Deficiency of Antigen-Presenting Cell Invariant Chain Reduces Atherosclerosis in Mice

Jiusong Sun, PhD*; Karsten Hartvigsen, PhD*; Meng-Yun Chou, PhD; Yadong Zhang, PhD; Galina K. Sukhova, PhD; Jie Zhang, PhD; Marco Lopez-Ilasaca, MD, PhD; Cody J. Diehl, BA; Niva Yakov, PhD; Dror Harats, MD; Jacob George, MD; Joseph L. Witztum, MD; Peter Libby, MD; Hidde Ploegh, PhD; Guo-Ping Shi, DSc

Background—Adaptive immunity and innate immunity play important roles in atherogenesis. Invariant chain (CD74) mediates antigen-presenting cell antigen presentation and T-cell activation. This study tested the hypothesis that CD74-deficient mice have reduced numbers of active T cells and resist atherosclerosis.

Methods and Results—In low-density lipoprotein receptor–deficient (Ldlr<sup>−/−</sup>) mice, CD74 deficiency (Ldlr<sup>−/−Cd74<sup>−/−</sup></sup>) significantly reduced atherosclerosis and CD25<sup>+</sup>-activated T cells in the atheroma. Although Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> mice had decreased levels of plasma immunoglobulin (Ig) G1, IgG2b, and IgG2c against malondialdehyde-modified LDL (MDA-LDL), presumably as a result of impaired antigen-presenting cell function, Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> mice showed higher levels of anti–MDA-LDL IgM and IgG3. After immunization with MDA-LDL, Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> mice had lower levels of all anti–MDA-LDL Ig isotypes compared with Ldlr<sup>−/−</sup> mice. As anticipated, only Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> splenocytes responded to in vitro stimulation with MDA-LDL, producing Th1/Th2 cytokines. Heat shock protein-65 immunization enhanced atherosclerosis in Ldlr<sup>−/−</sup> mice, but Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> mice remained protected. Compared with Ldlr<sup>−/−</sup> mice, Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> mice had higher anti–MDA-LDL autoantibody titers, fewer lesion CD25<sup>+</sup>-activated T cells, impaired release of Th1/Th2 cytokines from antigen-presenting cells after heat shock protein-65 stimulation, and reduced levels of all plasma anti–heat shock protein-65 Ig isotypes. Cytofluorimetry of splenocytes and peritoneal cavity cells of MDA-LDL– or heat shock protein-65–immunized mice showed increased percentages of autoantibody-producing marginal zone B and B-1 cells in Ldlr<sup>−/−Cd74<sup>−/−</sup></sup> mice compared with Ldlr<sup>−/−</sup> mice.

Conclusions—Invariant chain deficiency in Ldlr<sup>−/−</sup> mice reduced atherosclerosis. This finding was associated with an impaired adaptive immune response to disease-specific antigens. Concomitantly, an unexpected increase in the number of innate-like peripheral B-1 cell populations occurred, resulting in increased IgM/IgG3 titers to the oxidation-specific epitopes. (Circulation. 2010;122:808-820.)

Key Words: adaptive immunity ■ atherosclerosis ■ innate immunity ■ invariant chain

Antigen presenting cells (APCs) and T cells, key regulators of innate and adaptive immunity, may participate in human atherogenesis. Accumulating data suggest that atherosclerosis involves aspects of autoimmunity. Indeed, autoimmune diseases (eg, systemic lupus erythematosus or rheumatoid arthritis) may accelerate atherosclerosis. Systemic lupus erythematosus patients have a 4- to 8-fold higher risk of developing atherosclerosis than those who do not have systemic lupus erythematosus. Patients with rheumatoid arthritis also have an enhanced risk of atherosclerotic events. In general, T cells that mediate adaptive responses appear to play a proatherogenic role, and in some experimental models, T-cell deficiency produces up to an 80% reduction in atherosclerosis. However, emerging evidence also suggests that certain T-cell populations such as T regulatory subtypes may confer atheroprotective properties. In contrast, B cells appear to play an overall atheroprotective role, at least in part by secreting antibodies to disease-specific antigens (or “self-antigens”). Among such antigens, oxidized low-density lipoprotein (oxLDL), heat shock proteins (HSP),...
and β2-glycoprotein-1 appear to be prominent. For example, B-cell deficiency or splenectomy, which associate with absent levels of antibodies to oxLDL, leads to acceleration of atherosclerosis, which B-cell replacement can reverse in the case of splenectomy. Furthermore, immunization of LDL receptor–deficient (Ldlr−/−) rabbits and mice with apolipoprotein E–deficient (ApoE−/−) mice with malondialdehyde-modified LDL (MDA-LDL) suppressed atherosclerosis. Although other mechanisms may also function, current data suggest that the reduced atherosclerosis in MDA-LDL–immunized animals stems from increased production of atheroprotective oxLDL autoantibodies. Among these, innate immunoglobulin (Ig) M natural antibodies against “oxidation–specific” epitopes of oxLDL appear to be prominent. Evidence to support this derives from studies of the natural antibody E06, an anti-oxLDL IgM cloned from ApoE−/− mice. Such natural antibodies derive in large part from B-1 cells. Furthermore, in contrast to activation of B-2 cells, activation of B-1 cells occurs independently of cognate T-cell help. E06 binds to the phosphocholine head group of oxidized phospholipids, as found on oxLDL but not on native LDL. It bears the T15 idiotype, which binds the phosphocholine moiety present on the cell wall of Streptococcus pneumoniae, as shown previously. Indeed, immunization of cholesterol-fed Ldlr−/− mice with heat-inactivated S pneumoniae led to a nearly monocolonal increase in plasma E06/T15 titers and atheroprotection. In ApoE−/− mice, treatment with an IgM with the T15 idiotype reduced vein graft atherosclerosis. In a recent study, Ldlr−/− mice deficient in serum IgM were shown to have substantially larger and more complex atherosclerotic lesions, suggesting that IgM antibodies as a class possess antitherogenic properties. Indeed, plasma IgM antibodies to oxLDL correlate inversely with carotid and coronary atherosclerosis in humans. In contrast, the role of IgG isotypes generated by adaptive immune responses to oxidation–specific epitopes is more complex. In general, in epidemiological studies in humans, IgG isotypes to oxLDL appear to correlate positively with manifestations of cardiovascular diseases, whereas immunization with oxLDL epitopes in murine models that generate elevations of IgG, particularly IgG1, usually is associated with atheroprotection. Such IgG1 usually is associated with Th2 help, and whether the benefit results purely from the antibodies themselves or from the associated Th2 cytokine help generally regarded as atheroprotective remains unclear. The antibodies might influence atherogenesis by their ability to inhibit uptake of oxLDL by macrophages, attenuation of proinflammatory effects of oxidized lipids, or conceivably activation of the inhibitory Fcy receptor IIb. In contrast, immunization with other disease-related epitopes such as HSP65 has been associated with a mixed IgG response and worsening atherosclerosis. Interpreting such immunization studies can prove difficult because they involve not only changes in adaptive humoral immunity but also major changes in T-cell function, which in turn profoundly modulate lesion formation.

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Adaptive immunity to disease-specific antigens and associated APC antigen presentation and T-cell activation may directly play detrimental roles in atherosclerosis. Within APCs, antigens must undergo full processing and presentation by major histocompatibility class II (MHC-II) molecules to CD4+ T cells. In human APCs, this process requires the involvement of a 45-kDa type II transmembrane protein (41 kDa in mice) called invariant chain (CD74). CD74 is usually synthesized in excess over MHC-II, ensuring that in the endoplasmic reticulum all MHC-II Αα and Αβ heterodimers are associated with invariant chains. In the endoplasmic reticulum, CD74 functions as a chaperone to assist the MHC-II complex in folding properly, preventing the early binding of peptides to MHC-II, and determining the targeting and transport of MHC-II complexes to the appropriate organelles (lysosome/endosome), where CD74 molecules are proteolytically removed and antigenic peptides are loaded onto class II molecules. If CD74 is incompletely processed because of the inhibition or absence of protease activities, MHC-II will fail to present antigens to the APC surface. These APCs will fail to activate T cells, and mice will produce fewer antibodies against immunogens; even the total T- or B-cell numbers are not affected. In the case of a complete absence of CD74 (Cd74−/−) in C57BL/6 mice, MHC-II Ααββ is expressed predominantly as free Αα and Αβ chains in APCs, confirming that the Ααββ assembly also depends strongly on CD74 coexpression. As a result, Cd74−/− mice have greatly reduced levels of thymic and peripheral CD4+ T cells. Concomitantly, the thymi or peripheries of Cd74−/− mice often have greater CD8+ T-cell numbers than those of wild-type control mice. Although Cd74−/− mice have fewer peripheral mature B cells than wild-type control mice, double mutation of CD74 and MHC-II resulted in no difference in the number of mature B cells compared with wild-type control mice, suggesting that B-cell development requires neither CD74 nor MHC-II. CD4+ T-cell numbers comprise the predominant T lymphocyte type in human and murine atherosclerotic lesions, and their activation contributes to experimental atherogenesis. In mice, the absence of CD4+ T cells significantly reduced atherosclerosis. Adoptive transfer of CD4+ T cells isolated from atherosclerotic ApoE−/− mice into T-cell–deficient ApoE−/−/scid/scid mice restored the atherosclerosis phenotypes. Reconstitution of antigen (oxLDL)-specific CD4+ T cells yielded even more atherosclerosis than in mice that received naïve CD4+ T cells, an observation of particular interest because nearly 10% of the T-cell clones from human atherosclerotic plaques responded to oxLDL. Thus, both APCs and T cells modulate human and mouse atherogenesis.

This study tested the hypotheses that APCs lose their antigen presentation activity in the absence of CD74, thereby rendering them unable to activate T cells, and that impaired antigen presentation and defective T-cell activation attenuate atherogenesis in Ldlr−/− mice.
Methods

Mice and Atherosclerosis Model

*CD74*−/− mice (C57BL/6) were crossbred with *Ldlr*−/− mice (C57BL/6, Jackson Laboratories, Bar Harbor, Me) to generate both *Ldlr*−/−*CD74*−/− and *Ldlr*−/−*Cd74*−/− mice. Male *Ldlr*−/−*CD74*−/− and *Ldlr*−/−*Cd74*−/− littermates consumed an atherogenic diet for 12 to 26 weeks (D12108, Research Diet, New Brunswick, NJ) starting at 8 weeks of age. At the end of each experiment, mouse plasma was collected for lipoprotein and Th1 and Th2 cytokine measurements, aortic arches were harvested for frozen section preparation and lesion characterization, and thoracic-abdominal aortas were harvested for lipid deposition analysis. Aortic arch lesion grade, intima area, thoracic-aortic en face preparation, Oil Red O staining, lesion area calculation, lesion macrophage (Mac-3) staining, and smooth muscle cell (α-actin) staining were performed as we previously reported.33 Lesion total CD4+ T-cell enumeration used anti-CD4 monoclonal antibody (1:90, Pharmingen, San Diego, Calif) immunostaining, and detection of lesion-activated T cells used an anti-CD25 monoclonal antibody (1:70, Pharmingen). Lesion CD8+ T cells, NK.1.1 T cells, CD1d+ cells, and dendritic cells were detected with rat anti-mouse CD8 monoclonal (1:100, Pharmingen), mouse NK.1.1 monoclonal (PK136, 1:250, Novus Biologicals, Littleton, Colo), mouse CD1d monoclonal (1:100, AbD Serotech, Raleigh, NC), and hamster anti-mouse CD11c (1:50, Pharmingen), respectively. All murine studies were preapproved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Immunogen Preparation

Human LDL and MDA-LDL were prepared as previously described34 and contained <1.5 mg lipopolysaccharides/mg protein (apolipoprotein B). MDA modification of BSA (MDA-BSA) was carried out in a manner similar to that for MDA-LDL, except the reaction continued for 24 hours instead of 3 hours. Recombinant *Mycobacterium bovis* HSP65 was expressed in

Plasma Lipoprotein and Cytokine Determination

Plasma lipoproteins, including total cholesterol, LDL, high-density lipoprotein, and triglycerides, were determined with enzymatic assay kits according to the manufacturer’s instructions (Pointe Scientific Inc, Canton, Mich). Plasma cytokines, including interleukin (IL)-2 (BD Biosciences, San Jose, Calif), IL-4, IL-5 (Pierce Endogen, Rockford, Ill), IL-10, and interferon-γ (IFN-γ) (PeproTech Inc, Rocky Hill, NJ), were measured with ELISA kits according to the manufacturer’s instructions.

Plasma Antibody Isotype Determination

Plasma antibody titers to native LDL, MDA-BSA, and HSP65 were determined by chemiluminescent ELISA as previously described.13 In brief, 96-well Microtiter plates (Thermo Labsystems, Franklin, Mass) were coated with various antigens at 5 μg/mL. The plates were blocked with 1% BSA in PBS, serially diluted plasma was added; and the plates were incubated for 1.5 hours at room temperature. Bound plasma Ig isotypes were detected with various anti-mouse Ig isotype-specific alkaline phosphatase conjugates, LumiPhos 530 (Luminen, Southfield, Mich) solution, and a Dynex Luminometer (Dynex Technologies, Chantilly, Va). The following goat alkaline phosphatase-conjugated secondary Ig isotype-specific antibodies were used: anti-mouse IgG (μ-chain specific) (Sigma) and anti-mouse IgG1, IgG2b, IgG2c, and IgG3 (Jackson ImmunoResearch Laboratories, West Grove, Pa). Data were expressed as relative light units counted per 100 milliseconds.

Antigen Recall Assay

Splenocyte and lymph node cell preparation and antigen recall assays were performed as described previously.31 Splenocytes from *Ldlr*−/− and *Ldlr*−/−*Cd74*−/− mice immunized with MDA-LDL, CFA/PBS, or PBS were plated on a 96-well plate (8×10^5 cells/200 μL per well) with human native LDL or MDA-LDL at various concentrations in RPMI for 3 days, followed by determination of antigen-specific cytokine measurement as above.

Fluorescence-Activated Cell Sorting Analysis

At the time of death, peritoneal cavity cells and splenocytes were prepared and resuspended in a staining buffer containing PBS and 1% BSA. After blocking with an anti-Fcy receptor monoclonal antibody (Pharmingen) specific for various surface markers [FITC–anti-CD11b/Mac-1, PE–anti-CD5, PerCP-Cy5.5–anti-CD19; PE–anti-mouse CD43, FITC–anti-mouse IgM (II/41)] or APC–anti-mouse CD21 in 100 μL staining buffer for 30 minutes at 4°C. More than 0.5×10^5 cells per sample were analyzed. Peritoneal B-1 cells were identified as CD19+ /CD11b+ /CD5- . Splenic B-1 cells were identified as CD19+ /IgM+ /CD43+ , marginal zone (MZ) B cells as CD19+/CD21+ /CD23- , and follicular B cells as CD19+/CD21-/CD23-.

Statistical Analysis

Unpaired Student *t* test and nonparametric Mann-Whitney *U* test were used to examine statistical significance, depending on the normality of the data sets. Values of *P* < 0.05 were considered statistically significant. Bonferroni posthoc correction was used when multiple comparisons were made to minimize type I error; the corrected *P* value is indicated in the respective figure. Data from antibody titers are presented in a descriptive way without performing statistical significance tests.

Results

Reduced Atherosclerosis and CD25+ Activated T-Cell Numbers in CD74-Deficient Mice

T-cell activation by disease-specific antigens may enhance atherogenesis. We tested the hypothesis that a deficiency of CD74 would impair APC antigen presentation and T-cell activation and therefore attenuate atherogenesis. Remarkably, after 12 or 26 weeks of an atherogenic diet,
**Figure 1.** Atherosclerotic lesion characterizations in Ldr<sup>−/−</sup> and Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice that consumed an atherogenic diet for 12 and 26 weeks, including aortic arch lesion grade (A), thoracic-abdominal aortic lipid deposition with representative aortas shown on the right (C), lesion Mac-3<sup>+</sup> macrophage–positive area (D), NK-1.1<sup>+</sup> T-cell number (E), CD4<sup>+</sup> T-cell number (F), CD1d-positive area (G), CD8<sup>+</sup> cell number (H), CD11c<sup>+</sup> dendritic cell number (I), and CD25<sup>+</sup> activated T-cell number (J). Mann-Whitney U test. Values of *P*<0.05 were considered statistically significant. The number of mice in each group is indicated in parentheses.

Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice developed significantly smaller atherosclerotic lesions than did Ldr<sup>−/−</sup> mice. In the aortic arches, Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had reduced lesion grade (Figure 1A) and intima area (Figure 1B) compared with Ldr<sup>−/−</sup> mice at both time points. In thoracic-abdominal aortas, Oil Red O staining demonstrated that Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had significantly smaller aortic lipid depositions or lesion areas than did Ldr<sup>−/−</sup> mice at the 26-week time point (Figure 1C). Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had significantly reduced several lesion inflammatory cell contents, including Mac-3<sup>+</sup> macrophage–positive area and CD4<sup>+</sup> T-cell numbers and absence of NK1.1<sup>+</sup> T cells, compared with Ldr<sup>−/−</sup> mice at either the 12-week or 26-week time point (Figure 1D through 1F), although we did not detect significant changes in CD1d-positive area, CD8<sup>+</sup> T cells, or lesion dendritic cells (Figure 1G through 1I). Consistent with our hypothesis, Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had greatly reduced lesional content of CD25<sup>+</sup> cells, most likely consistent with a decreased presence of activated T cells compared with Ldr<sup>−/−</sup> mice (Figure 1J).

Plasma lipid levels often are associated with atherosclerotic lesion progression. On a chow diet, Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had plasma total cholesterol levels similar to Ldr<sup>−/−</sup> mice, although LDL cholesterol levels were slightly lower (Table I in the online-only Data Supplement). After 12 and 26 weeks of an atherogenic diet, the 2 groups had significantly elevated yet equal levels of total and LDL cholesterol. However, Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had significantly lower plasma triglyceride levels at both time points but also lower high-density lipoprotein cholesterol at the 26-week time point. It is possible that reduced plasma triglyceride levels in Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice were secondary to attenuated atherosclerosis of these mice.

Plasma cytokine ELISA did not reveal significant differences in IFN-γ, IL-5, and IL-10 levels between Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> and Ldr<sup>−/−</sup> mice at both the 12- and 26-week time points. However, Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had higher plasma IL-4 levels than Ldr<sup>−/−</sup> mice at both time points (Figure I in the online-only Data Supplement), suggesting a Th2 bias in atherosclerotic CD74-deficient mice.

**Plasma Anti–MDA-LDL Antibody Isotypes**

In humans and mice, plasma IgG antibodies against ox-LDL often correlate positively with lesion development, whereas IgM antibodies correlate negatively. We anticipated lower titers of adaptive anti–MDA-LDL IgG1, IgG2b, and IgG2c levels in Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice than in Ldr<sup>−/−</sup> mice. Consistent with this hypothesis, Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice had lower anti–MDA-LDL IgG2b and IgG2c titers than did Ldr<sup>−/−</sup> mice at both the 12- and 26-week time points (Figure 2). Ldr<sup>−/−</sup> Cd74<sup>−/−</sup> mice also had lower IgG1 titers at the 12-week time point, although overall both groups of mice had much lower levels of IgG1 than IgG2b or IgG2c. The reduced plasma anti–MDA-LDL IgG titers would correspond with impaired antigen presentation in CD74-deficient mice. To our surprise, at both time points, we observed higher titers of
IgM to MDA-LDL in \( Ldlr^{-/-}Cd74^{-/-} \) mice and, similarly, higher titers of IgG3, although no statistical test was performed (Figure 2). In uninfected mice, IgM is largely considered to represent natural antibodies generated by innate-like B-1 and MZ B cells, the production of which is believed to occur independently of antigen-specific T-cell help. Such B-1 cells/MZ B cells also secrete IgG3. Thus, these data suggested an unexpected stimulation of B-1/MZ B cells. Notably, elevated IgM titers to epitopes of oxLDL play a role in atheroprotection.\(^{15,16} \)

**MDA-LDL Immunization and Antibody Production**

The reduced titers of anti–MDA-LDL IgG2b and IgG2c in hyperlipidemic \( Ldlr^{-/-}Cd74^{-/-} \) mice suggested reduced humoral adaptive responses resulting from impaired antigen presentation, whereas in contrast, the increased anti–MDA-LDL IgM and IgG3 titers indicated enhanced B-1/MZ cell activation, consistent with enhancement of innate-like responses. To examine whether such phenotypes are associated solely with the atherogenic diet or the development of atherosclerosis, we immunized \( Ldlr^{-/-}Cd74^{-/-} \) and \( Ldlr^{-/-} \) mice with MDA-modified human LDL without feeding them an atherogenic diet. We have previously shown that the adaptive response to MDA-LDL is MHC-II restricted.\(^{11} \)

Before immunization, \( Ldlr^{-/-}Cd74^{-/-} \) mice had similar adaptive anti–MDA-LDL Ig levels but higher innate IgM and IgG3 compared with \( Ldlr^{-/-} \) mice. However, after MDA-LDL immunization, \( Ldlr^{-/-}Cd74^{-/-} \) mice had lower titers of all anti–MDA-LDL IgG isotypes than \( Ldlr^{-/-} \) mice. For example, IgG1 titers showed a robust increase in response to immunization in the \( Ldlr^{-/-} \) mice (Figure 3A, left) as previously reported,\(^{10,11} \) but \( Ldlr^{-/-}Cd74^{-/-} \) mice showed no increase at all (right).
LDL, in which the absence of IL-5 enhanced atherosclerosis in immunocompetent mice after immunization with murine MDA-LDL in a gender-specific manner (Figure 3A and 3B, right). Consistent with this hypothesis, Ldlr−/− mice immunized with MDA-LDL developed significantly smaller lesion grade and intima sizes than Ldlr+/− mice (Figures 5A and 5B). Notably, HSP65–HSP65-specific T cells in blood and in atherogenic diet, and increased HSP65 expression in intimal and/or MZ B cells.

CD74 deficiency not only led to impaired adaptive antibody production in vivo but also resulted in reduced Th1 and Th2 cytokine production in vitro. Although we did not detect significant differences in plasma Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10) cytokine levels between MDA-LDL–immunized Ldlr−/−Cd74−/− and Ldlr−/− mice (data not shown), we did detect lower medium Th1 cytokine levels (IL-2, IFN-γ) in splenocytes from MDA-LDL–immunized Ldlr+−/− mice (data not shown), which was also observed in the Ldlr−/−Cd74−/− mice (right). These data affirm an important role of CD74-mediated antigen presentation to disease-specific antigens (ie, MDA-LDL) in atherogenesis and again document higher IgM levels, supporting the hypothesis that CD74 deficiency enhances activation of innate-like B-1- and/or MZ B cells.

Similarly, IgG2b, IgG2c, and even IgG3 showed no response to immunization above that seen in response to the CFA/IFA or PBS control, although we did not perform statistical analysis (Figures II through IV in the online-only Data Supplement). In contrast, in response to immunization, IgM titers to MDA-LDL showed a modest increase in the Ldlr+/− mice (Figure 4), which again document higher IgM levels, supporting the hypothesis that CD74 deficiency enhances activation of innate-like B-1 and/or MZ B cells.

Figure 4. Culture medium cytokine levels after stimulation with MDA-LDL or native LDL in splenocytes from MDA-LDL–immunized Ldlr−/−Cd74−/− and Ldlr−/− mice. Each data point is mean ± SE of 6 mice. No statistical test was performed.

CD74 Deficiency Reduced Atherosclerosis After HSP65 Immunization

Previous studies have suggested that animals or humans exposed to infectious agents may develop antibody responses to bacterial HSPs that then cross-react with human HSP expressed on the surface of cells undergoing stress such as endothelial cells, which can lead to arthritis and exacerbation of atherosclerosis. Indeed, in human populations, increased levels of plasma anti-HSP65 antibody correlate with intima thickening and lesion calcification, and atherosclerotic plaques contain activated CD4+ T cells against human HSP65. In contrast to reduced atherosclerosis in Ldlr−/− or Apoe−/− mice after immunization with MDA-modified homologous LDL, immunization of hyperlipidemic Ldlr−/− mice and rabbits with heterologous mycobacterial HSP65 aggravated atherosclerosis. In rabbits, plasma titers against HSP65 have been shown to increase rapidly after initiation of an atherogenic diet, and increased HSP65 expression in intimal cells and the presence of HSP65–specific T cells in blood and in atherosclerotic lesions have been documented, consistent with exacerbation of lesion formation as a consequence of an adaptive response to HSP. Therefore, we predicted that the enhanced atherosclerosis seen with HSP immunization in Ldlr−/− mice would be attenuated in Ldlr−/−Cd74−/− mice. Consistent with this hypothesis, Ldlr−/−Cd74−/− mice immunized with HSP65 developed significantly smaller lesion grade and intima sizes than Ldlr−/− mice (Figure 5A and 5B). Ldlr−/−Cd74−/− mice also had fewer lesion CD4+ T cells and CD25+–activated T cells than did Ldlr−/− mice (Figure 5C and 5D). Notably, HSP65 immunization increased lesion grade significantly, as was originally reported, and lesion CD4+ T cells in Ldlr−/− mice were more than in those immunized with PBS (Figure 5B and 5C). In contrast, HSP65 immunization did not change these lesion parameters in Ldlr−/−Cd74−/− mice.

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In HSP65-immunized mice, we did not detect any significant differences in plasma lipoprotein levels (total cholesterol, LDL, high-density lipoprotein, and triglycerides) (Table I in the online-only Data Supplement) and Th1 (IFN-γ) or Th2 (IL-4, IL-5, IL-10) cytokine levels between the 2 groups of mice (data not shown).

Consistent with impaired antigen presentation and atherogenesis, \( Ldlr^{-/-}Cd74^{-/-} \) mice had greatly blunted IgG isotype responses to HSP immunization compared with \( Ldlr^{-/-} \) mice (Figure 6). Nonimmunized mice had extremely low titers to HSP65 for all Ig isotypes. To affirm impaired antigen presentation and reduced adaptive immunity in \( Ldlr^{-/-}Cd74^{-/-} \) mice, we prepared splenocytes and lymph node cells from \( Ldlr^{-/-}Cd74^{-/-} \) mice and stimulated these cells with mycobacterial HSP65. In this antigen recall assay, cells from \( Ldlr^{-/-}Cd74^{-/-} \) mice had significantly reduced production of both Th1 and Th2 cytokines compared with those from \( Ldlr^{-/-} \) mice (Figure 7).

**CD74 Deficiency Leads to Increased Autoantibody-Producing B-1 Cells**

In uninfected mice, plasma IgM and, to an extent, IgG3 are largely considered products of B-1/MZ B cells. Thus, we hypothesized that the increased IgM and IgG3 in \( Ldlr^{-/-}Cd74^{-/-} \) mice occurred as a result of expanded B-1/MZ B-cell populations. To test this hypothesis, we used fluorescence-activated cell sorter analysis to analyze cell preparations from the spleens and the peritoneal cavities, which are rich in B-1 cells. Although the spleens of \( Ldlr^{-/-}Cd74^{-/-} \) mice had fewer B cells than did \( Ldlr^{-/-} \) mice (Table II in the
online-only Data Supplement), they had a significantly higher percentage of both B-1 cells and MZ B cells than did those from Ldlr<sup>−/−</sup> mice, regardless of whether mice were immunized with PBS, MDA-LDL, or HSP65 (Figure 8A and 8B). These data coincide with the observations of enhanced plasma IgM anti–MDA-LDL natural antibodies (Figures 2 and 3). In contrast, Ldlr<sup>−/−</sup>Cd74<sup>−/−</sup> mice had fewer follicular B cells than Ldlr<sup>−/−</sup> mice, consistent with the impaired T-cell activation in Ldlr<sup>−/−</sup>Cd74<sup>−/−</sup> mice after MDA-LDL (Figure 3) or HSP65 (Figure 6) immunization. Similar to the spleen,
Figure 8. Flow cytometry analysis for CD19+ splenocytes and peritoneal cavity cells from Ldlr−/− and Ldlr−/− Cd74−/− mice immunized with PBS, MDA-LDL, or HSP65. A, Percentages of IgM+CD19+CD43+ B-1 cells, CD19+CD21+CD23+ MZ-B cells, and CD19+CD21+CD23+ follicular B cells (FO-B) in spleen. B, Representative flow cytometry analysis of splenocytes from MDA-LDL–immunized Ldlr−/− and Ldlr−/− Cd74−/− mice. C, Percentage of CD19+ cells in peritoneal cavity: CD11b+ B-1 and CD11b− B-2 cells. The number of mice in each group is indicated inside the bar; data are mean±SE. Student t test. Values of P<0.05 were considered statistically significant. D, Representative flow cytometry scans of peritoneal cavity cells from PBS-immunized Ldlr−/− and Ldlr−/− Cd74−/− mice. Total B-1 cells [CD5+ (B-1a) and CD5− (B-1b)] are counted.
peritoneal cavities in Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice contained significantly more B-1 cells and fewer B-2 cells than those in Ldlr\(^{-/-}\) mice under all tested immunization conditions (Figure 8C and 8D).

**Discussion**

There are now considerable data that, once established, adaptive immune responses significantly modulate atherogenesis. In particular, APCs play a key role in processing antigens for delivery by MHC-II molecules to enable CD4\(^{+}\) T-cell activation, a central mediator of both cellular and humoral adaptive immune responses. The invariant chain, CD74, is essential to intracellular MHC-II trafficking and antigen presentation.\(^{31}\) Therefore, we predicted that the absence of CD74 would attenuate atherogenesis. We now demonstrate that diet-induced atherosclerosis was reduced in CD74-deficient Ldlr\(^{-/-}\) mice, which reflects the importance of "endogenous" disease-specific antigen (eg, MDA-LDL) presentation and subsequent T-cell activation in the pathogenesis of atherosclerosis. We further show that such antigen presentation was also rate limiting in response to an "exogenous" disease-related antigen, bacterial HSP65. These data affirm the important role that CD74 plays in presenting both endogenous and exogenous disease-specific antigens leading to T-cell-mediated activation of adaptive immune responses that adversely affect atherosclerosis. Because immune responses to HSP are activated in cholesterol-fed animals even in the absence of exogenous immunization with HSP,\(^{43}\) these data strongly emphasize the importance of adaptive immune responses to endogenous disease-specific antigens in the pathogenesis of atherosclerosis.

Although the mechanisms by which the absence of CD74 and subsequent impairment of CD4\(^{+}\) T-cell activation reduced atherosclerosis are likely to be complex, our data point to a number of contributing mechanisms. First, the absence of CD74 clearly restricted MHC-II antigen presentation and T-cell activation. This is evidenced by decreased expression of MHC-II (I-Ab) on APCs of splenic cells, markedly decreased CD4\(^{+}\) T cells in the spleen, blunted antigen-specific Th1/Th2 cytokine production in splenic cultures, and greatly blunted or absent IgG responses to MDA-LDL (an MHC-II–restricted antigen).\(^{11}\) Previously, it has been reported that in the absence of CD74, there were reduced peripheral CD4\(^{+}\) T cell numbers and heightened Th1 immune responses, although the CD4\(^{+}\) T cells from CD74-deficient mice were not intrinsically impaired because they proliferated no differently than those from wild-type mice in responding to immunogens.\(^{26,45}\) In our study, not only was there a marked reduction in the percentage of CD4\(^{+}\) T cells in the spleens of Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice (Table II in the online-only Data Supplement), but importantly, we also noted significantly reduced CD4\(^{+}\) T cells in the atherosclerotic lesions of Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice, concomitant with reduced expression of CD25, most likely reflecting decreased CD4\(^{+}\) T-cell activation. CD4\(^{+}\) T cells constitute the main T lymphocytes in human and mouse atherosclerotic lesions.\(^{27,28}\) and previous studies have demonstrated an overall proatherogenic role for such CD4\(^{+}\) T cells.\(^{29–31}\) NK1.1\(^{+}\) T cells are also essential to atherogenesis, and their activations are CD1d dependent. Mice lacking CD1d demonstrate reduced NK1.1\(^{+}\) T-cell activation and impaired glycolipid \(\alpha\)-galactosylceramide–induced atherosclerosis.\(^{46}\) We have previously shown that CD74 deficiency not only affects CD1d-mediated antigen presentation but also exhibits defective thymic NK1.1\(^{+}\) T-cell–positive selection,\(^{47}\) which may explain the absence of NK1.1\(^{+}\) T cells in atherosclerotic lesions from Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice (Figure 1E), although the mechanism by which CD74 controls NK1.1\(^{+}\) T cell maturation in thymus remains untested. In addition to impaired antigen presentation and T-cell activation, lack of NK1.1\(^{+}\) T cells in Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice may contribute to reduced atherosclerosis in these mice.

Second, changes in APC content and activities in Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice also likely contributed to attenuated atherosclerosis. Cytofluorimetry of splenocytes from Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice revealed a reduction in the number of peripheral total B220\(^{+}\) B cells compared with Ldlr\(^{-/-}\) mice, although the reduction of B cells was not as profound as that of CD4\(^{+}\) T cells. Splenic cell MHC class II (I-A\(^{\beta}\)) expression, found on professional APCs (such as dendritic cells, macrophages, and B cells), also fell significantly, consistent with a reduced number of B cells (Table II in the online-only Data Supplement) and enhanced MHC-II retention within the intracellular organelles as a result of CD74 deficiency.\(^{23}\) This would lead to decreased CD4\(^{+}\) T-cell activation and explain the impaired splenic Th1 and Th2 cytokine production from both MDA-LDL (Figure 4) and HSP65 (Figure 7) immunization antigen recall assays. This in turn would explain the absent generation of plasma IgG1, IgG2b, and IgG2c titers against MDA-LDL (Figure 3 and Figures II and III in the online-only Data Supplement) and HSP65 (Figure 6) in the antigen-immunized Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice compared with the increases observed in Ldlr\(^{-/-}\) mice. Presumably, this also explains the greatly blunted IgG responses to MDA-LDL in the cholesterol-fed Ldlr\(^{-/-}\) Cd74\(^{-/-}\) mice.

Third, it is not clear what role such IgG titers to epitopes of oxLDL might play because their impact may be complex, as noted earlier. IgG titers to oxidation-specific epitopes have in general been found to be positively correlated with atherosclerosis in both murine models and humans, but immunization of mice with oxLDL or models of oxLDL is also associated with marked increases in Th2-biased IgG1 titers and atheroprotection.\(^{4,14,18,27}\) Furthermore, some studies have shown that direct infusion of such antibodies is associated with atheroprotection.\(^{48}\) In contrast, there are considerable data that IgM titers, which in large part represent innate natural antibodies, are associated with atheroprotective properties in both murine models and humans, as discussed in the introductory section.\(^{11,13,14,19}\) IgM and IgG3 are generated by innate-like B-1 cells and splenic MZ B cells. Because activation of these cells is independent of cognate T-cell help, it was not anticipated that their levels would be decreased by CD74 deficiency. Surprisingly, however, we found that IgM and IgG3 titers to MDA-LDL were actually increased in the
Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice, likely indicative of enhanced innate-like activity of B-1 cells, which might also contribute to the reduced atherosclerosis observed in the Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice.\(^{15}\) Therefore, the present study is consistent with the notion that increased circulating IgM protects mice from atherogenesis.\(^{14}\)

In noninfected mice, plasma IgM is thought to represent predominantly the products of innate-like B-1 cells and splenic MZ B cells as a percent of B cells (Figure 8). The reasons for the increased innate-like B-1 cell populations in CD74-deficient mice are unknown. The defect of adaptive immunity caused by CD74 deficiency appears to have promoted compensatory mechanisms leading to augmented innate-like B-1 cell compartment. Such compensatory mechanisms may also explain why Ldlr\(^{-/-}\)/Cd74\(^{-/-}\)-mouse spleens had increased percentages of CD8\(^{+}\) T cells compared with the spleens of Ldlr\(^{-/-}\) mice (Table II in the online-only Data Supplement). Similar phenomena occur in other mutant mice.\(^{50}\) The mechanisms by which reduced B220\(^{+}\) B cells and CD4\(^{+}\) T cells apparently led to increased percentages of B-1 cells and CD8\(^{+}\) T cells and the role of CD74 deficiency in the augmented innate immune responses merit further investigation.

Besides reduced atherosclerosis, Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice differed from Cd74\(^{-/-}\) single knockout mice in Th1/Th2 cytokine production by lymphocytes. Topilski et al\(^{45}\) reported that mice lacking Cd74 develop preferential Th1 immune response after keyhole limpet hemocyanin immunization; ie, CD4\(^{+}\) T cells from Cd74\(^{-/-}\) mice released high amounts of IFN-\(\gamma\), but not IL-4, after antigenic stimulation compared with those from wild-type control mice. However, Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice immunized with MDA-LDL (Figure 4) or HSP65 (Figure 7) developed opposite phenotypes. After either immunization protocol, splenocytes and/or lymph node cells from Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice responded poorly to MDA-LDL, native LDL, or HSP65 in IL-2 and IFN-\(\gamma\) production. In contrast, splenocytes from Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice responded to MDA-LDL or LDL by releasing IL-4 (Figure 4) and responded to HSP65 by producing IL-4, IL-5, and IL-10 (Figure 7). One explanation asserts that HSP65, like human HSP60, preferentially induced B-2 cell activities, which activated more Th2 cells.\(^{51}\) The same held true in MDA-LDL–immunized Ldlr\(^{-/-}\) mice, which resulted in a very strong Th2 bias and was associated with reduced atherosclerosis.\(^{15}\) Preferential Th2 response and reduced atherosclerosis in Ldlr\(^{-/-}\)/Cd74\(^{-/-}\) mice also concur with prior studies that proinflammatory Th1 response favors atherosclerotic plaque formation, whereas the Th2 response and enhanced IgG1 humoral immunity were associated with reduced atherosclerosis.\(^{52}\) Altered Th1/Th2 balance toward Th2 such as in atherosclerosis-prone mice on a Balb/c genetic background also attenuated plaque formation.\(^{53}\)

## Conclusions

CD74 deficiency decreased adaptive immune responses to endogenous and exogenous disease-specific antigens, which led to attenuation of atherosclerosis. Surprisingly, CD74 deficiency also led to enhanced immune responses by innate-like B-1 and MZ B cell populations and consequently natural antibody IgM and likely IgG3 production, which also may have contributed to atheroprotection. The mechanisms by which such enhancement of B-1/MZ B cell activation occurs are unclear and will be the subject of future investigation.

## Acknowledgments

We thank Eugenia Shvartz for technical assistance and Joan Perry for editorial assistance.

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## Disclosures

None.

## References


CLINICAL PERSPECTIVE

CD4+ T cells are the main T-cell subtypes in human atherosclerotic lesions, and their activation requires antigen presentation from antigen-presenting cells. Major histocompatibility class II molecules mediate this process in which their folding, intracellular trafficking, and peptide loading require the chaperones from invariant chains called CD74. The present study tested the hypothesis that mice lacking CD74 have reduced atherosclerosis as a result of altered T-cell activation. Using an atherogenic diet–induced mouse atherosclerosis model in low-density lipoprotein receptor–deficient (Ldlr+/−) mice, we found that mice lacking CD74 (Ldlr+/−Cd74+/−) had protection from atherosclerosis. Although Ldlr+/− mice had enhanced atherosclerosis after immunization with proatherogenic heat shock protein-65, Ldlr+/−Cd74+/− mice remained resistant, suggesting an important role of CD74 in adaptive immunity. To our surprise, Ldlr+/−Cd74+/− mice had higher serum autoantibody levels (immunoglobulin M and immunoglobulin G3 against autoantigen malondialdehyde-modified LDL) than Ldlr+/− mice, even without antigen immunization, suggesting enhanced innate-like immunity in mice lacking the invariant chain. Indeed, the absence of CD74 led to increased numbers of marginal zone B cells in the spleen and B1 cells in the peritoneal cavity, where B1 cells are enriched. These cells provide the predominant source of autoantibodies. Given the findings that proatherogenic antigen immunization promotes atherogenesis, increased autoantibody production after autoantigen vaccination reduces atherogenesis, and deficiency of CD74 impairs adaptive immunity but enhances innate-like immunity, this study provides clinical implications that regulation of invariant chain expression or processing can directly affect the progression of atherogenesis.
Deficiency of Antigen-Presenting Cell Invariant Chain Reduces Atherosclerosis in Mice
Jiusong Sun, Karsten Hartvigsen, Meng-Yun Chou, Yadong Zhang, Galina K. Sukhova, Jie Zhang, Marco Lopez-Ilasaca, Cody J. Diehl, Niva Yakov, Dror Harats, Jacob George, Joseph L. Witztum, Peter Libby, Hidde Ploegh and Guo-Ping Shi

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# SUPPLEMENTAL MATERIAL

## Supplemental Tables

### Supplemental Table 1. Scrum lipid levels.

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<th>Genotype</th>
<th>Diet / Immunization</th>
<th>n</th>
<th>Total Cholesterol (mg/dL, mean±SE)</th>
<th>HDL (mg/dL, mean±SE)</th>
<th>LDL (mg/dL, mean±SE)</th>
<th>TG (mg/dL, mean±SE)</th>
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<tr>
<td>LDL&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Chow</td>
<td>9</td>
<td>462.0 ± 33.8</td>
<td>62.1 ± 1.7</td>
<td>399.7 ± 27.8</td>
<td>168.7 ± 35.8</td>
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<td>LDL&lt;sup&gt;−/−&lt;/sup&gt; Cd74&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>10</td>
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<td>278.9 ± 18.3</td>
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<td>0.736</td>
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<tr>
<td>LDL&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Western diet (12 weeks)</td>
<td>10</td>
<td>1087.5 ± 49.2</td>
<td>57.4 ± 5.2</td>
<td>918.7 ± 60.2</td>
<td>380.6 ± 33.0</td>
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<td>LDL&lt;sup&gt;−/−&lt;/sup&gt; Cd74&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>Western diet (26 weeks)</td>
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<td>1503.4 ± 70.3</td>
<td>66.7 ± 6.2</td>
<td>1339.6 ± 84.8</td>
<td>466.0 ± 65.1</td>
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<td>1042.2 ± 127.4</td>
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<td>0.020</td>
<td>0.076</td>
<td>0.037</td>
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<td>LDL&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>PBS**</td>
<td>11</td>
<td>1429.6 ± 64.3</td>
<td>85.6 ± 6.8</td>
<td>1210.3 ± 104.3</td>
<td>641.5 ± 46.5</td>
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<td>LDL&lt;sup&gt;−/−&lt;/sup&gt; Cd74&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>0.381</td>
<td>0.558</td>
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* P < 0.05 was considered significant. Student t test. ** Mice consumed a Western diet for 12 weeks.

### Supplemental Table 2. T cell and B cell percentages (mean ± SE) in splenocytes.

<table>
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<tr>
<th>Genotype</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD6&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt;</th>
<th>I-A&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>17.15±1.45</td>
<td>19.59±0.57</td>
<td>32.18±2.34</td>
<td>54.17±0.99</td>
<td>73.15±1.50</td>
</tr>
<tr>
<td>LDL&lt;sup&gt;−/−&lt;/sup&gt; Cd74&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3.75±0.26</td>
<td>3.03±0.64</td>
<td>34.17±0.24</td>
<td>42.48±0.34</td>
<td>46.40±0.24</td>
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<tr>
<td>P value*</td>
<td>0.024</td>
<td>0.0003</td>
<td>0.646</td>
<td>0.011</td>
<td>0.007</td>
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</table>

* P < 0.05 was considered statistically significant, Student t test.
Supplemental Figures

Supplemental Figure 1. Plasma cytokine levels. Mice consumed a Western diet for 12 and 26 weeks. Plasma samples were prepared and cytokine levels determined by ELISA (Methods). The number of mice in each group is indicated in parentheses. P < 0.05 was considered statistically significant, Mann-Whitney U test.

Supplemental Figure 2. Plasma IgG2b titers that bound to MDA-LDL in Ldr<sup>-/-</sup>Cd74<sup>-/-</sup> and Ldr<sup>-/-</sup> mice pre- and post-immunization with MDA-LDL, FA, or PBS. Each data point is mean ± SE of six mice.
Supplemental Figure 3. Plasma IgG2c titers that bound to MDA-LDL in Ldlr⁻/⁻Cd74⁻/⁻ and Ldlr⁻/⁻ mice pre- and post-immunization with MDA-LDL, FA, or PBS. Each data point is mean ± SE of six mice.

Supplemental Figure 4. Plasma IgG3 titers that bound to MDA-LDL in Ldlr⁻/⁻Cd74⁻/⁻ and Ldlr⁻/⁻ mice pre- and post-immunization with MDA-LDL, FA, or PBS. Each data point is mean ± SE of six mice.