

Supplemental Methods

Animals

Targeted deletion of the GX sPLA₂ gene was performed by InGenious Targeting Laboratory, Inc. (Stony Brook, NY) using embryonic stem cells derived from C57BL/6 mice. The targeting vector contained a Neo cassette that was trapped inside exon 1 upstream of the translation start signal, replacing 564 bp of the GX sPLA₂ gene. Male and female GX sPLA₂^{+/-} mice were crossed to generate wild-type (WT) and GX sPLA₂-deficient (GX KO) mice for these studies. The Lexington Kentucky Veterans Administration Medical Center Institutional Animal Care and Use Committee approved all animal procedures.

Mouse peritoneal macrophages (MPMs). Resident MPMs were collected by peritoneal lavage with 5 ml ice-cold phosphate-buffered saline (PBS) and used immediately for preparation of total RNA. For measurements of [³H]-AA release, mice were injected intraperitoneally with a sterile solution (1 ml) of 1% Biogel 100 (Bio-Rad) in PBS to elicit MPMs. After 96 h MPMs were collected by peritoneal lavage and seeded in 24-well dishes at a density of $\sim 1.0 \times 10^6$ cells/well in complete medium (DMEM media containing 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine) supplemented with 25 ng/ml macrophage colony-stimulating factor (Calbiochem). Cells were allowed to attach overnight, and then washed three times with complete media. For indoxam treatments, biogel-elicited MPMs were isolated as described above and then incubated in complete medium containing 20 μ M indoxam or vehicle for 16 h prior to harvesting the cells for RNA preparation. For cellular cholesterol measurements, MPMs were allowed to attach overnight and extracted as previously described¹. Briefly, 1 ml hexane-isopropanol 3:2 (v/v) was added, and the cells were incubated at room temperature on a shaking platform for 1 h. Supernatants were dried in a Freezedry System (Freezone 4.5; Labconco, Kansas City, MO), and then 1 ml 1% Triton X-100 in chloroform was added. Samples were dried again and dissolved in H₂O. Aliquots were assayed for total and free cholesterol content using a colorimetric kit (Wako, Richmond, VA).

Stable overexpression of FLAG-tagged GX sPLA₂ and the catalytically inactive mutant H46Q.

Murine J774 macrophage-like cells were obtained from the American Type Culture Collection and maintained in complete medium. C-terminal Flag-tagged cDNA for mouse GX sPLA₂ (NCBI gene name : Pla2g10 and accession number NM_011987) and catalytically inactive GX sPLA₂ mutant H46Q was constructed as described earlier². J774 cells were transfected with pcDNA3 vector (“J774-C cells”) or vector encoding wild-type GX sPLA₂ (“J774-GX cells”) or H46Q (“J774-H46Q cells”) using Nucleofector Solution V (Amaxa, Gaithersburg, MD) and program X-001 of the nucleofector device (Amaxa) following the manufacturer’s protocol (1 µg DNA per 1x10⁶ cells). Stable transformants were selected and then maintained in complete media supplemented with 500 µg/ml G418 (Gibco). Single cell isolates were expanded and then analyzed by immunoblotting as described below to identify clones with comparable wild-type GX sPLA₂ and H46Q protein expression. Phospholipase activity secreted by cultured cells was quantified using mixed micelles containing 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) as described previously³. All experiments with stably transfected cells were performed with at least two independently derived clones.

RNA extraction and qPCR- Total RNA was isolated from J774 cells or MPMs using RNeasy Mini kit (Promega). 0.2-1 µg of RNA was reverse transcribed into cDNA using a reverse-transcription system (Promega). After 4-fold dilution, 5 µl was used as a template for real-time PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (Applied Biosystem) and IQ 5 real-time PCR machine (I Cycler, BioRad). Quantification was performed in duplicate using the standard curve method and normalizing to 18S. The primers used for various genes are provided on Supplemental table 1.

Immunoblotting- FLAG-tagged fusion proteins (GX sPLA₂ and H46Q mutant of GX sPLA₂) were detected by Western blot analysis using anti-FLAG M2 monoclonal antibody (Stratagene, La Jolla, CA). mABCA1 antibody was a gift from Dr. M.R. Hayden (University of British Columbia, Vancouver, Canada). LXR α was detected by Western blot using polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Beta-actin was detected by Western blot using monoclonal antibody from Sigma.

Cholesterol efflux assay

HDL₃ (d=1.13-1.18) was isolated from human plasma by density gradient ultracentrifugation according to established techniques⁴. J774-C, J774-GX, and J774-H46Q cells (~60% confluent) were incubated with complete DMEM containing 0.2 µCi/ml³ H-cholesterol (35–50 Ci/mmol, Amersham Biosciences) for 48 h. At the end of the incubation, cells were washed at least 3 times with PBS containing 0.1% BSA, and then incubated overnight with DMEM containing 0.2% BSA and 5 µM LXR agonist (T0901317 Cayman Chemical, Ann Arbor, Michigan). Cells were washed twice with PBS containing 0.1% BSA and then incubated in efflux media (DMEM with 0.2% BSA) with or without apoA1 (10µg/ml) or HDL₃ (50µg/ml) for 5 h. Lipid efflux was calculated as the percentage of ³H-cholesterol in the media divided by the total amount of ³Hcholesterol in the media and cells. For efflux assay in MPMs, mice were injected with 1% Biogel 100 in PBS to elicit MPMs. After 96 h MPMs were collected by peritoneal lavage and seeded in 24-well dishes at a density of ~1.0 x 10⁶ cells/well in complete medium supplemented with 25 ng/ml macrophage colony-stimulating factor. Cells were allowed to attach overnight, and then washed three times with complete media. Efflux assay in these cells were then carried out as described above for J774 cells.

Arachidonic acid release assay

J774 cells or MPMs were plated in 12-well plates (70-80% confluent) in 1 ml complete medium (DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin) containing 0.1 µCi [³H]arachidonate (200 Ci/mmol; American Radiochemicals Inc, Saint Louis, MO) and incubated for 20–24 h. The cells were then washed three times with complete medium followed by 6 h incubation in complete medium. Where indicated, 0.1 µg/ml mGX sPLA2 was added during the 6 h incubation period. The amount of tritium associated with cells and released into the medium was determined by scintillation counting.

Reporter assays

RAW 264.7 cells or Human embryonic kidney-293 (HEK293) cells (the American Type Culture Collection) were grown in complete medium (DMEM media containing 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine). LXRE luciferase reporter and mLXR α plasmids were both gifts from Dr. Peter Tontonoz, University of California, Los Angeles. Cells were plated in 12-well plates to 90% confluence and then transfected with pTK-3xLXRE-Luc reporter construct (0.5 μ g) along with plasmids encoding mLXR α (0.5 μ g) and renilla luciferase (pRL-Tk, 0.01 μ g; Promega) and either mGX sPLA₂ expression vector or the corresponding pcDNA3 empty control vector (0.5 μ g) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Fresh medium with or without additives (T0901317 or 22(R) HC) was added to cells for the time period as indicated in the figure legends. The volume of vehicle (DMSO) added to all wells was kept constant. To assess NF- κ B activation, RAW264.7 cells were transfected with mGX sPLA₂ expression vector or the corresponding pcDNA3 empty control vector along with pNF- κ B-luc (Clontech) and plasmids encoding mLXR α and renilla luciferase as described above. After 6 h, fresh medium was added to the cells and incubations were continued for an additional 18 h with 100ng/ml LPS, with or without 1 μ M T0901317. Transfection efficiency was normalized using renilla luciferase activity generated by cotransfection of pGL4.74[hRluc/TK] (pRL-Tk, Promega).

RNA silencing with Small Interfering RNA (siRNA)

A set of pre-designed, double-stranded synthetic oligonucleotides directed to mouse LXR- α with the sequences 5'-GGAGUGUCGACUUCGCAAATT (SENSE), 5'-UUUGCGAAGUCGACACUCCTG (Antisense) or LXR- β 5'-AGACAGAAUGCAUCACGUUTT(Sense), 5'-AACGUGAUGCAUUCUGUCUCG (Antisense) were purchased from Ambion (Austin, Texas). For control siRNA, validated non-targeting siRNA (Ambion) of equal concentration was used. The oligonucleotides (6 μ g per 1×10^6 cells) were transfected into J-774 cells using Nucleofector Solution V (Amaxa, Gaithersburg, MD) and program X-001 of the nucleofector device (Amaxa) following the

manufacturer's protocol. For silencing LXR- α and LXR- β 3.0 μg of each oligonucleotide per 1×10^6 cells was used. Total siRNA concentration in all the wells were maintained at 6 μg with control siRNA. Cells were treated with 1 μM T0901317, 24 h after transfection for another 20 h before RNA preparation.

Construction of chimeric expression plasmid containing LXR-ligand binding domain

An expression vector containing the Gal4 DNA binding domain (DBD) fused to the ligand-binding domain (LBD) of LXR α (amino acids 142-447) was used for LXR-LBD assay. The fragment containing LXR-ligand binding domain was obtained from mouse LXR α cDNA (Gift from Dr. Peter Tontonoz, University of California, Los Angeles) by PCR using forward and reverse primers ,5'-AATTAGCGATCGCCGACACCTACATGCGGCGG-3' (F) and 5'-TGACGATGCCGTTTAAACTCACTCGTGGACATCCCA-3' (R) containing the PME1 and SGF1 sites, respectively. LXR-LBD region was then cloned into the expression plasmid pFN26A(BIND) (Promega, Madison, WI) which carries an upstream Gal4 DBD. The correct sequence of the construct was confirmed by sequencing (Davissequencing, Davis, CA).

Gal4-Ligand Binding Domain assay

Human embryonic kidney-293 (HEK293) cells were transiently transfected using Lipofectamine as described under reporter assay. Each well received 0.35 μg of a vector pGL4.35 (luciferase reporter construct containing nine tandem copies of GAL4 response element (GAL4RE), Promega, Madison, WI), 0.35 μg of vector pFN26A(BIND)hRluc (Promega, Madison, WI) carrying LXR-LBD with either mGX sPLA₂ expression vector or the corresponding pcDNA3 empty control vector (0.05 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 20 h, fresh medium with or without additives (T0901317 or 22(R)HC) was added to the cells and then incubated for 24 h. The volume of vehicle (DMSO) added to all wells was kept constant. GRLBD assay was carried out as described above except that the vector pFN26A(BIND)hRluc was replaced by 0.35 μg of pBIND-GR vector (Promega, Madison, WI) which carries LBD of glucocorticoid receptor. Transfection efficiency was normalized using renilla luciferase activity generated by coexpression of renilla by pFN26A(BIND)hRluc vector.

Supplemental Figure legends

Supplemental Figure I. (A) MPMs from WT mice were treated with 20 μ M indoxam or vehicle for 16 h prior to RT-PCR. Data (means \pm SEM; n = 4) are expressed relative to WT MPM values treated with vehicle control. (B) Expression of ABCA1 in J774 cells treated exogenously with increasing doses of purified recombinant GX sPLA₂ for 20 h. Data that are not significantly different ($p \geq 0.05$) are indicated with the same letter.

Supplemental Figure II. (A) Relative mRNA expression of LXR target genes in J774-GX cells relative to J774-C cells. Data are means \pm SEM. *, $p < 0.05$ compared to corresponding control cells. (B) Relative mRNA expression of genes involved in cholesterol metabolism in J774-GX cells relative to J774-C cells. Data are means \pm SEM. (C) Western blot analysis of LXR α and β actin in J774-C and J774-GX cells. (D) Relative mRNA expression of genes involved in LXR pathway in J774-GX cells relative to J774-C cells. Data are means \pm SEM. ***, $p < 0.001$ compared to corresponding control cells.

Supplemental Figure III. Expression of LXR α (A) and LXR β (B) in J774-C and J774-GX cells transfected with either a control siRNA (scr), siRNA targeting LXR α , siRNA targeting LXR β or siRNAs targeting both LXR α and LXR β for 24 h, and then treated with 1 μ M T0901317 for an additional 20 h, prior to RT-PCR. Data are means \pm SEM (n = 4) and are representative of 2 experiments; Data that are not significantly different ($p \geq 0.05$) are indicated with the same letter.

Supplemental Figure IV. (A) Reporter activity in HEK293 cells transiently transfected with pTK-3xLXRE-Luc reporter construct along with plasmids encoding mLXR α , renilla luciferase, and either mGX sPLA₂ expression vector, H46Q or the corresponding pcDNA3.0 empty expression vector. Twenty h after transfection, cells were incubated for 24 h with fresh media containing the indicated concentrations of T0901317 dissolved in a constant volume of DMSO. Data are means \pm SEM (n= 3) and are

representative of at least 3 independent experiments. Group of data sharing the same alphabets are not statistically different, while different letters indicate significant differences. (B) The Gal4-driven luciferase reporter construct was cotransfected into HEK293 cells with an expression vector containing Gal4 DNA-binding domain fused to ligand-binding domain of glucocorticoid receptor and expression plasmid for GX sPLA₂ or vector control. After 24 h of transfection, the cells were treated with fresh media containing 10 μM of dexamethasone (DEX) for further 24 h before analyzing the cell lysates for luciferase activity. Data are means ± SEM (n= 3) and are representative of at least 2 independent experiments. (C) GX sPLA₂ does not alter the *trans*-repressive effect of LXR on NF-κB activation. Reporter activity in RAW 264.7 cells transiently transfected with NF-κB-luc reporter construct along with plasmids encoding mLXRα, renilla luciferase, and either mGX sPLA₂ expression vector (RAW-GX) or the corresponding pcDNA3.0 empty expression vector (RAW-C). After 6 h, fresh medium was added to the cells and incubations were continued for an additional 18 h with 100ng/ml LPS, with or without 1 μM LXR agonist (T0901317). Data are means ± SEM (n=4) and are representative of 2 independent experiments. Group of data sharing the same alphabets are not statistically different, while different letters indicate significant differences.

Supplemental Table 1

QPCR primer sequences

Gene	Accession Number	Primer
mAbca1	NM_013454	5'-AGCCAGAAGGGAGTGTCAGA-3' 5'-CATGCCATCTCGGTAAACCT-3'
mAbcg1	NM-009593	5'-TTCCCCTGGAGATGAGTGTC-3' 5'-CAGTAGGCCACAGGGAACAT-3'
mSREBP-1c	NM 011480	5'-GGA GCC ATG GAT TGC ACA TT-3' 5'-GGC CCG GGA AGT CAC TGT-3'
mLXR α	NM-013839	5'-AGGAGTGTCGACTTCGCAA-3' 5'-CTCTTCTTGCCGCTTCAGTTT-3'
mLXR β	NM-009473	5'-AAGCAGGTGCCAGGGTTCT-3' 5'-TGCATTCTGTCTCGTGGTTGT-3'
m18s	NR_003278	5'-CGGCTACCACATCCAAGGAA-3' 5'-GCTGGAATTACCGCGGCT-3'
SRA	NM_031195	5'-AAG AAG AAC AAG CGC ACG TGG AAC-3' 5'-AGG AGG CCC TTG AAT GAA GGT GAT-3'
LDLR	Z19521	5'-AGG CTG TGG GCT CCA TAG G-3' 5'-TGC GGT CCA GGG TCA TCT-3'
apoE	AK159517	5'-TCT GAC CAG GTC CAG GAA GAG-3' 5'-CAT CAG TGC CGT CAG TTC TTG-3'
ACAT	NM 144784	5'-CCA GAT GTG GTG GTG AAA GA-3' 5'-ATT CGT GCC AAT GGC TTA AC-3'
SR-B1	AK145588	5'-GGC TGC TGT TTG CTG CG-3' 5'-GCT GCT TGA TGA GGG AGG G-3'
CD36	NM 007643	5'-TGG AGC TGT TAT TGG TGC AG-3' 5'-GTT CTT TGC CAC GTC ATC TG-3'
HMGCoAR	NM_008255	5'-CTT GTG GAA TGC CTT GTG ATT G-3' 5'-AGC CGA AGC AGC ACA TGA T-3'
LPL	NM_008509	5'-ATC CAT GGA TGG ACG GTA ACG-3' 5'-CTGGATCCCAATACTTCGACC-3'
MMP9	NM 013599	5'-CAA TCC TTG CAA TGT GGA TG-3' 5'-AGT AAG GAA GGG GCC CTG TA-3'
PLPT	NM_011125	5'-CCT GAG GGT ATC AAC TTC GTG C-3' 5'-CGGTTCTTGTCAATCACTTCTCG-3'
OSC	NM_146006	5'-GCTGCATGTGGTGTATGGAC-3' 5'-GAGAAACGTGCTCCTGGAAG-3'
ASC2	NM_019825	5'-CCCACCTCACCAACAAGTCT-3' 5'-CCCTGGGAAAGTGGGTTATT-3'
SULT2B1	NM_017465	5'- TACACCCGACCAGTTCCTTC -3' 5'-AGCCCTTGATGTGGTCAAAC-3'

Supplemental references

1. Boyanovsky BB, Shridas P, Simons M, van der Westhuyzen DR, Webb NR. Syndecan-4 mediates macrophage uptake of group V secretory phospholipase A2-modified LDL. *J. Lipid Res.* 2009;50:641-650.
2. Shridas P, Bailey WM, Boyanovsky BB, Oslund RC, Gelb MH, Webb NR. Group X secretory phospholipase A2 regulates the expression of steroidogenic acute regulatory protein (StAR) in mouse adrenals. *J. Biol. Chem.* 2010; 285:20031-20039.
3. Wooton-Kee CR, Boyanovsky BB, Nasser MS, de Villiers WJS, Webb NR. Group V sPLA2 Hydrolysis of Low-Density Lipoprotein Results in Spontaneous Particle Aggregation and Promotes Macrophage Foam Cell Formation. *Arterioscler Thromb Vasc Biol.* 2004;24:762-767.
4. Strachan AF, de Beer FC, van der Westhuyzen DR, Coetzee GA. Identification of three isoform patterns of human serum amyloid A protein. *Biochem J.* 1988;250:203-207.

Figure 1

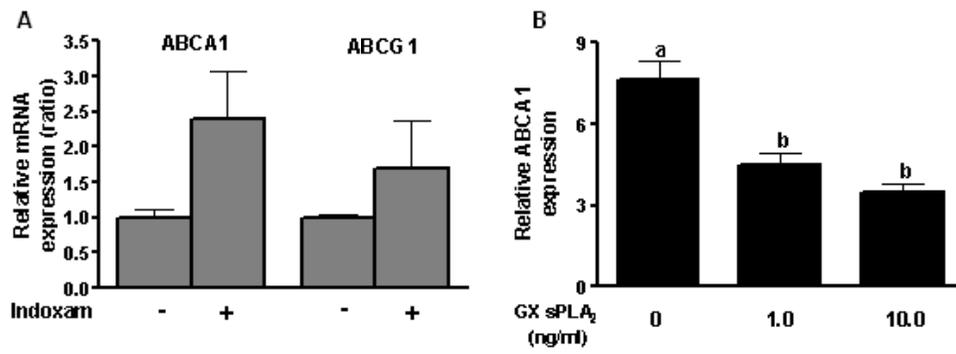


Figure II

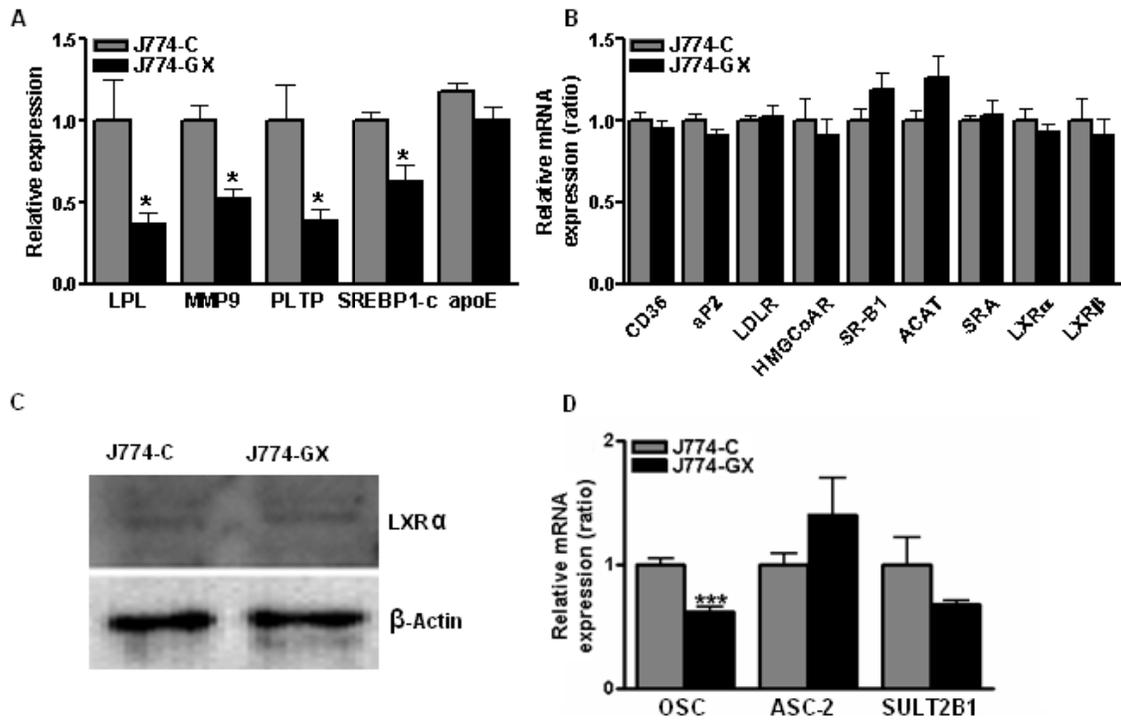


Figure III

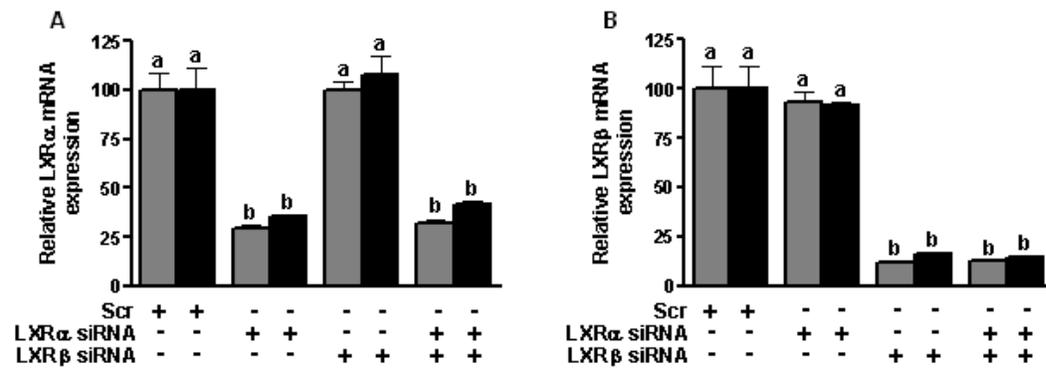


Figure IV

