Expanded Methods

Analysis of lesions. Mouse hearts were perfused *in situ* with 10ml of Dulbecco's phosphate buffered saline (PBS) and fixed in 10% buffered formalin. Oil Red-*O* stained sections from the aortic root were analyzed at each time point according to the methods of Plump and coworkers.[1] Duplicate sections were obtained 100 µm apart for 400 µm and the average lesion area reported in mm^2/section. Innominate arteries were removed after 24 weeks of high-fat diet and immediately frozen in OCT compound. Duplicate sections were obtained 100 µm apart for 700-800 µm and the average lesion area reported in mm^2/section. Hearts used for immunohistochemical analyses were frozen immediately after isolation. 5µm sections through the valve leaflets from five FcγRIII double knockouts and four $LDLR^{-/-}$ mice were analyzed after 24 weeks of high-fat diet from hearts that were not used for lesion analysis. Cryosections were air dried for 1-2 hours then fixed in -20° acetone. For analysis, sections were first treated with an endogenous enzyme block followed by a serum-free protein block (each from DAKO) before staining with anti-CD68 (rat IgG2a, Serotec) or an isotype matched negative control. Antibody binding was detected with goat anti-rat Fab'_2 conjugated to alkaline phosphatase (Jackson Immunotech) and a substrate kit from Vector Labs. Slides were counterstained prior to analysis.

Sections of the aortic root, the aortic arch, and the innominate artery from five mice of each strain were analyzed for presence of T-cells after 14 weeks of high-fat diet, and four of each strain after 24 weeks. In each case a total of 10

serial transverse sections were obtained. The aortic arch was sectioned just proximal to the aortic root to where the innominate artery emerges. The innominate artery was sectioned beginning just proximal to its branch point from the arch. Sections were fixed in -20° acetone then stained with either APC conjugated anti-CD3 alone (BD-Pharmingen, #553066) or in combination with FITC-anti-B220. Analysis was with a Nikon *eclipse* 80i immunofluorescence microscope interfaced with NIS *Elements* software.

Analyses of plasma for lipids and antibody levels. Mice were fasted for 4-5 hours prior to euthanasia. Prior to cardiac perfusion, a blood sample was obtained via cardiac puncture and anticoagulated by addition of disodium EDTA (5 mM/L final concentration, pH 7.5). Samples were divided into those treated with sodium azide and stored at 4°C (used for lipid analyses 3-4 days later), while remaining portions were stored immediately at -80° and used at a later date for analyses of anti-OxLDL antibodies. Total plasma cholesterol and triglycerides were determined by enzymatic assay (Raichem, Columbia, MD). Total IgG levels in plasma were determined with a commercial kit (Pierce Chemical, Rockland, (IL). Antibody titers to OxLDLs were determined by chemiluminescent immunoassay as described.[2] In the case of OxLDLs, either malondialdehyde-LDL (MDA-LDL) or copper OxLDL were used for ligands. IgG1 and IgG2c antibodies were detected with isotype specific mAbs conjugated to alkaline phosphatase (BD-Pharmingen, San Diego, CA). The IgG2c titers were determined with an IgG2a specific mAb that crossreacts with IgG2c but not to

IgG1. IgG2c is the structural and functional IgG2a homolog expressed in C57BL/6 mice [3-5].

Flow Cytometry. Single cell suspensions of spleen cells were obtained by standard procedures that included lysis of red blood cells. To determine the CD4-CD8 ratio, freshly isolated cells were enumerated with a hemacytometer then stained on ice with FITC-anti-mouse-CD4 and PE-anti-mouse CD8 (BD-PharMingen). Analysis was with a Becton-Dickinson FACScan® or FACS Canto flow cytometer. Lymphocytes were gated based on forward versus side scatter and the mean fluorescence intensity (MFI) of CD4+ and CD8+ cells was recorded after analyzing 50,000 cells.

For cytokine analysis by intracellular staining, peripheral blood mononuclear cells isolated with Histopaque (Sigma), or purified CD4+ splenocytes isolated with an AutoMACS isolation column (Miltenyi Biotec), were restimulated for 6 hours at 37° with a leukocyte activation cocktail containing phorbol ester, ionomycin, and brefeldin A (eBiosciences). Cells were stained on ice with PE-Cy5-anti-mouse CD4 (eBiosciences) and permeabilized in saponin buffer (eBiosciences). After washing, the cells were stained for 45 minutes with 5µg/ml of FITC-anti-mouse IL-10 (rat IgG2b), FITC-anti-mouse IL-4 (rat IgG1), or PE-anti-mouse IFN-γ (rat IgG1) (all from eBiosciences). For flow cytometry, CD4+ cells were gated and the fluorescence associated with 50,000-100,000 total cells was recorded. Isotype-matched negative controls were included to determine background fluorescence.

Real-Time RT-PCR. For analysis of cytokine mRNA levels, mice were perfused with 10ml of PBS and aortas were isolated from the arch to the iliac bifurcation, washed in cold PBS, and homogenized on ice in Trizol reagent. Total RNA was obtained according to the manufacturer's instructions and frozen at -80°. It was subsequently reverse transcribed using reverse transcriptase from Invitrogen. Primers for IL-10 and IFN-γ were obtained from SA Biosciences. Real-time RT-PCR was done using master mix #PA-011 (SA Biosciences). Gene expression in each case was normalized to GAPDH, and data are presented as fold-increase relative to mRNA levels obtained from an aorta from a chow-fed C57BL/6 mouse.

Statistical Methods. All data were analyzed using Prism software and shown as means ± standard deviation. Unpaired Student's t test was used to compare groups of data that were normally distributed and of similar variance; otherwise the nonparametric Mann-Whitney test was used. In each case $p < 0.05$ was taken to indicate statistical significance.

References.

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phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins *J Clin Invest*, 1999, *103*: 117-128.

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Supplement Figure 1.
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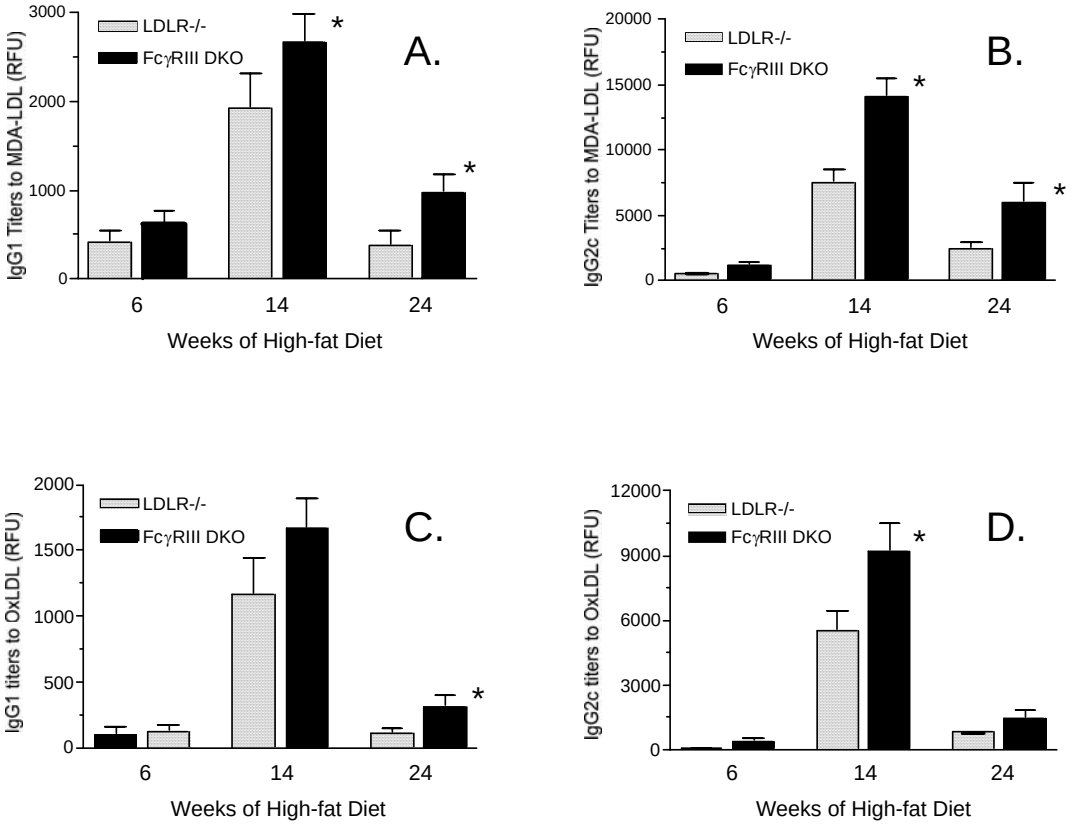

Supplemental Figure 1. Increased levels of relative titers of anti-OxLDL isotypes in FcγRIII$^{-/-}$ x LDLR$^{-/-}$ double knockout mice. Levels of anti-MDA-LDL IgG1 and IgG2c (A and B) or anti-copper OxLDL IgG1 and IgG2c (C and D) after 6, 14, or 24 weeks of high-fat diet in LDLR$^{-/-}$ and FcγRIII$^{-/-}$ x LDLR$^{-/-}$ double knockouts. In A, the differences in IgG1 titers between strains were statistically significant at 14 and 24 weeks (p = .04 for each) for n = 15 LDLR$^{-/-}$ and 14 FcγRIII double knockouts at 14 weeks, and n = 14 and 11 at 24 weeks. For IgG2c (B), p = .001 and .03 respectively for 14 and 24 weeks. In figures C and D, the differences between strains (asterisks) were each statistically significant at p = .03. RFU refers to relative fluorescence intensity. Shown are the means ± SEM.

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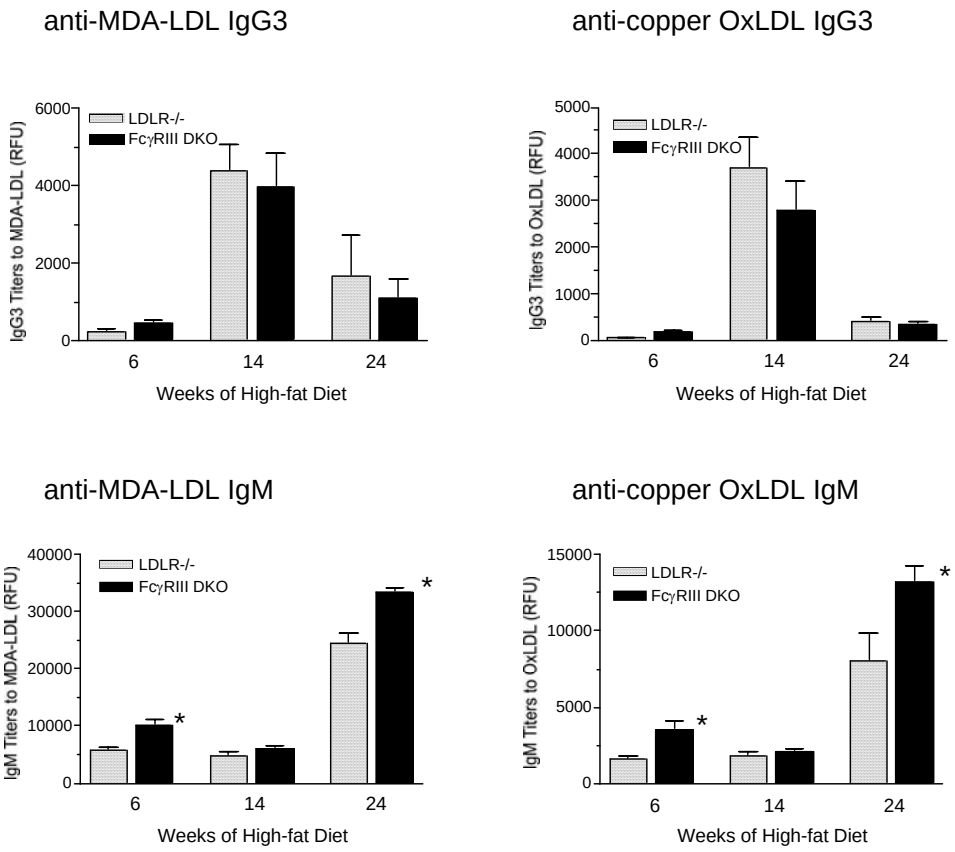
Supplemental Figure 2.



Supplemental Figure 2. Relative titers of anti-OxLDL IgM in FcγRIII$^{-/-}$ x LDLR$^{-/-}$ double knockout mice. Levels of anti-OxLDL IgG3 (top figures) and anti-OxLDL IgM (bottom figures) were determined after 6, 14, or 24 weeks of high-fat diet of high-fat diet in LDLR$^{-/-}$ and FcγRIII$^{-/-}$ x LDLR$^{-/-}$ double knockouts. The same mice were analyzed as in sFigure 1. In the top figures there were no differences in anti-OxLDL IgG3 titers between strains. In the bottom figures, the differences between strains for anti-MDA-LDL IgM at 6 and 24 weeks were each statistically significant (p = .002 and p = .0008, respectively). For anti-copper OxLDL IgM, the differences between strains at 6 and 24 weeks were also statistically significant (p = .009 in each case).