EXTENDED METHODS SECTION

**Cell culture and materials.** Murine macrophage-like cell line J774A.1 (ATCC) was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Cellgro) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Omega Scientific) and 50 µg/ml gentamicin (Gybco). A murine fibroblast cell line stably overexpressing human 15-lipoxygenase 1 was cultured in 10% FBS/DMEM/gentamicin with 0.5 mg/ml G418 (Calbiochem) to maintain selection. CHO cell lines constitutively expressing an NF-κB reporter together with TLR4 or TLR4/MD-2 were produced as described 2 and maintained in DMEM/F12 with 10% FBS.

TLR4−/− and MyD88−/− mice were kindly provided by Dr. Shizuo Akira from Research Institute for Microbial Diseases, Osaka University 3,4. (We also thank Drs. Eyal Raz and Peter Tobias for providing knockout mice.) Resident peritoneal cells were harvested from 8-10 week old female mice of either wild type (C57BL/6), TLR4−/− or MyD88−/−. Macrophages were selected by attachment to culture plates for 3 hours and maintained in 10% heat-inactivated FBS/DMEM supplemented with 50 µg/ml gentamicin. Four hours before stimulation, macrophages were preincubated in serum-free media.

LPS from *Salmonella minnesota* Re 595 was prepared as described previously 5. The phosphoinositide 3-kinase (PI3K) inhibitors wortmannin and LY294002 were from Calbiochem and the MEK inhibitor U0126 was from Cell Signaling Technology. Antibodies and standards for mouse cytokine ELISA were from R&D Systems (JE/MCP-1, IL-6 and MIP-2) and from BD Pharmingen (TNFα). Antibodies used in Western Blot were purchased from Cell Signaling Technology.

**LDL isolation and modification.** LDL (density=1.019-1.063g/ml) was isolated from plasma of normolipidemic donors by sequential ultracentrifugation 6. Contamination of native and modified LDL preparations by bacterial LPS was assessed with a LAL kit (BioWhittaker). LDL preparations with LPS higher than 50 pg/mg protein were discarded. Because in most experiments LDL was used at a final concentration of 50 µg/ml, the LPS contamination in experimental samples was kept below 2.5 pg/ml, a concentration that has no reported biological activity when applied on murine cells.

For oxidation, the LDL was diluted to 1 mg protein/ml with EDTA-free PBS and incubated with 10 µM CuSO4 for 18 hours at 37°C. This procedure resulted in profound LDL oxidation and resulting preps are referred to in the text as OxLDL. The extent of LDL oxidation...
was assessed by measuring thiobarbituric acid reactive substances \(^7\) and LDL binding to monoclonal autoantibody EO6 (specific to oxidized PC-containing phospholipids or oxidized phospholipid-protein adducts) and EO14 (specific to MDA-lysine epitopes) \(^8\).

**Minimally modified (oxidized) LDL.** To produce mmLDL, we incubated 50 µg/ml LDL in serum-free DMEM for 18 hours with a murine fibroblast cell line overexpressing 15-lipoxygenase \(^1\). We have previously documented that this procedure generates mmLDL, i.e. it binds to native LDL receptors but not to scavenger receptors \(^1,9-12\). MmLDL contains early lipid peroxidation products \(^9,11\) but it does not contain any measurable thiobarbituric acid reactive substances or EO6-reactive phospholipid oxidation products above that of “native LDL” \(^12\). The mmLDL modification appeared to be very reproducible and a successful modification was documented in a biological assay in which mmLDL induced spreading of J774 macrophages in cell culture \(^11,12\).

**NF-κB activation assays.** Four different assays were used to assess NF-κB translocation and activation.

**A. Immunocytochemistry and imaging.** Cells were treated with 50 µM modified LDL or 10 ng/ml LPS for 15 minutes and fixed with 3.7% paraformaldehyde. Then cells were permeabilized with 0.4% Triton X-100, blocked in 5% dry milk containing 0.8 µg/ml Fc block (Pharmingen) and stained with a rabbit anti-p65 antibody (Santa Cruz Biotechnology), followed by an FITC-conjugated F(ab′)\(_2\) fragment donkey anti-rabbit Ig G (H+L) antibody (Jackson ImmunoResearch). F-actin was stained by TRITC-conjugated phalloidin (Sigma) and cell nuclei were stained blue with Hoechst 33342 (Sigma). Images were captured by deconvolution microscopy \(^13\) using a DeltaVision deconvolution microscopic system operated by SoftWorx software (Applied Precision) as described \(^11,14\).

**B. Chemiluminescent assay for p65-DNA binding.** J774 nuclear extracts were isolated using a Nuclear Extraction kit (Active Motif). NF-κB activation was measured using a sensitive Active Motif’s TransAM NF-κB p65 assay in an ELISA format \(^15,16\) where specific DNA oligos representing NF-κB response elements were immobilized on a microtiter plate. Binding of p65 to immobilized DNA was then measured by the binding of a specific p65 antibody using a Dynex chemiluminescent plate reader. This antibody only recognizes p65 bound to DNA, but not free p65.
**C. NF-κB reporter gene assay.** NF-κB activity was also assessed using CHO cells bearing inducible membrane CD25 under the transcriptional control of a human E-selectin promoter containing NF-κB binding sites, and stably transfected with either TLR4 or TLR4/MD-2, as described². Following 24 hour incubation with 50 µg/ml of modified LDL or 100 ng/ml LPS, the cells were stained with a phycoerythrin-conjugated anti-CD25 antibody (BD Pharmingen) and analyzed by FACS.

**D. p65 phosphorylation** in J774 lysates was assessed by western blotting as described below.

**Phosphorylation of signaling proteins (Western Blot).** Cells were lysed on ice with a lysis buffer containing protease and phosphatase inhibitors. Protein content was determined with a BCA kit (Pierce) and equal protein amounts of the cell lysates were run on a 4-12% Bis-Tris SDS-PAGE with MOPS buffer (Invitrogen) and then transferred to a PVDF membrane (Invitrogen). The blots were stained with appropriate antibodies against specific phosphorylated proteins, secondary antibodies conjugated with alkaline phosphatase and a WesternBlue stabilized alkaline phosphatase substrate (Promega). Since most treatments were for only 15 minutes, we assumed no changes occurred in total protein expression and gels were loaded with equal amounts of cell lysate proteins.

**PI3K activity assay.** Macrophage cell lysates (prepared as above) were immunoprecipitated with PY20 anti-phosphotyrosine antibody (Transduction Labs) and protein G agarose (Upstate Biotech) overnight at 4°C. PI3K activity was measured as described¹⁷ in a reaction with phosphatidylinositol (Sigma) in the presence of [γ-32P]ATP. After TLC separation, phosphatidylinositol-3-phosphate was visualized by autoradiography.

**Real-time PCR.** Total RNA was isolated from cells lysates using QIAshredder and RNase kits (Qiagen) and reverse transcribed with Superscript II RT (Invitrogen). Resulting cDNA was treated with RNase H (Invitrogen) and subjected to real-time kinetic PCR using an Applied Biosystems ABI Prism 7700 Sequence Detection System with software version 1.6.3, as performed in Genomic Core, Center for AIDS Research, UCSD. Primers and probes were as published¹⁸ and as follows: **MIP-2** (CXCL2): 5’ GGC TGT TGT GGC CAG TGA A 3’ (forward), 5’ AAG CTC TGG ATG TTC TTG AAG TCA 3’ (reverse), 5’ CAA TGC CTG AAG ACC CTG CCA AGG 3’ (probe); **IFNβ**: 5’ CTG GAG CAG CTG AAT GGA AAG 3’
(forward), 5’ TCC GTC ATC TCC ATA GGG ATC T 3’ (reverse), 5’ TCA ACC TCA CCT ACA GGG CGG ACT TC 3’ (probe).

**Cytokine ELISA assays.** Cytokines in culture media were measured according to standard antibody manufacturers’ protocols, except that chemiluminescent detection was employed, as previously described from our lab.\(^1\)

References


