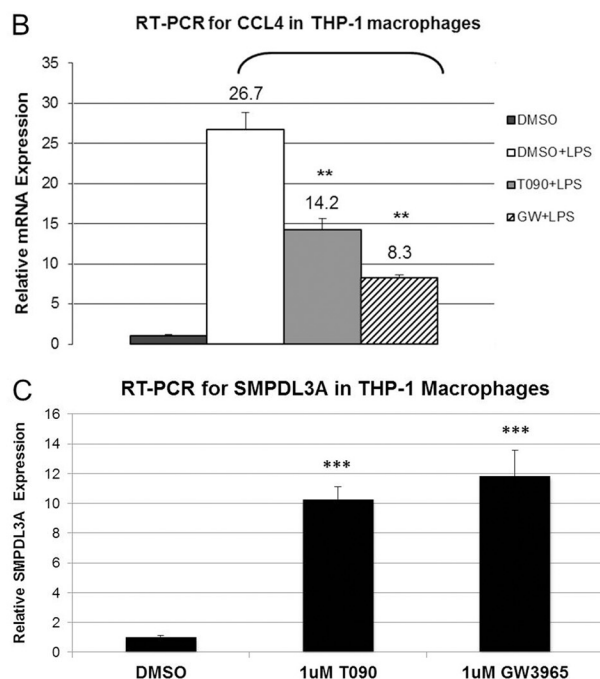
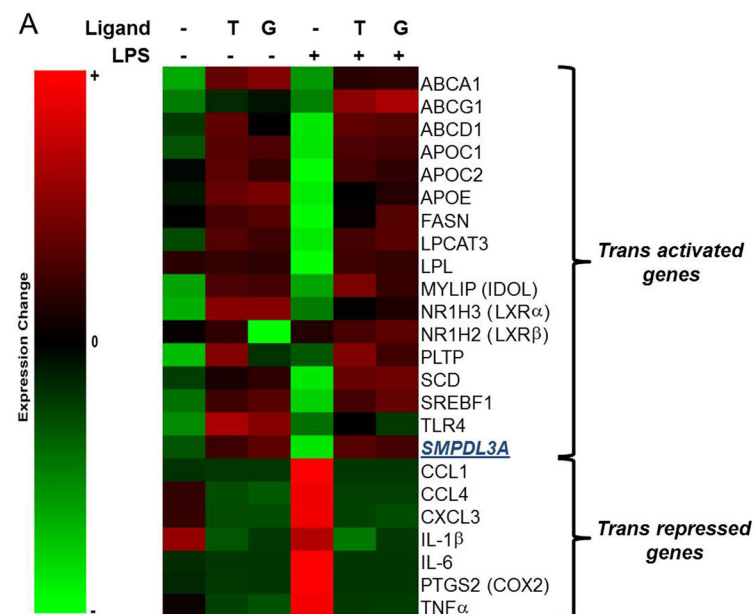
**SMPDL3A Protein Analysis.** HEK293 cells were plated in 35-mm dishes at  $5 \times 10^6$  cells/dish the day before transfection. Each transfection mix contained either 5  $\mu$ g of an empty control vector (OriGene, Rockville, MD) or 5  $\mu$ g of a Myc-DDK-tagged SMPDL3A plasmid (OriGene) with 30  $\mu$ l of Lipofectamine 2000 (Invitrogen) in Opti-MEM Reduced Serum Medium. Twenty-four hours after transfection, both HEK293 cells and THP-1-macrophages treated with the LXR ligand were lysed in ice-cold radioimmunoprecipitation assay buffer, supplemented with protease inhibitor cocktail, and sonicated on a cup horn (Thermo Fisher Scientific, Waltham, MA) for 2 min with 30-s bursts. Cell lysates were cleared by centrifugation for 10 min at 14,000 rpm at 4°C.

Protein concentration in the lysates was determined by Bradford assay. Western blots were performed by resolving 100  $\mu$ g of protein from the cell lysates by SDS-polyacrylamide gel electrophoresis, blotting to nitrocellulose, and probing overnight at 4°C with 1.7  $\mu$ g/ml polyclonal anti-SMPDL3A antibody produced in mouse (Sigma-Aldrich) followed by incubation with 1:2000 diluted donkey anti-mouse HRP conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). As a loading control, the blot was cut and stained with 1:5000 diluted, HRP-conjugated, anti- $\beta$ -actin antibody (GenScript, Piscataway, NJ). HRP activity was visualized using chemoluminescence (Invitrogen).

**Gel Mobility Shift Assays.** Purified human full-length LXR $\alpha$ , LXR $\beta$ , and RXR $\alpha$  were purchased from ProteinOne (Rockville, MD). Single strands containing the LXRE (underlined) in human SMPDL3A (5'-GAAGGAAGAGGGGTTACTGGAGTTCAGTG-GTCTGAA-3') were biotinylated using the Biotin 3' End DNA Labeling Kit (Thermo Fisher Scientific). Double-stranded oligonucleotides were annealed via denaturation at 95°C for 1 min followed by incubation at 70°C for 30 min. The labeled DNA was

incubated with 20 ng of the purified receptors in 10 mM Tris, pH 7.5, 60 mM KCl, 0.02 mM EDTA, 2% glycerol, and 1 mM dithiothreitol for 30 min at room temperature. DNA-protein complexes were resolved on a 6% polyacrylamide gel, electroblotted to nylon membrane, UV cross-linked for 10 min, and incubated with streptavidin-HRP in blocking buffer for 15 min (LightShift Chemoluminescent EMSA Kit; Thermo Fisher Scientific).

**Chromatin Immunoprecipitation Assays.** THP-1 cells were differentiated as described above in 150-mm dishes for 24 h. T0901317 was added in media supplemented with delipidated fetal bovine serum, and the cells were incubated for 16 h. The cells were treated with 1% formaldehyde for 10 min at room temperature. Unreacted formaldehyde was neutralized with 0.125 M glycine. Cells were washed twice with ice-cold PBS and scraped in ice-cold PBS containing protease inhibitors. The cells were collected and washed twice with PBS by centrifugation at 700g for 5 min at 4°C. The cell pellets were lysed in ice-cold radioimmunoprecipitation assay buffer with protease inhibitors, and the chromatin was sheared via sonication on a cup horn for 6 min with 30-s bursts to yield an average DNA fragment length of approximately 500 base pairs. Lysates were clarified by centrifugation at 12,500g for 5 min at 4°C, diluted 1:5 with chromatin immunoprecipitation (ChIP) dilution buffer (Novus Biologicals, Inc., Littleton, CO), and incubated overnight at 4°C with the following antibodies: anti-LXR $\alpha$  (Abcam Inc., Cambridge, MA) or mouse IgG (Invitrogen) as a control. Immunoprecipitation and DNA purification were performed according to the Novus Biologicals protocol (ChromataChIP Kit). The isolated DNA was used for quantitative PCR with POWER SYBR Mix (Applied Biosystems) and the following primers: *hSMPDL3A*: forward, ACTCTGTGAGTCTTCACACCT; reverse, CTGAGAGGAGGCAGGAGAGTT. For the control genes, the following primers were used: *hABCA1*: forward, ACGTGCTTCT-GCTGAGTGA; reverse, ACCGAGCGCAGAGGTTACTA; *h36B4*: forward, ACGCTGCTGAACATGCTCAA; reverse, GATGCTGC-CATTGTCGAACA (as described by Phelan et al., 2008).



**Fig. 1.** A, results of the genome-wide analysis of human genes in THP-1 macrophages. Transactivation of known LXR target genes by T0901317 (T) and GW3965 (G) and LXR-mediated transrepression of LPS-induced genes. Cells were pretreated with either 1  $\mu$ M T0901317 or GW3965 for 1 h and then incubated for 8 h with either LPS-containing or plain media. *SMPDL3A* is identified as a novel LXR target gene. B, regulation of *CCL4* by LXRs confirmed via RT-PCR (THP-1 macrophages treated the same way as for the genome-wide analysis). C, relative expression of the *SMPDL3A* gene in THP-1 macrophages treated with a 1  $\mu$ M concentration of either LXR ligand for 8 h. Data represent means  $\pm$  S.D. ( $n = 4$ ). \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , as determined by Student's  $t$  test.



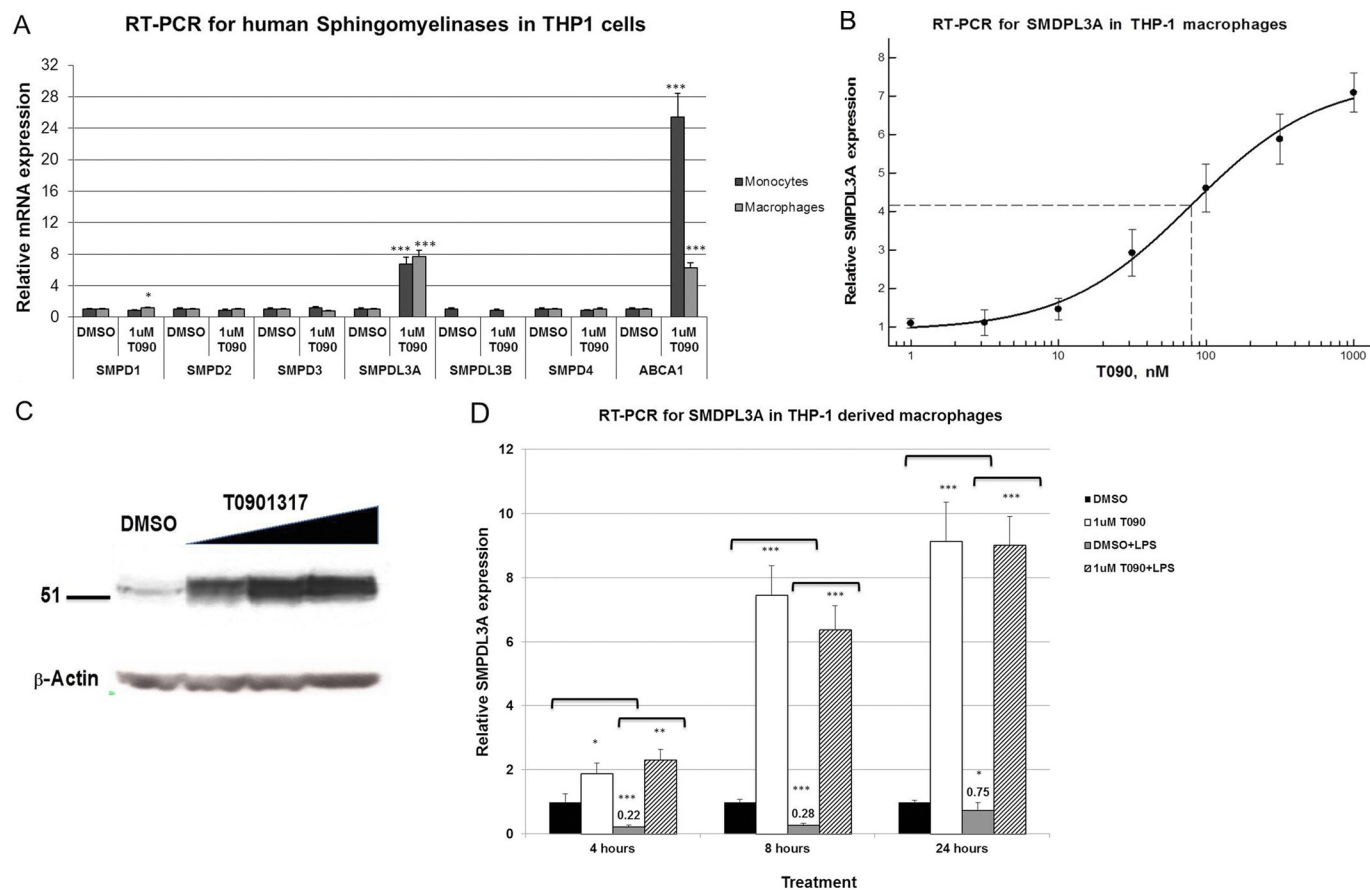
## Results

**Genome-Wide Gene Expression Analysis and Validation by RT-PCR.** We used genome-wide microarray gene expression technology to identify novel LXR-regulated genes in THP-1-derived macrophages. Cells were treated with two LXR synthetic agonists, T0901317 (Schultz et al., 2000) and GW3965 (Collins et al., 2002) in either the presence or the absence of LPS. Treatment with LPS induced more than 400 genes, many of which are known to be regulated by LXRs. Almost all of these genes to some degree were down-regulated upon cotreatment with the LXR ligands. Specifically, proinflammatory genes that were induced by LPS, tumor necrosis factor- $\alpha$ , and IL-6 were mildly reduced (30–40%) by both T0901317 and GW3965 (Fig. 1A). In addition, the expression of chemokine (C-C motif) ligand 4 (CCL4), also known as macrophage inflammatory protein 1 $\beta$ , was modestly reduced by both LXR ligands as had been observed previously in murine macrophages (Joseph et al., 2003). Both LXR agonists up-regulated in common 18 genes. These genes include most of the known LXR target genes, including *ABCA1*, *APOE*, and *NR1H3* (*LXR $\alpha$* ) (Venkateswaran et al., 2000; Laffitte et al., 2001; Li et al., 2002). In addition, one novel gene was identified in this fashion, *SMPDL3A*. In the

absence of LPS, both T0901317 and GW3965 induced an ~4- to 5-fold increase in expression of the *SMPDL3A* gene. Treatment with LPS lowered *SMPDL3A* expression by half. Both T0901317 and GW3965 were able to increase gene expression even in the presence of LPS. The negative effect of LPS on gene expression was also observed for other genes, such as the ABC transporters, apolipoproteins, and the genes involved in lipid synthesis, but not for the LXR genes.

We validated the microarray results for the *CCL4* and *SMPDL3A* genes by quantitative RT-PCR. The down-regulation of the *CCL4* gene by both T0901317 and GW3965 in the presence of LPS was confirmed by RT-PCR. As shown in Fig. 1B, both compounds significantly reduced the *CCL4* mRNA levels. In addition, treatment of macrophages with either T0901317 or GW3965 induced the gene expression of *SMPDL3A* by several fold (Fig. 1C), confirming the findings from the microarray chip.

**Expression of *SMPDL3A* Is Induced by LXR Agonists.** *SMPDL3A* may be functionally related to other sphingomyelinases. Several of these, e.g., *SMPD1* and *SMPD2* and *SMPDL3B*, were shown to be up-regulated by LXR activation in mouse skin keratinocytes (Chang et al., 2008). We measured the effect of the T0901317 treatment on the ex-



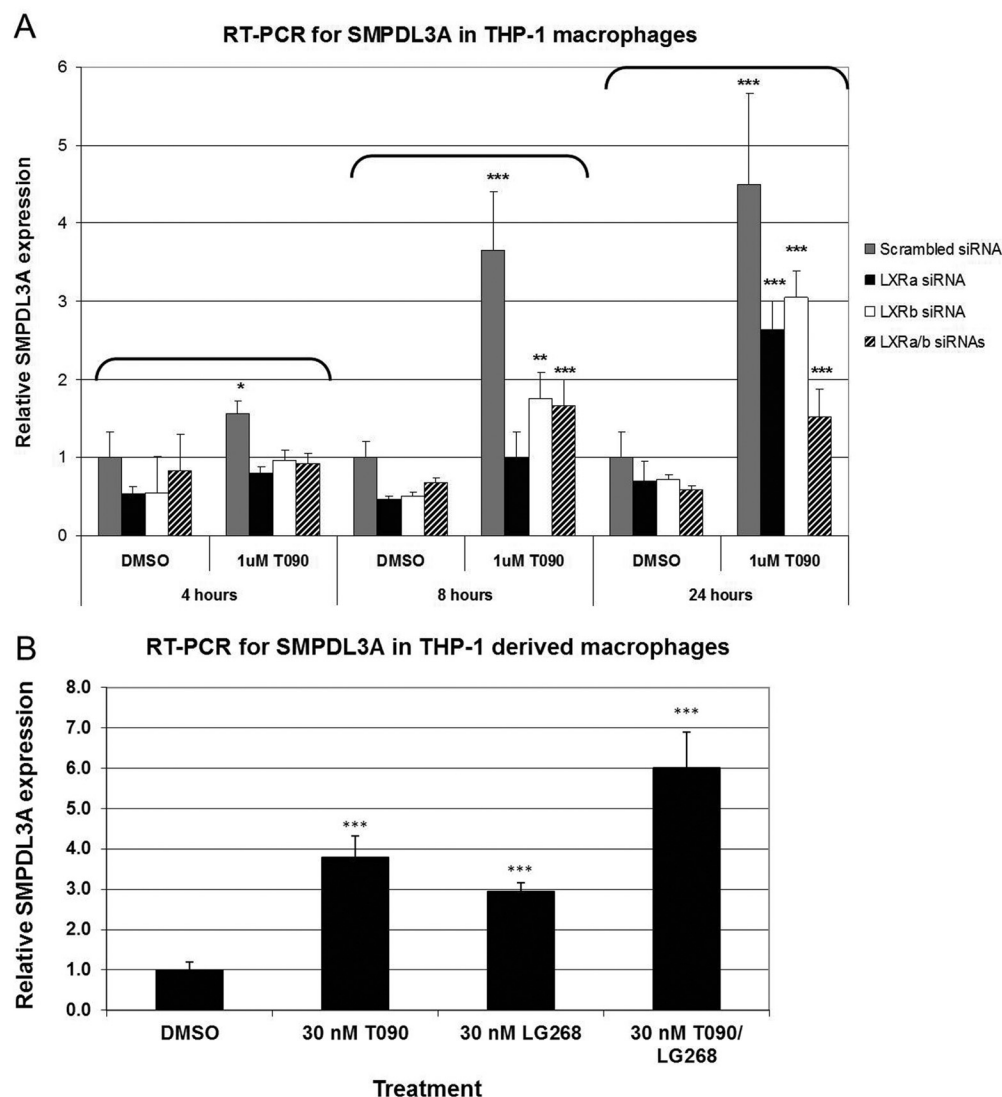
**Fig. 2.** Regulation of the *SMPDL3A* gene by LXRs in THP-1 cells. The relative expression of all genes analyzed was measured by real-time PCR. A, relative expression of human sphingomyelinases in THP-1 cells (monocytes versus derived macrophages) treated with 1  $\mu$ M T0901317 (T090) for 24 h. B, concentration-dependent *SMPDL3A* gene induction by T0901317 in THP-1 macrophages. The extrapolated  $EC_{50}$  is ~80 nM. C, Western blot analysis of *SMPDL3A* expression in THP-1-derived macrophages treated with either DMSO (lane 1) or T0901317 at 50, 500, and 5000 nM for 24 h (lanes 2–4). Total cell lysates (100  $\mu$ g) were resolved by SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and stained with 1.7  $\mu$ g/ml anti-SMPDL3A followed by donkey anti-mouse-HRP (1:2000). D, time-dependent *SMPDL3A* gene up-regulation by 1  $\mu$ M T0901317 with and without 100 ng/ml LPS. For each time point, the DMSO+LPS data ( $\Delta\Delta Ct$ ) were normalized to DMSO data ( $\Delta Ct$ ), and the T090+LPS data were in turn normalized to the DMSO+LPS data. Data represent means  $\pm$  S.D. ( $n = 4$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  as determined by Student's  $t$  test.

pression of the sphingomyelin phosphodiesterase family in THP-1 macrophages. Because none of these genes was differentially regulated by the LXR agonists in the genome-wide gene expression analysis (data not shown), we analyzed the mRNA levels of four sphingomyelinases and the analog of *SMPDL3A*, *SMPDL3B*, by RT-PCR in THP-1 cells. Of interest, *SMPDL3A* seems to be the only gene related to the sphingomyelinase phosphodiesterase family that is induced by the LXR agonists in both THP-1 monocytes and the PMA-differentiated macrophages (Fig. 2A).

The activation of the *SMPDL3A* gene expression was also confirmed to be concentration-dependent for T0901317, with a calculated  $EC_{50}$  value of  $\sim 80$  nM (Fig. 2B), which corresponds to the cellular potency of T0901317 typically observed in Gal4-LXR reporter assays (data not shown). In addition, the levels of *SMPDL3A* protein increased in a concentration-proportional manner upon T0901317 treatment of THP-1 macrophages (Fig. 2C). Specificity of the antibody used for analysis of *SMPDL3A* protein expression was demonstrated by immunostaining of the cell lysate from HEK293 cells transiently transfected with a vector encoding the full-length human *SMPDL3A* fused to the Myc-DDDDK tag at the C terminus (Supplemental Fig. 1).

To discern the dynamics of *SMPDL3A* gene induction by LXR agonists, *SMPDL3A* mRNA levels were measured 4, 8, and 24 h after addition of T0901317 to THP-1 macrophages with and without stimulation with LPS (Fig. 2D). LPS suppressed basal expression of *SMPDL3A* by 78 and 72% at 4 and 8 h, respectively, similar to the effect observed in the genome-wide gene expression analysis. The suppressive effect of LPS on the baseline *SMPDL3A* expression was significantly reduced at 24 h, with only a 25% decrease in expression levels versus those of controls. T0901317 was able to increase gene expression at all time points in a time-dependent manner, regardless of whether THP-1 macrophages were stimulated with LPS. Significant induction is seen within 4 h, reaching a near-maximal effect at 8 h.

Knockdown of LXRs in THP-1-derived macrophages reduces the expression of the *SMPDL3A* gene. To examine whether the transcriptional regulation of the *SMPDL3A* gene is indeed mediated by LXRs, we monitored the gene expression of *SMPDL3A* over time in THP-1 macrophages incubated with an LXR ligand (T0901317) and transfected with siRNA for LXR $\alpha$ , LXR $\beta$ , or both (Fig. 3A). The absolute mRNA levels of *SMPDL3A* (normalized to the scrambled siRNA controls) were reduced after the LXR isoforms were



**Fig. 3.** A, LXR-mediated induction of the *SMPDL3A* gene. THP-1 macrophages were treated with either scrambled siRNA or LXR $\alpha/\beta$  siRNA for 24 h and incubated with the LXR ligand for 4, 8, and 24 h. B, treatment with a 30 nM concentration of either T0901317 or LG100268 for 18 h leads to *SMPDL3A* gene induction. Data represent means  $\pm$  S.D. ( $n = 4$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , as determined by Student's  $t$  test. LG268, LG100268.

silenced individually or together, implying possible involvement of both LXR isoforms in the regulation of SMPDL3A. Treatment with T0901317 led to significant up-regulation of SMPDL3A over time when individual LXR isoforms were knocked down, reflecting overlap in functions of LXR $\alpha$  and LXR $\beta$  for induction of the SMPDL3A gene expression. Less pronounced but still significant stimulation of SMPDL3A expression by T0901317 was observed in the double-knock-down experiment. This residual activation is probably due to incomplete silencing of both LXR isoforms by approximately ~75% and 85% for LXR $\alpha$  and LXR $\beta$ , respectively, as assessed by RT-PCR analysis (Supplemental Fig. 2).

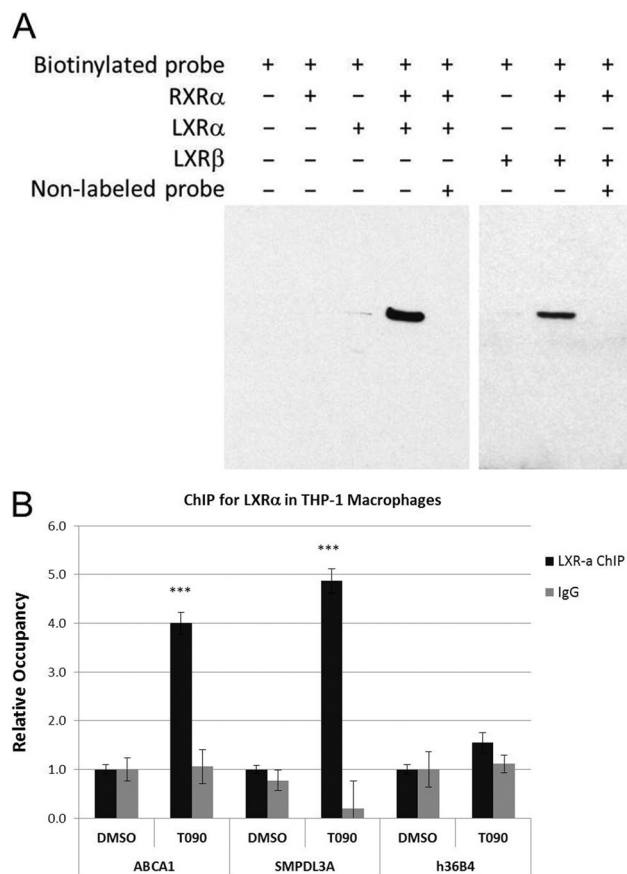
**Both RXR and LXR Ligands Induce SMPDL3A Gene Expression.** LXRs require RXRs as obligate heterodimer partners to bind to their cognate response elements, which can be activated by both RXR and LXR ligands (Willy et al., 1995). To test whether the *SMPDL3A* gene can be induced by an RXR ligand, we treated THP-1 macrophages with a known RXR agonist, LG100268 (Boehm et al., 1995). THP-1 macrophages were treated with suboptimal concentrations of T0901317 and LG100268 (Fig. 2B) (Li et al., 2002). Each compound significantly induced *SMPDL3A* gene expression (Fig. 3B). When compounds were applied together, the extent of the *SMPDL3A* induction appeared to be the sum of the effects seen with either RXR or LXR agonist alone.

Similar to LXRs, the peroxisome proliferator-activated receptor  $\gamma$  functions as a heterodimer with RXRs (Bardot et al., 1993; Gearing et al., 1993). We treated THP-1 macrophages with rosiglitazone, a known peroxisome proliferator-activated receptor  $\gamma$  agonist (Lehmann et al., 1995) and did not observe any significant induction of the *SMPDL3A* gene expression (Supplemental Fig. 3).

**LXR Directly Interacts with LXR Response Element in SMPDL3A Promoter Region.** We identified a putative LXRE in the promoter region of the *SMPDL3A* gene (Pehkonen et al., 2012) based on sequence homology to a consensus LXRE motif (Sandelin and Wasserman, 2005). Duplex oligonucleotides containing the base pairs 2105 to 2120 of the *SMPDL3A* gene (NC\_000006.11, bp 123109971...123130865 of chromosome 6) exhibited specific binding to a heterodimer of the full-length LXR $\alpha/\beta$  and RXR $\alpha$  proteins. None of the proteins was recruited by the DNA duplex as either monomers or homodimers (Fig. 4A). The labeled DNA can be displaced from the protein-DNA complex with the unlabeled duplex DNA having the same sequence.

To demonstrate direct interaction of LXR with the promoter region of *SMPDL3A* within a cell, we conducted ChIP in THP-1 macrophages with LXR $\alpha$ -specific antibodies and analyzed the abundance of the LXRE DNA by real-time PCR (Fig. 4B). Treatment of the cells with the LXR agonist T0901317 led to a significant increase in the amount of the *SMPDL3A* LXRE DNA in the immunoprecipitated material, similar to the DNA of a known LXR target, *ABCA1*.

**LXRs Regulate the *SMPDL3A* Gene in a Cell Type-Specific Fashion in Human Cells.** Next, we investigated the expression levels of the *SMPDL3A* gene in multiple human tissues. The expression levels of the *SMPDL3A* gene across all human tissues were normalized to spleen tissue, which showed the lowest level of expression. Kidney, colon with mucosa lining, placenta, lung, and liver showed the highest relative expression of the gene (Fig. 5A). We then analyzed various immortalized cell lines derived from the

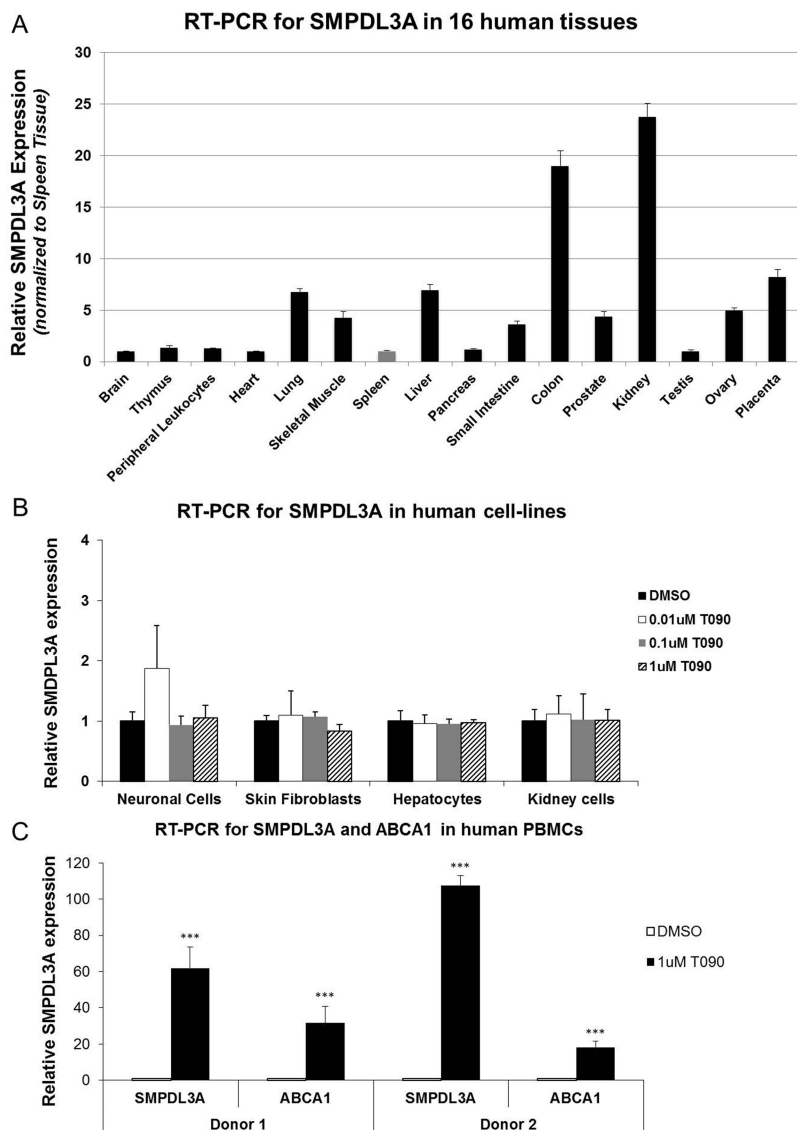


**Fig. 4.** Identification of a DR-4-type LXRE within the human *SMPDL3A* gene. **A**, EMSAs using purified full-length LXRs and RXR $\alpha$  with the DR-4-LXRE sequence identified within the *SMPDL3A* gene (2095–2130). Both LXR $\alpha$ :RXR $\alpha$  and LXR $\beta$ :RXR $\alpha$  bind to the LXRE (lanes 4 and 7, respectively). Excess of nonbiotinylated probe (200 $\times$ ) abolishes the interaction of the LXR:RXR heterodimers with the Biotin LXRE (lanes 5 and 8). **B**, ChIP assays for THP-1 macrophages. Treatment with 5  $\mu$ M T0901317 (T090) for 24 h increases the occupancy of LXR $\alpha$  within the *SMPDL3A* gene. The known LXR target gene, *ABCA1*, was included in the analysis as a positive control. The ChIP signal was normalized to a nonspecific DNA region spanning the *36B4* gene. Data represent means  $\pm$  S.D. ( $n = 4$ ). Data are from a representative experiment repeated three times with similar results. \*\*\*,  $p < 0.001$ , as determined by Student's  $t$  test.

tissues expressing low and high levels of *SMPDL3A*. As shown in Fig. 5B, no *SMPDL3A* gene induction by T0901317 was observed in human H4 neuroglioma cells, CCD 1112 skin fibroblasts, HepG2 hepatocytes, and HEK293 kidney cells. Control studies showed that known LXR target genes were robustly induced in these cell lines by T0901317: *ABCA1* in neuronal cells, HEK293 cells, and skin fibroblasts and *SREBP1c* in HepG2 cells (Supplemental Fig. 4). To rule out the possibility that regulation of *SMPDL3A* by LXRs is restricted to an immortalized cell line, such as THP-1 from acute monocytic leukemia, we measured the effects of T0901317 on human PBMCs isolated from two healthy donors. As shown in Fig. 5C, treatment with T0901317 led to robust *SMPDL3A* and *ABCA1* gene induction in the PBMCs from both donors.

***SMPDL3A* Is Not Induced by LXRs in Mice.** In contrast to human monocytes, *Smpdl3a* gene induction was not observed in RAW264.7 mouse macrophages treated with T0901317 (Fig. 6A). We also analyzed expression levels of *Smpdl3a* in blood, liver, and intestine in mice treated with





**Fig. 5.** Cell type-specific induction of the *SMPDL3A* gene by LXRs. A, relative expression of the *SMPDL3A* gene across a panel of human tissues (Clontech Human MTC Panels I and II). Data were normalized to spleen tissue (lowest expression). B, *SMPDL3A* mRNA levels in several cell lines treated with three different concentrations of T0901317 for 24 h. C, significant induction of the *SMPDL3A* and *ABCA1* (control) genes by T0901317 in human peripheral blood mononuclear cells isolated from two healthy donors. Data represent means  $\pm$  S.D. ( $n = 4$ ). \*\*\*,  $p < 0.001$ , as determined by Student's  $t$  test.

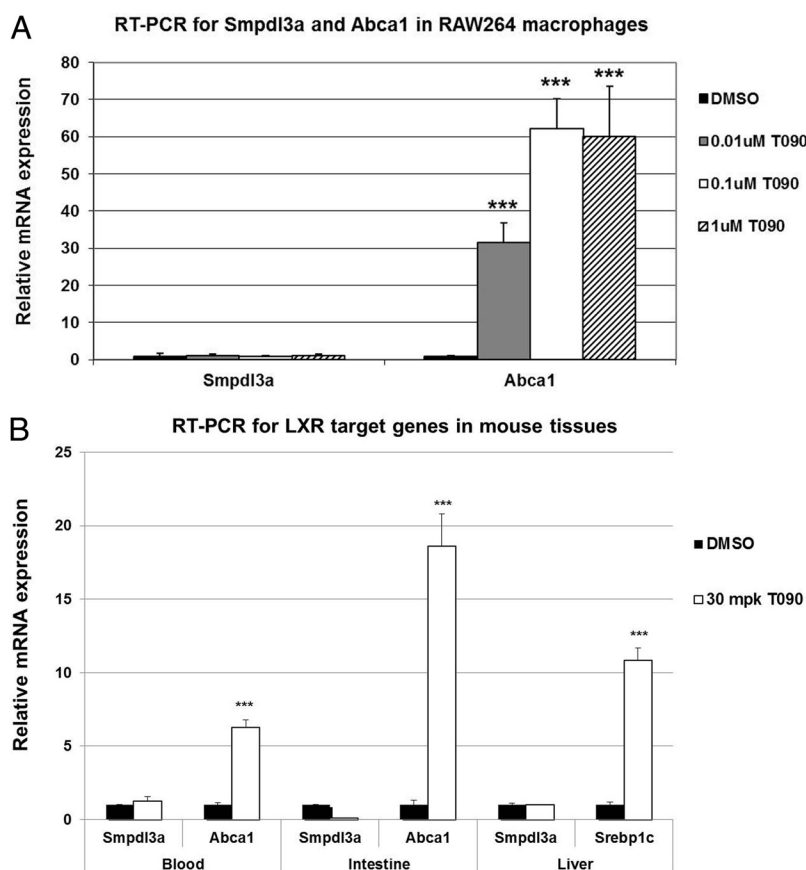
either vehicle or 30 mg/kg T0901317 for 4 days. Although T0901317 treatment strongly induced known target genes, *Abca1* and *Srebp1c*, the expression levels of *Smpdl3a* remained unaffected by the LXR ligand in blood, intestine, or liver (Fig. 6B). These data imply that regulation of the *SMPDL3A* gene by LXR occurs in human monocytes and macrophages but does not occur in murine tissues.

## Discussion

To identify novel LXR target genes in THP-1 macrophages, we analyzed genome-wide expression profiles of 44,000 genes using microarray gene expression analysis in THP-1 macrophages with and without stimulation of inflammatory response with LPS. The validity of the gene expression results was supported by robust induction of the genes that had been previously ascribed to regulation by LXRs. Anti-inflammatory properties of LXRs in THP-1 macrophages seem to be not as strong as those observed with steroidal glucocorticoid receptor agonists (Auphan et al., 1995). However, the overall down-regulation of the expression of several cytokines, including chemokines such as CCL1, CCL4, and chemokine

(C-X-C motif) ligand 3 reflects an LXR-mediated transrepression of proinflammatory genes *tuning down* the attraction of additional monocytes to atherosclerotic foam macrophages.

We confirmed the microarray findings by real-time PCR quantitation of *SMPDL3A* mRNA in THP-1 macrophages treated with two known LXR ligands, T0901317 and GW3965. We saw strong induction of the gene expression by both LXR agonists, demonstrating that the *SMPDL3A* gene is indeed the target of LXR transcriptional activity in THP-1 cells. The induction of the *SMPDL3A* gene by the RXR agonist LG100268 alone and its additive effect with the LXR-mediated transcription of the gene further supports direct regulation of the *SMPDL3A* gene by LXR/RXR heterodimers. While working on the manuscript, we became aware of the recent study (Pehkonen et al., 2012) in which two LXR peaks within the transcription start site of the *SMPDL3A* gene were detected in THP-1 macrophages by ChIP-sequencing analysis. We used this information for identifying an LXRE sequence within the region pinpointed by the ChIP-sequencing study and demonstrated direct interaction of LXR with this region by EMSA and ChIP analyses. To investigate the cell-specific activity, we also performed ChIP anal-



**Fig. 6.** Analysis of gene expression for *Smpdl3a* in mouse tissues. A, treatment with T0901317 (T090) at various concentrations for 24 h does not induce gene expression of *Smpdl3a* in RAW264.7 macrophages as opposed to the control LXR target gene, *Abca1*. B, gene expression analysis of *Smpdl3a* in tissues collected from mice treated with either vehicle or 30 mg/kg T0901317 for 4 days. Data represent means  $\pm$  S.D. ( $n = 4$ ). \*\*\*,  $p < 0.001$ , as determined by Student's  $t$  test.

ysis in HepG2 cells and observed that LXR is recruited to the SMPDL3A promoter in response to an LXR ligand (data not shown), indicating that the cell-specific activation by LXRs must lie in the differential recruitment of cofactors to LXR, similar to what has been described for estrogen receptors (Shang and Brown, 2002). Taken together, these results unequivocally prove that LXR has an active role in transcriptional control of *SMPDL3A* gene expression.

LPS seems to suppress the basal expression of SMPDL3A at least by 2-fold, and this effect diminishes over time. We have also observed a similar effect in murine macrophage-like cells (RAW264.7) upon treatment with LPS (data not shown). Similar down-regulation of sphingomyelinase activity by pertussis toxin, also a Toll-like receptor 4 ligand, had been described by Wang et al. (2007), who showed that the pertussis toxin treatment prolongs macrophages survival by inhibiting acid sphingomyelinase activity. The effect of the LXR agonists on SMPDL3A expression does not depend on the stimulation of THP-1 macrophages with LPS. T0901317 induced *SMPDL3A* gene expression with and without LPS. Knockdown of both LXR isoforms followed by the treatment with T0901317 showed significant reduction in the expression levels of SMPDL3A, demonstrating that the gene is under direct control of LXR, and both LXR isoforms contribute to the stimulation of *SMPDL3A* gene expression. Collectively, the data indicate that *SMPDL3A* is a direct target of LXR.

We were intrigued by the observation that *SDMPL3A* was the only gene belonging to the sphingomyelinase family to be regulated by LXRs. This may suggest that SMPDL3A has functions other than sphingomyelinase and phosphodiester-

ase activities. The fact that the protein levels of SMPDL3A increase in a concentration-dependent fashion in the T0901317-treated THP-1 macrophages implies a functional role of SMPDL3A in leukocytes.

The cell type specificity of the SMPDL3A regulation by LXRs is also a very interesting phenomenon. The expression of the *SMPDL3A* gene seems to be controlled by LXRs in monocytes and macrophages, immortalized cells derived from monocytic leukemia, and primary cell cultures from healthy donors. However, no LXR-mediated induction of the *SMPDL3A* gene was observed in immortalized cell lines derived from kidney, liver, skin fibroblasts, and neuroglioma. Expression of SMPDL3A is not restricted only to leukocytes. The gene is widely expressed among human tissues. The significantly higher gene expression levels of *SMPDL3A* in kidneys and colon may suggest a particular functional role of this gene in epithelial cells. Further analysis of SMPDL3A expression in primary cell cultures will be helpful in assessing the significance of the tissue-specific regulation of this gene by LXRs. The induction of the *SMPDL3A* gene by LXRs may be species-specific, because no increase in gene expression could be observed in murine macrophages (RAW264.7) and no changes were detected in three different tissues, including blood, collected from the mice treated with T0901317. Further work on elucidation of the functions of *SMPDL3A* and the role of LXR-mediated induction of this gene in human monocytes and macrophages is in progress.

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## Authorship Contributions

Participated in research design: Noto, Bukhtiyarov, McKeever, McGeehan, and Lala.

Conducted experiments: Noto and Shi.

Performed data analysis: Noto.

Wrote or contributed to the writing of the manuscript: Noto, Bukhtiyarov, McKeever, McGeehan, and Lala.

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