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Deficiency of Antigen Presenting Cell Invariant Chain Reduces Atherosclerosis in Mice

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Abstract

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

Methods and Results—In low-density lipoprotein receptor-deficient mice ($Ldlr^{-/-}$), CD74 deficiency ($Ldlr^{-/-}Cd74^{-/-}$) significantly reduced atherosclerosis and $CD25^{+}$ activated T cells in the atheromata. While $Ldlr^{-/-}Cd74^{-/-}$ mice had decreased levels of plasma IgG1, IgG2b, and IgG2c against malondialdehyde-modified LDL (MDA-LDL), presumably due to impaired APC function, $Ldlr^{-/-}Cd74^{-/-}$ mice showed higher levels of anti-MDA-LDL IgM and IgG3. After immunization with MDA-LDL, $Ldlr^{-/-}Cd74^{-/-}$ mice had lower levels of all anti-MDA-LDL immunoglobulin (Ig) isotypes compared with $Ldlr^{-/-}$ mice. As anticipated, only $Ldlr^{-/-}$ splenocytes responded to in vitro stimulation with MDA-LDL, producing Th1/Th2 cytokines. Heat shock protein-65 (HSP65) immunization enhanced atherogenesis in $Ldlr^{-/-}$ mice, but $Ldlr^{-/-}Cd74^{-/-}$ mice remained protected. Compared with $Ldlr^{-/-}$ mice, $Ldlr^{-/-}Cd74^{-/-}$ mice had higher anti-MDA-LDL autoantibody titers, fewer lesion $CD25^{+}$ activated T cells, impaired release of Th1/Th2 cytokines from APC after HSP65-stimulation, and reduced levels of all plasma anti-HSP65 Ig isotypes. Cytofluorimetry of splenocytes and peritoneal cavity cells of MDA-LDL- or HSP65-immunized mice showed increased percentages of autoantibody-producing marginal zone-B and B-1 cells in $Ldlr^{-/-}Cd74^{-/-}$ mice compared to $Ldlr^{-/-}$ mice.

Conclusion—Invariant chain deficiency in $Ldlr^{-/-}$ mice reduced atherosclerosis. This finding was associated with an impaired adaptive immune response to disease-specific antigens. Concomitantly, there occurred an unexpected increase in the number of innate-like peripheral B-1 cell populations, resulting in increased IgM/IgG3 titers to the oxidation-specific epitopes.

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DISCLOSURES
The authors have declared that no conflict of interest exists.

Keywords

Invariant chain; atherosclerosis; innate immunity; adaptive immunity; autoantibody

INTRODUCTION

Antigen presenting cells (APC) and T cells, key regulators of innate and adaptive immunity, may participate in human atherogenesis. Accumulating data suggest that atherosclerosis involves aspects of autoimmunity.[1] Indeed, autoimmune diseases (e.g., systemic lupus erythematosus [SLE] or rheumatoid arthritis) may accelerate atherosclerosis. SLE patients have a four- to eight-fold higher risk of developing atherosclerosis than those who do not have SLE.[2] Patients with rheumatoid arthritis also have enhanced risk of atherosclerotic events.[3] In general, T cells that mediate adaptive responses appear to play a pro-atherogenic role, and in some experimental models, T cell deficiency produces up to an 80% reduction of atherosclerosis.[4] However, emerging evidence also suggests that certain T cell populations, such as T regulatory subtypes, may confer atheroprotective properties.[5] 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 LDL (oxLDL), heat shock proteins (HSP), and β2-glycoprotein-1 appear prominent.[6] For example, B cell deficiency[7] or splenectomy,[8] 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 low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) rabbits and mice[9–11] or apolipoprotein E-deficient (*Apoe*$^{-/-}$)[12] mice with malondialdehyde-modified LDL (MDA-LDL) suppressed atherogenesis. 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.[9–12] Among these, innate IgM natural antibodies against "oxidation-specific" epitopes of oxLDL appear prominent.[13] 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 (PC) head group of oxidized phospholipids (oxPL), as found on oxLDL but not on native LDL. It bears the T15 idiotype, which binds the PC moiety present on the cell wall of *Streptococcus pneumoniae*, as shown previously.[14] Indeed, immunization of cholesterol-fed *Ldlr*$^{-/-}$ mice with heat-inactivated *S. pneumonia* led to a near monoclonal increase in plasma E06/T15 titers and atheroprotection.[15] In *Apoe*$^{-/-}$ mice, treatment with an IgM with the T15 idiotype reduced vein graft atherosclerosis.[16] 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 anti-atherogenic properties.[17] Indeed, plasma IgM antibodies to oxLDL correlate inversely with carotid and coronary atherosclerosis in humans.[14,18,19] 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,[14,18] whereas immunization with oxLDL epitopes in murine models that generate elevations of IgG, particularly IgG1, usually associate with atheroprotection. Such IgG1 usually associates 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 Fcγreceptor IIb. In contrast, immunization with other disease-related epitopes, such as HSP65, has 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.

Adaptive immunity to disease-specific antigens and associated APC antigen presentation and T cell activation may directly play detrimental roles in atherosclerosis.[4] Within APC, antigens must undergo full processing and presentation by major histocompatibility class II (MHC-II) molecules to $CD4^+$ T cells. In human APC, 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 the MHC-II, ensuring that in the endoplasmic reticulum all MHC-II Aαand Aβ heterodimers associate with invariant chains. In the endoplasmic reticulum, CD74 functions as a chaperone to assist the MHC-II complex to fold properly, to prevent the early binding of peptides to MHC-II, and to determine 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.[20] If CD74 is incompletely processed due to the inhibition or absence of protease activities, MHC-II will fail to present antigens to the APC surface. These APC 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.[21] In the case of complete absence of CD74 ($Cd74^{-/-}$) in C57BL/6 mice, MHC-II $A\alpha^b$ $A\beta^b$ is predominantly expressed as free $A\alpha^b$ and $A\beta^b$ chains in APC, confirming that $A\alpha^b$ $A\beta^b$ assembly also depends strongly on CD74 co-expression.[22] 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.[23–25] 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 number of mature B cells compared to wild-type control mice, suggesting that B cell development requires neither CD74 nor MHC-II.[26] $CD4^+$ cells comprise the predominant T lymphocyte type in human and murine atherosclerotic lesions,[27,28] and their activation contributes to experimental atherogenesis. In mice, absence of $CD4^+$ T cells significantly reduced atherosclerosis.[29] Adoptive transfer of $CD4^+$ T cells isolated from atherosclerotic $Apoe^{-/-}$ mice into T cell-deficient $Apoe^{-/-}/scid/scid$ mice restored the atherosclerosis phenotypes.[30,31] In $Apoe^{-/-}/scid/scid$ mice, reconstitution of antigen (oxLDL)-specific $CD4^+$ T cells yielded even more atherosclerosis than in mice that received naive $CD4^+$ T cells,[31] an observation of particular interest since nearly 10% of the T cell clones from human atherosclerotic plaques responded to oxLDL.[32] Thus, both APC and T cells modulate human and mouse atherogenesis.

This study tested the hypothesis that APC 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)[26] were crossbred with $Ldlr^{-/-}$ mice (C57BL/6, Jackson Laboratories, Bar Harbor, ME) to generate both $Ldlr^{-/-}Cd74^{-/-}$ and $Ldlr^{-/-}$ mice. Male $Ldlr^{-/-}Cd74^{-/-}$ and $Ldlr^{-/-}$ 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 and oil red-O staining and lesion area calculation, lesion macrophage (Mac-3) 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) immunostaining, and detection of lesion-activated T cells used an anti-CD25 monoclonal antibody (1:70, Pharmingen). Lesion $CD8^+$ T cells, NK1.1 T cells, $CD1d^+$ cells, and dendritic cells were detected using rat anti-mouse CD8 monoclonal (1:100, Pharmingen), mouse NK1.1 monoclonal (PK136, 1:250, Novus Biologicals, Littleton, CO), mouse CD1d monoclonal (1:100, AbD Serotec), and hamster anti-mouse CD11c (1:50, Pharmingen), respectively. All murine studies were pre-approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Immunogen preparation

Human LDL and MDA-LDL were prepared as previously described[34] and contained <1.5 ng lipopolysaccharides/mg protein (ApoB). MDA modification of bovine serum albumin (MDA-BSA) was carried out similarly to that for MDA-LDL, except the reaction continued for 24 hrs instead of 3 hrs. Recombinant *Mycobacterium bovis* HSP65 was expressed in *E. coli* and purified to >95% as fully described previously.[35]

MDA-LDL immunization

Eight-week-old $Ldlr^{-/-}$ and $Ldlr^{-/-}Cd74^{-/-}$ mice received intrainguinally the primary immunization with 50 μg human MDA-LDL in 50 μl PBS emulsified in an equal volume of complete Freund's adjuvant (CFA, Sigma). Mice were boosted intraperitoneally with 25 μg of antigen in incomplete Freund's adjuvant (IFA) after 2 and 4 weeks. CFA (or IFA)/PBS and PBS alone were used for separate immunization controls. Mice then received a recall boost intraperitoneally with 25 μg of antigen in PBS. Three days after the recall boost, plasma samples were collected for antigen-specific antibody titer measurement and splenocytes isolated for antigen recall assay.[11]

HSP65 immunization

Eight-week-old $Ldlr^{-/-}$ and $Ldlr^{-/-}Cd74^{-/-}$ mice were fed a Western diet while immunized with 25 μg of HSP65 in 12.5 μl PBS to the hind footpad emulsified in an equal volume of IFA. Mice were boosted twice with the same amount of antigens to the alternate hind footpad 3 and 6 weeks following the primary immunization. Twelve weeks after the first immunization, aortic arches were harvested for atherosclerotic lesion characterization and splenocytes and lymph node cells collected for antigen recall assay as described previously.[21,35] IFA and PBS immunizations served as experimental controls.

Plasma lipoprotein and cytokine determination

Plasma lipoproteins, including total cholesterol, LDL, high-density lipoprotein (HDL), and triglyceride, were determined using enzymatic assay kits according to the manufacturer's instructions (Pointe Scientific, Inc.). Plasma cytokines, including interleukin-2 (IL2) (BD Biosciences), IL4, IL5 (Pierce Endogen), IL10, and interferon-γ(IFN-γ) (PeproTech Inc.), were measured using ELISA kits according to the manufacturer's instructions.

Plasma antibody isotype determination

Plasma antibody titers to native LDL, MDA-LDL, MDA-BSA, and HSP65 were determined by chemiluminescent ELISA as previously described.[11,15] In brief, 96-well MicrotiterR plates (Thermo Labsystems, Franklin, MA) 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 incubated for 1.5 hrs at room temperature. Bound plasma Ig isotypes were detected with various anti-mouse Ig isotype-specific alkaline phosphatase conjugates, LumiPhos 530 (Lumigen, Southfield, MI) solution, and a Dynex Luminometer (Dynex Technologies,

Chantilly, VA). The following goat alkaline phosphatase-conjugated secondary Ig isotype-specific antibodies were used: anti-mouse IgM (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 (RLU/100 ms).

Antigen recall assay

Splenocyte and lymph node cell preparation and antigen recall assays were performed as described previously.[21] 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/well) with human native LDL or MDA-LDL at various concentrations in RPMI for 3 days followed by determination of sentinel Th1 and Th2 cytokines in the medium. Splenocytes and lymph node cells from HSP65-immunized *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice were stimulated with different concentrations of purified HSP65 for 3 days followed by cytokine measurement as above.

FACS

At time of sacrifice, peritoneal cavity cells and splenocytes were prepared and resuspended in a staining buffer containing PBS and 1% BSA. After blocking with an anti-Fcγreceptor monoclonal antibody for 15 min at 4°C, 10^6 cells were stained with antibodies (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 min at 4°C. More than 0.5×10^5 cells per sample were analyzed. Peritoneal B-1 cells were identified as CD19$^+$/CD11b$^+$/CD5$^{lo\text{-}to\text{-}int}$ and B-2 cells 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 the statistical significance, depending on the normality of the data sets. $P<0.05$ was considered statistically significant. Bonferroni post-hoc correction was used when multiple comparisons were made in order to minimize Type I error; the corrected P value is indicated in the respective figures. 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, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice developed significantly smaller atherosclerotic lesions than did *Ldlr*$^{-/-}$ mice. In the aortic arches, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had reduced lesion grade (Figure 1A) and intima area (Figure 1B) compared with *Ldlr*$^{-/-}$ mice at both time points. In thoracic-abdominal aortas, oil red-O staining demonstrated that *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had significantly smaller aortic lipid depositions or lesion areas than did *Ldlr*$^{-/-}$ mice at the 26-week time point (Figure 1C). *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had significantly reduced several lesion inflammatory cell contents, including Mac-3$^+$ macrophage-positive area and CD4$^+$ T cell numbers and absence of NK1.1$^+$ T cells, compared with *Ldlr*$^{-/-}$mice at either 12-week or 26-week time point (Figure 1D-F), although we did not detect significant changes of CD1d-positive area, CD8$^+$ T cells, or lesion dendritic cells (Figure 1G-I).

Consistent with our hypothesis, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had greatly reduced lesional content of CD25^{+} positive cells, most likely consistent with decreased presence of activated T cells compared with *Ldlr*$^{-/-}$ mice (Figure 1J).

Plasma lipid levels often associate with atherosclerotic lesion progression.[33] On a chow diet, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had plasma total cholesterol levels similar to *Ldlr*$^{-/-}$ mice, though LDL cholesterol levels were slightly lower (Suppl Table 1). After 12 and 26 weeks of an atherogenic diet, the two groups had significantly elevated yet equal levels of total and LDL cholesterol. However, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had significantly lower plasma triglyceride levels at both time points but also lower HDL cholesterol at the 26-week time point. It is possible that reduced plasma triglyceride levels in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice were secondary to attenuated atherosclerosis of these mice.

Plasma cytokine ELISA did not reveal significant differences in IFN-γ, IL5, and IL10 levels between *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ and *Ldlr*$^{-/-}$ mice at both the 12- and 26-week time points. However, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had higher plasma IL4 levels than *Ldlr*$^{-/-}$ mice at both time points (Suppl Figure 1), suggesting a Th2 bias in atherosclerotic CD74-deficient mice.

Plasma anti-MDA-LDL antibody isotypes

In humans and mice, plasma IgG antibodies against oxLDL often correlate positively with lesion development, whereas IgM antibodies correlate negatively.[11,18,36] We anticipated lower titers of adaptive anti-MDA-LDL IgG1, IgG2b, and IgG2c levels in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice than in *Ldlr*$^{-/-}$ mice. Consistent with this hypothesis, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$mice had lower anti-MDA-LDL IgG2b and IgG2c titers than did *Ldlr*$^{-/-}$ mice at both the 12- and 26-week time points (Figure 2). *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ 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.[26] 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 are 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 due to impaired antigen presentation, while 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 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-Class II restricted.[11] Before immunization, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had similar adaptive anti-MDA-LDL Ig levels but higher innate IgM and IgG3, compared to *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 panels) as previously reported,[10,11] but *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice showed no increase at all (right panels). 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 (Suppl Figures 2–4). In contrast, in response to immunization, IgM titers to MDA-LDL showed a modest increase in the *Ldlr*$^{-/-}$ mice (Figure 3B, left panels), which was also observed in the *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice (right panels). We also tested responses to human LDL (the heterologous carrier) and to MDA-BSA (the hapten), and identical IgM and IgG isotype antibody responses, as described above to MDA-LDL, were noted (data not shown). These data affirm an important role of CD74-mediated antigen presentation to disease specific antigens (i.e., 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.

CD74 deficiency not only led to impaired adaptive antibody production in vivo, but also resulted in reduction of Th1 and Th2 cytokine production in vitro. Although we did not detect significant differences in plasma Th1 (IFN-γ) and Th2 (IL4, IL5, IL10) cytokine levels between MDA-LDL-immunized *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (data not shown), we did detect lower medium Th1 and Th2 cytokine levels (IL2, IFN-γ, IL4, IL5, and IL10) in splenocytes from MDA-LDL-immunized *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice than those from *Ldlr*$^{-/-}$ mice in an MDA-LDL recall assay (Figure 4). Even when native human LDL served as a stimulus, *Ldlr*$^{-/-}$ splenocytes released more IL2 and IL5 than those of *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice (Figure 4). These observations with the male *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice appear different, at least with respect to IL5, from what we observed previously in immune-competent mice following immunization with murine MDA-LDL,[11] in which absence of IL5 enhanced atherosclerosis in female *Ldlr*$^{-/-}$ mice and atheroprotective immunization of female *Ldlr*$^{-/-}$ mice with MDA-LDL increased serum IL5 levels. However, the atheroprotective phenotypes in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice, either immunized with MDA-LDL or not, did not accompany concomitant increase of serum IL5 (data not shown). Although the exact cause of these unexpected observations remains unknown, gender differences between the studies may have influenced the outcomes.

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 cell 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[37] and lesion calcification,[38] and atherosclerotic plaques contain activated CD4^{+} T cells against human HSP60.[39] In contrast to reduced atherosclerosis in *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice after immunization with MDA-modified homologous LDL,[10–12] immunization of hyperlipidemic *Ldlr*$^{-/-}$ mice and rabbits[40–42] with heterologous *Mycobacterial* HSP65 aggravated atherosclerosis. In rabbits, plasma titers against HSP65 have been shown to increase rapidly after initiation of an atherogenic diet[43] and increased HSP65 expression in intimal cells and the presence of HSP65-specific T cells in blood and in atherosclerotic lesions have been documented,[42] 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 those of *Ldlr*$^{-/-}$ mice (Figure 5A/B). *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice also had fewer lesion CD4^{+} T cells and CD25^{+} activated T cells than did *Ldlr*$^{-/-}$ mice (Figure 5C/D). Notably, HSP65 immunization increased significantly lesion grade, as was originally reported,[41] and lesion CD4^{+} T cells in *Ldlr*$^{-/-}$ mice were also more than those immunized with PBS (Figure 5B/C). In contrast, HSP65 immunization did not change these lesion parameters in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice. In HSP65-immunized mice, we did not detect any significant differences in plasma lipoprotein levels (total cholesterol,

LDL, HDL, and triglyceride) (Suppl Table 1) and Th1 (IFN-γ) or Th2 (IL4, IL5, IL10) cytokine levels between the two 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 to *Ldlr*$^{-/-}$mice (Figure 6). Non-immunized 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 IgG3, to an extent – are largely considered as products of B-1/MZ B cells.[44] 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 utilized FACS to analyze cell preparations from the spleens and the peritoneal cavities, which are rich in B-1 cells.[44] Although the spleens of *Ldlr*$^{-/-}$*Cd74*$^{-/-}$mice had fewer B cells than did *Ldlr*$^{-/-}$ mice (Suppl Table 2), they had a significantly higher percentage of both B-1 cells and MZ B cells than did those from *Ldlr*$^{-/-}$ mice, regardless of whether mice were immunized with PBS, MDA-LDL, or HSP65 (Figure 8A/B). These data coincide with the observations of enhanced plasma IgM anti-MDA-LDL natural antibodies (Figures 2/3). In contrast, *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice had fewer follicular B cells than *Ldlr*$^{-/-}$ mice, consistent with the impaired T cell activation in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$mice after MDA-LDL (Figure 3) or HSP65 (Figure 6) immunization. Similar to the spleen, peritoneal cavities in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice contained significantly higher B-1 cells and lower B-2 cells than those in *Ldlr*$^{-/-}$ mice under all tested immunization conditions (Figure 8C/D).

DISCUSSION

There is 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.[21] 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 (e.g. 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 impact 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 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-A^b) on APC of splenic cells, markedly decreased $CD4^+$ 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, as 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 (Suppl Table 2), 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 reflective of 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 restricted $NK1.1^+$ T cell activation and impaired glycolipid α-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 lesion from *Ldlr*$^{-/-}$*Cd74*$^{-/-}$mice (Figure 1E), although a 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 also 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 of peripheral total $B220^+$ B cell numbers compared with those from *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^b) expression, found on professional APCs (such as dendritic cells, macrophages and B cells), also fell significantly, consistent with reduced B cell number (Suppl Table 2) as well as enhanced MHC class II retention within the intracellular organelles due to 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. In turn, this would explain the absent generation of plasma IgG1, IgG2b, and IgG2c titers against MDA-LDL (Figure 3 and Suppl Figures 2/3) and HSP65 (Figure 6) in the antigen-immunized *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice compared to the increases observed in the *Ldlr*$^{-/-}$ mice. Presumably, this also explains the greatly blunted IgG responses to MDA-LDL in the cholesterol-fed *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice as well.

Third, it is not clear what role such IgG titers to epitopes of oxLDL might play, as their impact may be complex as noted in the Introduction. 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 are also associated with marked increases in Th2 biased IgG1 titers and atheroprotection.[4,14,18,27] Further, some studies have shown that direct infusion of such antibodies is associated with atheroprotection.[48] In contrast, there is 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 also discussed in the Introduction.[11,13,14,19] IgM, as well as 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, as well as IgG3 titers to MDA-LDL were actually increased in the *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice, likey 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 current study is consistent with the notion that increased circulating IgM protect mice from atherogenesis.[14]

In non-infected mice, plasma IgM are thought to predominantly represent the products of innate-like B-1 cells and MZ B cells.[49] IgG3 are also products of these cells though it is not clear how much they contribute to basal titers. In our study, both IgM and IgG3 increased even as other IgG isotypes decreased to disease related oxidation epitopes. Consistent with this, CD74 deficiency was associated with increased percentage of peritoneal 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 to those in *Ldlr*$^{-/-}$mice (Suppl Table 2). 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, i.e., CD4^{+} T cells from *Cd74*$^{-/-}$ mice released high amounts of IFN-γ, but not IL4, 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. Following either immunization protocol, splenocytes and/or lymph node cells from *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice responded poorly to MDA-LDL, native LDL, or HSP65 in IL2 and IFN-γproduction. In contrast, splenocytes from *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice responded to MDA-LDL or LDL by releasing IL4 (Figure 4) and responded to HSP65 by producing IL4, IL5, and IL10 (Figure 7). One explanation asserts that HSP65, like human HSP60, induced preferentially 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.[11] Preferential Th2 response and reduced atherosclerosis in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice also concurs with prior studies that pro-inflammatory Th1 response favors atherosclerotic plaque formation, whereas the Th2 response and enhanced IgG1 humoral immunity was associated with reduced atherogenesis.[52] Altered Th1/Th2 balance toward Th2, such as in Balb/c genetic background atherosclerosis-prone mice, also attenuated plaque formation.[53]

In conclusion, 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 consequent natural antibody IgM and likely IgG3 production, which may have contributed to atheroprotection as well. The mechanisms by which such enhancement of B-1/MZ B cell activation occurs are unclear and will be the subject of future investigation.

Clinical Perspective

CD4^{+} T cells are the main T cell subtypes in human atherosclerotic lesions and their activations require antigen presentation from antigen-presenting cells. Major

histocompatibility class II (MHC-II) molecules mediate this process in which their folding, intracellular trafficking, and peptide loading require the chaperones from invariant chains, called CD74. This current study tested the hypothesis that mice lacking CD74 have reduced atherosclerosis due to altered T cell activation. Using an atherogenic diet-induced mouse atherosclerosis model in low-density lipoprotein receptor-deficient mice (*Ldlr*$^{-/-}$), we found that mice lacking CD74 (*Ldlr*$^{-/-}$ *Cd74*$^{-/-}$) had protection from atherosclerosis. While *Ldlr*$^{-/-}$ mice had enhanced atherosclerosis after immunization with pro-atherogenic heat shock protein-65 (HSP65), *Ldlr*$^{-/-}$ *Cd74*$^{-/-}$ mice remained resistant, suggesting an important role of CD74 in adaptive immunity. To our surprises, *Ldlr*$^{-/-}$ *Cd74*$^{-/-}$ mice had higher serum autoantibody levels (IgM and IgG3 against autoantigen malondialdehyde-modified LDL, MDA-LDL) than those from *Ldlr*$^{-/-}$ mice, even without antigen immunization, suggesting enhanced innate-like immunity in mice lacking the invariant chain. Indeed, absence of CD74 led to increased numbers of marginal zone B cells in the spleens and B1 cells in the peritoneal cavity, where B1 cells are enriched. These cells provide the predominant sources of autoantibodies. Given the findings that pro-atherogenic 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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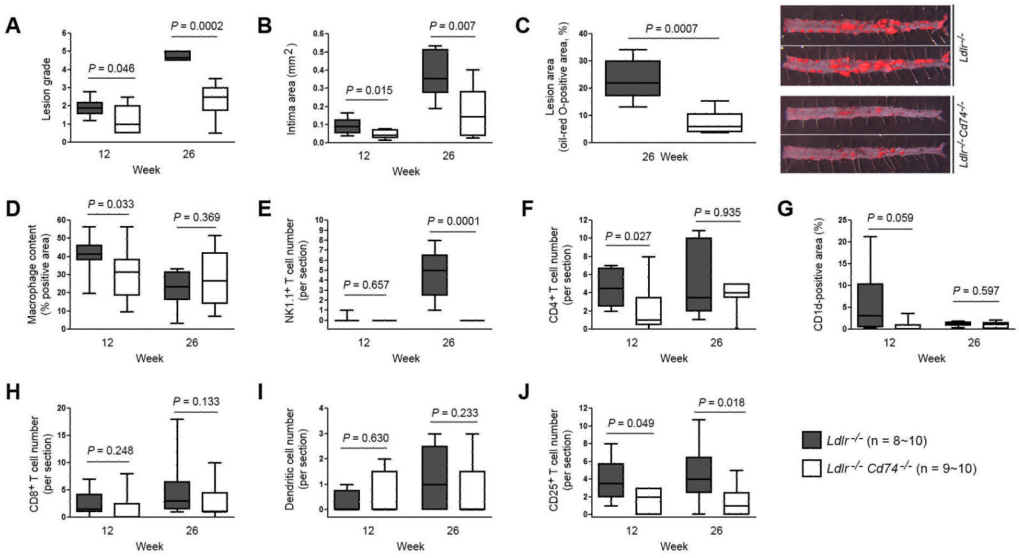

Figure 1.

Atherosclerotic lesion characterizations in *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ mice that consumed an atherogenic diet for 12 and 26 weeks, including aortic arch lesion grade (**A**), intima area (**B**), thoracic-abdominal aorta lipid deposition with representative aortas shown to the right panels (**C**), lesion Mac-3^{+} macrophage-positive area (**D**), NK-1.1^{+} T cell number (**E**), CD4^{+} T cell number (**F**), CD1d-positive area (**G**), CD8^{+} cell number (**H**), CD11c^{+} dendritic cell number (**I**), and CD25^{+} activated T cell number (**J**). Mann-Whitney *U* test. $P<0.05$ was considered statistical significant. The number of mice in each group is indicated in parentheses.

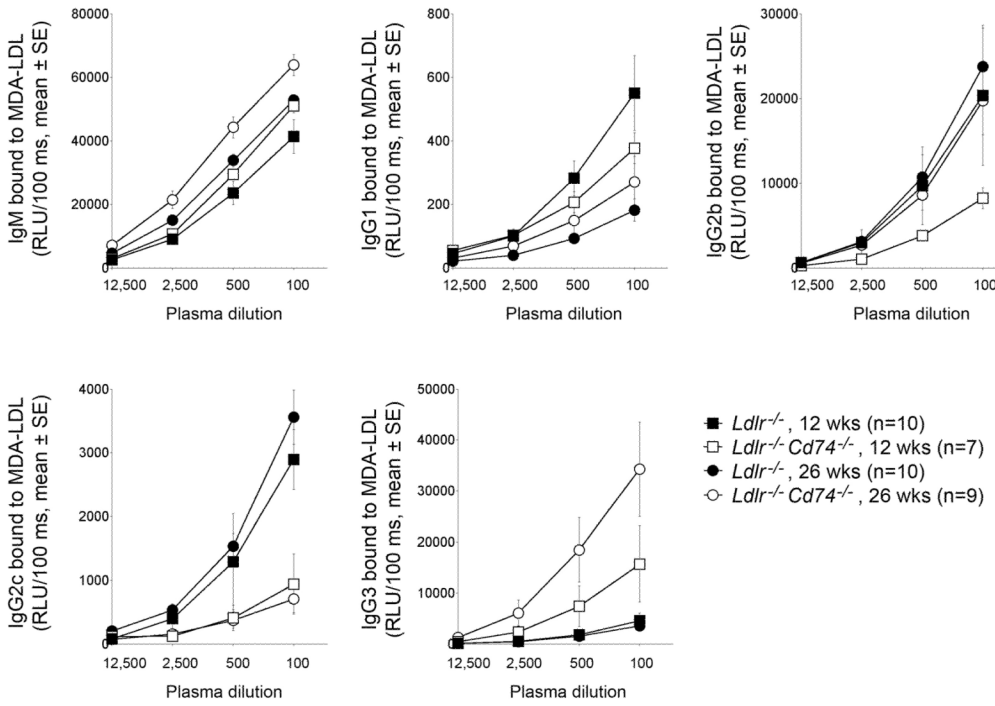

Figure 2.

Anti-MDA-LDL antibody titers (mean ± SE) in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ and *Ldlr*$^{-/-}$ mice that consumed an atherogenic diet for 12 and 26 weeks. The number of mice in each group is indicated in parentheses. No statistical test was performed.

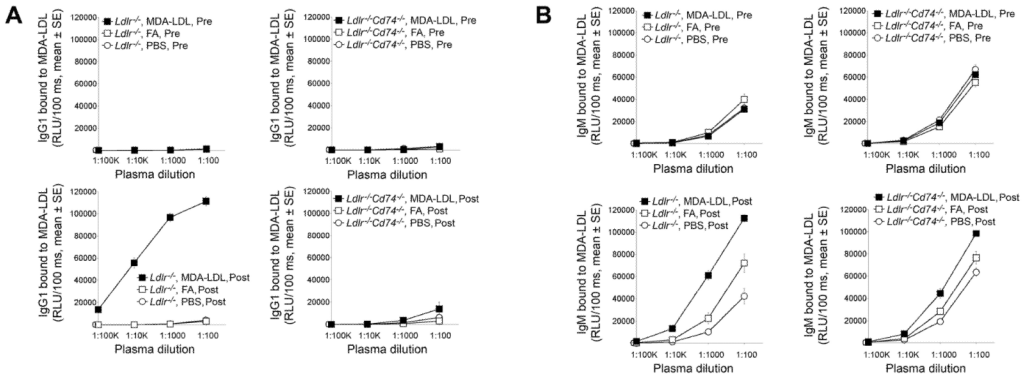

Figure 3.

Plasma IgG1 (**A**) and IgM (**B**) titers that bound to MDA-LDL in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$and *Ldlr*$^{-/-}$ mice pre- and post-immunization with MDA-LDL, IFA, or PBS. Each data point is mean ± SE of six mice. No statistical test was performed.

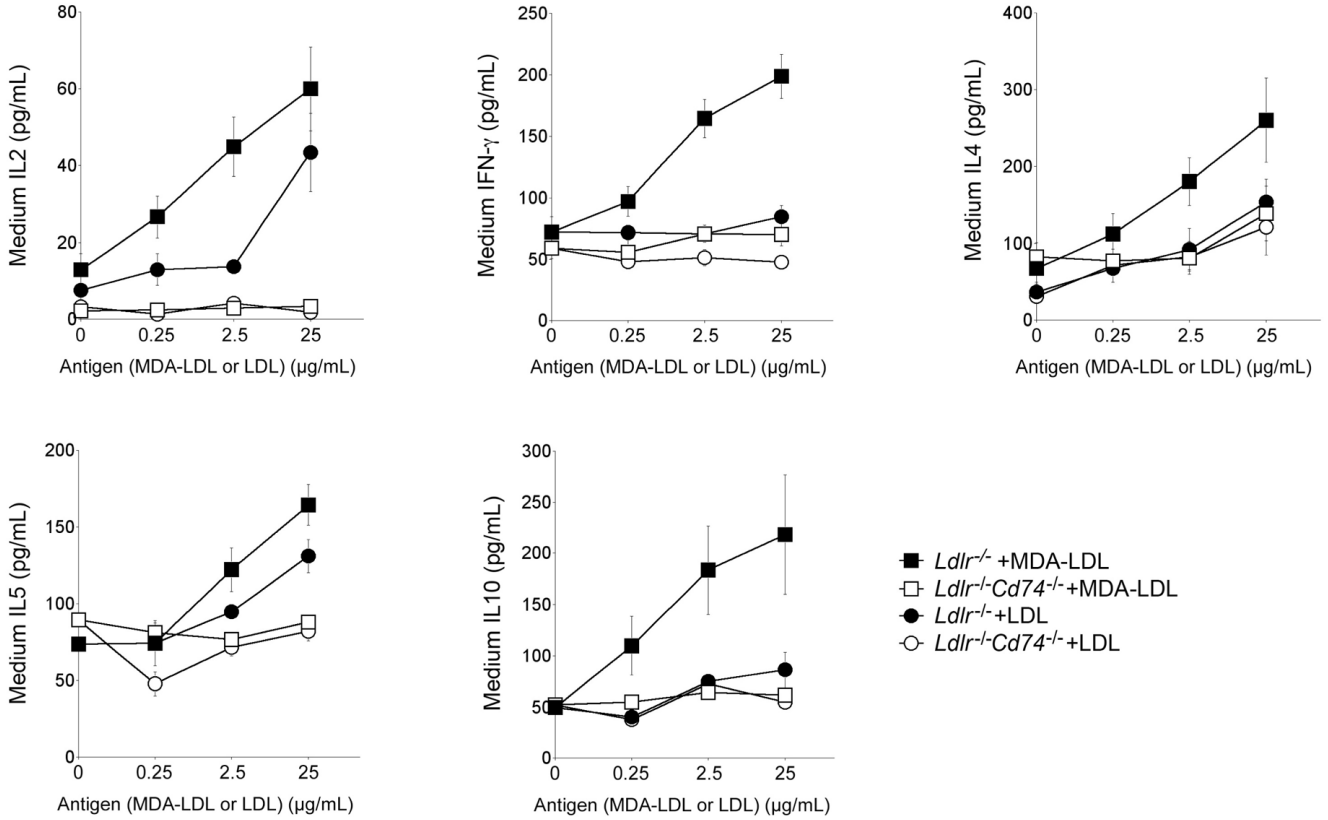

Figure 4.

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 six mice. No statistical test was performed.

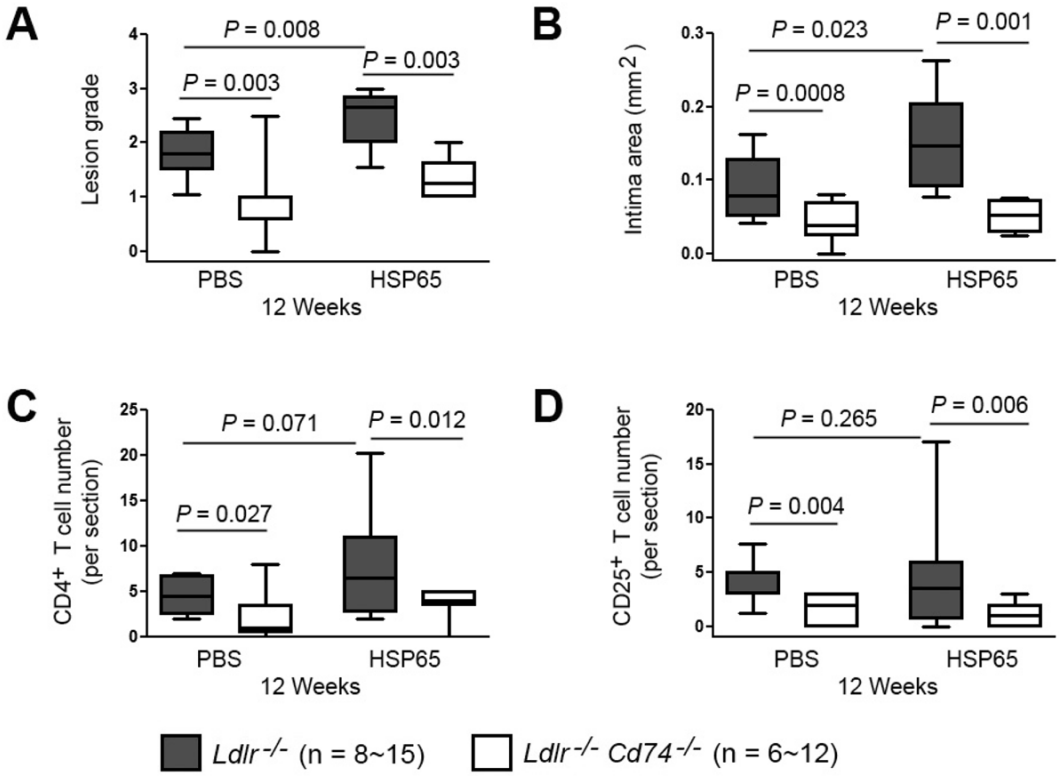

Figure 5.

Aortic arch lesion grade (**A**), intima area (**B**), lesion $CD4^+$ T cell number (**C**), and $CD25^+$ activated T cell number (**D**) in $Ldlr^{-/-}$ and $Ldlr^{-/-}Cd74^{-/-}$ mice immunized with PBS or HSP65 while consuming an atherogenic diet for 12 weeks. The number of mice in each group is indicated in parentheses. Mann-Whitney *U* test followed with Bonferroni post-hoc correction. $P < 0.016$ was considered statistical significant.

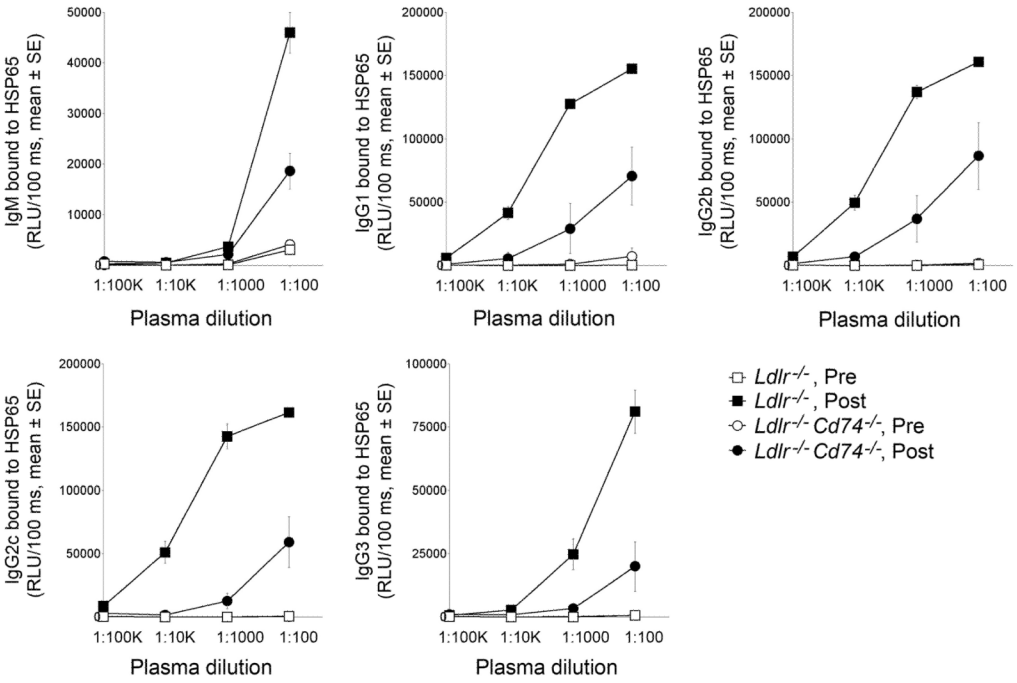

Figure 6.

Antibody levels to HSP65 in *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ (n=6) and *Ldlr*$^{-/-}$ (n=11) mice pre-(8 weeks old) and post-HSP65 immunization (mean ± SE). All mice consumed an atherogenic diet for 12 weeks after initial immunization and before harvesting. No statistical test was performed.

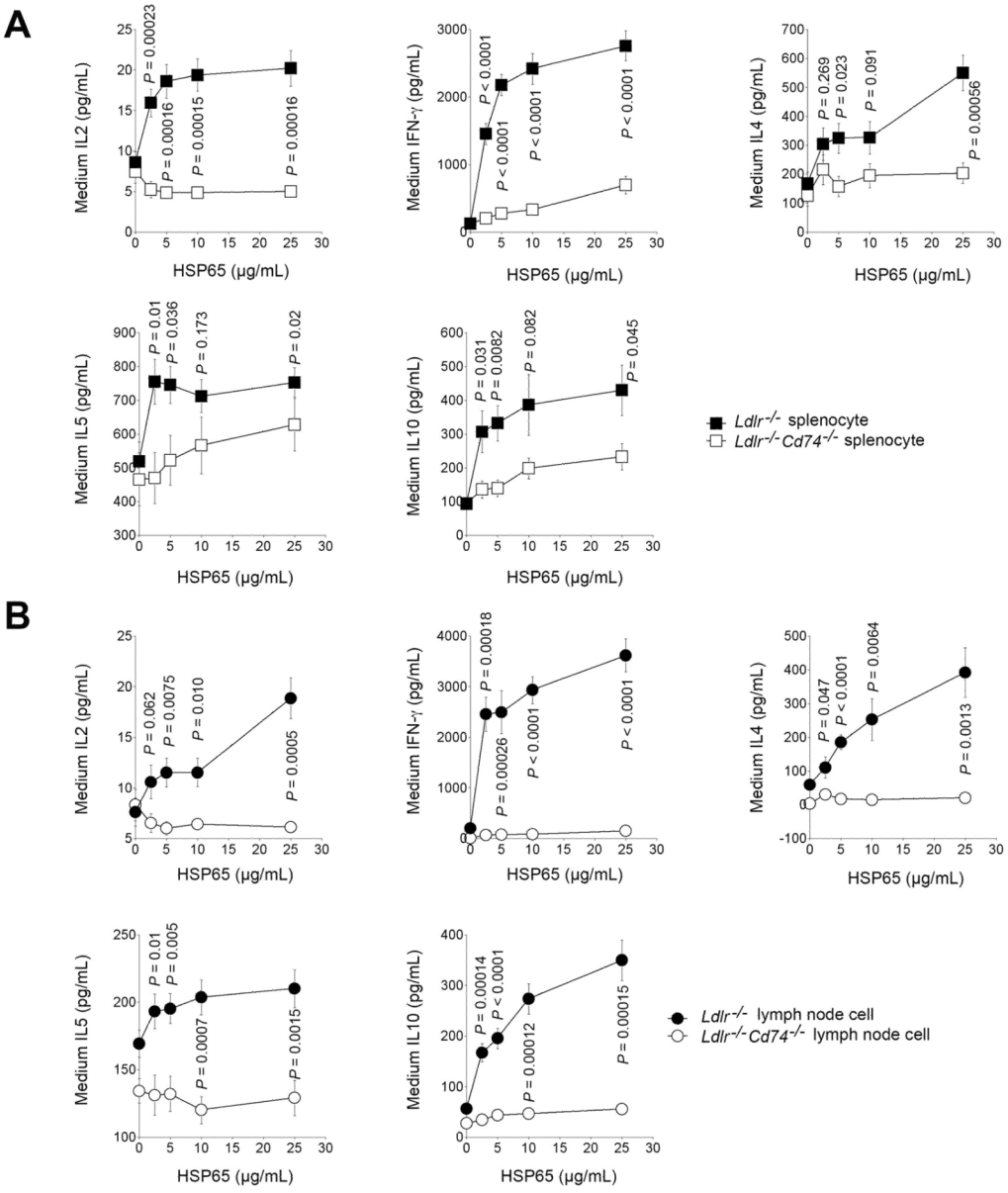

Figure 7.

HSP65 antigen recall assays of splenocytes and lymph node cells from HSP65-immunized *Ldlr*$^{-/-}$*Cd74*$^{-/-}$ (n=6) and *Ldlr*$^{-/-}$ (n=10) mice. Data are presented as mean ± SE. Unpaired student's *t* test followed with Bonferroni post-hoc correction. P < 0.0125 was considered statistical significant.

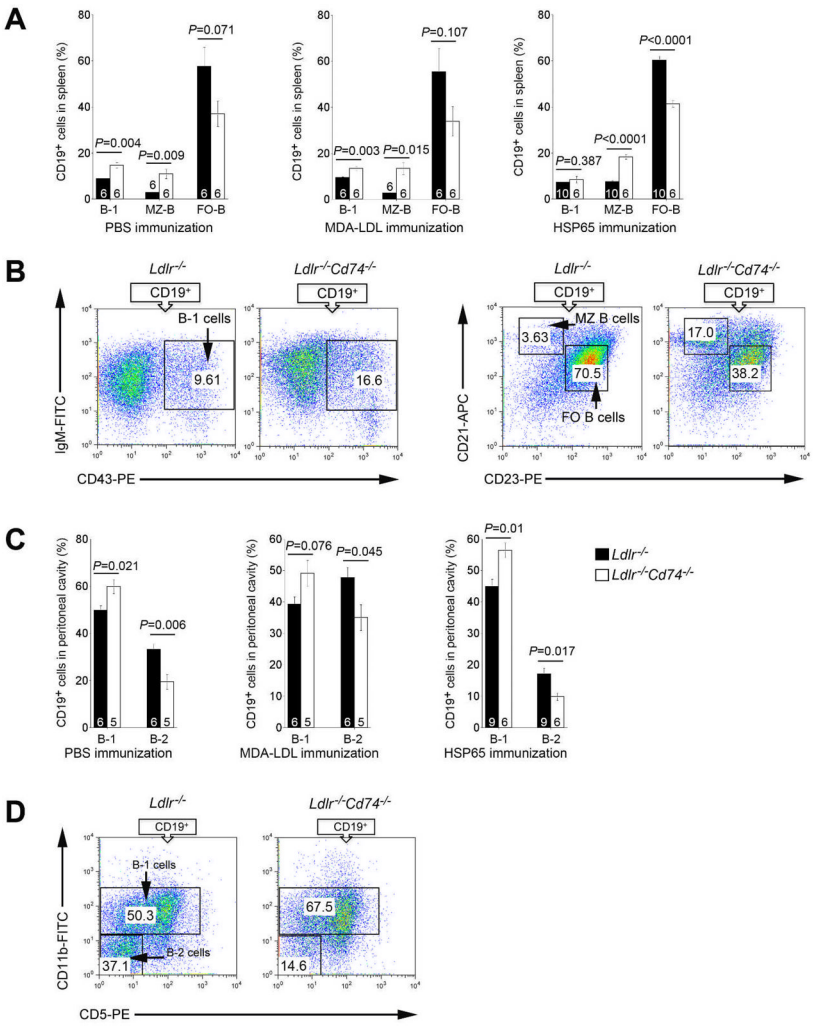

Figure 8.

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$^-$ marginal zone-B cells (MZ-B), 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, and data are mean±SE. Student *t* test, $P<0.05$ was considered statistical 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.