Materials and Methods

(Materials and Methods are available in the online-only Data Supplement)

Study design:

WT, LDLR$^{-/-}$, and apoE$^{-/-}$ mice were purchased from the Jackson laboratories and housed at the Vanderbilt University Medical Center. MΦLRP1$^{-/-}$ mice were generated by *Cre/lox* recombination, as previously described[1]. MΦLRP1$^{-/-}$/apoE$^{-/-}$ (DKO) mice were generated by crossing MΦLRP1$^{-/-}$ and apoE$^{-/-}$ mice[2, 3].

12-week old female LDLR$^{-/-}$ mice received lethal irradiation (900 rad) followed by transplantation of bone marrow from WT, MΦLRP1$^{-/-}$, apoE$^{-/-}$, or DKO mice. After transplantation, mice were fed a chow diet for 4 weeks to allow full recovery and then switched to a western-type diet (42% kcal from fat, Harlan). Adalimumab (Humira®, AbbVie Inc., Chicago, IL) administration was started concomitantly with the western-type diet at a dose of 2.2mg/kg twice a week via intraperitoneal injection. Control groups were injected with an equal dose of human IgG. A first cohort of mice (n=5 in each group) was sacrificed 4 weeks after western-type diet and treatment with adalimumab for preliminary evaluation of drug effects. A second cohort of mice (n=5-7 in each group) was sacrificed 10 weeks after western-type diet and treatment with adalimumab. Blood, hearts and aortas were collected. Animal care and experimental procedures were performed according to the regulation of the Institutional Animal Care and Usage Committee of Vanderbilt University, which approved the study protocol.

Plasma TNFα levels were evaluated using an ELISA kit from R&D System Inc. (Minneapolis, MN). In preliminary experiments using plasma samples from cohort 1 mice, Adalimumab did not affect ELISA values when we compared the ELISA results before and after immunoprecipitation with protein A/G magnetic beads (New England Biolabs) for adalimumab (data not shown). Plasma TNFα levels at the end point of experiments were determined according to the manufacturer's protocol for the ELISA kit.

Atherosclerosis and plasma lipid analyses:

Hearts and aortas were embedded in OCT, and snap-frozen for further analysis. Frozen sections of 10 μm thickness were stained with oil red O, as previously described[3]. Images were captured using the KS300 imaging system (Kontron Elektronik GmbH).

Cholesterol and triglyceride levels were determined in pooled plasma from mice fasted for 5hr by enzymatic colorimetric assays using Cholesterol Reagent and Triglycerides GPO Reagent kits from Raichem (San Diego, CA).

Quantification of apoptosis, efferocytosis, and necrosis in atherosclerotic lesions:

Serial 5 μm cryo-sections of the aortic sinus area were used as described previously[1, 3]. Apoptotic cells in atherosclerotic lesions were detected using the *in situ* cell death detection kit (TMR red, Roche, Germany) following the manufacturer's instructions. The same sections were also stained for macrophage markers using a rabbit anti-CD68 biotin antibody (Calbiochem) and Streptavidin-AlexaFluor 488 (Invitrogen). Free apoptotic cells and bodies were defined as those not associated with CD68-positive macrophages with an intact nucleus. The extent of necrosis in

atherosclerotic lesions was quantitated using Image J software by measuring the cellular area negative for Harris's hematoxylin/eosin staining (H&E) in the intima. At least two sections from each mouse were analyzed and all the experimental mice (n=5-7) were used for analysis.

Sequential staining of CD68 expressing macrophages and arginase -1 and arginase-2 positive macrophages, ly6C positive monocytes, and MCP-1 and VCAM-1 expression in the lesion:

Rat anti-mouse ly6C antibody conjugated to biotin (Pharmingen) and Streptavidin-AlexaFluor 647 (Invitrogen) were used for analysis of lesion ly6C monocyte expression. Two-5μm sequential cryo-sections on the same glass slide were used for each staining to determine M1 (arginase-2 positive) and M2 (arginase-1 positive) macrophages in lesions. One section was stained for arginase-1(Gene Tex) and CD68 (Calbiochem), the other section was stained for arginase-2 (Proteintech Group) and CD68 (Calbiochem). Briefly, sections were fixed in cold acetone for 10min, then washed with PBS for 2 times, blocked in background buster (Innovex) at 37 ^{o}C for 1hr, then incubated with anti-arginase-1 or anti-arginase-2 antibody and anti-CD68 antibody conjugated to biotin at 4 ^{o}C for overnight. The next morning, sections were washed with PBS for 3 times, then incubated with Alexa Fluor 594 goat-anti rabbit IgG (Invitrogen) and streptavidin Alexa Fluor 488 (Life technologies) at 37 ^{o}C for 1 hr. For MCP-1 staining, a goat polyclonal IgG MCP-1 antibody was purchased from Santa Cruz (sc-1784), and Alexa Fluor488 donkey-anti-goat IgG (Invitrogen) was used as secondary antibody. For VCAM-1 staining, rat anti-mouse CD106 (VCAM-1) was purchased from Millipore (CBL-1300), and Alexa Fluor488 goat-anti-rat IgG (Invitrogen) was used as secondary antibody. Slides were washed and cell nuclei were counterstained with Hoechst (Vector Labs, Burlingame CA). Images were captured using Olympus IX81 microscope and analyzed using Adobe Photoshop CC (Mac) software. For quantification of M1 or M2 macrophages, arginase-2 (or -1, red) and CD68 (green) double positive cells were visualized with separated color channel filters using Adobe Photoshop CC (Mac) software. For quantification of MCP-1 expression in endothelial cells, multi-channel images were split first and the density (green component only) of MCP-1 expression in endothelial cells was measured using Image J software. Blind analysis was performed for all images. At least two sections from each mouse were analyzed and all the experimental mice (n=5~7) were used for analysis.

Flow-cytometry analysis of blood inflammatory ly6C^{hi} monocytes:

Similar to the procedure we used before [3, 4], blood was collected via retro-orbital vein plexus in sodium citrate (10mM Sodium citrate, 13mM glucose, pH 6.5). One-hundred μl aliquots of blood were blocked for 5 minutes at room temperature with 1μl Fc receptor block (BD Biosciences). FITC fluorochrome tagged rat anti-mouse CD90.2, B220, GR1 (Pharmingen) and NK cells (Caltag) were used to discriminate other cell populations from monocytes [3]. Monocytes were fluorescently labeled with rat anti-mouse CD11b-PE (Pharmingen) and rat anti-mouse ly6C conjugated to biotin (Pharmingen) for 20 min at RT in the dark. Cells were washed and then incubated with streptavidin-linked AlexaFluor 647 (Invitrogen). Red blood cells were lysed using BD Pharm Lys^{TM} buffer (BD Biosciences). Surface labeled cells were then washed and fixed with 4% PFA. Labeled cells were analyzed on a MACSquant seven-color flow

cytometer (Miltenyi Biotec) and data analyzed with FlowJo software (Tree Star). Blood ly6C^{hi} monocytes were determined according to Covarrubias et al [4], and detailed information is provided in Supplemental Figure *II*. All experimental mice (n=5~7) were included in this analysis.

Statistical Analysis

All measurements passed D'Agostino & Pearson omnibus normality test (alpha=0.05). For images, data are collected from at least two sections for each mouse, and all experimental mice were included (n=5~7 for each group). Data are expressed as mean ± SEM. Differences between mean values were determined by two-way ANOVA with Bonferroni's post-test as indicated.

Methods for Supplemental Figures

Binding of Adalimumab to human and mouse TNFα proteins:

There was no available data on the effectiveness of adalimumab, a human mAB, in the mouse when our study was started. Alignment of soluble murine and human TNFα amino acid sequences showed 78% (122 of 157 amino acids) identity. In addition, the amino acid sequence within the binding domain of TNFα was more than 90% identical between adalimumab and infliximab, a monoclonal antibody known to bind to mouse TNFα [5]. Moreover, we showed that adalimumab avidly binds to murine TNFα (Supplemental Figure *I*). TNFα functions as a homotrimer [6, 7]. Our Western blots (Supplemental Figure *I*) using adalimumab detected both monomer and dimer forms for TNFα, consistent with the report from Tang et al for the Western blots under the similar immunoblotting conditions [7]. The dosage used in our study was determined according to effects on blood TNFα as tested in cohort 1. The administration of adalimumab in our study was similar to a recent report of using adalimumab to suppress TNFα signaling in mice [8].

Immunoblotting of LRP1 for bone marrows and intraperitoneal macrophages:

Bone marrows were collected from WT, MΦLRP1$^{-/-}$, apoE$^{-/-}$ and DKO mice (n=5 for each genotype). Intraperitoneal macrophages were collected from WT mice (n=5) 3 days post intra-peritoneal injection of 3% thiolycollate. Cells were washed with ice cold PBS for 2 times, then lysed in Pierce RIPA Buffer (Prod# 89901, Thermo Scientific) with protease inhibitors (Cat# P2714, Sigma) and phosphatase inhibitor cocktail 2 (Cat# P5726, Sigma) according to protocols provided by manufactures. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Prod# 23225, Thermo Scientific). Western blots were performed as previously reported [9]. Antibody for LRP1 was from Novus (NBP1-40726); antibody for β-actin was from Santa Cruz (sc-47778).

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