SUPPLEMENTAL METHODS

Cell culture

Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation [1]. Monocyte differentiation to resting macrophages (RM) was induced by 6 days of culture in RPMI 1640 medium (Invitrogen, France) supplemented with gentamicin (40 µg/mL), L-glutamine (2 mmol/L) (Sigma-Aldrich, France) and 10% pooled human serum (Abcys, France). Alternatively differentiated macrophages (M2) were obtained by stimulating monocytes with recombinant human IL-4 (15 ng/mL) (Promocell, Germany) for 6 days. Classical differentiated (M1) macrophages were obtained by differentiating monocytes in the presence of IL-1β (10 ng/ml) or TNFα (10 ng/ml) for 6 days.

Where indicated, the LXR agonists T0901317 (T09, 1 µmol/L) and 22(R)-hydroxycholesterol (22OH, 10 µmol/L), the PPARγ agonist GW1929 (600 nmol/L) or the 15-LOX inhibitors cinnamyl-3,4-dihydroxy-a-cyanocinnamate (CDC, 5 µg/ml) and R04508159 (10 µmol/L), were added for 24h in medium without serum.

RNA extraction and analysis

Total cellular RNA was extracted from macrophages using Trizol (Life Technologies, France). RNA extraction from LCM-isolated samples was performed using the Picopure RNA extraction kit (MDS Analytical Technologies). RNA quality was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies). Only samples displaying a RNA Integrity Number (RIN) $\geq$ 6, indicating RNA integrity in the frozen tissue, were used for RNA analysis after LCM. RNA was amplified in two rounds using the ExpressArt TRinucleotide mRNA amplification Nano kit (AmpTec GmbH).

For quantitative PCR (Q-PCR), RNA was reverse transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies, France) and cDNAs were quantified on a MX4000 apparatus (Stratagene) using specific primers (online table 1). mRNA levels were normalized to cyclophilin mRNA.

Immunohistochemical analysis and laser capture microdissection (LCM)

Immediately after endarterectomy, carotid atherosclerotic plaques were frozen in liquid nitrogen. Serial 8 µm-cryosections were stained with Oil Red O for detection of lipids. For immunohistochemical analysis, endogenous peroxidase activity was quenched and sections stained with monoclonal anti-human CD68 (Dako Corporation), goat polyclonal anti-human MR, anti-human LXRα (SantaCruz Biotechnology) or rabbit polyclonal anti-human ABCA1 (Novus) or anti-human 15-LOX (abcam) antibodies followed by detection with appropriate biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Immunostains were visualized using the AEC substrate-chromogen system.

For double immunostaining, MR and 15-LOX were stained with N-Histofine Simple Stain MAX PO and N-Histofine Simple Stain AP, respectively (Nichirei Biosciences Inc.). MR was revealed by peroxydase and AEC which give a red/brown precipitate and 15-LOX by alkaline phosphatase and BCIP/NBT yielding a blue colour.

Macrophage populations were defined as expressing (CD68+MR+) or not (CD68+MR-) the mannose receptor (MR) protein. Adjacent cryosections of atherosclerotic plaques containing CD68+MR+ and CD68+MR- macrophage-rich areas were dehydrated in a series of graded alcohols, cleared in xylene and prepared for laser capture microdissection (LCM), performed on an ArcturusXT Microdissection Instrument with CapSure Macro LCM Caps (MDS Analytical Technologies). Macrophage-rich areas were captured from 3 adjacent 14 µm-sections and pooled for RNA extraction.

Cholesterol efflux

Macrophages were cholesterol-loaded by incubation with [^{3}H]cholesterol-AcLDL (50 µg/mL) for 48h. Cells were washed in PBS and cholesterol efflux measured by adding fresh medium containing or not 10 µg/ml of apoAI or HDL_3 for 24h. [^{3}H]-cholesterol radioactivity was measured by scintillation counting in centrifuged medium and in cells after extraction of lipids with hexane/isopropanol (3v:2v).

Cellular cholesterol measurement

Human macrophages were and cholesterol-loaded by incubation with [^{3}H]cholesterol-AcLDL (50 µg/ml) in RPMI 1640 medium for 48 hours. For cholesterol distribution analysis, intracellular lipids were separated by thin layer chromatography (TLC) in petroleum ether/ diethyl ether/ acetic acid (180:20:10, vol:vol:vol). Spots corresponding to CE and FC were scraped and radioactivity measured by scintillation counting and the ratio CE/total radioactivity was calculated.

Measurement of cholesteryl ester formation

Cholesteryl ester formation within the cells was assessed by measuring the incorporation of [^{14}C]-oleate into cholesteryl esters. Macrophages were cholesterol-loaded with AcLDL (50 µg/ml) in RPMI 1640 medium for 48 hours and subsequently incubated for 2h with 1 µCi [^{14}C]-oleic acid per ml of medium presented to the cells as BSA-sodium oleate complex [2]. At the end of the assay, cells were washed and lipids extracted with hexane/isopropanol. [^{14}C]-oleic acid incorporation into cholesteryl esters was measured on lipid extracts after separation by TLC in hexane/diethyl ether/acetic acid (90:10:1, vol:vol:vol). Spots corresponding to cholesteryl oleate and oleic acid were scraped and radioactivity measured by scintillation counting.

***In vitro* phagocytosis assay**

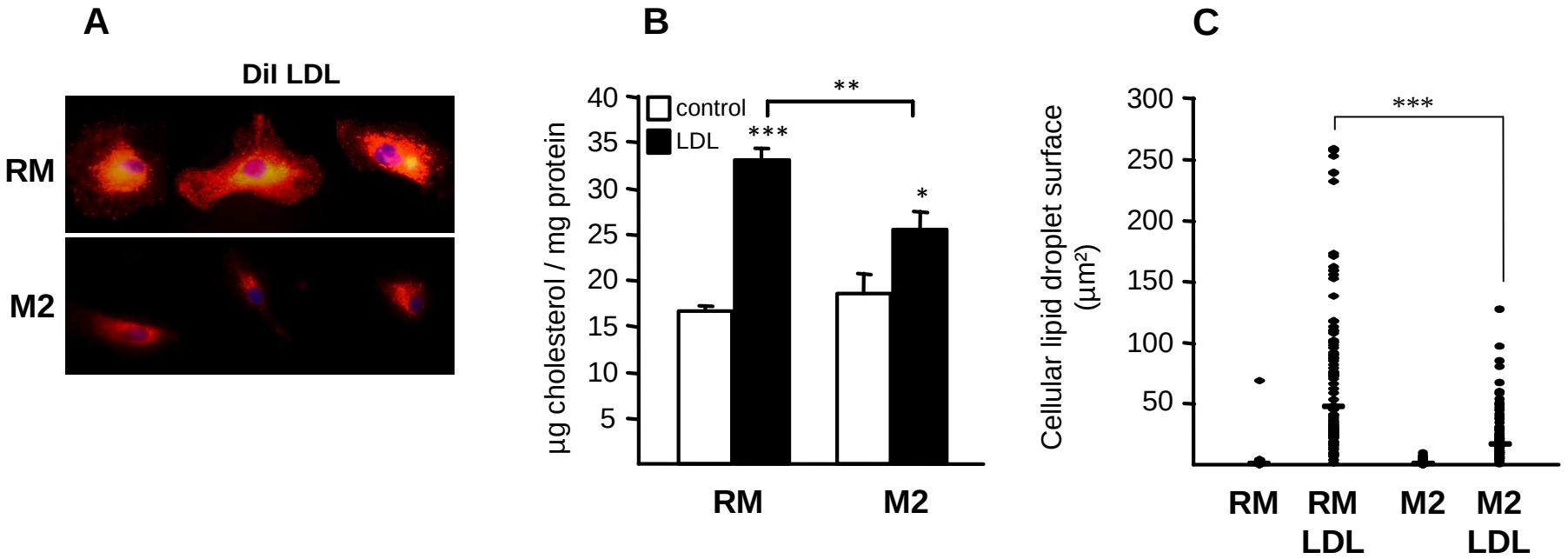
Phagocytosis tests were performed using apoptotic cells or latex beads. Jurkat cells were cultured in RPMI with 10% FCS, 1% glutamine and 1% gentamicin at a concentration of $2x10^6$ cells/mL, submitted to UV light (254 nm) for 15min and then cultured at 37°C in 5% CO_2 for 4h. Apoptosis was measured by flow cytometry using double staining Annexin V (AnnV) and propidium iodide (PI) to discriminate apoptotic from necrotic cells. Apoptotic cells, defined as AnnV+/PI-, were labeled with the red fluorescent dye PKH26 (Sigma). In other experiments, 2µm-fluorescent blue-green latex beads (Molecular Probes) were used. Apoptotic cells or latex beads were added to macrophages for 1h at 37°C. Where indicated, a thrombospondin 1 (TSP-1) blocking antibody (Lab Vision Corporation) was added 30min before phagocytosis assays. Macrophages (20000/condition) were then washed to remove non-phagocytosized material, scraped in PBS with 1% ethylene diamine tetraacetic acid (EDTA) and then analyzed on a Facscalibur flow cytometer (BD Biosciences) using Cell Quest software. Data analysis was performed with FlowJo software. Results are given as a percentage of fluorescent macrophages which have engulfed apoptotic cells or beads, reported to the total macrophage population. Each experiment was performed three times.

REFERENCES

1. Chinetti G, Griglio S, Antonucci M, Pineda Torra I, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B. Activation of peroxisome proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. *Journal of Biological Chemistry*. 1998;273:25573-25580.
2. Schmitz G, Niemann R, Brennhausen B, Krause R, Assmann G. Regulation of high density lipoprotein receptors in cultured macrophages: role of acyl-CoA: cholesterol acyltransferase. *EMBO Journal*. 1985;4:2773-2779.

Online Table I. Primer sequences

AMAC-1	forward	5’- AGC TCT GCT GCC TCG TCT AT-3’
	reverse	5’-CCC ACT TCT TAT TGG GGT CA-3’
MR	forward	5'-CGA GGA AGA GGT TCG GTT CAC C-3'
	reverse	5'-GCA ATC CCG GTT CTC ATG GC-3'
IL-1Ra	forward	5'-TTG AGC CTC ATG CTC TGT TC-3'
	reverse	5'-CAG TGA TGT TAA CTG CCT CCA G-3'
CPT-1	forward	5’-ACA GTC GGT GAG GCC TCT TAT GAA-
	reverse	5’-TCT TGC TGC CTG AAT GTG AGT TGG-3’
LXRα	forward	5'-AGG GCT GCA AGG GAT TCT TCC -3'
	reverse	5'-TCT GAC AGC ACA CAC TCC TCC C-3'
LXRβ	forward	5’-CTC AGT CCA GGA GAT CGT GG-3’
	reverse	5’-CAC TCT GTC TCG TGG TTG TAG-3’
LOX-1	forward	5’-GGT CCT TTG CCT GGG ATT AGT AG-3’
	reverse	5’-CTG GTG AGT TAG GTT TGC TTG C-3’
CD36	forward	5'-TCA GCA AAT GCA AAG AAG GGA GAC-3'
	reverse	5'-GGT TGA CCT GCA GCC GTT TTG-3'
ApoE	forward	5’-TCA GCT CCC AGG TCA CCC AG-3’
	reverse	5’-GCG CCG GCT GCA CCT TG-3’
SR-A	forward	5’-GAT TGG GAA CAT TCT CAG ACC TT-3’
	reverse	5’-CTT GTC CAA AGT GAG CTG CCT T-3’
ABCA1	forward	5’-AAG GTC TTG TTC ACC TCA GCC ATG AC-3’
	reverse	5’-GTG AAC AGC TCC AGC TCC TCC AC3-3’
ACAT-1	forward	5’-AGT TGA CAG CAG AGG CAG AG -3’
	reverse	5’-GGA TAA AGA GAA TGA GGA GGG-3
LAL	forward	5'-GCA ACA GCA GAG GAA ATA C-3'
	reverse	5'-GAG AAT GAC CCA CAT AAT ACA C-3'
MMP-9	forward	5'-GAC GAT GAC GAG TTG TGG TCC-3'
	reverse	5'-CCT CGA AGA TGA AGG GGA AG-3'
C1qa	forward	5'-GGA AGA ACC GTA CCA GAA CCA C-3'
	reverse	5'-AGA CGA TGG ACA GGC AGA TTT C-3'
C1qb	forward	5'-AGT TCG GAG AGA AGG GAG ACC-3'
	reverse	5'-GAT TTT CTG GGT GGC CTT GTA G-3'
C1qc	forward	5'-CAA GCC AAC ACA GGC TGC TAC-3'
	reverse	5'-CTT CTG CCC TTT GGG TCC TC-3'
GAS-6	forward	5'-GAA AGT GAA CAC GAG GAT GCA G-3'
	reverse	5'-AGC CAC GAC TTC TAC TTC CCA G-3'
MERTK	forward	5'-GCT TCC TTC AGC ATA ACC AGT G-3'
	reverse	5'-TTC ATG CTC TCA GGC TGC TTA G-3'
TSP-1	forward	5'-GCT GCA GAA TGT GAG GTT TGT C-3'
	reverse	5'-GCC AAT GTA GTT AGT GCG GAT G-3'
CD47	forward	5'-GAA GGT GAA ACG ATC ATC GAG C-3'
	reverse	5'-TGT CCC CAG AAC AGG AGT ATA GC-3'
cyclophilin	forward	5'-GCA TAC GGG TCC TGG CAT CTT GTC C-3'
	reverse	5'-ATG GTG ATC TTC TTG CTG GTC TTG C-3'



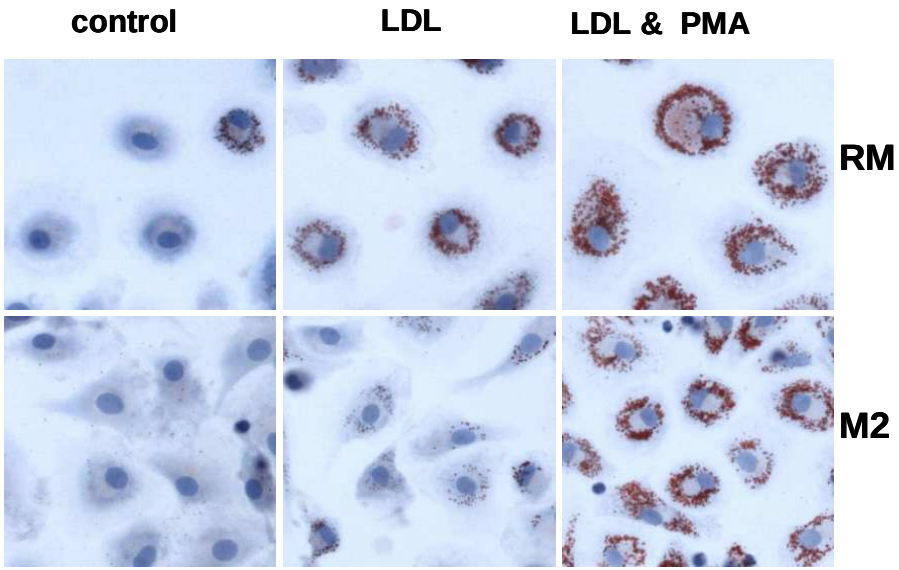
Online Figure I. Lower accumulation of native LDL in M2 macrophages

Panel A. RM or M2 macrophages were incubated with DiI-native LDL (0.2 mg/ml) for 24 hours and visualized using a fluorescent microscope.

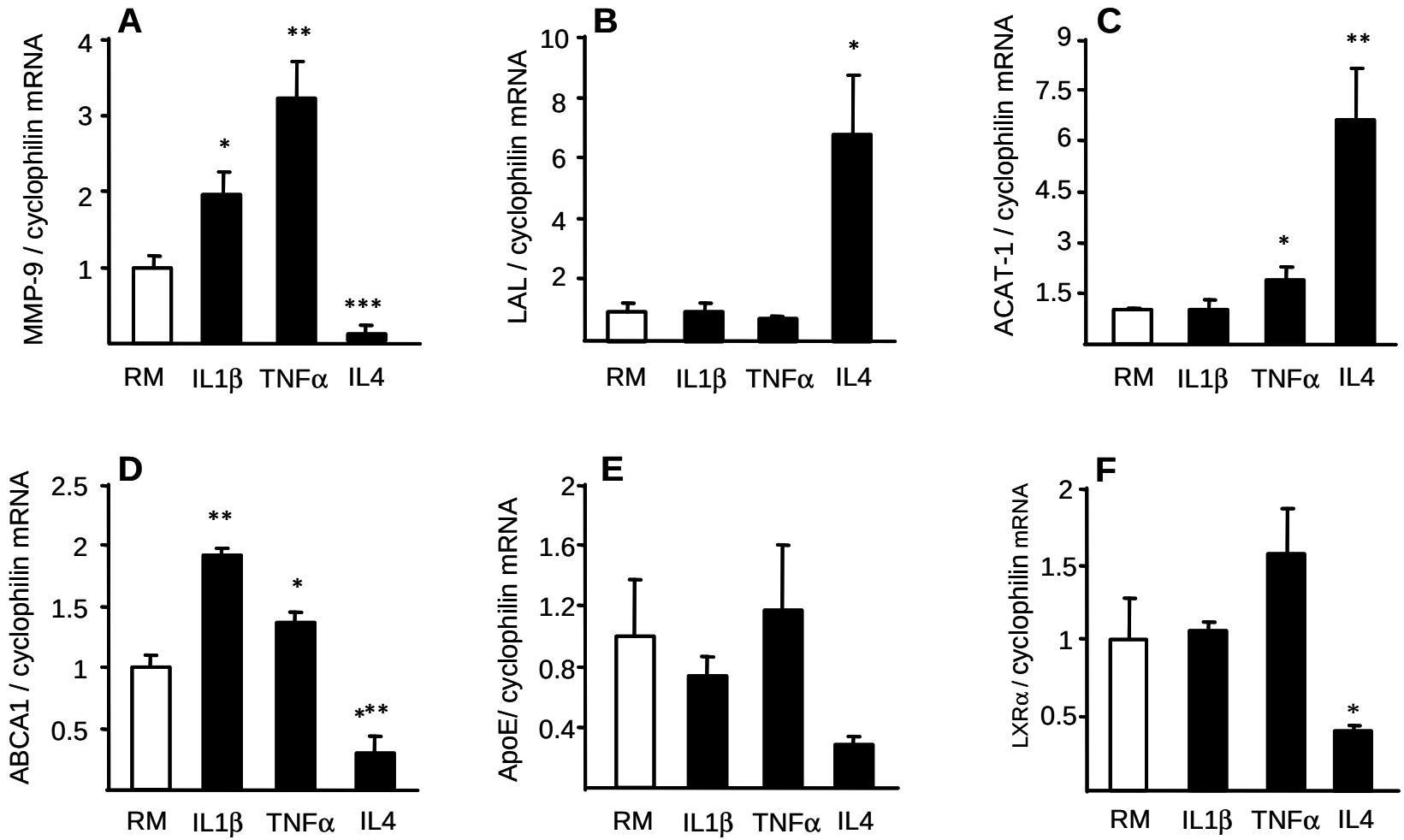
Panel B. RM or M2 macrophages were incubated with native LDL (1 mg/ml) for 24 hours; lipids were extracted and cellular cholesterol determined. Results are the mean ± SD of triplicate determinations, representative of 3 independent experiments. Statistically significant differences are indicated (t-test; *p< 0.05, **p< 0.01, ***p< 0.001).

Panel C. RM and M2 macrophages were loaded with native LDL (1 mg/ml) for 24 hours.Oil Red O staining was performed and lipid droplet surface measured using Quips software (Leica) . Median values are indicated. Statistically significant differences are indicated (t-test; ***p< 0.001).

Supplement Material

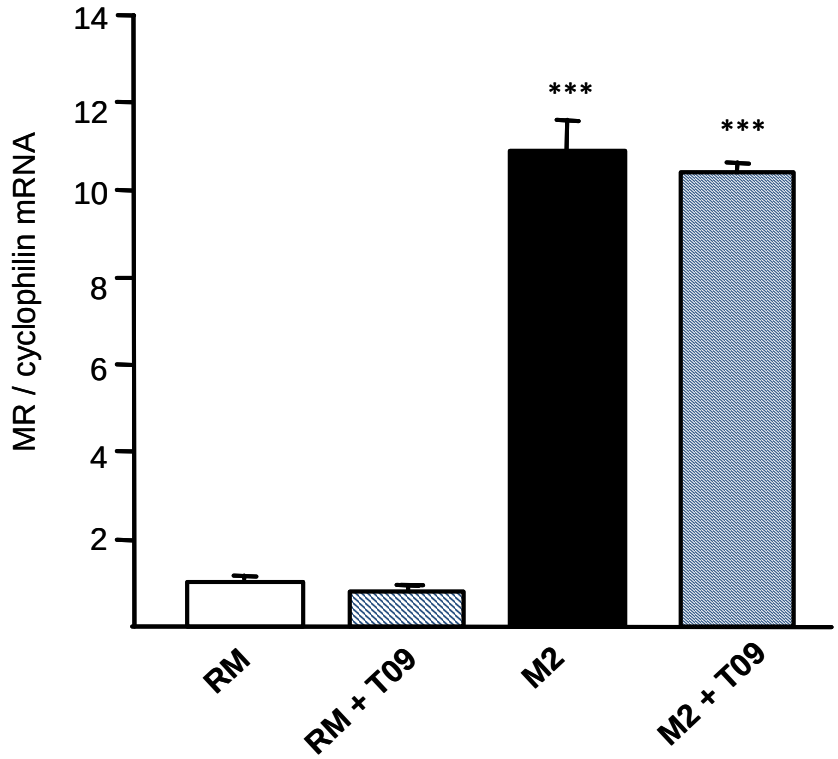


Online Figure II. Lower accumulation of native LDL in M2 macrophages is not due to a defective endocytosis
RM and M2 macrophages were loaded with native LDL (1 mg/ml) for 24 hours in the absence or in the presence of PMA (1 µg/ml). Oil Red O staining was performed.

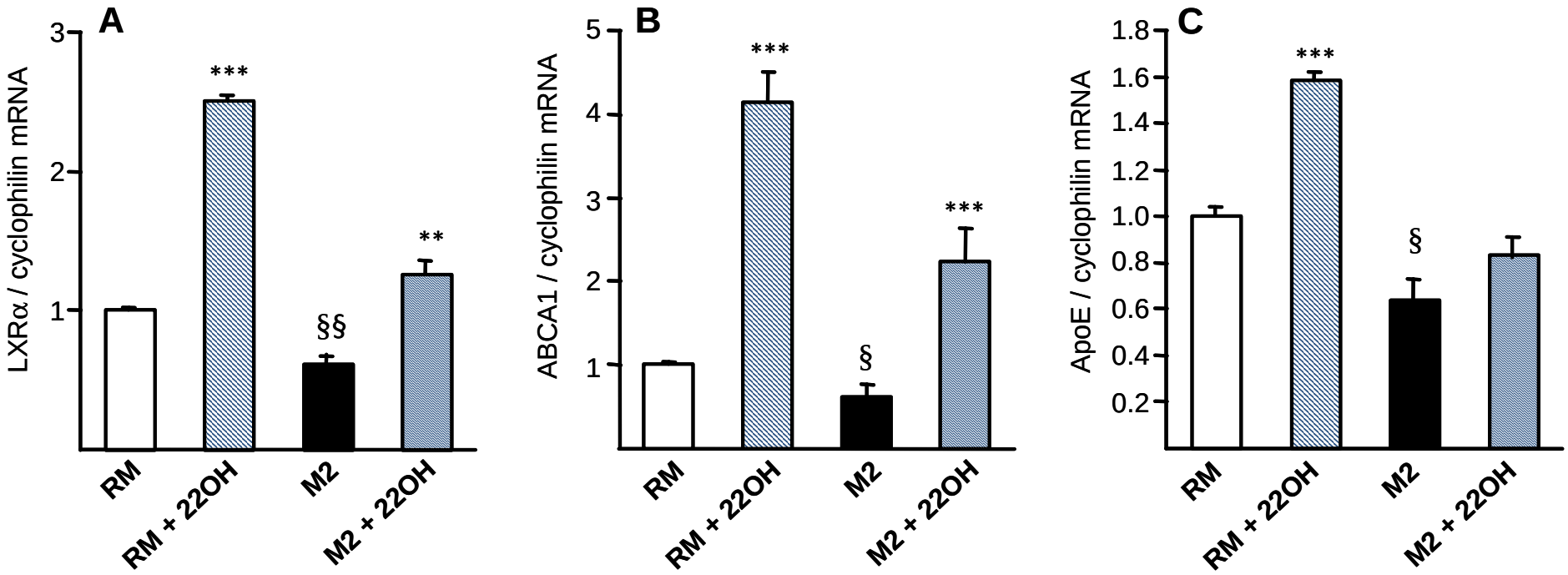


Online Figure III. Regulation of gene expression in M1 and M2 macrophages

Q-PCR analysis of MMP-9 **(A)**, LAL **(B)**, ACAT-1 **(C)**, ABCA1 **(D),** ApoE **(E)** and LXRα **(F)** was performed on RM, M2 and M1 macrophages obtained by differentiation in the presence of IL-1β and TNFα. mRNA levels was normalized to cyclophilin mRNA and expressed relative to RM set at 1.
Results are means ± SD of three independent experiments. Statistically significant differences are indicated (t-test; *p< 0.05, **p< 0.01, ***p< 0.001).

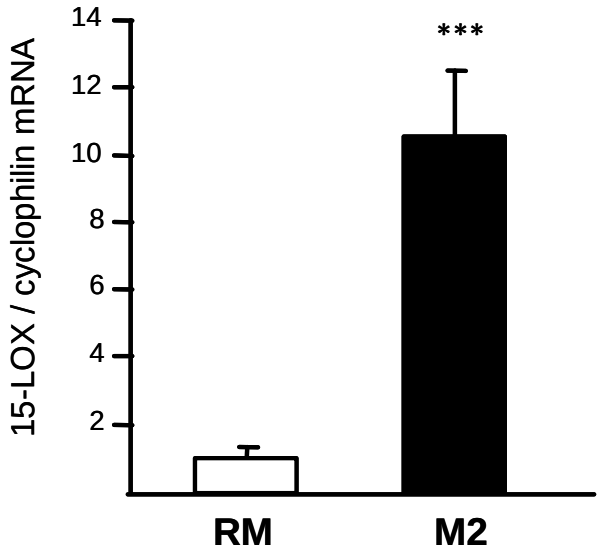


Online Figure IV. LXR activation does not affect the alternative macrophage differentiation marker MR
Q-PCR analysis of MR was performed in RM or M2 macrophages in the absence or in the presence of the T0901317 compound (T09, 1 µmol/L). mRNA levels was normalized to cyclophilin mRNA and expressed relative to RM set at 1. Results are means ± SD of three independent experiments. Statistically significant differences are indicated (t-test; ***p< 0.001).



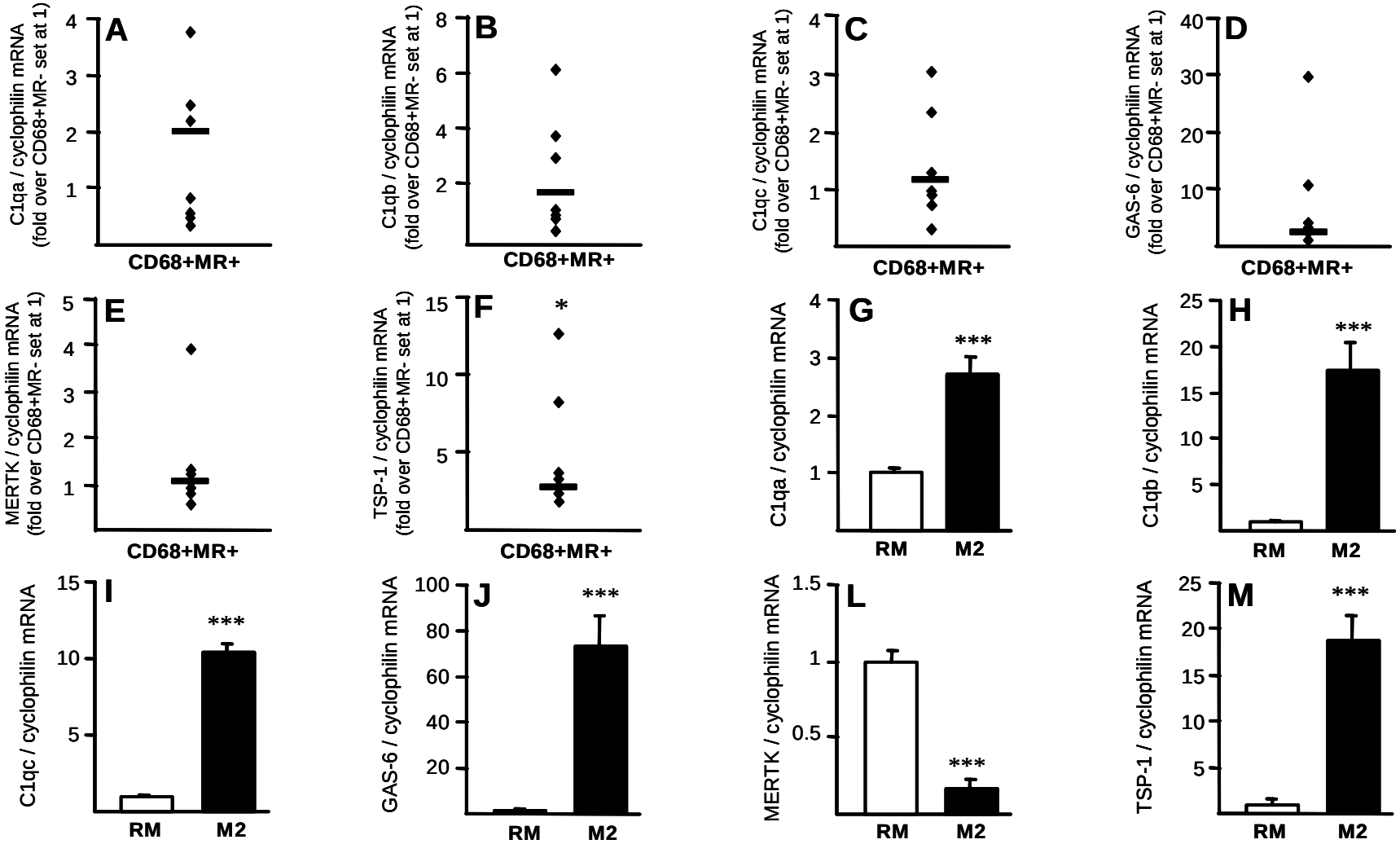
Online Figure V. M2 macrophage response to a natural LXR ligand
Q-PCR analysis of LXRa **(A)**, ABCA1 **(B)** and ApoE **(C)** was performed in RM or M2 macrophages treated or not with the 22-hydroxycholesterol (22OH, 10 µmol/L). mRNA levels was normalized to cyclophilin mRNA and expressed relative to RM set at 1. Results are means ± SD of three independent experiments. Statistically significant differences are indicated (t-test; RM vs M2 § $p< 0.05$, §§ $p< 0.01$;
and 22OH treated vs control **$p< 0.01$, ***$p< 0.001$).

Supplement Material



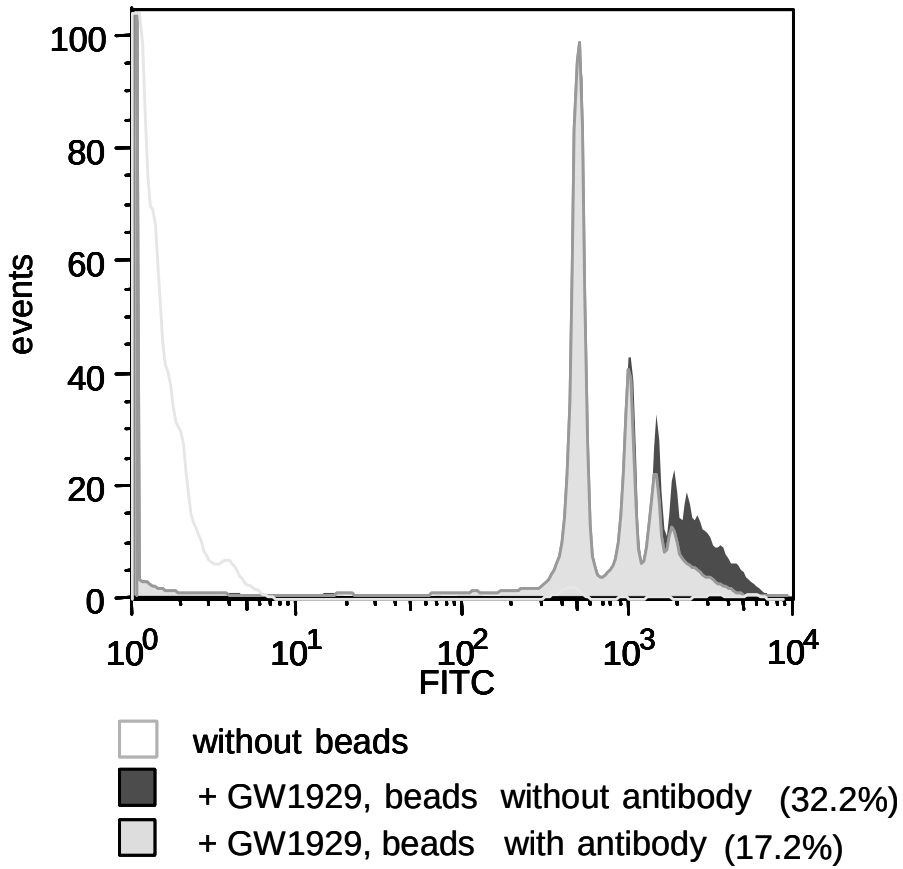
Online Figure VI. 15-LOX gene expression is higher in alternative M2 macrophages
Q-PCR analysis of 15-LOX was performed in RM and M2 macrophages. mRNA levels was normalized to cyclophilin mRNA and expressed relative to RM set at 1. Results are means ± SD of three independent experiments. Statistically significant differences are indicated (t-test; ***p< 0.001).

Supplement Material



Online Figure VII. Expression of genes involved in phagocytosis is altered in alternative macrophages *in vitro* and *in vivo*.

Q-PCR analysis of C1qa, C1qb, C1qc, GAS-6, MERTK and TSP-1 in LCM-isolated CD68+MR- and CD68+MR+ macrophage-rich areas from 7 samples **(A-F)** or in RM and M2 IL-4-differentiated macrophages **(G-M)**. For *in vitro* analysis, mRNA levels were normalized to cyclophilin mRNA and expressed as mean ± SD relative to RM set at 1, from three independent experiments. Statistically significant differences are indicated (t-test; ***p< 0.001).
For *in vivo* analysis, mRNA levels were normalized to cyclophilin mRNA and expressed relative to the levels in CD68+MR- area set at 1.
Each point corresponds to a single atherosclerotic plaque. The median value is shown. Statistically significant differences are indicated (t-test; *p< 0.05).



Online Figure VIII. PPARγ-enhanced phagocytic activity of alternative macrophages is mediated by TSP-1.
Phagocytosis of fluorescent beads was measured in M2 macrophages treated with GW1929 (600 nmol/L) for 24h in the absence or in the presence of a TSP-1 blocking antibody added 30 minutes before phagocytosis assay.