Supplement Material

SI MATERIALS AND METHODS

Reagents

LPS (Escherichia coli O111:B4) was obtained from Sigma. The antibodies against RARα, GAPDH, SRC3, GSK3β, pGSK3β-Tyr216 and β-actin were purchased from Santa Cruz Biotechnology. ABCA1, ABCG1 and SR-B1 specific antibodies were obtained from Novus Biologicals and the pGSK3β-Ser9, LaminB antibodies were purchased from Cell Signaling. [^{3}H]cholesterol (40 Ci/mmol) was obtained from Perkin-Elmer.

Mice and cell culture

C57BL/6 Wild type (WT) mice were purchased from the Jackson laboratory. IRAK-1 deficient mice from C57BL/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. Tollip deficient mice from C57BL/6 background were provided by Dr. Jürg Tschopp from the University of Lausanne at Switzerland. IRAK-M deficient mice were provided by Dr. Richard Flavell from Yale University School of Medicine. The WT and GSK3β deficient murine embryonic fibroblast cells were obtained from Dr. James Woodgett at Ontario Cancer Institute. All mice were housed and bred at Derring Hall animal facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. BMDMs were isolated from the tibias and femurs of mice by flushing the bone marrow with Dulbecco's modified Eagle's medium (DMEM). The cells were cultured in untreated tissue culture dishes with 50 ml DMEM containing 30% L929 cell supernatant. On the third day of culture, the cells were fed with an additional 20 ml fresh medium and cultured for another additional 3 days. Cells were harvested

with phosphate-buffered saline (PBS), resuspended in DMEM supplemented with 1% fetal bovine serum, and allowed to rest overnight before further treatments.

Analysis of protein and mRNA

Isolation of whole-cell lysates, as well as cytoplasmic and nuclear extracts, was performed as described earlier [1]. Briefly, untreated or treated BMDMs (WT, IRAK-1 deficient and Tollip deficient) were rinsed in PBS and then lysed on ice in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) for 30 min followed by addition of 10% Triton X-100. The samples were centrifuged for 10 min at 5,000 rpm, and the supernatant fractions were discarded. Pellets containing the intact nuclei were lysed and solubilized with a high-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.4 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice followed by centrifugation at maximum speed for 20 min. The supernatant was saved as the nuclear extract. Western blotting analysis of the protein samples was performed as described previously. Immunoblots were developed by using the Amersham ECL Plus chemiluminescent detection system (GE Healthcare). The intensities of the bands were quantified using the Fujifilm Multi Gauge software and then normalized against β-ACTIN or GAPDH levels.

Total RNA was prepared from untreated or LPS-treated BMDM cells using TRIzol (Invitrogen) according to the manufacturer's protocol. Reverse transcription (RT) was carried out using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), and subsequent real-time RT-PCR analyses were performed using the SYBR green supermix on an IQ5 thermocycler (Bio-Rad). The relative levels of *Sr-b1, Abca1*, *Abcg1* and *ApoE* transcripts were calculated using the $\Delta\Delta C_t$ method after normalizing with *Gapdh* as the internal control.

Analysis of cholesterol export

Macrophages were radiolabeled with 2 μCi/ml of ^{3}H-cholesterol for 18 h in medium containing 1% fetal bovine serum. Subsequently, the cells were washed with PBS and equilibrated with 0.2% bovine serum albumin overnight before treatment with LPS (50pg/ml) for 18 h in the presence of apoA1 or HDL, isolated from human plasma. After incubation, the medium was removed, spun at 13,000g for 20 min to remove cell debris, and counted for ^{3}H radioactivity using a scintillation counter. The remaining cells were washed with PBS and lysed in 0.5% sodium dodecyl sulfate (SDS) and 0.1 N NaOH. Efflux was calculated using the following formula: percent efflux = 100 × cpm_{media}/(cpm $_{media}$ + cpm_{cell}), where cpm_{media} is the counts per minute calculated for all the conditioned media and cpm_{cell} is the counts per minute in the entire cell lysate.

Foam cell formation

Macrophages were plated in 24-well plates and incubated with 50 μg/mL OxLDL in the presence or absence of LPS 50 pg/mL, rinsed three times with DPBS, and then fixed at room temperature for 10 min with 10% phosphate buffered formalin. Macrophages were then rinsed once in 60% isopropanol and stained 3 min at 37^{o}C with 1 mL of filtered Oil Red O. Oil Red O solution was prepared by dissolving Oil Red O in isopropanol to obtain a 2% solution (w/v). Oil Red O was diluted by adding 2 parts water to 3 parts Oil Red O and then filtered (0.45 μm). Positively stained cells were observed through light microscope. The foam cells were counted under different viewing fields, and their percentages out of total cells were represented in the figure.

Chromatin-immunoprecipitation analysis

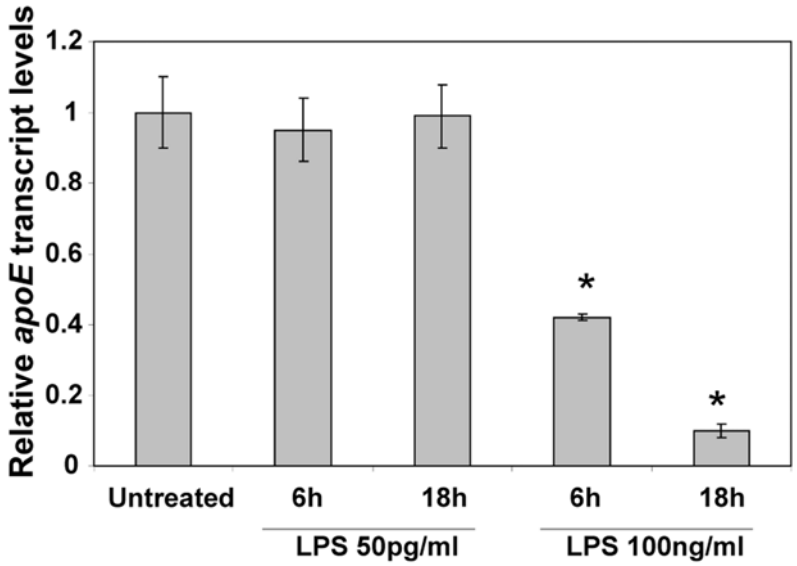
Murine BMDMs were either untreated or treated with 50 pg/ml LPS followed by cross-linking with 1% formaldehyde in complete medium for 15 min with gentle rocking at room temperature. Cells were then washed twice with ice-cold PBS and treated with glycine solution for 5 min to stop the cross-linking reaction. Cells were then lysed in buffer containing SDS and protease inhibitor cocktail. Samples were sonicated six times with 30-s pulses at 4°C followed by centrifugation to collect the sheared chromatin. The sheared chromatin was used to set up immunoprecipitation reactions with specified antibodies using the CHIP-IT Express kit (Active Motif) as per the manufacturer's recommendations. The immunoprecipitated DNA fragments were analyzed by PCR using the primers spanning the binding sites of the specified transcription factors on the promoter regions of *Sr-b1*, *Abca1* and *Abcg1*.

Statistical analysis

Statistical significance was determined using the unpaired two-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant. Data are expressed as mean +/- SD.

Reference:

1. Maitra U, Gan L, Chang S, Li L. Low-dose endotoxin induces inflammation by selectively removing nuclear receptors and activating ccaat/enhancer-binding protein δ. *J Immunol*. 2011;186:4467-4473



Supplementary Figure I. Low dose LPS fails to modulate the expression of ApoE.

WT BMDM cells were treated with either low dose LPS (50 pg/mL) or high dose (100 ng/mL) for the indicated time periods and the expression levels of *ApoE* transcript was measured by real-time RT-PCR assays and standardized against *Gapdh* levels. Each experiment was performed in triplicate. *, $P < 0.05$ compared with the untreated sample. Error bars represent SD.