Materials and Methods

Macrophages and foam cells: Mixed peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque 1.077 (Sigma Diagnostics, St. Louis, MO) and washed twice with PBS containing 0.02% EDTA (Sigma Diagnostics, St. Louis, MO). Subsequently, monocytes were isolated using a negative selection monocyte isolation kit and LS columns (both Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of monocytes was >97% as determined by flow cytometry using anti-CD14 antibody (Figure S1A).

Monocytes were cultured in Macrophage Serum-Free Medium (Invitrogen, Carlsbad, CA) in the presence of 1% media supplement nutridoma-SP (Roche Molecular Biochemicals, Indianapolis, IN), penicillin (100 u/mL), streptomycin (1 mg/mL, both Sigma Diagnostics, St. Louis, MO) and macrophage colony-stimulating factor (M-CSF) or CXCL4 (100 ng/mL, both Peprotech, Rocky Hill, NJ) for 6 days. This standard macrophage cell culture medium has a final glucose concentration of 17.5 mM. Macrophages were then incubated with minimally modified LDL (mmLDL, a kind gift from Joseph Witztum, San Diego, CA) or oxidized LDL (oxLDL, Biomedical Technolgies, Stoughton, MA) at a concentration of 100 μg/mL for up to 48 hours to induce foam cell formation. Controls were incubated with medium only.

Foam cell formation was verified by oil red O staining. Therefore, cells were fixed in 1 % paraformaldehyde over night at 4°C. Cells were then washed twice with PBS and incubated with 70% isopropanol for 2 minutes. Oil red O staining was performed with 0.5%

oil red O (Sigma Diagnostics, St. Louis, MO) in 60% isopropanol for 10 minutes followed by counterstaining with Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO) for 5 minutes (Figure S1B).

For experiments under normo- and hyperglycemic conditions, cells were cultured in glucose-deficient medium (glucose-free RPMI 1640 (Invitrogen, Carlsbad, CA) +10% dialyzed FCS (HyClone, Logan, UT)) supplemented with either 30 mM D-glucose or 5 mM D-glucose plus 25 mM L-glucose as osmotic control (both Sigma, St.Louis, MO).

Aldose reductase activity assay: Aldose reductase enzyme activity was measured spectrophotometrically by determination of the decrease in absorbance at 340 nm in absence and presence of glyceraldehyde as substrate reflecting the consumption of NADPH [1-3] in either cell lysates or cell culture supernatants [2]. The total assay volume of 1000 µl contained 730 µl sodium phosphate buffer (100 mM, pH 7.0), 50 µl NADPH (1.6 mM, both Sigma Diagnostics, St. Louis, MO), 20 μl diphenylhydantion (10 mM), and 100 μl of enzyme solution. To start the reaction, 10 μl DL-glyceraldehyde (100 mM, Sigma Diagnostics, St. Louis, MO) were added and the absorption at 340 nm was measured at 25°C for 3 minutes. Glyceraldehyde was dedimerized for 10 mins at 85°C before assay. Diphenylhydantoin was added to inhibit unspecific metabolization of glyceraldehydes by aldehyde dehydrogenase. Absorbance values were corrected for blanks containing all components of the reaction except the substrate. AR activity was normalized to protein content as measured photometrically [4] and expressed in mU/µg (nmol NADPH oxidized per minute per µg protein).

Oxidative stress assay: Macrophages were exposed to 100 μg/mL oxLDL for 48 hours, with or without pre-treatment with 0.2 μM epalrestat for 24 hours. Cells were then washed with PBS and incubated with 2'7'-dichlorofluoresceine diacetate (H_2DCFDA, 10 μM, Invitrogen, Carlsbad, CA) at 37°C for 30 minutes. After an additional wash step with PBS, cells were harvested by scraping and fluorescence was assessed using a FACS Calibur instrument (BD Biosciences, Franklin Lakes, NJ). Results were expressed as fold change of geometric means over unstimulated cells

Immunohistochemistry: Five μm paraffin sections of coronary arteries were used. Following heat induced antigen retrieval using antigen unmasking solution (Vector Laboratories, Burlingame, CA) tissue sections were either incubated with antibodies against aldose reductase (a kind gift from Dr. Aruni Bhatnagar, University of Kentucky, Louisville, KY; for later experiments SC-17732, Santa Cruz Biotechnology, Santa Cruz, CA), CD 68 (clone KP-1, Santa Cruz Biotechnology, Santa Cruz, CA) or α-smooth muscle actin (clone alpha sm-1, Novocastra Laboratories, Norwell, MA). Antibodies were detected using the ABC method (Vector Laboratories). Antibodies were visualized using DAB (DAKO corporation, Carpinteria, CA). Sections were then counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI). Sections were analyzed for light microscopy using an Olympus BX51 microscope and Image Pro Plus 3.0 software (Media Cybernectics, Silver Spring, MD).

For fluorescence microscopy was performed after heat induced antigen retrieval using antigen unmasking solution. Sections were co-incubated with either antibodies against

aldose reductase (SC-17732) and CD68-Alex Fluor-488 (clone KP-1, both Santa Cruz Biotechnology, Santa Cruz, CA) or aldose reductase and smooth muscle α-actin-Cy3 conjugated (clone IA-4, Sigma Diagnostics, St. Louis, MO). For CD68 and aldose reductase, aldose reductase was detected using donkey anti-Goat Alexa Fluor 555 (Molecular Probes, Carlsbad, CA). For SMA and aldose reductase, AR was detected using Donkey anti-Goat Alexa Fluor 488 (Molecular Probes, Carlsbad, CA). All slides were coverslipped using Vector Shield Hardset with DAPI (Vector Laboratories, Burlingname, CA). Sections were analyzed by fluorescence microscopy (Olympus BX51 microscope) and Image Pro Plus 3.0 software (Media Cybernectics, Silver Spring, MD).

For quantification of AR positive macrophages in atherosclerotic plaques and adventitia AR positive and CD68 positive cells were counted in at least four fields of vision at a magnification of 400X and the proportion of AR positive cells out of CD68 positive cells was calculated.

Statistical analysis of gene arrays: The exact details of gene array data analysis have been described previously [5]. Briefly, after exclusion of internal controls, gene expression was normalized and log-transformed. The local pooled error (LPE) test for differential expression discovery under two conditions and the heterogeneous error model (HEM) for differential expression discovery under multiple conditions [6] were applied using the open-source statistical software R (www.r-project.com).

Supplementary tables

Table S1: Genes most strongly up- or downregulated by oxLDL in monocyte-derived macrophages. Given are Affymetrix H133A gene chip probe set ID, gene symbols, gene titles, false discovery rate (FDR) and fold change in oxLDL versus untreated cells in macrophages generated with either MCSF or CXCL4.

Probe set ID	Gene Symbol	Gene Title	FDR	Fold change MCSF	Fold change CXCL4
Cytokines, chemokines and their receptors					
207533_at	CCL1	chemokine (C-C motif) ligand 1	<0.001	0.34	0.04
204103_at	CCL4	chemokine (C-C motif) ligand 4	0.028	0.54	0.57
212659_s_at	IL1RN	interleukin 1 receptor antagonist	0.009	2.02	2.56
216243_s_at	IL1RN	interleukin 1 receptor antagonist	0.015	1.94	1.79
212657_s_at	IL1RN	interleukin 1 receptor antagonist	0.009	1.92	1.84
206975_at	LTA	lymphotoxin alpha (TNF superfamily, member 1)	0.015	0.17	0.04
207339_s_at	LTB	lymphotoxin beta (TNF superfamily, member 3)	<0.001	0.37	0.13
218856_at	TNFRSF21	tumor necrosis factor receptor superfamily, member 21	<0.001	4.22	3.22
214228_x_at	TNFRSF4	tumor necrosis factor receptor superfamily, member 4	0.009	0.43	0.31
207426_s_at	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	0.021	0.45	0.26
206508_at	TNFSF7	tumor necrosis factor (ligand) superfamily, member 7	0.009	0.41	0.45
206365_at	XCL1	chemokine (C motif) ligand 1	<0.001	0.41	0.36

206366_x_at	XCL1	chemokine (C motif) ligand 1	<0.001	0.37	0.17
Genes related to lipid metabolism					
203504_s_at	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	<0.001	2.37	1.71
203505_at	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	<0.001	2.21	2.62
204567_s_at	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	<0.001	2.79	2.73
211113_s_at	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	<0.001	2.78	2.66
209122_at	ADFP	adipose differentiation-related protein	<0.001	2.28	3.60
213553_x_at	APOC1	apolipoprotein C-I	<0.001	2.92	1.79
208964_s_at	FADS1	fatty acid desaturase 1	0.009	0.45	1.00
208962_s_at	FADS1	fatty acid desaturase 1	<0.001	0.30	1.18
208963_x_at	FADS1	fatty acid desaturase 1	<0.001	0.21	0.69
202218_s_at	FADS2	fatty acid desaturase 2	<0.001	0.21	0.44
202539_s_at	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	<0.001	0.23	0.31
202540_s_at	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	<0.001	0.17	0.29
221750_at	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	<0.001	0.27	0.31
202068_s_at	LDLR	low density lipoprotein receptor (familial hypercholesterolemia)	<0.001	0.15	0.46
202067_s_at	LDLR	low density lipoprotein receptor (familial hypercholesterolemia)	<0.001	0.05	0.18
Genes related to oxidative stress					
212859_x_at	MT1E	metallothionein 1E (functional)	<0.001	3.73	3.32
217165_x_at	MT1F	metallothionein 1F (functional)	<0.001	4.51	4.37
213629_x_at	MT1F	metallothionein 1F (functional)	<0.001	4.24	3.89
204745_x_at	MT1G	metallothionein 1G	<0.001	5.07	5.37
206461_x_at	MT1H	metallothionein 1H	<0.001	4.99	4.10

211456_x_at	MT1H	metallothionein 1H	<0.001	3.68	3.50
217546_at	MT1K	metallothionein 1K	0.009	21.34	11.19
204326_x_at	MT1L	metallothionein 1L	<0.001	5.58	6.16
208581_x_at	MT1X	metallothionein 1X	<0.001	3.22	3.15
212185_x_at	MT2A	metallothionein 2A	<0.001	2.75	2.68
216609_at	TXN	thioredoxin	<0.001	2.84	2.44
214205_x_at	TXNL2	thioredoxin-like 2	<0.001	2.49	2.53

Supplementary figures

Figure S1:

(A) Purity of monocytes obtained by negative isolation as determined by FACS staining for CD14. Forward side scatter (FSC – SSC) plot as well as histogram for FL1 are shown.
(B) Foam cell formation after exposure of monocyte-derived macrophages to oxLDL (100 µg/ml) for 48 hours as determined by oil-O-red staining. Controls were treated with medium for 48 hours.

Figure S2:

(A) AR gene expression in PBMC, monocytes, macrophages and foam cells. PBMC were isolated from healthy donors by gradient, monocytes by negative bead isolation. Macrophages were generated either by six days treatment with M-CSF or CXCL4, cells were then incubated with medium, mmLDL or oxLDL (100 µg/mL each) for an additional two days as indicated. Gene expression was determined by Affymetrix gene chip analysis. (means+SEM, * $P<0.05$, *** $P<0.0001$)
(B) Time course of AR gene expression during foam cell formation from macrophages generated with CXCL4 for 6 days. Cells were exposed to oxLDL (100 µg/mL) for 0, 2, 4, 6, 12, 24, and 48 hours (means±SEM, *** $P<0.001$ versus unstimulated)
(C) AR activity as determined by photometrically measuring NADPH consumption at 340 nm in monocyte-derived macrophages (6 days CXCL4) with and without exposure to oxLDL (100 µg/mL) for two days (means+SEM, ** $P<0.01$, * $P<0.05$)

Figure S3:

Gene expression of the pro-inflammatory mediators (A) TNFα (TNFA), (B) IL-6 (IL6), and (C) IL-1β (IL1B) during foam cell formation. Macrophages generated with M-CSF were exposed to vehicle (unst.) or 100 µg/ml oxLDL for 48 hours and gene expression was determined by Affymetrix gene array.

Figure S4: Proportion of AR positive macrophages in plaque and adventitia in seven different donors (n.d. = not determined).

References

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