Supplemental Material

Supplemental Methods

Mice

We developed a mouse model that specifically lacks macrophage LRP expression (MΦLRP$^{-/-}$) as previously described[1]. MΦLRP$^{-/-}$ mice were mated with apoE$^{-/-}$ mice to obtain apoE$^{-/-}$/ MΦLRP$^{-/-}$ mice. Prior to mating, both MΦLRP$^{-/-}$ and apoE$^{-/-}$ mice were backcrossed for at least 10 generations onto the C57BL/6 background. All offspring were genotyped by polymerase chain reaction (PCR) for apoE, *loxP* and *Cre*. Animal care and experimental procedures were performed in accordance with the regulation of the Institutional Animal Care and Usage Committee of Vanderbilt University.

Atherosclerosis Analyses

Recipient LDLR$^{-/-}$ mice (12 weeks old) were lethally irradiated (9.5 Gy) using a cesium gamma source and transplanted with 5×10^6 bone marrow cells from female apoE$^{-/-}$(n=10) or apoE$^{-/-}$MΦLRP$^{-/-}$ (DKO) mice (n=10) through injection into the retro-orbital venous plexus, as previously described[2, 3]. After four weeks on a normal chow diet, the mice were placed on a western-type diet for 8 weeks. Twelve weeks after bone marrow transplantation, the extent of atherosclerosis was examined. For experiments comparing atherosclerosis in apoE$^{-/-}$ (n=12) versus apoE$^{-/-}$MΦLRP$^{-/-}$ mice (n=12), eight to ten-week old female mice were fed a western-type diet for 8 weeks before the extent of atherosclerosis was measured. For analysis of atherosclerosis, frozen sections of 10 μm thickness were taken in the region of the proximal aorta starting from the end of the aortic sinus and for 300 μm distally as previously described [3, 4]. Sections were stained with oil red O and counterstained with hematoxylin. Quantitative analysis of lipid-stained lesions was performed on 15 alternate cryosections starting just beyond the end of the aortic sinus. The lipid-stained lesions were measured by digitizing morphometry, and reported in μm^2 per lesion. *En face* preparations of whole aortas were also stained with oil

red O. Both oil red O-stained cross-sections of the proximal aorta (10-μm cryosections) and *en face* preparations of whole aortas were analyzed using the KS300 imaging system (Kontron Elektronik GmbH) as described previously[3, 4].

Plasma Lipid Analyses

Cholesterol and triglyceride were determined in serum from mice fasted for 4h by enzymatic colorimetric assays using Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem, San Diego, Calif). Plasma lipoprotein cholesterol profiles were determined by separation on a Superose 6 column by fast-performance liquid chromatography as previously described[5].

In vitro Analyses of Macrophage Apoptosis

Peritoneal macrophages were collected in PBS three days after peritoneal injection of 3% thioglycollate in wildtype (WT), LRP1$^{-/-}$, apoE$^{-/-}$ or apoE$^{-/-}$LRP$^{-/-}$ mice. The macrophages were seeded in Laboratory-Tek chamber slides (Nalge Nunc International) at 0.5x10^6 cells in DMEM with 10% FBS. The cells were then incubated for 16h in serum-free DMEM alone or containing lipopolysaccharide (LPS, 50ng/ml). Cell death was then determined by TUNEL (Tdt-mediated dUTP nick end labeling) staining using the in situ cell death detection kit (Roche), according to the manufacturer's instructions. TUNEL-positive cells were quantitated in triplicate chamber slide wells, with cells being counted in 8 fields per well.

Analyses of Atherosclerotic Lesion Apoptosis, Efferocytosis, and Necrosis

For the lesions in recipient LDLR$^{-/-}$ mice, the TUNEL staining was performed on five-micron proximal aortic cryosections using the in situ cell death detection kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. The TUNEL positive cells were counted in four serial sections per mouse and normalized by the total oil red O stained lesion area. For analysis in lesions of apoE$^{-/-}$ and apoE$^{-/-}$MΦLRP$^{-/-}$ mice, apoptotic cells were detected in five-micron proximal aortic cryosections by TUNEL after Triton X-100 treatment using the in situ cell death detection kit, TMR red (Roche, Mannheim,Germany) following the manufacturer's

instructions. The nuclei were counterstained with Hoechst, and images of the sections were taken using fluorescence microscopy. Four serial sections from each mouse were stained, and the number of apoptotic cells per section were quantitated and normalized to the lesion area as determined by oil red-O staining. We analyzed the efferocytosis in lesions essentially as we recently reported[6], following previously published procedures[7, 8]. The same sections that were stained with TMR red TUNEL and Hoechst were also stained for macrophage cytoplasm using a rabbit antimacrophage antibody (AIA31240, Accurate Chemical and Scientific Corp.), goat anti-rabbit Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Inc.). The free versus macrophage associated apoptotic cells or bodies were counted in five sections per mouse. Apoptotic cells or bodies were counted as free when they were not associated with or in close proximity to viable macrophages that were detected as Alexa Fluor 488 stained macrophage cytoplasm surrounding a Hoeschst-stained nucleus. Apoptotic cells or bodies that were associated with macrophage cytoplasmic debris, but not in contact or close proximity with viable macrophages were counted as free. To determine lesion necrosis, five serial sections from each mouse were stained with Harris's hematoxylin and eosin (H&E). Necrosis was quantitated by measuring the H&E negative acellular area in the intima versus total intimal area.

Analyses of Lesion MOMA-2, Ly-6C, and CCR2.

To analyze lesion macrophage content, serial 5-μm cryosections of the proximal aortas were fixed in acetone and incubated with rabbit antibodies against the mouse macrophage marker MOMA-2 (1:300 dilution) overnight at 4°C (Accurate Chemical & Scientific Corp., Westbury, NY, USA). The sections were treated with secondary biotinylated goat antibodies to rabbit IgG (Pharmingen, San Diego, California, USA) and incubated with avidin-biotin complex labeled with alkaline phosphatase (Vector Laboratories, Burlingame, California, USA). The enzyme activity was visualized with Fast Red TR/Naphthol AS-NX substrate (Sigma Chemical Co.). To quantify lesion macrophage content, areas stained with MOMA-2, were measured using the Imaging System KS300 (Kontron Elektronik GmbH) on 5 sections per mouse.

To analyze the lesion content of Ly-6C^{high} pro-inflammatory monocyte markers Ly-6C and CCR2, serial 5-μm cryosections of the proximal aortas were fixed in acetone, washed with PBS, and then blocked with PBS/4%BSA for 30 minutes at room temperature. The sections were then incubated for 1 hour at 37º with rat anti-mouse Ly6-c biotin (BD Pharmingen #557359) or rabbit anti-mouse CCR2 monoclonal (Ab Cam #ab32144) primary antibodies diluted 1:50 in PBS/1%BSA. After washing with PBS, the sections were incubated in either Streptavadin-AlexaFluor 488 (Invitrogen #S11223) or goat anti-rabbit AlexaFluor 647 (Invitrogen #A21244) at a dilution of 1:150 in PBS/1%BSA for 30 minutes at 37º. Then the sections were washed in PBS and mounted with Vectashield containing DAPI (Vector Labs #H1200) to illuminate nuclei. CCR2 positive and negative cells in the lesion were then counted in 4 sections per mouse.

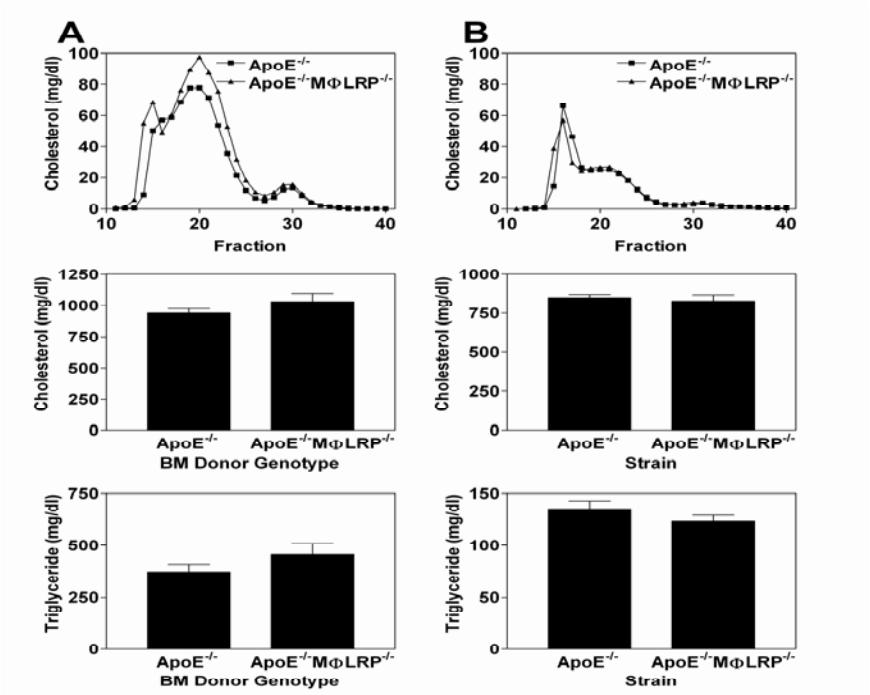
Flow Cytometry Analyses of Blood and Spleen Ly-6C^{high} Pro-inflammatory Monocytes.

WT (n=10), apoE$^{-/-}$ (n=10), MΦLRP$^{-/-}$ (n=8), or apoE$^{-/-}$MΦLRP$^{-/-}$ (n=10) mice were put on a Western Diet for 8 weeks. Mice were then sacrificed and whole blood was collected via cardiac puncture and preserved in a sodium citrate solution (10mM sodium citrate, 13mM glucose, pH 6.5) prior to antibody staining for flow cytometry. Spleens were homogenized by disruption in sterile PBS pH 7.4 through a 70μM mesh screen. Splenocytes were then centrifuged to pellet the cells and subsequently resuspended at a concentration of 10 million cells per 1ml in PBS/2%FBS pH 7.4. One hundred microliters of either splenocytes (one million cells) or of whole blood per test mouse were added to 5ml FACS tubes containing 2μl Fc Block (Pharmingen Catalog #553142) and mixed gently by pipetting. Then 1μl each of rat anti-mouse CD90.2, B220, GR1 (Pharmingen #553003, #553087 and 553126, respectively) and NK cells (Caltag RM7901), all with the FITC fluorochrome tag was added to each sample, as well as rat anti-mouse CD11b-PE (Pharmingen #557397) and rat anti-mouse Ly6-C conjugated with biotin (Pharmingen #557359). Cells were then incubated for 20 minutes at room temperature in the dark before being diluted to 4ml in PBS/2%FBA and pelleted by centrifugation. Cells were then resuspended in 100μl PBS/2%FBS before the addition of streptavadin-linked AlexaFluor 647

(Invitrogen #S32357) for another 20 minutes at room temperature in the dark. Cells were washed and the pellets were resuspended in 1x Permaflow (Invirion # 55001) and incubated for 1h at room temperature to fix the cells and lyse red blood cells. Cells were washed twice and resuspended in PBS for flow cytometric analysis. Cells were analyzed and gated for monocytes, as defined as CD90.2, B220, GR1-FITC-negative, CD11b-PE-positive cells. These cells were then gated for high levels of Ly6-C biotin-streptavadin-AlexaFluor 647.

Statistical Analysis

Differences between mean values were determined by one-way ANOVA (Bonferroni's post test) and Mann-Whitney test using GraphPad PRISM. Prior to using one-way ANOVA (Bonferroni's post test) to test for significance, the normality of the sample populations was tested by the Kolmogorov-Smirnov test. $p<0.05$ was considered to be significant.



Supplemental Figures 1A and 1B. Plasma lipid and lipoprotein profile analysis of mouse plasma. **A**. FPLC profiles and plasma cholesterol and triglyceride levels in LDLR$^{-/-}$ mice reconstituted with apoE$^{-/-}$ or apoE$^{-/-}$ MΦLRP$^{-/-}$ bone marrow (BM, n=10 per group). **B**. FPLC profiles and plasma cholesterol and triglyceride levels in apoE$^{-/-}$ and apoE$^{-/-}$MΦLRP$^{-/-}$ mice (n=12 per group).

Supplemental References

1. Overton CD, Yancey PG, Major AS, Linton MF, Fazio S. Deletion of Macrophage LDL Receptor-Related Protein Increases Atherogenesis in the Mouse. Circ Res. 2007;100:670-677.
2. Fazio S, Babaev VR, Murray AB, Hasty AH, Carter KJ, Gleaves LA, Atkinson JB, Linton MF. Increased atherosclerosis in C57BL/6 mice reconstituted with apolipoprotein E null macrophages. Proc. Natl. Acad. Sci. USA. 1997;94:4647-4652.
3. Linton MF, Atkinson JB, Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. Science. 1995;267:1034-1037.
4. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis. 1987;68:231-240.
5. Zhang W, Yancey PG, Su Y, Babaev V, Zhang Y, Fazio S, Linton M. Inactivation of macrophage scavenger receptor class B type I promotes atherosclerotic lesion development in apolipoprotein E-deficient mice. Circulation. 2003;108:2258-2263.
6. Yancey PG, Blakemore J, Ding L, Fan D, Overton CD, Zhang Y, Linton MF, Fazio S. Macrophage LRP-1 controls plaque cellularity by regulating efferocytosis and Akt activation. Arterioscler Thromb Vasc Biol. 2010;30:787-795.
7. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol. 2005;25:1256-1261.
8. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice. Arterioscler Thromb Vasc Biol. 2008;28:1421-1428.