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MOL #78865

Title page

"Regulation of Sphingomyelin Phosphodiesterase, acid-like 3A gene (SMPDL3A) by Liver X Receptors"

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Running Title page

Regulation of SMPDL3A by LXRs

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ABBREVIATIONS: ABCA1, ATP-binding cassette (ABC) transporter A1; DBCCR1, deleted in bladder cancer chromosome region 1; LXR, liver X receptor; LXRE, liver X receptor response element; PBMCs, peripheral blood mononuclear cells; RXR, retinoid X receptor; SMPDL3A, sphingomyelin phosphodiesterase acid-like 3A; SREBP1c, sterol regulatory element-binding protein-1c; RT-PCR, real-time polymerase chain reaction; ChIP, chromatin immunoprecipitation; HEK, human embryonic kidney; EMSA, electrophoretic mobility shift assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LG100268, 2-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)cyclopropyl]pyridine-5-carboxylic acid; 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-

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Abstract

Liver X receptors alpha (LXRα) and beta (LXRβ) 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 employed 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 (T0901317 and GW3965). This screen identified the sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A) gene as a novel LXR regulated gene, with an LXR response element (LXRE) 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 SMDPL3A is LXR-dependent and is restricted to human blood cells with no induction observed in mouse cellular systems.

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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 (RCT) via direct gene upregulation 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 Tontonoz, 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 (FAS) and stearoyl CoA desaturase (SCD) (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 (TNFα), interleukins (IL-1β, IL-6), cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NFκB) 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 NFκB, that modulate the expression of inflammatory genes (*transrepression*) (Ghisletti et al., 2007). An additional therapeutic indication for LXRs is in Alzheimer's disease (AD). LXR activation has been shown to increase the levels of the Apolipoprotein E (ApoE) in murine and human macrophages (Mak et al., 2002) and in rat brain, where increased ApoE and its higher lipidation due to induction

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of ABCA1 had been positively associated with amyloid Aβ 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). Additionally, in human macrophages, LXRα, but not LXRβ, 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). Additionally, Toll-like Receptor 4 (TLR4) is upregulated 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 employed 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 (direct repeats separated by any 4 nucleotides) LXRE within its promoter. LXRs activate the expression of the SMDPL3A 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 based on its sequence similarity with acid Sphingomyelinase, while its function has not been characterized so far other than its increased expression and association with DBCCR1

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(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.

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Material and Methods

Cell Culture and Transfections. THP-1 cells were maintained in RPMI 1640 medium with Glutamax (Invitrogen, catalog # 61870127) supplemented with 10% fetal bovine serum (Hyclone, catalog # SH30531), 50 μM β-mercaptoethanol and antibiotics [penicillin (50 U/ml)-streptomycin (50 μg/ml), Invitrogen, catalog # 15070063] at 37°C under 5% CO2. Twenty-four hours before treatment, THP-1 cells were plated in 96-well plates at a density of 5 x 10^4 cells/well in presence of 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma catalog # 79346). Cell were then incubated with LXR ligands in RPMI 1640 medium with Glutamax supplemented with 10% delipidated-fetal bovine serum (Hyclone, catalog # SH3085502HI) and antibiotics.

HepG2 cells were maintained and routinely propagated in minimal essential medium (Invitrogen, catalog # 0820234DJ) supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% CO2.

CCD 1112 foreskin fibroblasts were maintained and routinely propagated in Iscove's Modified Dulbecco's Medium (Invitrogen, catalog # 12440046) supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% CO2.

H4, HEK293 and RAW264.7 cells were maintained and routinely propagated in Dulbecco's modified Eagle's medium (Invitrogen, catalog # 10566032) supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% CO2. H4, HEK293, RAW264, CCD 1112 and HepG2 were plated in 96-well plates at a density of 4 x 10^4 cells/well and incubated for 24 hours with LXR ligands in their respective medium supplemented with 10% delipidated-fetal bovine serum and antibiotics.

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For siRNA studies, 24 hours before transfection, THP-1 cells were plated in 96-well plates at a density of 5 x 10^4 cells/well and differentiated with PMA, as described above. Each transfection was carried out with either 30 nM of scrambled siRNA (Invitrogen, catalog # AM4635,) or 30 nM LXR-specific siRNA (LXRα, catalog # 4390824-s19568; LXRβ, catalog # 4390824-s14685, Invitrogen) using 0.4 μl Lipofectamine RNAiMAX (Invitrogen, catalog # 13778075) in Opti-MEM Reduced Serum Medium (Invitrogen, catalog # 31985062).

Twenty-four hours after transfection, cells were washed once with DPBS and treated with the LXR agonist in RPMI 1640 medium with Glutamax supplemented with 10% delipidated-fetal bovine serum and antibiotics.

The LXR ligands GW3965 and TO901317 were purchased from Tocris (catalog # 2474 and 2373). The RXR ligand LG100268 was purchased from Toronto Research Chemicals, Inc. catalog # L397650.

Gene expression microarray analysis. THP-1 cells were differentiated as described above and plated in 35mm-dishes at 5 x 10^6 cells/dish. Twenty-four hours later, cells were pre-treated for 1 hour with either DMSO or 1μM T090 in delipidated FBS-containing media and then incubated with either plain media or LPS-containing media (100 ng/ml) for an additional 8 hours (LPS purchased from Sigma catalog# L2654).

Total RNA was isolated and purified using RNeasy columns (Qiagen). Reverse transcription and hybridization on two Agilent Human GE 4x44K v2 Microarrays were carried out by MOgene LC (St. Louis, Missouri).

Analysis of the SMDPL3A expression in cells and tissues. For all cells treated in 96-well format, RNA was isolated and purified using ABI Prism 6100 Nucleic Acid

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PrepStation (Applied Biosystems). cDNA was synthesized and subjected to real-time PCR using One-Step RT-PCR reagents (Applied Biosystems). Gene expression analysis was carried out according to the method described by Bookout and Mangelsdorf (Bookout AL and Mangelsdorf DJ, 2003).

cDNA from Human MTC™ Panels I and II were purchased from Clontech (catalog# 636742 and 636743) and subjected to real-time PCR.

Whole blood from human donors was purchased from AllCells (catalog# WB001).

Human PBMCs were isolated from the whole blood collected into EDTA-containing tubes using standard Ficoll-Paque gradient centrifugation. Briefly, 15 ml of blood was transferred to 50ml-tubes, diluted with 15mL of DPBS and underlayed with 10 ml of Lymphocyte Separation Medium (9.4% Sodium Diatrizoate, 6.2% Ficoll, MP Biomedicals, LLC, catalog # 50494X). The tubes were centrifuged for 60 min at 800 × *g*, with no brake. The cell interface layer was harvested carefully, and the cells were washed three times with PBS (sedimented for 10 min at 800 × *g*) and resuspended in RPMI 1640 medium with Glutamax supplemented with 10% fetal bovine serum and antibiotics before counting. Cells were plated on 6-well plates at a density of 3×10^6 cells/well and treated with the LXR ligand for 24 hours. 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 (carried out in our testing facility, Costa Mesa, California).

Male C57BL/6 mice (Charles River Laboratories) of approximately 11-13 weeks of age were given a single daily dose, administered by oral gavage, of either vehicle (1% Polysorbate-80 and 0.5% natrosol) or T090 at 30 mg/kg for four consecutive days. Four

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animals per group were used. Four hours after the last treatment (day four), blood was collected and preserved in RNAlater (Qiagen, catalog # 76104) 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 Ribo Pure™-Blood kit (Invitrogen, catalog # AM1928). 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 35mm-dishes at 5 x 10^6 cells/dish the day before transfection. Each transfection mix contained either 5 μg of an empty control-vector (OriGene, catalog # PCMV6XL4) or 5 μg of a Myc-DDK-tagged SMDPL3A plasmid (OriGene, catalog # RC204332) with 30 μl of Lipofectamine 2000 (Invitrogen, catalog # 11668019) 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 cold RIPA buffer, supplemented with protease inhibitors cocktail, and sonicated on a cup horn (Fisher Scientific) for 2 minutes with 30 second-bursts. Cell lysates were cleared by centrifugation for 10' at 14,000 rpm at 4^{o}C.

Protein concentration in the lysates was determined by Bradford assay.

Western Blots were carried out by resolving 100 μg of protein from the cell lysates by SDS-PAGE, blotting to nitrocellulose, and probing overnight at 4ºC with 1.7 μg/ml polyclonal anti-SMPDL3A antibody produced in mouse (Sigma, catalog # SAB1400412) followed by the incubation with 1:2,000 diluted donkey anti-mouse-HRP conjugate (Jackson ImmunoResearch Laboratories, catalog # 715-035-151). As a loading control,

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the blot was cut and stained with 1:5,000 diluted, HRP-conjugated, anti-β-actin antibody (GenScript, catalog # A00730). HRP activity was visualized using chemoluminescence (Invitrogen, Cat.#WP20005).

Gel Mobility Shift Assays. Purified human full-length LXRα, LXRβ, and RXRα were purchased from Protein One (catalog# P1045, P1046 and P1022, respectively). Single strands containing the LXRE response element in human SMDPL3A (5'-GAAGGAAGAG***GGGTTACTGGAGTTCA***GTGGTCTGAA-3') were biotinylated using the Biotin 3' End DNA Labeling Kit (Thermo Scientific, catalog# 89818). Double-stranded oligonucleotides were annealed via denaturation at 95°C for 1 minute followed by incubation at 70°C for 30 minutes. 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 DTT for 30 min at room temperature. DNA-protein complexes were resolved on a 6% polyacrylamide gel, electro blotted to nylon membrane, UV-crosslinked for 10 minutes and incubated with Streptavidin-HRP in blocking buffer for 15 minutes (LightShift Chemoluminiscent EMSA Kit, Thermo Scientific, catalog# 20148).

Chromatin Immunoprecipitation Assays. THP-1 cells were differentiated as described above in 150-mm dishes for 24 hours. T090 was added in media supplemented with delipidated-FBS and the cells were incubated for 16 h. The cells were treated with 1% formaldehyde for 10 minutes at room temperature. Unreacted formaldehyde was neutralized with 0.125 M glycine. Cells were washed twice with ice-cold PBS and scraped in cold PBS containing protease inhibitors. The cells were collected and washed twice with PBS by centrifugation at 700 x *g* for 5 minutes at 4°C. The cell pellets were lysed in cold RIPA buffer with protease inhibitors and the

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chromatin was sheared via sonication on a cup horn for 6 minutes with 30 second-bursts to yield an average DNA fragment length of approximately 500 bp. Lysates were clarified by centrifugation at 12,500 x *g* for 5 min at 4°C, diluted 1:5 with ChIP dilution buffer (Novus Biologicals) and incubated overnight at 4°C with the following antibodies: anti-LXRα (Abcam, catalog# ab41902) or mouse IgG (Invitrogen, catalog# 100005292) as a control. Immunoprecipitation and DNA purification were carried out according to the Novus Biologicals protocol (ChromataChIP Kit, NBP1-71709). The isolated DNA was used for quantitative PCR with POWER SYBR mix (Applied Biosystems, catalog# 4367659) and the following primers: *hSMPDL3A*-F-ACTCTGTGAGTCTTCACACCT, *hSMPDL3A*-R- CTGAGAGGAGGCAGGAGAGTT. For the control genes, the following primers were used: *hABCA1*-F-ACGTGCTTTCTGCTGAGTGA, *hABCA1*-R-ACCGAGCGCAGAGGTTACTA, *h36B4*-F-ACGCTGCTGAACATGCTCAA, *h36B4*-R-GATGCTGCCATTGTCGAACA (as described by Phelan et al., 2008).

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Results

Genome Wide Gene Expression Analysis and validation by real time-PCR (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 (T090) (Schultz et al., 2000) and GW3965 (Collins JL 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 downregulated upon co-treatment with the LXR ligands. Specifically, pro-inflammatory genes that were induced by LPS, TNFα and IL-6, were mildly reduced (30%-40%) by both T090 and GW3965 (Fig. 1A). Additionally, the expression of chemokine (C-C motif) ligand 4 (CCL4), also known as macrophage inflammatory protein 1β (MIP-1β), was modestly reduced by both LXR ligands as had been previously observed in murine macrophages (Joseph et al., 2003). Both LXR agonists upregulated in common 18 genes. These genes include most of the known LXR target genes, including *ABCA1, APOE* and *NR1H3 (LXRα)* (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 T090 and GW3965 induced ~4-5-fold increase in expression of the SMPDL3A gene. Treatment with LPS lowered SMPDL3A expression by half. Both T090 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.

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We validated the microarray results for the CCL4 and SMPDL3A genes by quantitative RT-PCR. The downregulation of the CCL4 gene by both T090 and GW3965 in the presence of LPS was confirmed by RT-PCR. As shown in figure 1B, both compounds significantly reduced the CCL4 mRNA levels. In addition, treatment of macrophages with either T090 or GW3965 induced the gene expression of SMDPL3A by several folds (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 upregulated by LXR activation in mouse skin keratinocytes (Chang et al., 2008). We measured the effect of the T090 treatment on the expression of the sphingomyelin phosphodiesterase family in THP-1 macrophages. Since 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. Interestingly, SMPDL3A appears 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 T090, with a calculated EC_{50} value of ~80 nM (Fig. 2B), which corresponds to the cellular potency of T090 typically observed in Gal4-LXR reporter assays (data not shown). Additionally, the levels of SMPDL3A protein increased in a concentration-proportional manner upon T090 treatment of THP-1 macrophages (Fig. 2C). Specificity of the antibody used for analysis of SMPDL3A

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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 Myc-DDDDK tag at the C-terminus (Supplemental Figure 1).

In order to discern the dynamics of the SMPDL3A gene induction by LXR agonists, SMPDL3A mRNA levels were measured 4, 8 and 24 hours after addition of T090 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 hours, respectively, similar to the effect observed in the genome-wide gene expression analysis. The suppressive effect of LPS on the base-line SMPDL3A expression was significantly reduced at 24 hours, with only 25% decrease in expression levels versus controls. T090 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 hours reaching near maximal effect at 8 hours.

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 SMDPL3A over time in THP-1 macrophages incubated with an LXR ligand (T090) and transfected with siRNA for LXRα, LXRβ or both (Fig. 3A). The absolute mRNA levels of SMPDL3A (normalized to the scrambled siRNA-controls) were reduced after silencing the LXR isoforms individually or together, implying possible involvement of both LXR isoforms in the regulation of SMDPL3A. Treatment with T090 led to significant upregulation of SMPDL3A over time when individual LXR isoforms were knocked down, reflecting

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overlap in functions of LXRα and LXRβ for induction of the SMPDL3A gene expression. Less pronounced but still significant stimulation of SMPDL3A expression by T090 was observed in the double-knockdown experiment. This residual activation is most likely due to incomplete silencing of both LXR isoforms – by approximately ~75% and 85% for LXRα and LXRβ, respectively, as assessed by RT-PCR analysis (Supplemental Figure 2).

Both Retinoid X Receptor (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 SMDPL3A 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 sub-optimal concentrations of T090 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 (PPARγ) functions as a heterodimer with RXRs (Bardot et al., 1993; Gearing et al., 1993). We treated THP-1 macrophages with Rosiglitazone, a known PPARγ agonist (Lehmann et al., 1995), and did not observe any significant induction of the SMPDL3A gene expression (Supplemental Figure 3).

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LXR directly interacts with LXR response element in SMPDL3A promoter region.

We identified a putative LXR response element (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 2105-2120 of the SMPDL3A gene (NC_000006.11, bp 123109971…123130865 of Chromosome 6) exhibited specific binding to a heterodimer of the full-length LXRα/β and RXRα 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.

In order to demonstrate direct interaction of LXR with the promoter region of SMPDL3A within a cell we conducted chromatin Immunoprecipitation (ChIP) in THP-1 macrophages with LXRα-specific antibodies and analyzed the abundance of the LXRE DNA by real-time PCR (Fig.4B). Treatment of the cells with 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 SMDPL3A 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

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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 tissues expressing low and high levels of SMPDL3A. As shown in Figure 5B, no SMPDL3A gene induction by T090 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 T090: ABCA1 in neuronal cells, HEK293 and skin fibroblasts and SREBP1c in HepG2 cells (Supplemental Figure 4). In order to rule out the possibility that regulation of SMDPL3A by LXRs is restricted to an immortalized cell line, such as THP-1 from acute monocytic leukemia, we measured the effects of T090 on human peripheral blood mononuclear cells (PBMCs) isolated from two healthy donors. As shown in Figure 5C, treatment with T090 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, the Smpdl3a gene induction was not observed in RAW264.7 mouse macrophages treated with T090 (Fig. 6A). We also analyzed expression levels of Smpdl3a in blood, liver and intestine in mice treated with either vehicle or 30 mg/kg of T090 for four days. While T090 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 SMPDL3A gene by LXR occurs in human monocytes and macrophages but does not occur in murine tissues.

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Discussion

In order to identify novel LXR target genes in THP-1 macrophages, we analyzed genome-wide expression profiles of forty-four thousand 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 appear to be not as strong as those observed with steroidal glucocorticoid receptor (GR) 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 (CXCL3) 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 the SMPDL3A mRNA in THP-1 macrophages treated with two known LXR ligands, T090 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 SMDPL3A gene by LXR/RXR heterodimers. While working on the manuscript we became aware of the recent study (Pehkonen et al., 2012) where two LXR peaks within the transcription start site of the SMDPL3A gene were detected in THP-1 macrophages by ChIP-Seq analysis. We used this information for identifying an LXRE sequence within the region pinpointed by the ChIP-Seq study and demonstrated direct interaction of LXR with this region by EMSA and ChIP analyses. To investigate

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the cell specific activity we also carried out ChIP analysis 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 the 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 appears to suppress the basal expression of SMPDL3A at least by two-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 (PTX), also a TLR-4 ligand, had been described by Wang et al. (2007), who showed that the PTX 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. T090 induced the SMPDL3A gene expression with and without LPS. Knockdown of both LXR isoforms followed by the treatment with T090 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 the 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 phospodiesterase activities. The fact that the protein levels of SMPDL3A increase in a concentration-dependent fashion in

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the T090-treated THP-1 macrophages implies a functional role of SMPDL3A in leukocytes.

The cell-type specificity of the SMDPL3A regulation by LXRs is also a very interesting phenomenon. The expression of the SMPDL3A gene appears 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 SMDPL3A 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, since no increase in gene expression could be observed in murine macrophages (RAW264.7) nor any changes had been detected in three different tissues, including blood, collected from the mice treated with T090.

Further work is in progress on elucidation of the functions of SMPDL3A and the role of LXR-mediated induction of this gene in human monocytes and macrophages.

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Acknowledgements

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Authorship Contributions

Participated in research design: Noto, Bukhtiyarov, McKeever, McGeehan, Lala.
Conducted experiments: Noto, Shi.
Contributed new reagents or analytic tools: n/a
Performed data analysis: Noto
Wrote or contributed to the writing of the manuscript: Noto, Bukhtiyarov, McKeever, McGeehan, Lala.

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Figure Legends

Fig. 1.

A, Results of the genome-wide analysis of human genes in THP-1 macrophages. Transactivation of known LXR target genes by T090 (T) and GW3965 (G) and LXR-mediated transrepression of LPS-induced genes. Cells were pre-treated with either 1μM T090 or GW3965 for 1 hour and then incubated for 8 hours 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 SMDPL3A gene in THP-1-macrophages treated with 1μM of either LXR ligand for 8 hours. Data represent mean ± SD (n=4). ** $p<0.01$, *** $p<0.001$ as determined by Student's *t*-test.

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 1μM T090 for 24 hours. B, Concentration-dependent SMDPL3A gene induction by T090 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 T090 at 50, 500 and 5,000 nM for 24 hours (lanes 2-4). 100 μg of total cell lysates were resolved by SDS-PAGE, blotted to nitrocellulose, and stained with 1.7 μg/ml anti-SMPDL3A followed by DAM-HRP (1:2,000)

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D, Time-dependent SMPDL3A gene up regulation by 1μM T090 with and without 100 ng/ml LPS. For each time point, the "DMSO+LPS" data (ΔΔCt) was normalized to "DMSO" data (ΔCt) and the "T090+LPS" data was in turn normalized to the "DMSO+LPS" data. Data represent mean ± SD (n=4). *p < 0.05, **p<0.01, ***p<0.001 as determined by Student's *t*-test.

Fig. 3. A, LXR-mediated induction of the SMPDL3A gene. THP-1 macrophages were treated with either scrambled siRNA or LXRα/β siRNA for 24 hours and incubated with the LXR ligand for 4, 8 and 24 hours. B, Treatment with either 30 nM of either T090 or LG100268 for 18 hours leads to SMDPL3A gene induction. Data represent mean ± SD (n=4). *p < 0.05, **p<0.01, ***p<0.001 as determined by Student's *t*-test.

Fig. 4. Identification of a DR-4 type LXRE within the human SMPDL3A gene.

A, Electromobility Shift Assays (EMSAs) using purified full-length LXRs and RXRα with the DR4-LXRE sequence identified within the SMPDL3A gene (2095-2130). Both LXRα:RXRα and LXRβ:RXRα bind to the LXRE (lanes 4 and 7, respectively). Excess of non-biotinylated probe (200X) abolishes the interaction of the LXR:RXR heterodimers with the Biotin-LXRE (lanes 5 and 8).

B, Chromatin Immunoprecipitation Assays (ChIP) for THP-1 macrophages. Treatment with 5μM T090 for 24hr increases the occupancy of LXRα within the SMDPL3A gene. The known LXR target gene, ABCA1, was included in the analysis as a positive control. ChIP signal was normalized to nonspecific DNA region spanning the 36B4 gene, and data represent mean ± SD (n=4). Data are from a representative experiment repeated three times with similar results. ***p<0.001 as determined by Student's *t*-test.

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Fig. 5. Cell type-specific induction of the SMDPL3A gene by LXRs.

A, Relative expression of the SMPDL3A gene across a panel of human tissues (Clontech Human MTC™ Panels I and II). Data normalized to spleen tissue (lowest expression) B, SMPDL3A mRNA levels in several cell-lines treated with three different concentrations of T090 for 24 hours. C, Significant induction of the SMPDL3A and ABCA1 (control) genes by T090 in human peripheral blood mononuclear cells isolated from two healthy donors. Data represent mean ± SD (n=4). ***p<0.001 as determined by Student's *t*-test.

Fig. 6. Analysis of gene expression for Smpdl3a in mouse tissues. A, Treatment with T090 at various concentrations for 24 hours does not induce the 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 mpk T090 for four days. Data represent mean ± SD (n=4). ***p<0.001 as determined by Student's *t*-test.

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Figure 1A

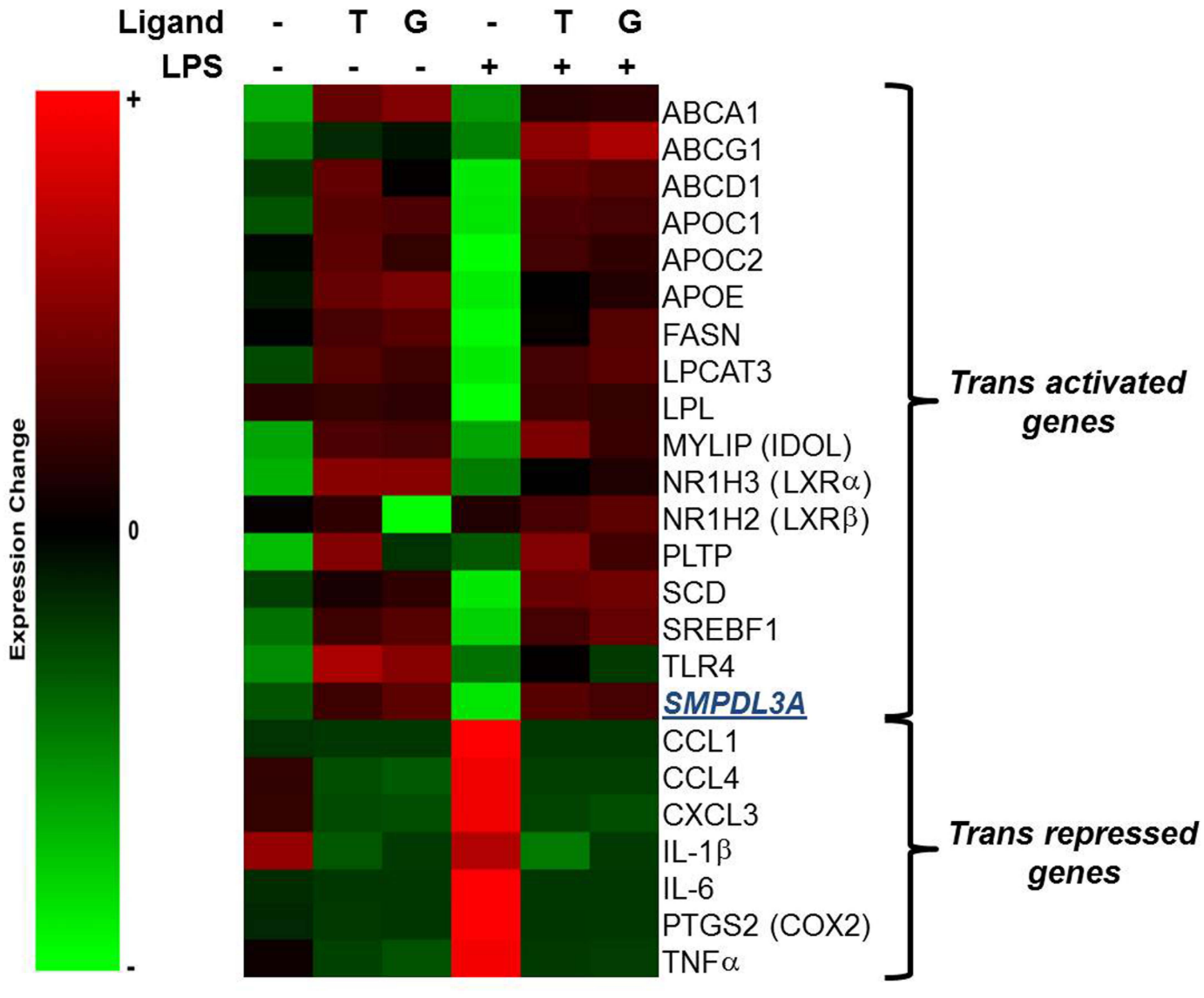

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Figure 1B

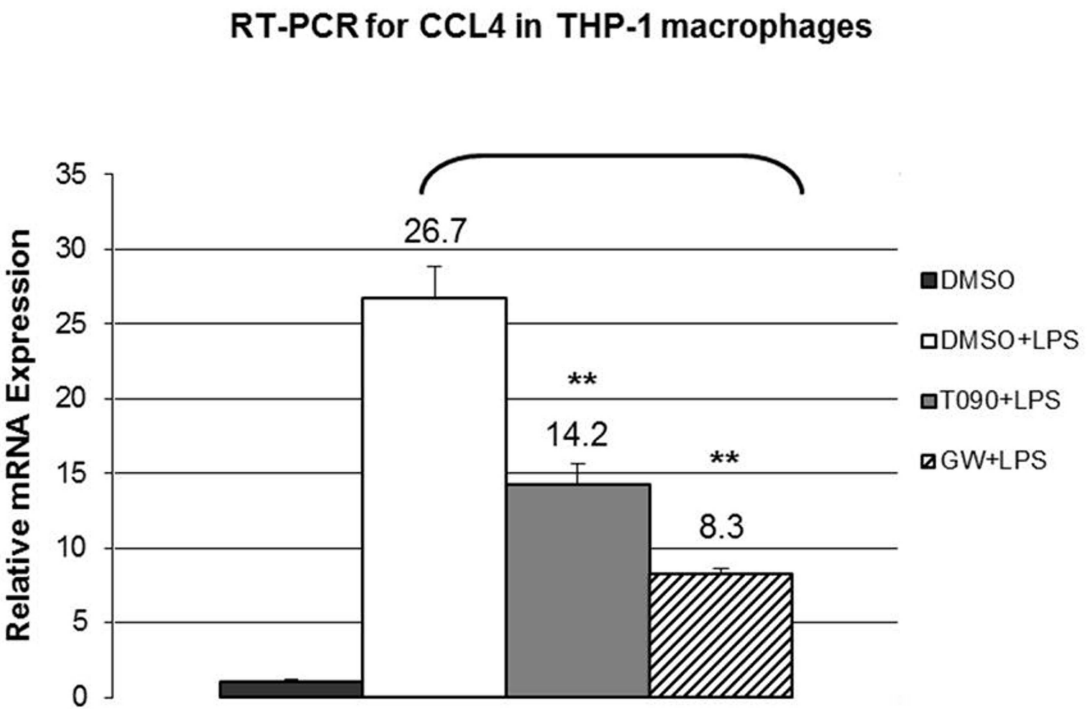
RT-PCR for CCL4 in THP-1 macrophages
Relative mRNA Expression
35
30
25
20
15
10
5
0
26.7
**
14.2
**
8.3
DMSO
DMSO+LPS
T090+LPS
GW+LPS

Figure 1C

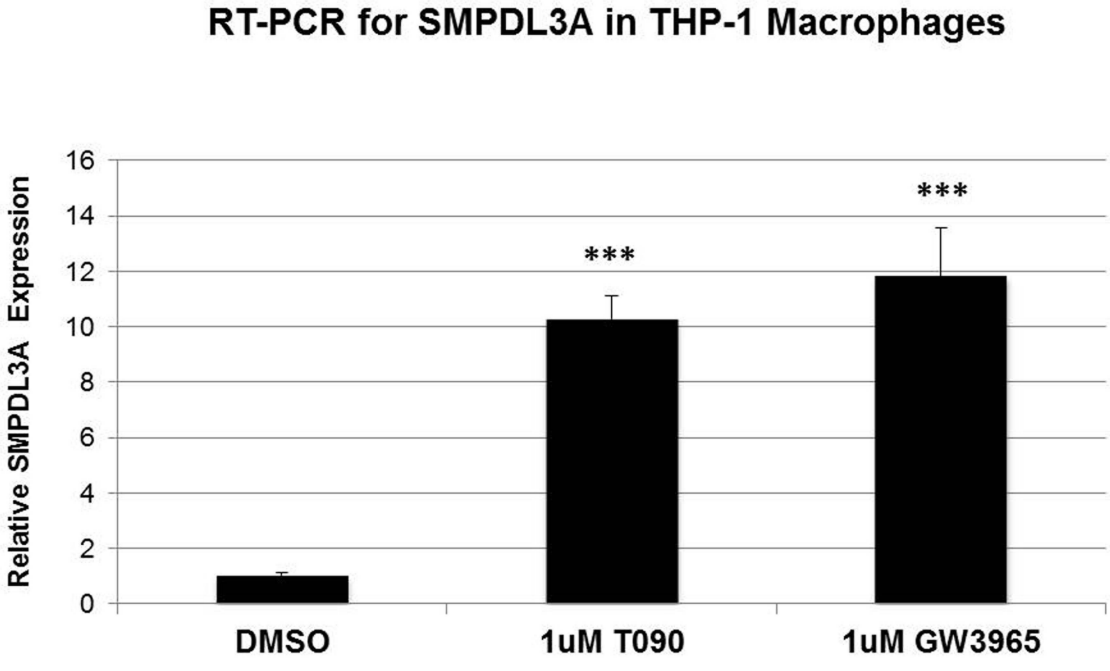
RT-PCR for SMPDL3A in THP-1 Macrophages
Relative SMPDL3A Expression
16
14
12
10
8
6
4
2
0
***
***
DMSO
1uM T090
1uM GW3965

Figure 2A

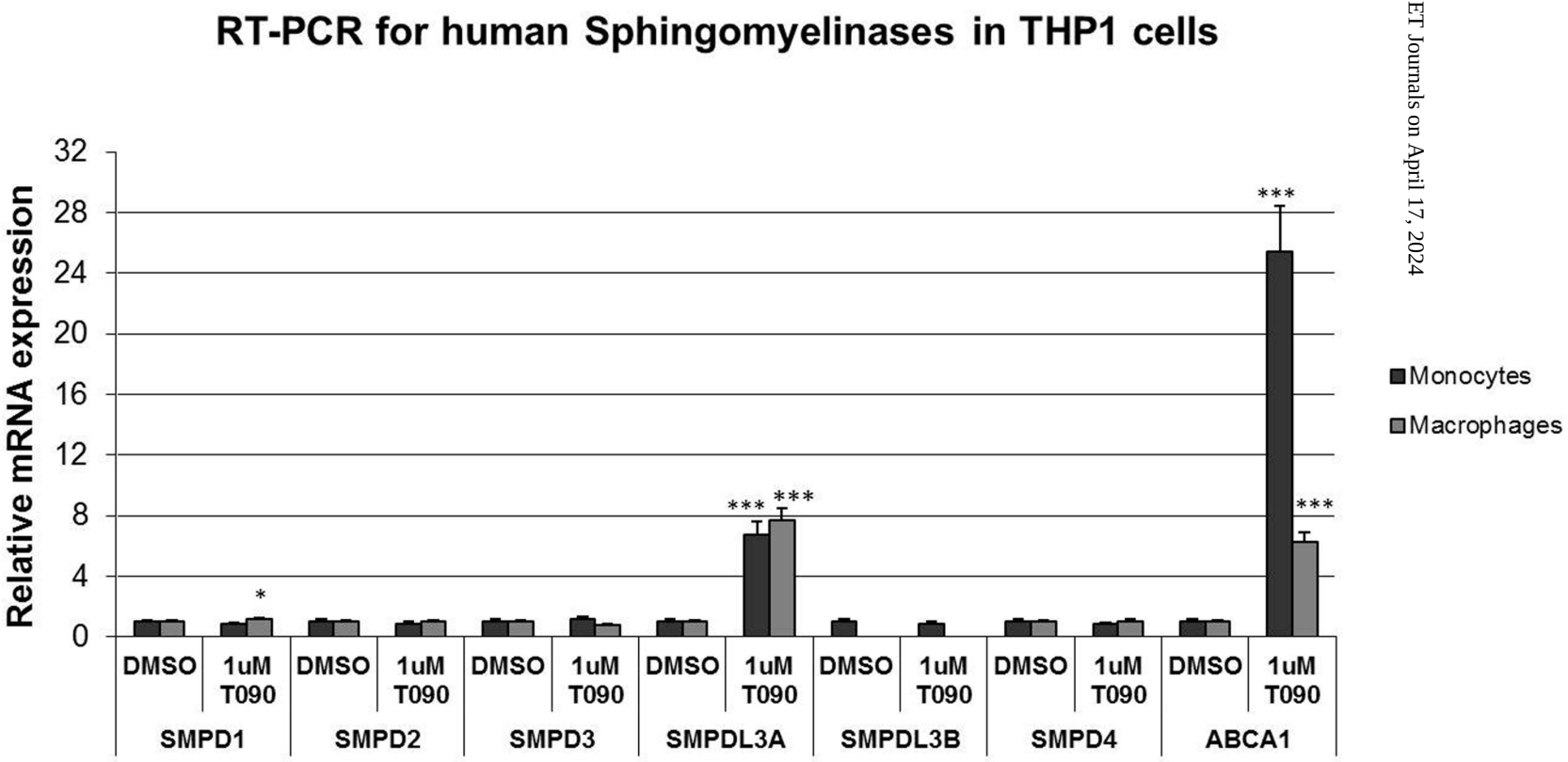

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Figure 2B

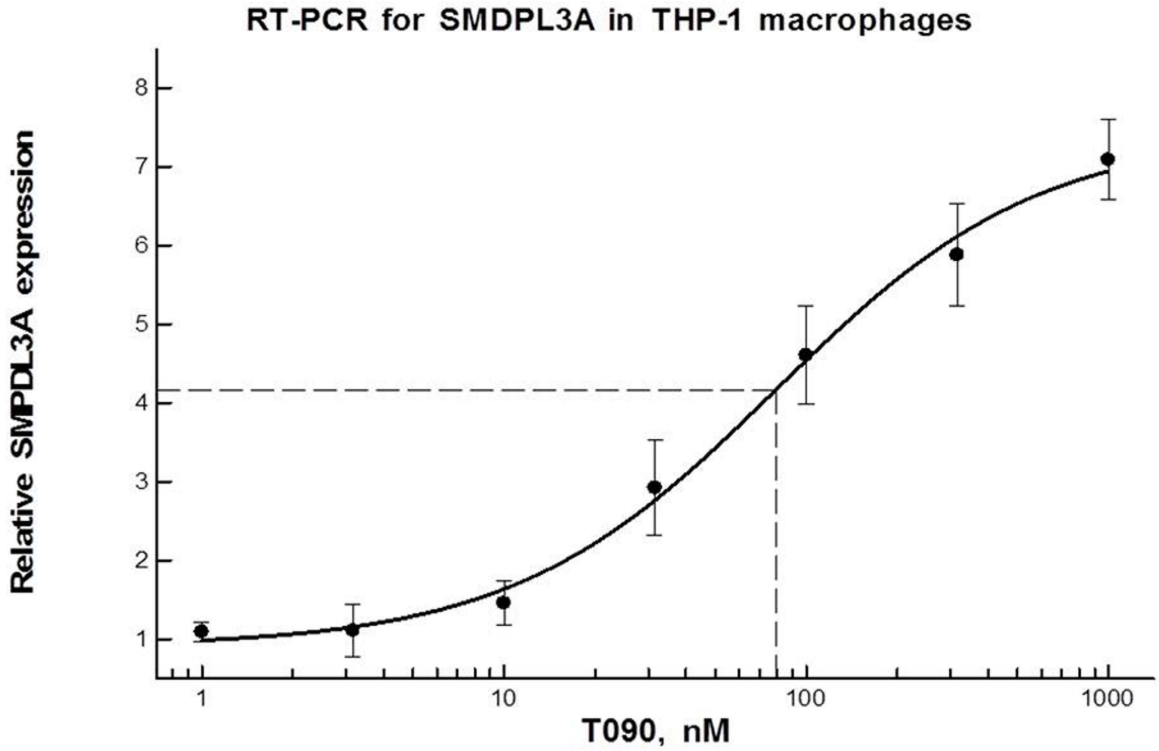
RT-PCR for SMDPL3A in THP-1 macrophages
Relative SMPDL3A expression
8
7
6
5
4
3
2
1
1
10
100
1000
T090, nM

Figure 2C

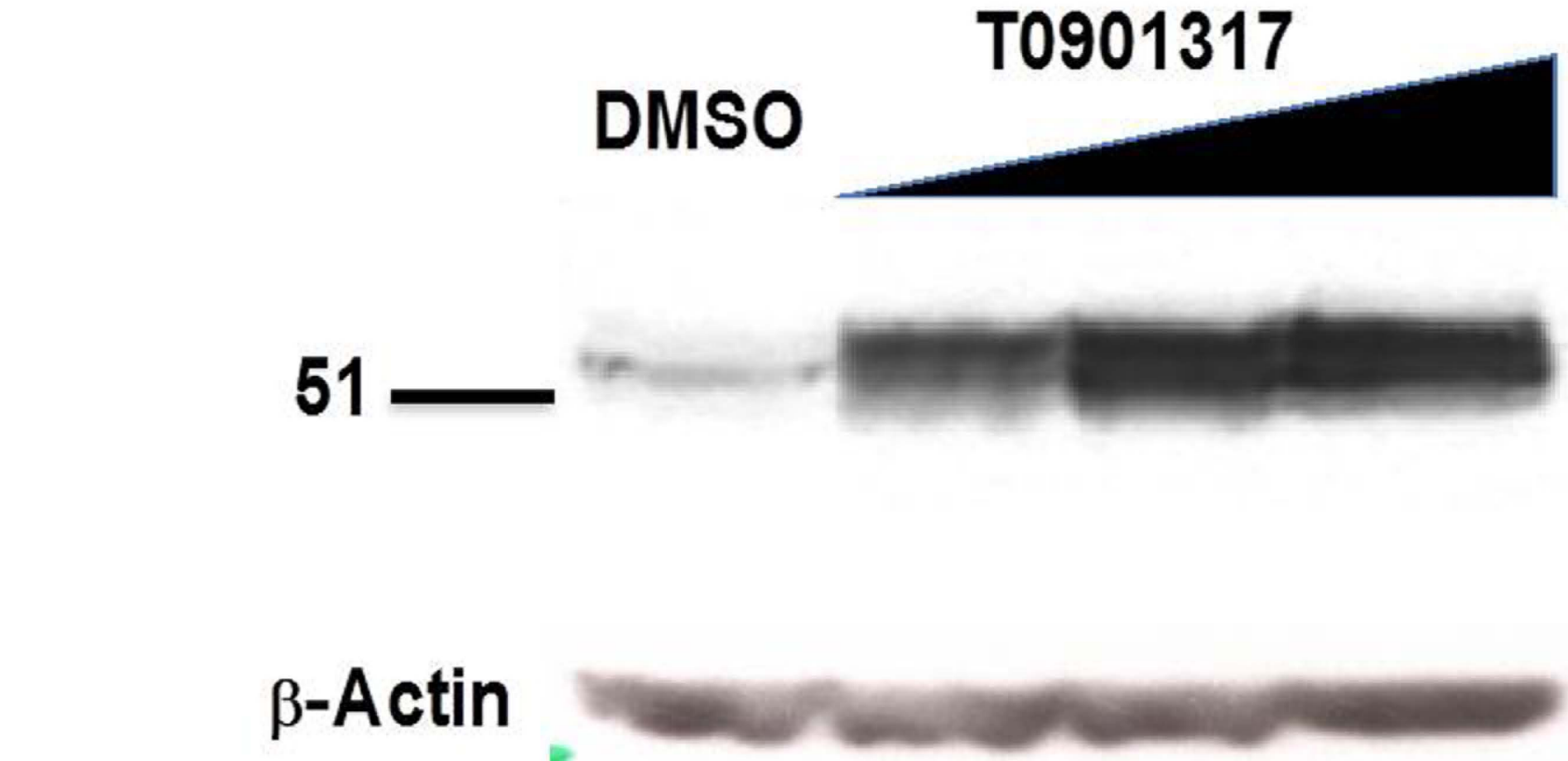

Figure 2D

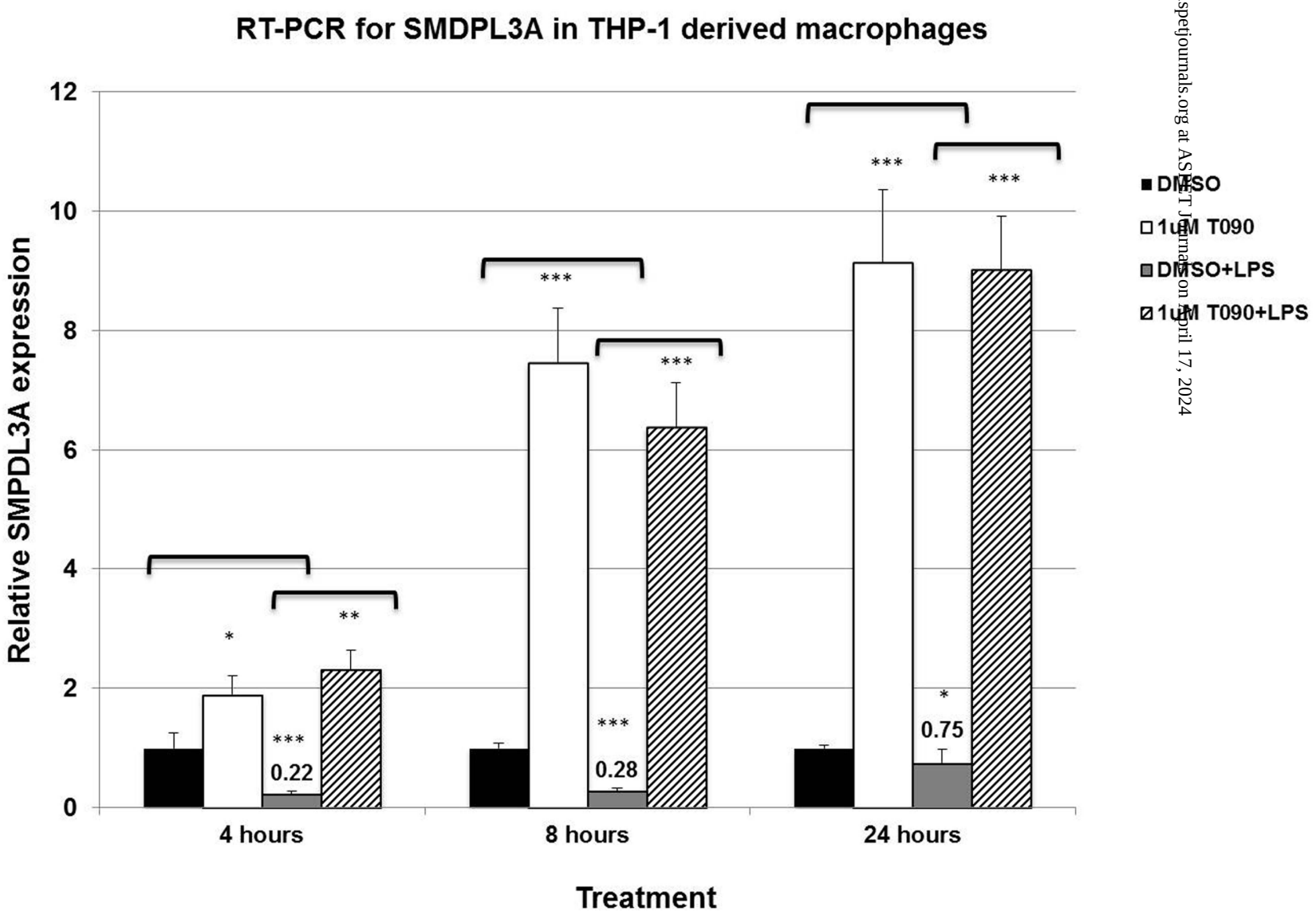

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Figure 3A

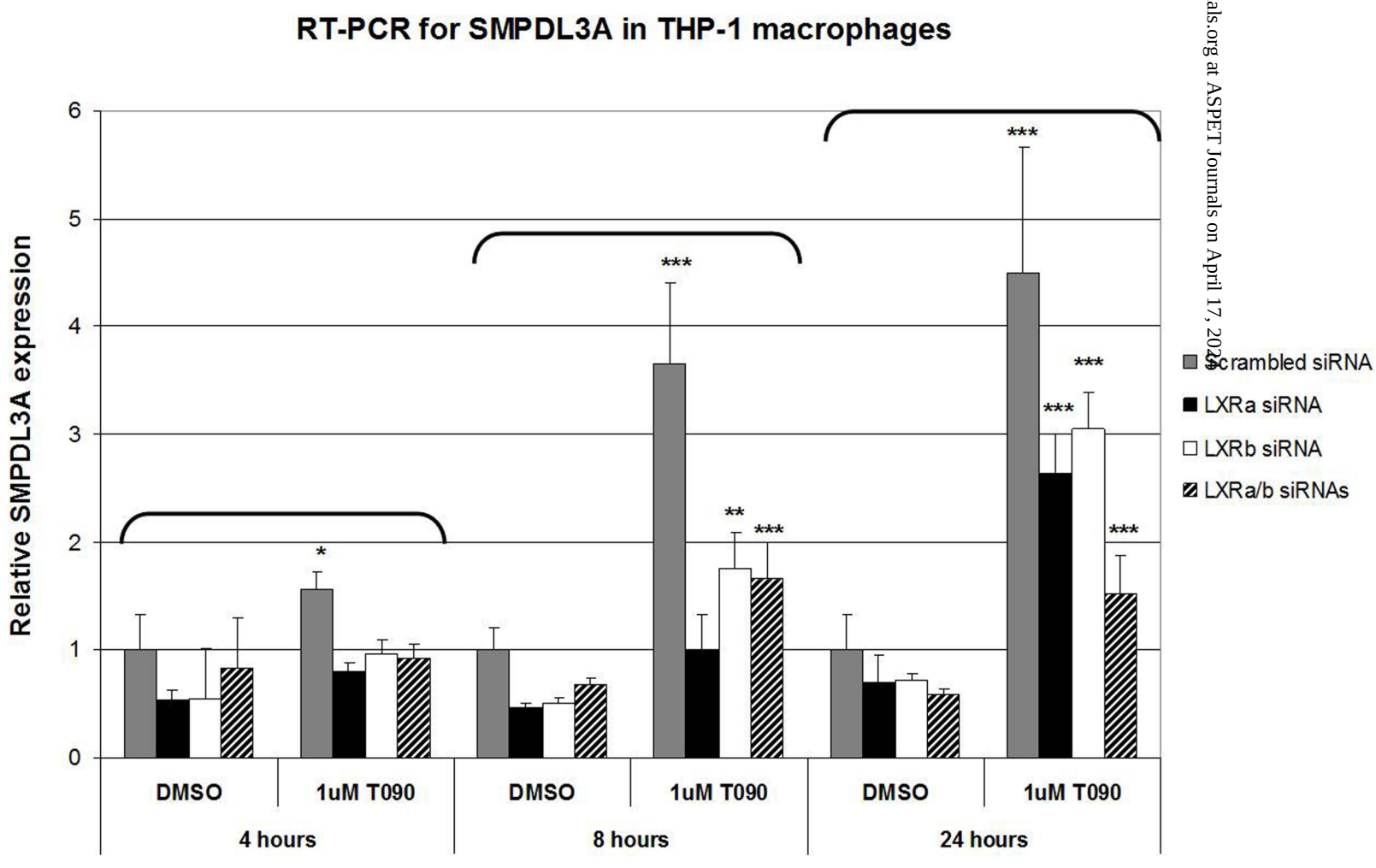

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Figure 3B

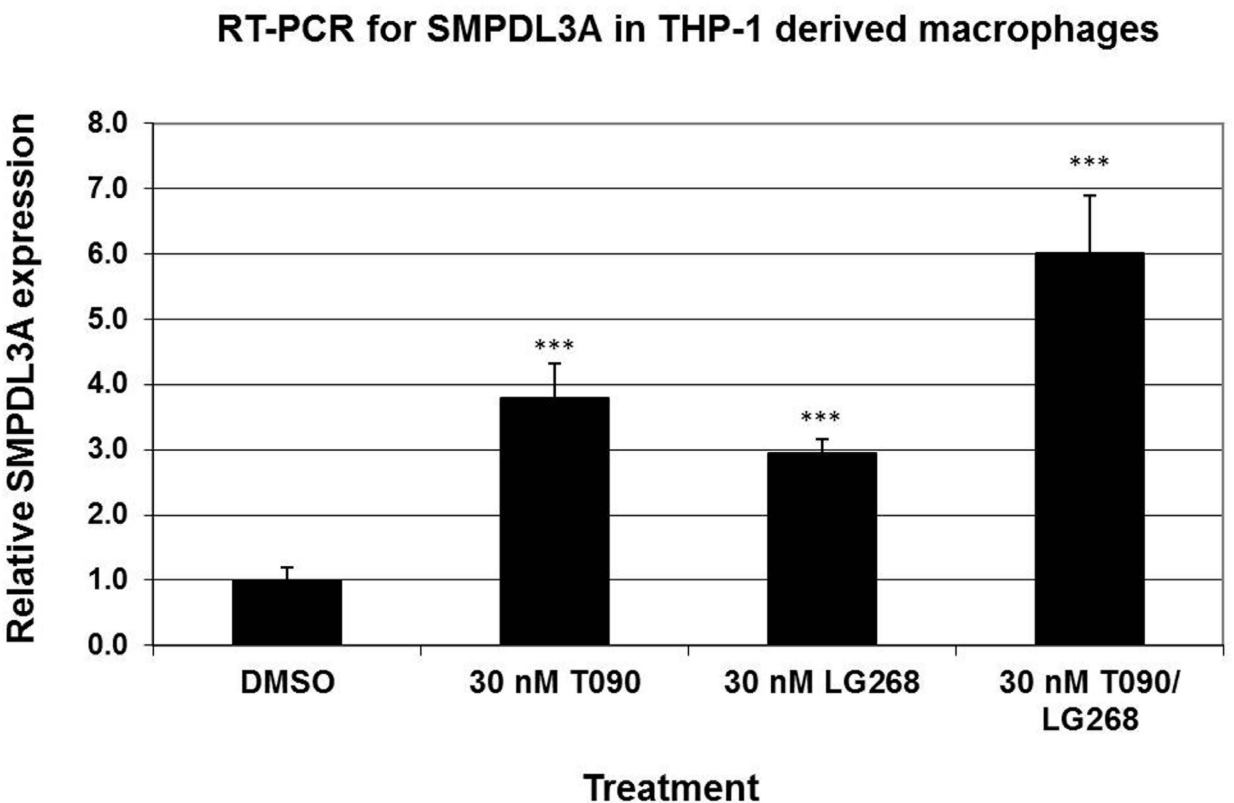
RT-PCR for SMPDL3A in THP-1 derived macrophages
Relative SMPDL3A expression
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
***
***
DMSO
30 nM T090
30 nM LG268
30 nM T090/ LG268
Treatment

Figure 4A

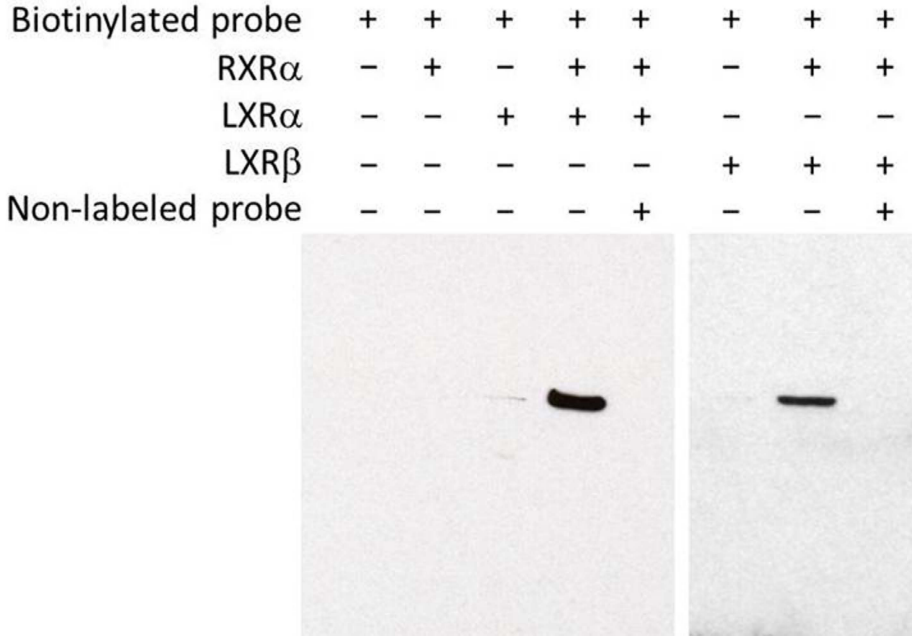

Figure 4B

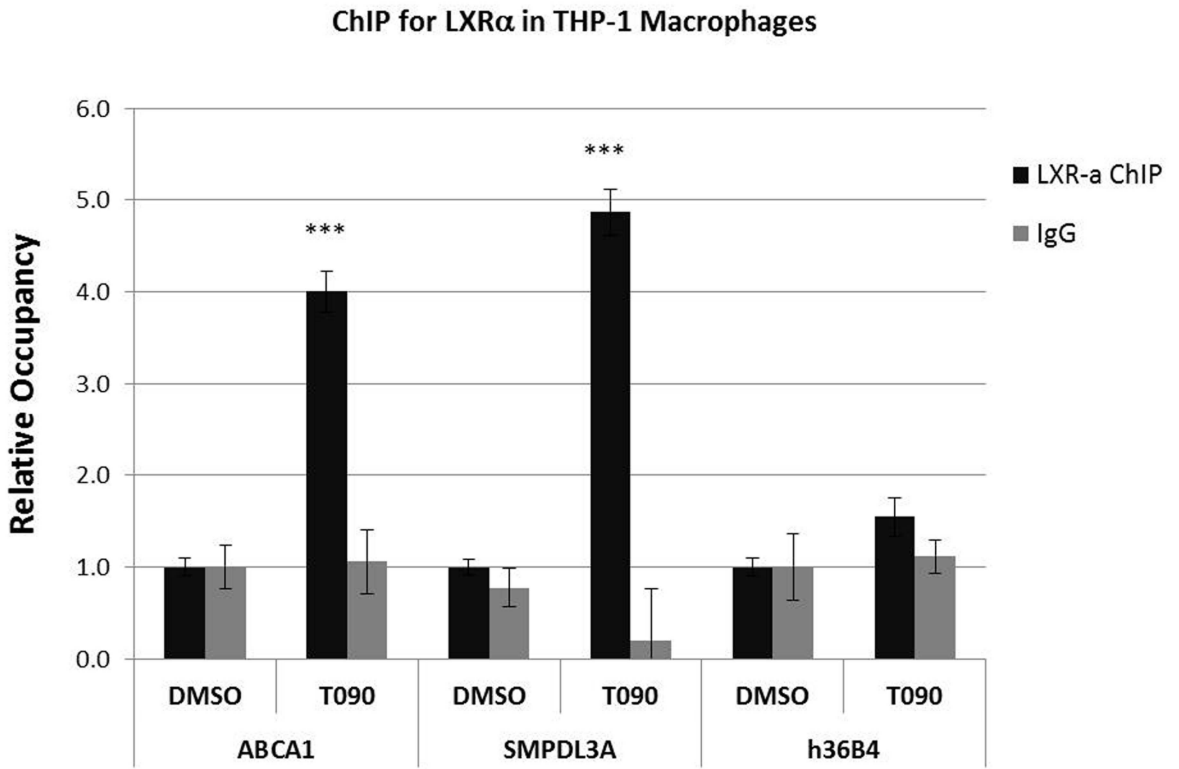
ChIP for LXRα in THP-1 Macrophages
Relative Occupancy
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
***
LXR-a ChIP
IgG
DMSO
T090
DMSO
T090
DMSO
T090
ABCA1
SMPDL3A
h36B4

Figure 5A

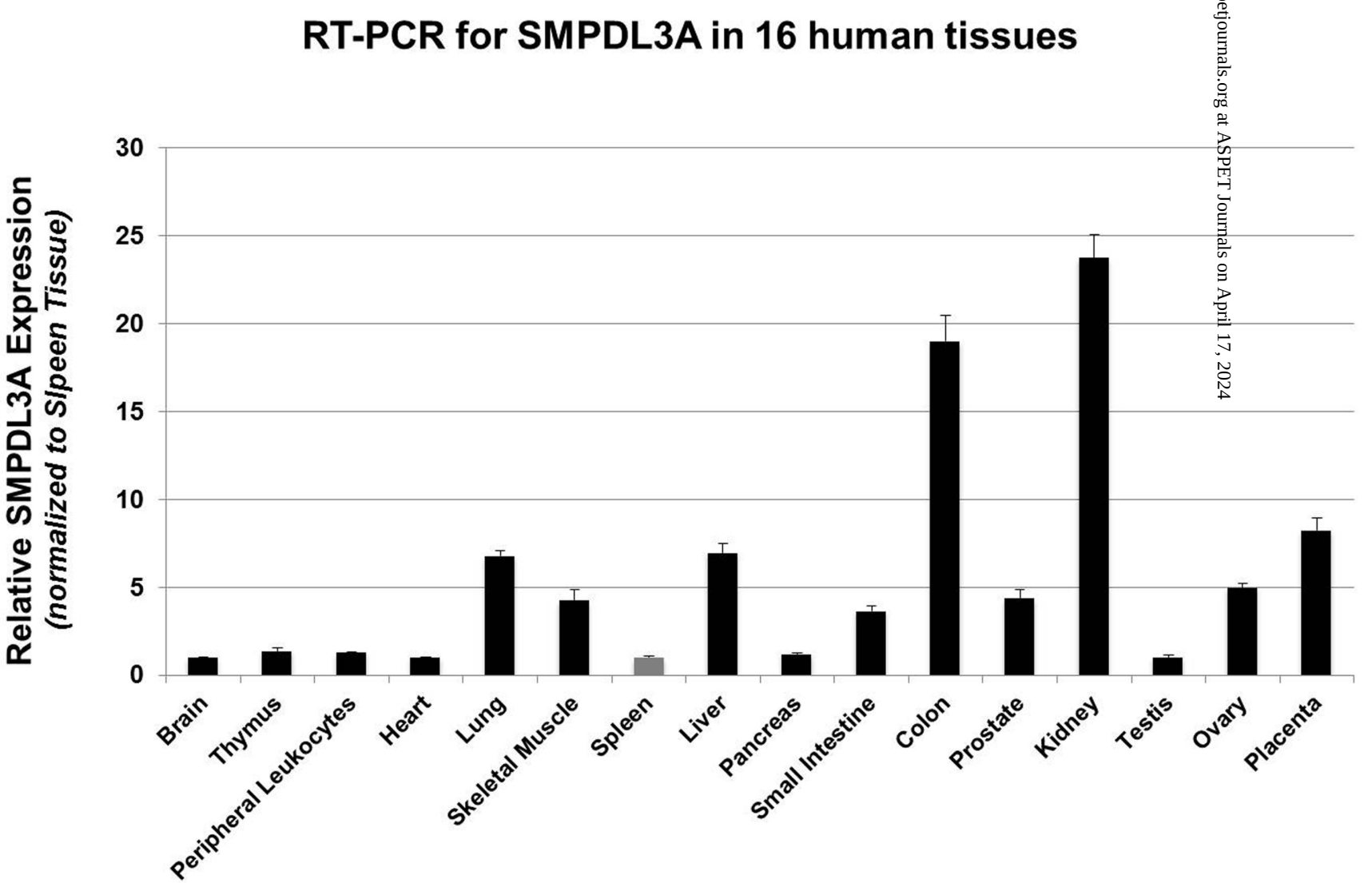

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Figure 5B

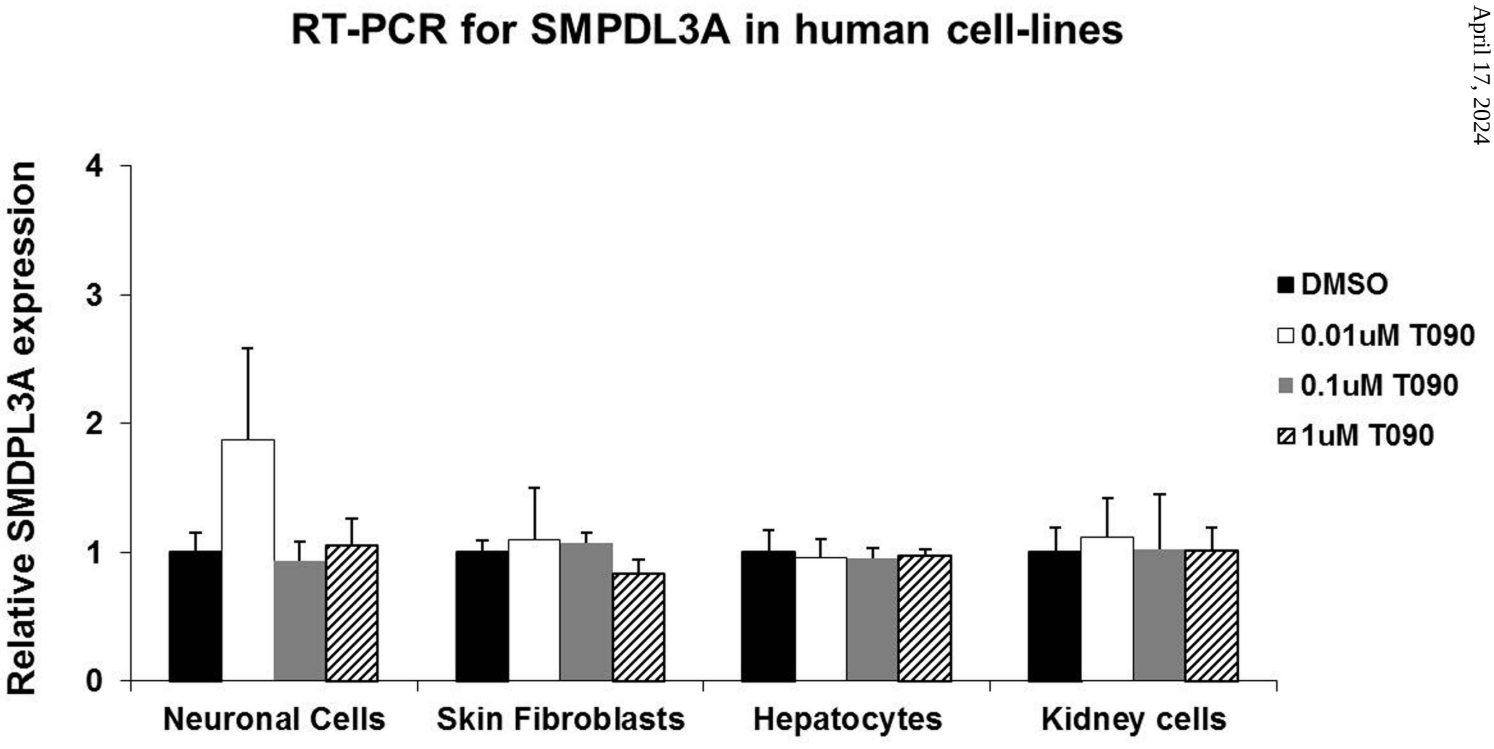

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Figure 5C

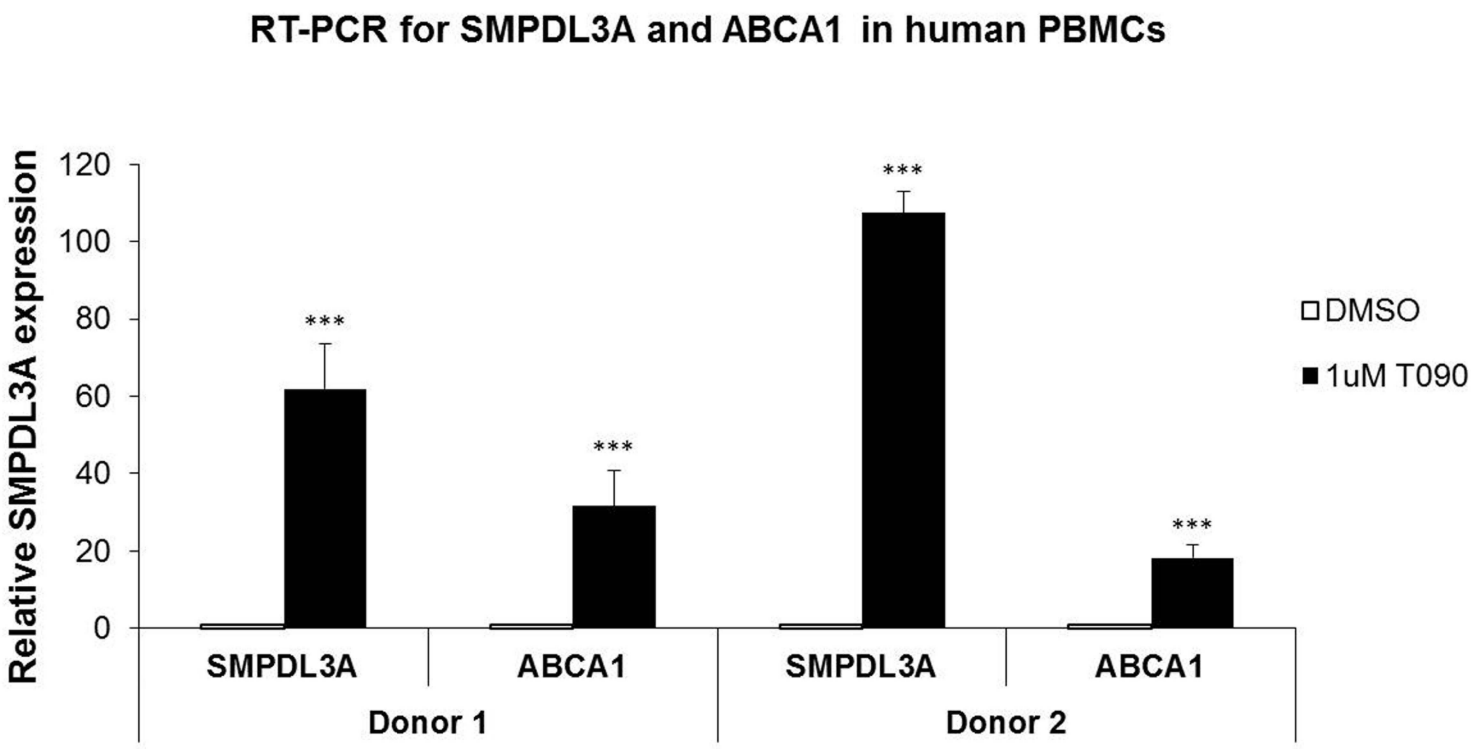

RT-PCR for SMPDL3A and ABCA1 in human PBMCs
Relative SMPDL3A expression
120
100
80
60
40
20
0
***
***
***
***
SMPDL3A
ABCA1
SMPDL3A
ABCA1
Donor 1
Donor 2
DMSO
1uM T090

Figure 6A

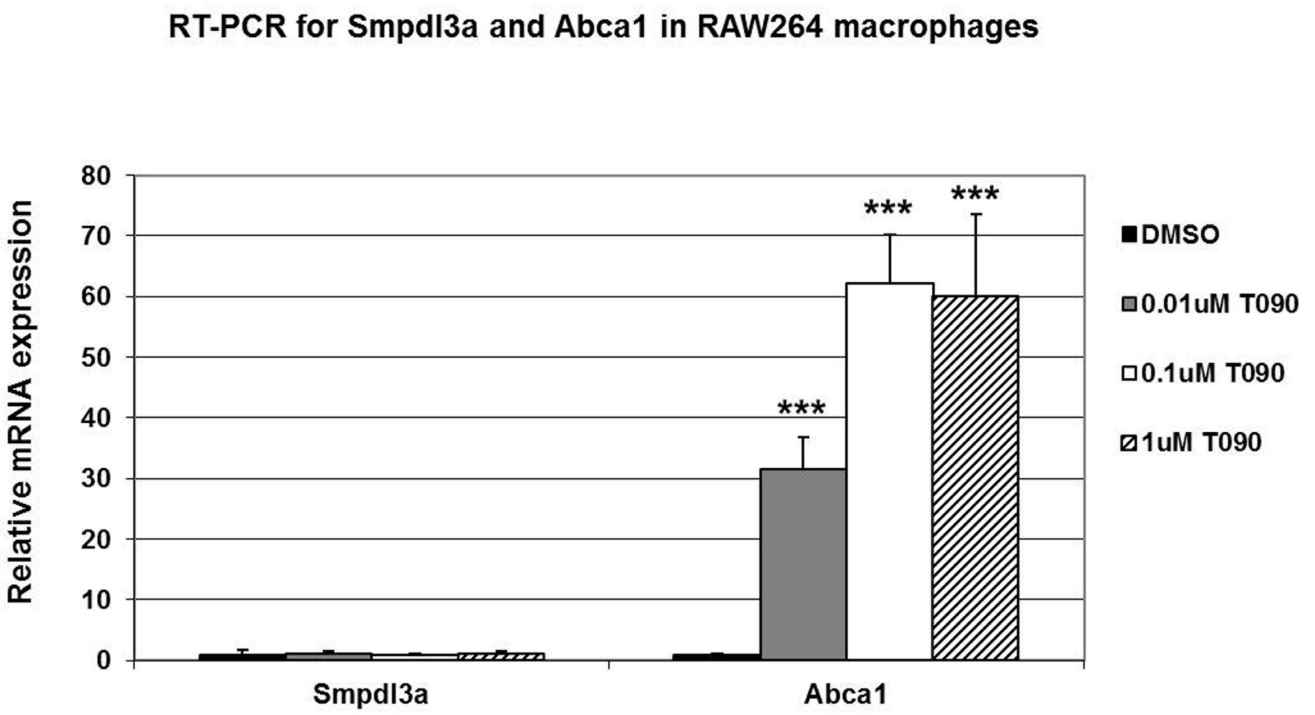
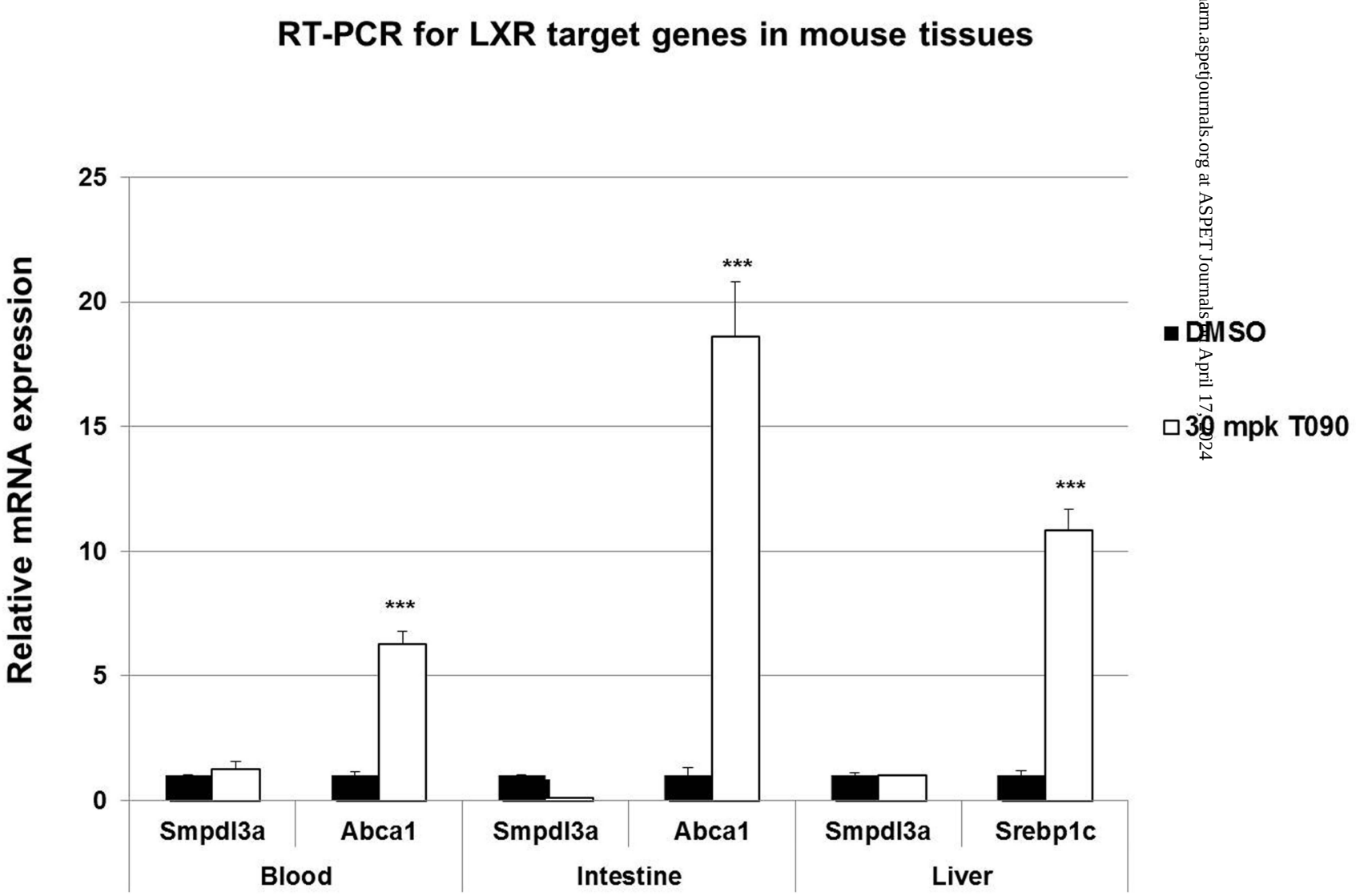

RT-PCR for Smpdl3a and Abca1 in RAW264 macrophages
Relative mRNA expression
80
70
60
50
40
30
20
10
0
***
***
***
Smpdl3a
Abca1
DMSO
0.01uM T090
0.1uM T090
1uM T090

Figure 6B



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