SUPPLEMENT MATERIAL

Macrophage Adipose Triglyceride Lipase Deficiency Attenuates Atherosclerotic Lesion Development in LDL Receptor Knockout Mice

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Materials and Methods

Animals and Bone Marrow Transplantation

Female ATGL KO mice and wild-type (WT) littermates (on a C57Bl/6 background) were housed and bred at the Medical University of Graz, Austria. Bone marrow of these mice was transplanted into female homozygous LDL receptor knockout (LDLr KO) mice (The Jackson Laboratory, Bar Harbor, ME) as described previously.[1] Briefly, irradiated recipients received 5 x 10^6 bone marrow cells by intravenous injection into the tail vein. The recipient mice were housed in sterilized filter-top cages and given unlimited access to food and water. They were maintained for 8 weeks on a sterilized regular chow, containing 4.3% (w/w) fat with no added cholesterol (RM3; Special Diet Services, Witham, UK). After a recovery period of 8 weeks, the animals were challenged with a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter; Special Diet Services, Witham, Essex, UK) for 9 weeks to induce atherosclerotic lesion development. Seventeen weeks after transplantation, the hematologic chimerism of the transplanted LDLr KO mice was determined in genomic DNA from bone marrow by PCR analysis (ATGL-fwd: 5'-AGAGAGAGAAGCTGAAGCCTGG-3'; ATGL-rev: 5'-GCCAGCGAATGAGATGTTCC-3'; ATGL-neo: 5'-CTGCGTGCAATCCATCTTGT-3'). These experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments, Austria, and the Leiden University, the Netherlands.

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Peritoneal and Bone Marrow-Derived Macrophage Isolation and Culturing

Peritoneal macrophages were obtained 3 days after i.p. injection of 3 ml 3% thioglycollate. Macrophages were cultured for 2 h in DMEM/10%LPDS/1%penicillin/ 1% streptomycin and subsequently used for measuring lipid parameters. To achieve foam cell formation of WT cells, macrophages were incubated with acetylated (ac)LDL for 24 h. LDL was isolated from human plasma by density gradient ultracentrifugation and LDL was acetylated as described.[2] For isolation of bone marrow-derived macrophages (BMDM), femurs and tibias were lavaged with phosphate-buffered saline (PBS). Bone marrow cells were plated in DMEM/20% FCS/1% penicillin/1% streptomycin and differentiated into macrophages by addition of 30% L929 cell-conditioned media (as a source of M-CSF) for 7 days. Macrophages were incubated for 24 h in the absence or presence of 50 μg/mL β-VLDL isolated according to Van Eck *et al*.[3] and subsequently lysed for mRNA extraction or fixed and stained with oil red O to visualize neutral lipid accumulation.

Electron Microscopy

Cells were plated on a Melinex Film (Groepl, Tulln, Austria). Cells were fixed in 0.06 M phosphate buffer (pH 7.2) containing 2.5 % glutardialdehyde for 90 min. Thereafter, cells were rinsed twice in 0.06 M phosphate buffer for 10 min and post-fixed in 1 % osmium tetroxide in the same buffer for 1 h. Then the cells were rinsed 4 times for 10 min in 0.06 M phosphate buffer and dehydrated in 50 %, 70 %, 90 %, and 100 % cold acetone for 20 min each. Thereafter, cells were infiltrated by 2:1, 1:1, 1:2 mixtures of 100 % aceton and agar 100 epoxy resin (Groepl) and pure agar 100 epoxy resin for 4 h. The cells were then

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placed in agar 100 epoxy resin at RT for 8 h, transferred into embedding molds and polymerized at 60°C for 48 h. Sections (75 nm) were cut with a Reichert Ultracut S Ultramicrotome and stained with lead citrate (5 min) and uranyl acetate (15 min). Images were taken on a Zeiss transmission electron microscope equipped with a plate camera system.

Lipid Analyses

Eight weeks after bone marrow transplantation, 100 μL of blood was drawn from each individual mouse by tail vein bleeding after an overnight fasting period. Upon sacrifice, 17 weeks after bone marrow transplantation, blood was collected by retro-orbital venous plexus puncture after an overnight fasting period. Lipid analyses were performed as described.[4] NEFA concentrations were assayed enzymatically (Diasys, Holzheim, Germany) according to the manufacturer's protocol. Lipoprotein profiles were determined by fractionation of 50 μL of pooled serum from two mice by fast protein liquid chromatography (FPLC) using a Superose 6 column (3.2 × 300 mm, Smart-System; Pharmacia, Uppsala, Sweden). Total cholesterol (TC) and TG content of the effluent was determined as described.[4]

Histological Analysis of the Aortic Root

To analyze the development of atherosclerosis at the aortic root, transplanted LDLr KO mice were euthanized after 9 weeks of WTD feeding. The arterial tree was perfused *in situ* with PBS (100 mm Hg) for 10 min via a cannula in the left ventricular apex. The heart plus aortic root and descending aorta were excised and stored in 3.7% neutral-

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buffered formalin (Formal-fixx; Shandon Scientific Ltd, Runcorn, UK). Serial sections (7 μm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion areas in oil red-O stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean lesion area (in μm^2) was calculated from 10 consecutive oil red-O stained sections, starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of macrophages using a rat MOMA-2 antibody, dilution 1:50 (Serotec Ltd, Oxford, UK). Goat anti-rat coupled to horse radish peroxidase (HRP) (1:100) (Dako, Glostrup, Denmark) was used as a secondary antibody and Nova red substrate (Vector Laboratories, Burlingame, CA) was used for HRP visualization. Collagen content of the lesions was determined after Masson's Trichrome staining (Sigma diagnostics, St Louis, MO). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of lesions was performed to determine the rate of apoptosis of macrophages in the atherosclerotic lesions using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). TUNEL-positive nuclei were visualized with Nova Red (Vector Laboratories), and sections were counterstained with 0.3% methylgreen. All quantifications were performed blinded by computer-aided morphometric analysis using the Leica image analysis system.

Western Blotting Analysis

Control and acLDL-loaded peritoneal macrophages were washed twice with Ripa buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 50% sodium deoxycholate) in

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the presence of 1 μg protease inhibitor cocktail (Sigma, Vienna, Austria). Forty μg of protein were separated on a 12.5% SDS polyacrylamide gel by electrophoresis and blotted onto nitrocellulose protran BA85 membranes (Whatman, Vienna, Austria). The blot was blocked with 5% BSA plus 0.1% Tween-20 and incubated with an anti-rabbit PARP antibody (1:800) (Cell Signaling, Vienna, Austria) and a monoclonal anti-mouse β-actin (1:1000) (SantaCruz, Heidelberg, Germany). The blot was incubated overnight at 4°C. The horseradish peroxidase-conjugated goat anti-rabbit (1:5000) (Santa Cruz) and rabbit anti-mouse antibodies (1:1000) (Dako, Glostrup, Denmark) were visualized by enhanced chemiluminescence detection (ECL Plus, Amersham Biosciences, Piscataway, NJ).

Cellular Cholesterol Efflux

BMDM were incubated with 0.5 μCi/mL ^{3}H-cholesterol in DMEM/0.2% fatty acid-free BSA and 30 μg/mL non-labeled cholesterol for 24 h at 37°C. Cholesterol efflux was determined after incubating the cells in DMEM/0.2% fatty acid-free BSA in the absence or presence of 10 μg/mL human apoAI (Calbiochem, La Jolla, CA, USA) or 50 μg/mL human HDL, isolated according to Redgrave *et al.*[5] Radioactivity in the medium and in the cells was determined by scintillation counting after 24 h of incubation. Cholesterol efflux is expressed as the percentage of total cell ^{3}H-cholesterol present in the medium after 24 h. Basal efflux to BSA (in the absence of acceptors) has been subtracted from the data shown.

mRNA Expression Analysis by Real Time PCR

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Total RNA from BMDM was isolated using the guanidinium thiocyanate (GTC) method[6] and reverse transcribed using a RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada). Relative mRNA expression was measured from the following genes: Scavenger receptor A (SR-A), scavenger receptor class B type 1 (SR-BI), CD36 receptor (CD36), LDLr, acyl-CoA:cholesterol acyltransferase 1 (ACAT-1), ATP-binding cassette sub-family A member 1 (ABCA1), ABCG1, apolipoprotein E (ApoE), hormone sensitive lipase (HSL), lipoprotein lipase (LPL), lysosomal acid lipase (LAL) and diacylglycerol acyltransferase 1 (DGAT1). The mRNA expression levels were assessed by real time PCR (ABI PRISM 7500; Applied Biosystems, Foster City, CA) using SYBR Green technology (Applied Biosystems). Housekeeping genes were used as a control. Primer sequences are available upon request.

Determination of Cytokine Concentrations

Murine monocyte chemoattractant protein 1 (MCP-1) serum levels were assayed using a MCP-1 instant ELISA kit (eBioscience, Hatfield, UK) according to the manufacturer's protocol. IL-6 concentrations (R&D Systems Europe Ltd, Abingdon, UK) were determined in supernatants of peritoneal macrophages cultured in 6-well plates in 300 µL DMEM/10% LPDS for 24h in the absence or presence of LPS (10 ng/mL).

White Blood Cell Content and Turnover Analyses

White blood cell (WBC) repopulation and content was analyzed using an automated Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex Corporation, Kobe, Japan). To determine the life span of WBC in the circulation, blood cells were

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biotinylated by tail vein injection of 3 mg EZ-Link Sulfo-NHS-biotin (Pierce, Rockford, IL) dissolved in 0.2 mL PBS. After 2.5 h circulation, 20 µL of blood was collected in ethylene diaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding for 100% biotinylated WBC determination. Twenty µL blood was drawn at set timepoints for turnover analysis. Blood samples were washed twice with erythrocyte lysis buffer to eliminate all red blood cells and WBC were subsequently labeled with phycoerythrin-conjugated streptavidin (1:500 in PBS) (Molecular Probes; Eugene, OR) and analyzed by flow cytometry (FACS Canto II; BD Biosciences, Mountain View, CA). WBC survival was determined by assessment of the number of biotinylated (streptavidin-labeled) WBC relative to the starting level, which was 100% biotinylated.

Flow Cytometry

Using sterile procedures, bone marrow cells were obtained by flushing femoral and tibial bones from donor mice with PBS. Subsequently, erythrocytes were lysed and the cells were analyzed on a FACS Canto II (BD Biosciences, Mountain View, CA). As a control, total bone marrow cells were gated for the life-gate, based on forward scatter/side scatter characteristics. The Lin^-, $Sca\text{-}1^+$, $cKit^+$ (LSK) population within the bone marrow was assessed by flow cytometry. Bone marrow cells were stained with FITC-labeled anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD19, anti-GR-1, anti-NK1.1, and anti-TER119 (all obtained from eBioscience, Vienna, Austria). These markers are further designated as Lin^-. The LSK population is assigned as the Lin^- population, double

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positive for PE-labeled anti-LY6A/E (Sca-1) and APC-labeled anti-CD117 (cKit) (both obtained from eBioscience).

Cell Migration

Cell migration assays were performed using 24-well transwell plates (5 µm pore size; Corning, Vienna, Austria). Two million peritoneal macrophages were added into the upper wells. The lower chambers were filled with DMEM/10% LPDS and 50 ng/mL MCP-1. After 4 h at 37°C, macrophages were analyzed by FACS (BD Biosciences, San Jose, CA) after staining with anti-F4/80 antibody (eBioscience, Vienna, Austria).

Statistical Analysis

Statistically significant differences among the means of different populations were tested using the unpaired Student's *t*-test (GraphPad InStat and GraphPad Prism software). The probability level for statistical significance was set at $p<0.05$.

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Legends

Figure I. Successful disruption of ATGL by bone marrow transplantation. RT-PCR on genomic DNA of bone marrow from ATGL KO → LDLr KO and WT → LDLr KO mice.

Figure II. Unchanged lipoprotein profiles in ATGL KO → LDLr KO mice. Fifty µl pools of 2 mice per genotype after 9 weeks of WTD feeding were subjected to FPLC. Distribution of serum TC and TG concentrations in 25 µl fractions was determined enzymatically. Values represent the means of $n \geq 5$ samples per group.

Figure III. Unchanged cholesterol efflux in ATGL KO → LDLr KO mice. (A) BMDM from both groups were incubated with 0.5 µCi/ml ^{3}H-cholesterol for 24 h. Cholesterol efflux to extracellular acceptors (10 µg/mL apoAI or 50 µg/mL HDL) was determined after 24 h. Data are expressed as the percentage of radioactivity released into the medium. Values represent the means of $n \geq 3$ samples ± SEM per group.

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Reference List

1. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT, Van Berkel TJ. Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol.* 2004;165:785-794.
2. Basu SK, Goldstein JL, Anderson GW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A.* 1976;73:3178-3182.
3. Van Eck M, Herijgers N, Yates J, Pearce NJ, Hoogerbrugge PM, Groot PH, Van Berkel TJ. Bone marrow transplantation in apolipoprotein E-deficient mice. Effect of ApoE gene dosage on serum lipid concentrations, (beta)VLDL catabolism, and atherosclerosis. *Arterioscler Thromb Vasc Biol.* 1997;17:3117-3126.
4. Out R, Hoekstra M, Hildebrand RB, Kruijt JK, Meurs I, Li Z, Kuipers F, Van Berkel TJ, Van Eck M. Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol.* 2006;26:2295-2300.
5. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem.* 1975;65:42-49.
6. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987;162:156-159.

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Supplemental Figures and Tables

Figure I

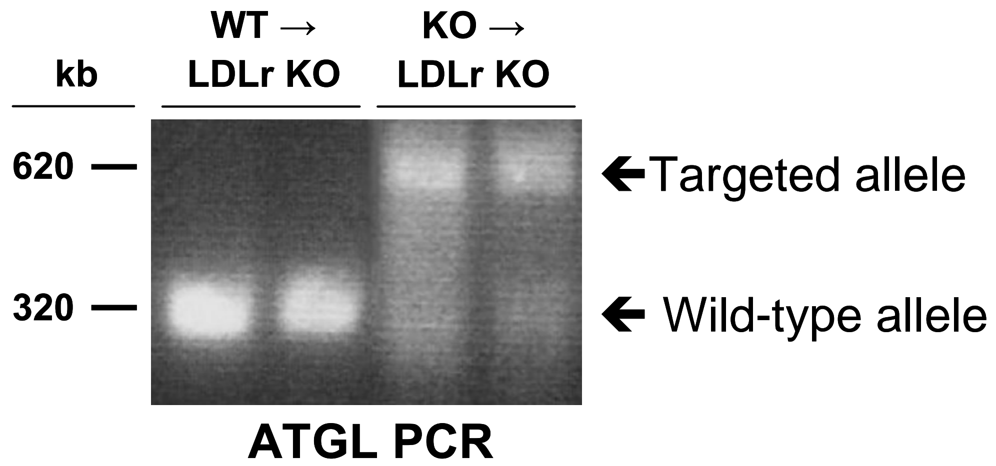

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Figure II

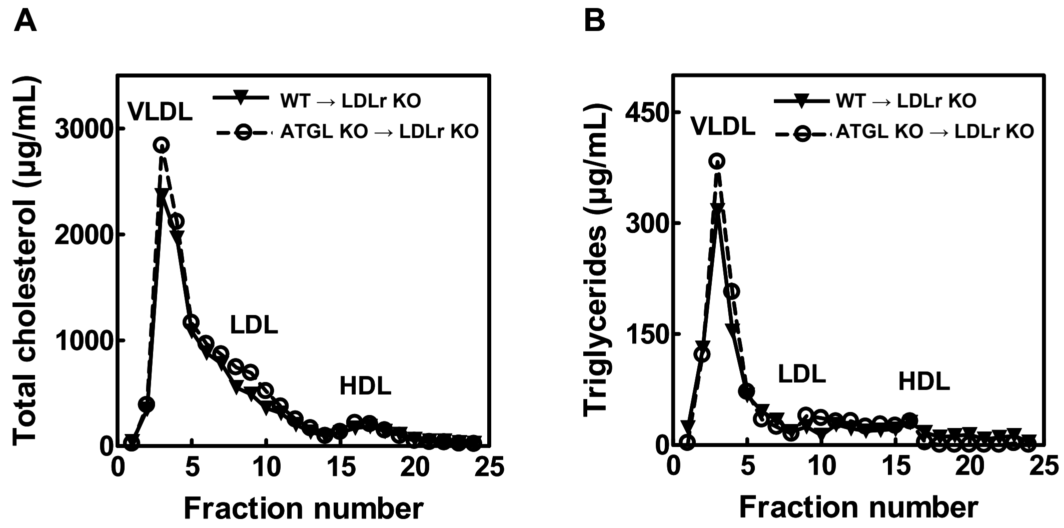

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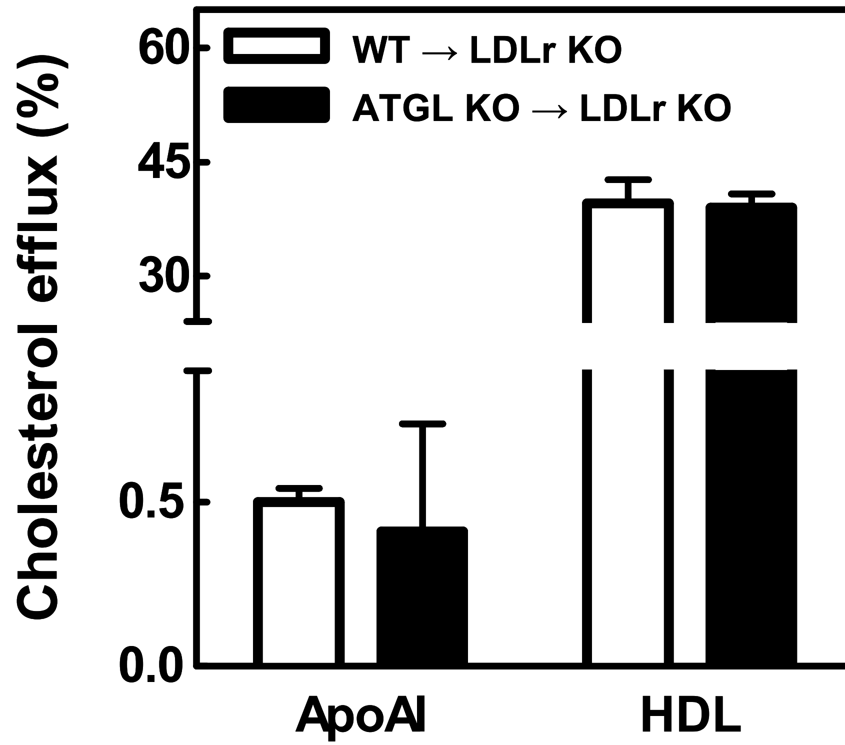
Figure III



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