Apolipoprotein A-I and its Role in Lymphocyte Cholesterol Homeostasis and Autoimmunity

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Objective—The purpose of this study was to determine the effects of an atherogenic diet on immune function in LDLr\(^{-/-}\), ApoA-I\(^{-/-}\) mice.

Methods and Results—When LDLr\(^{-/-}\), ApoA-I\(^{-/-}\) (DKO), and LDLr\(^{-/-}\) (SKO) mice were fed an atherogenic diet, DKO had larger peripheral lymph nodes (LNs) and spleens compared to SKO mice. LNs were enriched in cholesteryl and contain expanded populations of T, B, dendritic cells, and macrophages. Expansion of all classes of LN cells was accompanied by a \(\approx 1.5\)-fold increase in T cell proliferation and activation. Plasma antibodies to dsDNA, \(\beta_2\)-glycoprotein I, and oxidized LDL were increased in DKO, similar to levels in diet-fed Faslpr/lpr mice, suggesting the development of an autoimmune phenotype. Both LN enlargement and cellular cholesterol expansion were “prevented” when diet-fed DKO mice were treated with helper dependent adenovirus expressing apoA-I. Independent of the amount of dietary cholesterol, DKO mice consistently showed lower plasma cholesterol than SKO mice, yet greater aortic cholesterol deposition and inflammation.

Conclusions—ApoA-I prevented cholesterol-associated lymphocyte activation and proliferation in peripheral LN of diet-fed DKO mice. A \(\approx 1.5\)-fold increase in T cell activation and proliferation was associated with a \(\approx 3\)-fold increase in concentrations of circulating autoantibodies and \(\approx 2\)-fold increase in the severity of atherosclerosis suggesting a common link between plasma apoA-I, inflammation, and atherosclerosis. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: ApoA-I ■ cholesterol ■ lymphocytes ■ lymph node ■ atherosclerosis

High concentrations of plasma high-density lipoproteins (HDL) are a well-established negative risk factor for coronary heart disease (CHD). Apolipoprotein A-I (apoA-I), which makes up approximately 70% of HDL protein, is secreted by the liver and intestine and is essential for HDL formation and function. The formation of HDL depends on the ATP binding cassette transporter A1 (ABCA1) which effluxes cholesterol onto apoA-I whereas ABCG1, another member of the ABC transporter family, primarily effluxes cholesterol to HDL particles. Recent studies demonstrate that HDL apoA-I is an anti-inflammatory mediator modulating the progression of atherosclerosis through immune cell function. A direct link between immune cell function and lipoprotein metabolism was recently demonstrated when lymphotoxin and LIGHT produced by T cells were found to regulate plasma triglyceride levels. Moreover, monocyte populations may take on dendritic cell (DC)-like characteristics, migrate into an atherosclerotic lesion, become cholesteryl enriched, and migrate out, contributing to the stability of the plaque. Interestingly, hyperlipidemic apoE\(^{-/-}\) and LDLr\(^{-/-}\) mice show reduced migration of DCs between skin and LNs, but when HDL or apoA-I was administered normal migration was restored.

Based on these associations, it has been speculated that HDL apoA-I and autoimmunity are linked. Humans with autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and mouse models of these disorders are associated with increased atherosclerosis, with SLE and RA patients showing decreased HDL levels when compared to control subjects. The correlation among autoimmune disease, atherosclerosis, and plasma HDL apoA-I raises the possibility of a common link involving all 3 factors. We therefore undertook the current studies to investigate this link and found that apoA-I can modulate immune cell function by regulating cellular cholesterol balance, which in turn prevents LN cell expansion, activation, and the progression of atherosclerosis.
**Materials and Methods**

LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> (DKO), LDLr<sup>−/−</sup> (SKO), and B6.MRL-Faslpr/J (Faslpr/lpr) mice were fully backcrossed into the C57BL/6 background. All mice were housed at the Wake Forest University Medical Center. The Wake Forest University Medical Center Committee for Animal Care and Use approved all procedures. For a more detailed description of Materials and Methods see the material (available online at http://atvb.ahajournals.org).

**Results**

Cholesterol Accumulation and Expansion in Peripheral Lymph Nodes

After 10 weeks on an atherogenic diet, DKO mice acquired enlarged peripheral LNs, compared to SKO mice, shown in Figure 1A and 1A’, respectively. The enlargement of the DKO LNs was associated with increased Oil Red O staining.
(Figure 1C and 1C′), not seen in SKO mice. When fed an atherogenic diet for longer than 10 weeks, DKO mice develop skin lesions attributable to excessive scratching.13,14 To reduce the contribution of “open lesions” to the current studies, mice were fed diet for 10 weeks, at which time external skin abnormalities were not present as shown in supplemental Figure I. Although Figure 1 shows only brachial LNs, inguinal, axillary, and superficial cervical LNs were also enlarged in diet-Fed DKO mice. LNs from DKO weighed an average of 9.6±0.8 mg, while SKO LNs weighed 2.2±0.5 mg (n=5 to 10 mice per genotype, P<0.05).

Autoimmune disorders typically show enlargement of peripheral LN accompanied by immune cell expansion and the production of autoantibodies. In a mouse model of autoimmune, Faslpr/lpr, enlargement of both LNs and spleen15,16 production of autoantibodies. In a mouse model of autoimmune, peripheral LN accompanied by immune cell expansion and the production of autoantibodies. In a mouse model of autoimmune, Faslpr/lpr, enlargement of both LNs and spleen15,16 production of autoantibodies.

Although a large number of specific T cell subsets were counted, only DNT cells are shown, because all subsets increased in their esterified cholesterol (EC) content after 10 weeks on the atherogenic diet. Remarkably, DC contained the largest mass of TC compared to all other LN cell types. Figure 3A shows a statistical significant increase in the EC/TC ratio for B, T, and dendritic cells in DKO versus SKO mice. Although not statistically significant (P<0.08), DKO macrophages also appeared to follow this trend. The increase in the EC/TC ratio was attributable to an increase in the total mass of EC (Figure 3B), and not to a decrease in the mass of free cholesterol (Figure 3C).

**LN T and B cells Accumulate Cholesterol Ester in Response to Diet**

All cell types except macrophages showed a 2-fold increase in their esterified cholesterol (EC) content after 10 weeks on the atherogenic diet. Remarkably, DC contained the largest mass of TC compared to all other LN cell types. Figure 3A shows a statistically significant increase in the EC/TC ratio for B, T, and dendritic cells in DKO versus SKO mice. Although not statistically significant (P<0.08), DKO macrophages also appeared to follow this trend. The increase in the EC/TC ratio was attributable to an increase in the total mass of EC (Figure 3B), and not to a decrease in the mass of free cholesterol (Figure 3C).

**T Cell Activation**

The activation state and functionality of T cells, CD4⁺CD69high, and CD8⁺CD69high populations are shown in supplemental Figure IVA and IVB for LNs and IVC and IVD for spleen. These data show that the percent of CD69high cells was significantly higher in 10-week chow-fed Faslpr/lpr mice LNs compared to chow-fed DKO or SKO mice. However, in response to 10 weeks of the atherogenic diet both Faslpr/lpr and DKO mice showed a 1.5-fold increase in the percent of CD69high cells, suggesting a heightened activation state in response to dietary cholesterol.

**Proliferation of CD4⁺CD44high Cells**

Proliferation of effector CD4⁺CD44high and CD8⁺CD44high T cells was studied by measuring BrdU incorporation into LNs and spleens of 10-week diet-Fed DKO and SKO mice. Supplemental Figure V (top and bottom panels A through C) shows the results for LN and spleen for DKO mice which had a 1.5-fold more CD4⁺CD44high cells in their LNs, averaging 52.5%, versus 35.0% in SKO mice, whereas mice not receiving BrdU showed a background of 0.1%. BrdU incorporation. For CD8⁺CD44high cells, shown in supplemental Figure V (top and bottom panels D through F, for LN and spleen, respectively), incorporation was similar between genotypes. These data suggest that CD4⁺CD44high effector T cells in diet-Fed DKO mice undergo selective proliferation.
Circulating Autoantibodies in Response to Diet
In light of the increased numbers of activated and DNT cells in 10-week diet-fed DKO LNs the development of an autoimmune disorder was considered. Plasma autoantibodies for dsDNA shown in Figure 4 indicates that DKO, like Faslpr/lpr mice, had a 2-fold increase in autoantibodies levels. The relative levels for \( \text{H}2\text{-glycoprotein (H2-GPI)} \) and oxidized LDL (oxLDL) were measured in 10-week diet-fed DKO, SKO, and Faslpr/lpr mice and are shown in supplemental Figure VIA and VIB, respectively. These autoantibodies also showed increases similar to that seen in Faslpr/lpr mice.

LN Cholesterol Accumulation Is Prevented by Plasma ApoA-I
We next tested whether the presence of plasma apoA-I would prevent LN cholesterol accumulation. Four weeks before starting the atherogenic diet, DKO mice were injected with helper-dependent adenovirus expressing human apoA-I (HD Ad ApoA-I)\(^{21,22} \) whereas “Control” mice were injected with an equal amount of HD-Ad that did not carry the apoA-I gene. Figure 5A shows that the plasma apoA-I levels plateaued by 4 weeks and remained constant at \( \approx 40 \text{ mg/dL} \) to the end of the study. LN cholesterol content after 8 weeks on diet is shown in Figure 5B. Compared to control vector–treated mice, diet-fed DKO mice expressing apoA-I had normal levels of LN cholesterol, similar to TgWT ApoA-I mice that have plasma apoA-I concentrations of \( \approx 110 \text{ mg/dL} \). DKO mice expressing the mutant apoA-I, Tg L159R, have plasma levels of a mutant form of human apoA-I at \( \approx 10 \text{ mg/dL} \).\(^{23} \) In these mice LN cholesterol levels were intermediate between control and HD Ad ApoA-I mice, but this low level was sufficient to reduce LN cell expansion. Plasma autoantibodies to dsDNA (data not shown) in diet-fed HD Ad and TgWT ApoA-I mice were similar to those measured in diet-fed SKO mice, whereas diet-fed control (no
ApoA-I) DKO dsDNA autoantibody levels were similar to diet-fed DKO (Figure 4).

**Atherosclerosis Development Is Independent of Plasma Cholesterol**

Previous studies have shown that DKO and SKO mice fed diets containing the same amount of dietary cholesterol (0.1% cholesterol) have different total plasma cholesterol (TPC) levels. SKO mice have a 2.5-fold higher TPC than DKO mice, and yet the 2 genotypes had similar levels of aortic cholesterol accumulation. To quantify atherosclerosis when TPC was similar, the amount of dietary cholesterol fed to each genotype was adjusted based on their differential responses. DKO mice were fed 0.5% cholesterol, 5-fold more than the standard 0.1% diet, whereas SKO mice were fed the same base diet containing 0.05% cholesterol. Even with these manipulations, after 10 weeks DKO mice still had a lower TPC, 736±58 mg/dL, than did SKO mice, 1048±55 mg/dL. Both the VLDL and HDL particle diameters were significantly larger in diet-fed DKO compared to SKO mice, with no significant difference in LDL diameter between genotypes (see supplemental Table I). The plasma triglyceride level was not statistically different between DKO and SKO mice, 206±85 mg/dL versus 215±55 mg/dL (n=7 mice per genotype). Although HDL particles were larger in size in DKO mice they made up less than 5% to 10% of the d <1.21 g/mL mass compared to SKO mice. The increased HDL particle diameter likely indicates the presence of LDL/HDL transition particles enriched in apoE and devoid of apoA-I.

The extent of atherosclerosis was quantified by both morphometric evaluation of oil red O–stained aortic root and by measuring the total aortic cholesterol mass. The percent of lesion for DKO and SKO mice was 42.7±3.8% versus 33.4±3.4%, respectively (mean±SD of n=5, with P<0.05). These measurements were supported by total aortic cholesterol mass (shown in supplemental Figure VIIIB). Both male and female DKO mice had a statistically significant increase in total aortic cholesterol mass compared to male and female SKO mice. The aortas from DKO mice had a 3-fold increase in IL-1β mRNA after 8 and 16 weeks on an atherogenic diet when compared to matched SKO mice and shown in supplemental Figure VIIIC), suggesting a heightened inflammatory state in the DKO aortic environment.

**Discussion**

Our studies demonstrate that in response to an atherogenic diet DKO mice develop enlarged skin draining LNs that contain expanded populations of CE enriched lymphocytes. The cellular expansion and cholesterol loading affected the major classes of LN cells, including T cells, B cells, DCs, and to a lesser extent macrophages. Associated with these morphological changes was a 1.5-fold increase in the proliferation of CD4⁺CD44high T cells, but not CD8⁺CD44high T cells. Changes in T cell proliferation and activation occurred in conjunction with the production of plasma autoantibodies.
and inflammatory cell infiltrates in the dermis. In contrast, diet-fed SKO mice did not show an increase in LN size or cellularity even after consuming the atherogenic diet for 24 weeks (M. Zabalawi, unpublished observations). However, after long-term diet feeding 0.5% of SKO mice presented skin lesions that were not associated with LN enlargement, cholesterol accumulation, or cellular expansion as seen in diet-fed DKO mice. These studies demonstrate that plasma apoA-I prevented the phenotype by preventing LN enlargement, cholesterol loading, as well as accumulation of skin cholesterol in DKO mice. Taken together these results suggest a direct link between apoA-I, cholesterol metabolism, inflammation and autoimmunity. Based on these results we speculate that cholesterol accumulates in skin draining LNs under hypercholesterolemic conditions because of inefficient or nonexistent cellular cholesterol efflux. As cholesterol accumulates in lymphocytes, the lack of apoA-I slows or prevents cholesterol efflux from these cells and eventually leads to disruption in cellular function. Lymphocyte proliferation and activation, as well as cytokine production, exacerbates atherosclerosis and eventually leads to loss of self-tolerance and skin pathogenesis. LN immune cells play a vital role in atherosclerosis progression and regression from the formation of foam cells to the migration of DCs from atherosclerotic plaques to LNs, although the precise role apoA-I plays in these processes remain undefined. T and B cells, as well as DCs, are believed to migrate between atherosclerotic lesions and regional LNs and contribute to disease progression or regression. In one study, the migration of DCs in diet-fed apoE<sup>−/−</sup> and LDLr<sup>−/−</sup> mice showed impaired migration from the skin to the LNs, which was reversed by administering apoA-I.

In autoimmune disorders CD4<sup>+</sup>T cells are primarily responsible for the pathogenic anti-DNA autoantibody production in Fas<sup>−/−</sup> mice. In these studies proliferation of CD4<sup>+</sup>CD44<sup>high</sup> T cells, but not CD8<sup>+</sup>CD44<sup>high</sup> cells, was similar to that seen in a mouse model of SLE. In addition, CD69, an early T cell activation marker involved in signal transduction, cell proliferation, and cytokine secretion, was higher in SLE patients than in controls and it is also higher in our model. Another significant similarity characteristic of autoimmune disorders in mouse models of SLE was the increase in DNT cells. The defect in lpr mice is caused by insertion of a retroviral transposon into the second intron of fas that interferes with its ability to bind to the FAS ligand and mediate cell death through apoptosis. LNs in lpr mice enlarge in part from a massive expansion of DNT cells. As more T cells are produced the autoimmune process attacks tissues and organs including the skin, kidney, and joints. As in human SLE patients, one mechanism of tissue damage involves autoantibody and immune complex deposition, as well as increased infiltration of select tissues by lymphocytes.

Both human and animal studies have shown a convincing link between autoimmune disorders and atherosclerosis with a number of autoimmune “mouse models” exhibiting advanced atherosclerosis. Stanic et al showed that in a mouse model of autoimmunity, atherosclerotic progression was worsened by the lack of self-tolerance, whereas Gu et al, using quantitative trait analysis of the MRL/lpr autoimmune mouse, provided further evidence for a link between autoimmunity and HDL metabolism. In human patients suffering from autoimmune disorders HDL levels are significantly decreased. In light of these connections we compared the extent of atherosclerosis between SKO and DKO mice. In a previous study, DKO and SKO mice were fed 0.1% cholesterol and 10% palm oil for 16 weeks. This diet produced a 2.5-fold greater atherosclerosis, clearly demonstrating the protective effects of HDL apoA-I.
Although DKO mice share several common characteristics with autoimmune mouse models including skin lesions\(^1\) and advanced atherosclerosis, one aspect which remains unusual was the enormous accumulation of cholesterol in the skin. After 12 to 16 weeks on diet,\(^2\) diet-fed DKO mice die of inflammation induced by the massive skin cholesterol accumulation and its resulting pathogenesis as opposed to the renal failure and glomerulonephritis seen in 28- to 30-week Chow-fed Fas\(^{−/−}\) mice. The presence of activated T cells in DKO LN and skin (A. Wilhelm, unpublished observations) suggests that skin is the primary site of pathogenesis in DKO mice.

DKO mice accumulate 2.5-fold more whole body cholesterol compared to SKO mice\(^3\) fed the same diet, with skin being the primary site of cholesterol accumulation.\(^4\) However, the accumulation of cholesterol in the skin of diet-fed DKO mice is not unique, but similar in magnitude to that reported for diet-fed LXR\(^{−/−}\)-apoE\(^{−/−}\) mice that had a 2.5-fold increase in whole body cholesterol compared to apoE\(^{−/−}\) only mice with lipid accumulation occurring predominately in the skin.\(^5\) This unusual phenotype has been previously described in other mouse models that carry disruptions in cholesterol homoeostatic genes including, eg, ACAT1\(^{−/−}\), LDLr\(^{−/−}\) and ABCA1\(^{−/−}\), LDLr\(^{−/−}\).\(^6\) In ABCG1 mice this was accompanied by an increase in inflammatory cells and cytokines in the lungs that were largely absent from the plasma compartment.\(^6\) Despite this, the skin is a common tissue affected in autoimmune disorders such as SLE,\(^7\) SLE\(^{14}\), SLE\(^{16}\) but these lesions are not usually associated with cholesterol accumulation. Thus, it remains to be determined whether cholesterol-loaded inflammatory cells enter the skin in response to a specific stimulus or if a constant infiltration of cholesterol in the skin via LDL causes lymphocyte migration and activation, thereby triggering the resulting phenotype.

In conclusion, we have demonstrated that apoA-I prevents lymphocyte cholesterol accumulation, activation, and proliferation in skin draining LNs of diet-fed DKO mice. An increase in T cell activation and proliferation was linked to increased levels of circulating autoantibodies and a \(\approx 2\)-fold increase in aortic cholesterol accumulation. These results strongly suggest a common link between plasma apoA-I, inflammation, and atherosclerosis.

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**Disclosures**

None.

**References**


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Supplemental Materials

Materials and Methods

Animals and Diet

LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> and LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> mice were backcrossed fully into the C57BL/6 background as described previously<sup>1, 2</sup>. B6.MRL-Faslpr/J (Fas<sup>lpr/lpr</sup>) mice were obtained from Jackson Labs and are fully crossed into the C57BL/6 background. All mice were housed at the Wake Forest University Medical Center and all procedures were approved by the Wake Forest University Health Sciences Animal Care and Use Committee. In all studies except for atherosclerosis evaluation, mice from both genotypes were fed Purina chow or an atherogenic diet containing 0.1% cholesterol with 10% fat from palm oil, prepared in the Wake Forest University diet kitchen, beginning at 6 wks of age, as described previously<sup>1, 2</sup>. LDLr<sup>−/−</sup> mice respond to the 0.1% cholesterol + 10% palm oil fat diet by increasing their plasma cholesterol 2-2.5-fold higher than LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> mice fed the same amount of cholesterol, as has been described previously<sup>1, 2</sup>. Despite the higher plasma cholesterol, LDLr<sup>−/−</sup> mice still have similar atherosclerosis when compared to LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> mice<sup>1, 2</sup>. Therefore, to determine the effects of normalizing plasma cholesterol levels on atherosclerosis development, we attempted to normalize plasma cholesterol between genotypes by feeding the same base diet, but with each genotype receiving different amounts of cholesterol in the diet. LDLr<sup>−/−</sup> mice were fed a diet consisting of 0.05% cholesterol with 10% fat from palm oil while LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> mice received a diet consisting of 0.5% cholesterol with 10% fat from palm oil for 10 wks. No differences in body weights were noted between genotypes when compared to like genders and diets<sup>1</sup>. All mice were maintained in a temperature-controlled room with a 12 hr light/12 h dark cycle. Mice were fasted for 3 hrs prior
to being anesthetized for blood collection by cardiac puncture and subsequent blood processing as described previously\textsuperscript{1,2}.

**LN and Spleen Cell Isolation and Surface Staining**

At the time of sacrifice, the spleen and LN subsets including the axillary, brachial, superficial cervical and inguinal LNs were collected and placed in 10% RPMI. Cells were isolated by massaging the tissue over a mesh screen, as previously described\textsuperscript{3,4} then counting the total number of cells in the suspension using a hemocytometer. Antibodies used in the surface stain include rat anti-mouse CD3, CD4, CD8, CD62L, CD69, IgM, B220, CD11c, CD11b, and CD44 and were obtained from BD Biosciences. Rat anti-mouse F4/80 was obtained from eBioscience. Surface staining was performed, as described previously\textsuperscript{4}. Briefly, 1 x 10\textsuperscript{6} cells in 50 µl were stained with the appropriate antibodies at a 1:100 dilution for 30 min at 4ºC. Cells were then washed 3 times in PBS containing 2% FCS. Acquisition of samples was done using a FACSCalibur.

**Analysis of Immune Cell Populations**

Data was analyzed using FlowJo software (TreeStar, San Francisco, CA). Cell populations measured included effector/effector memory T cells (CD4\textsuperscript{+}CD62L\textsuperscript{low}, CD8\textsuperscript{+}CD62L\textsuperscript{low}, CD4\textsuperscript{+}CD44\textsuperscript{high}, CD8\textsuperscript{+}CD44\textsuperscript{high}), double negative T cells (CD3\textsuperscript{−}CD4\textsuperscript{−}CD8\textsuperscript{−}B220\textsuperscript{+}), activated T cells (CD4\textsuperscript{+}CD69\textsuperscript{high}, CD8\textsuperscript{+}CD69\textsuperscript{high}), naïve B cells (IgM\textsuperscript{+}B220\textsuperscript{+}), DCs (CD3\textsuperscript{−}CD11c\textsuperscript{+}, CD3\textsuperscript{−}CD11b\textsuperscript{−}CD11c\textsuperscript{+}) and macrophages (CD11c\textsuperscript{−}CD11b\textsuperscript{+}F4/80\textsuperscript{+}). Activation of T cells was assessed by measuring CD69\textsuperscript{high}, as the percent of the total CD4 or CD8 populations, respectively.

The total number of cells staining positive for a particular stain combination was calculated by using the percent of that population from the dot plots and the total number of cells for a given tissue. In order to assess the activation, the CD69\textsuperscript{high} cell data were expressed as the
percent of the total CD4 or CD8 populations, respectively. These percentages were obtained using the absolute number of CD4$^+$ or CD8$^+$ cells which was then divided by the absolute number of CD4$^+$CD69$^{\text{high}}$ or CD8$^+$CD69$^{\text{high}}$ cells, respectively. The results obtained in these studies were compared with data from Fas$^{\text{lpr/lpr}}$ mice, an autoimmune model known to have enlarged LNs and spleens with increased B cell and T cell populations$^4$.

To simplify the comparison among all cell populations between the two genotypes after both 4 and 10 wks of diet consumption, the data was calculated as the fold increase in a specific cell population for LDLr$^{-/-}$, ApoA-I$^{-/-}$ relative to the same cell population in LDLr$^{+/-}$ mice. All cell population numbers were calculated based on the absolute number of cells in a population for a given surface stain as described above. A value of 1.0 indicates equivalence in the absolute number of cells between two genotypes.

**Determination of Lymphocyte Cholesterol Mass by Mass Spectrometry**

From diet-fed LDLr$^{-/-}$ or LDLr$^{+/-}$, ApoA-I$^{-/-}$ mice their axillary, brachial, superficial cervical and inguinal LNs were collected. All four subsets of LNs from five mice were pooled for each genotype to create one sample per genotype. LN cells were then isolated as previously described$^3$ and prepared for sorting. Briefly, each sample was divided in two so that two different sorts could be performed. Cells in the first sort were stained with anti-CD3, IgM and B220 in order to obtain B and T cells. Cells in the second sort were stained with anti-CD3, CD11b and CD11c to obtain dendritic cells (DCs) and macrophages. The cells were stained for 30 min at 4ºC with antibodies at a 1:100 dilution. Approximately 80 x 10$^6$ cells were sorted for each set of stains for each genotype. The cell sorting was performed by a FACSCalibur. During sorting, CD3$^+$ cells were collected and identified as T cells. Cells staining IgM$^-$B220$^+$ were identified as naïve B
cells. DCs were those staining CD3^−CD11c^+ while macrophages were those cells staining CD3^−CD11c^−CD11b^+. After cells were sorted they were washed thoroughly in PBS and counted and subjected to lipid extraction. Cholesterol was extracted in chloroform:methanol (2:1), as described previously\(^1\),\(^2\) and 2 \(\mu\)g of \([^{13}\text{C}_2]\)cholesterol internal standard (in 20 \(\mu\)l ethanol) was added to each cell pellet. Cholesterol, together with other neutral lipids, was isolated from the lipid extract using a modification of the method of Kaluzny et al\(^5\). Briefly, each sample was dried under a stream of nitrogen, redissolved in 400 \(\mu\)l chloroform, and applied dropwise to a dry 3 mL Bakerbond aminopropyl solid-phase extraction cartridge. Neutral lipids were eluted in 4 mL of chloroform:isopropanol (2:1, \(v/v\)) without the use of suction. For free cholesterol (FC) determination, each neutral lipid fraction was dried under a stream of nitrogen, redissolved in 200 \(\mu\)l of hexane, and transferred to a glass autosampler vial for gas chromatography-mass spectrometry (GC-MS) analysis. One microliter of the sample was injected, without splitting, onto a 250 \(\mu\)m \(\times\) 30 m DB-1 capillary column (J&W Scientific/Agilent Technologies). Helium served as the carrier gas at a constant flow rate of 2.0 mL/min. The injector temperature was 280 °C. The temperature program was as follows: 150 °C for 1 min; increase at 25 °C/min to 280 °C; hold for 23 min. The MS interface was maintained at 280 °C. The MS used was a Thermo Finnigan single-quadrupole Trace MS, with a positive-ion electron impact ion source, set at electron ionization energy of 70 eV. Data was acquired in selected-ion monitoring mode, monitoring the molecular ions \(m/z\) 386.4 for cholesterol and \(m/z\) 388.4 for the \([^{13}\text{C}_2]\)cholesterol internal standard, with a dwell time of 0.125 sec. The internal standard peak area was corrected for the natural abundance of \(^{13}\text{C}\) by subtracting 7.29% of the cholesterol peak area. The calibration curve was linear from 50 pg to 25 ng of cholesterol injected.
Following the FC determination, the remaining sample was dried under a stream of nitrogen, redissolved in 1 mL of ethanol, mixed with 100 µl of 50% (w/w) aqueous potassium hydroxide, and saponified for 1 hour at 60 °C. Cholesterol was extracted with the addition of 1 mL water and 3 mL hexane. The hexane phase was evaporated, redissolved in 200 µl of hexane, and again analyzed by GC-MS as described above. Cholesteryl ester was calculated as the difference between free and total cholesterol and is expressed as ng per 10^6 cells extracted.

**Assessment of T cell Proliferation**

Mice were given drinking water containing 0.8 mg/ml bromodeoxyuridine (BrdU) for 6 days prior to sacrifice. Briefly, the spleen and LN were collected and cells stained with anti-CD8, CD4 and CD44 (BD Biosciences), then treated overnight with 1% paraformaldehyde and 0.5% Igepal in PBS in order to permeabilize the cells. Cells were then washed in PBS and incubated in PBS with 4.2 mM MgCl₂ and DNase I. Cells were stained with anti-BrdU FITC (BD Biosciences) washed and then analyzed by flow cytometry using FlowJo software (Treestar). Dot plots were created from the viable cells showing CD4 or CD8 versus CD44. To determine the percent of cells that had incorporated BrdU a gate was drawn for the CD4/8⁺CD44<sub>low</sub> cells and for the CD4/8⁺CD44<sub>high</sub> cells.

**Measurement of Plasma Autoantibodies**

Plasma anti-dsDNA and oxLDL autoantibodies were measured by ELISA, as described previously. ELISA for β2-glycoprotein I (β2-GPI) was performed by coating a 96-well Maxisorb plate with 10 mg/mL of purified β2-GPI in PBS. Plates were blocked and mouse serum was added at a 1:500 dilution and incubated overnight at 4°C. Plates were washed with 0.5% Tween/PBS and incubated with HRP conjugated goat anti-mouse IgG (Promega) for 1 hour at RT. Reactions were developed using the TMB substrate (BD Biosciences).
**Helper-Dependent Adenoviral ApoA-I Injection**

Helper dependent adenovirus expressing the human apoA-I gene (HD Ad ApoAI) or an empty vector (control) was constructed as described previously\textsuperscript{10, 11}. Both vectors were injected at 4.5x10\textsuperscript{12} particles/kg into the tail vein. Four wks after injection, the mice were started on the 0.1% cholesterol + 10% palm fat diet and continued for a total of 8 wks. Concentration of human plasma apoA-I was monitored throughout the study by ELISA\textsuperscript{12}. At the time of necropsy, LNs were collected, cleaned and extracted for total cholesterol, as described previously\textsuperscript{1, 2}. Comparisons were made to 8 wk diet-fed transgenic LDLr\textsuperscript{−/−}, ApoA-I\textsuperscript{−/−} mice expressing either human wild-type apoA-I (TgWT ApoA-I) or to LDLr\textsuperscript{−/−}, ApoA-I\textsuperscript{−/−} mice expressing the human helix 6 apoA-I mutant, L159R ApoA-I (TgL159R ApoA-I) which has been shown to have aberrant reverse cholesterol transport\textsuperscript{12}.

**Tissue Cholesterol Quantification and Histology**

For total aortic cholesterol determinations, male and female LDLr\textsuperscript{−/−} and LDLr\textsuperscript{−/−}, ApoA-I\textsuperscript{−/−} mice were fed 0.05% and 0.5% cholesterol containing diets, respectively with 10% palm oil as described, for 10 wks and then sacrificed. Aortas were placed in formalin and then cleaned of adventia before extraction. Total aortic cholesterol was quantified by GC, as previously described\textsuperscript{2, 13, 14}.

For all other studies, mice of both genotypes were fed a diet consisting of 0.1% cholesterol and 10% palm fat for 10 wks prior to tissue harvest. Following perfusion, the spleen and LN subsets including the axillary, brachial, superficial cervical and inguinal LNs were collected and their cholesterol content determined as described previously\textsuperscript{1, 2}. Total cholesterol in the LNs and spleen was expressed as mg per gram of wet weight.
For general tissue histology, mice of both genotypes were fed diet for 10 wks, and at the time of sacrifice, the LNs and spleen were collected and fixed in 10% formalin and infiltrated with 15% sucrose overnight at 4°C before being frozen in OCT\textsuperscript{1}. Tissue blocks were sectioned at -20 °C and slides were stained with Oil Red O in order to stain neutral lipids. Slides were then counterstained with Mayer’s Hematoxylin as described previously\textsuperscript{1}.

**Analysis of Plasma Cholesterol and Lipoprotein Particle Diameter**

Approximately 125 µl of plasma was brought to 1 mL with 1.0063 g/mL saline. Individual lipoprotein density classes were isolated by sequential density gradient at 40,000 rpm in a TFT 50.3 rotor at 14°C using a Beckman L870M ultracentrifuge. Density intervals were VLDL+ IDL d < 1.0223 g/mL, LDL = 1.0223 1.052 g/mL, and HDL = 1.052 1.21 g/mL. Lipoprotein particle diameters were determined by dynamic light scattering analysis using a Microtrac series 250 Ultrafine Particle Analyzer with a laser probe tip (UPA250; Microtrac, Clearwater, FL)\textsuperscript{15-17}. Following ultracentrifugation, samples were carefully removed from the rotor and uncapped. The laser probe was gently placed on the top layer of the supernatant fraction. Great care was taken to not mix the sample layer or allow air bubbles to form at the probe liquid interface. Due to the low lipoprotein content of some samples, triplicate 180 sec measurements were made for all samples. Average values for median particle diameter (in nm) expressed as area distributions were calculated for individual mice (n=4-5 mice per genotype), and mean values for each group calculated. Plasma was analyzed for total plasma cholesterol by enzymatic assay as described previously\textsuperscript{2}.

**Quantification of Aortic Root Lesion Area**

At the time of sacrifice each aorta were perfused with 5% paraformaldehyde and 5% sucrose in PBS. The aortas were cleaned of any fat and adventitia and embedded in OCT medium as
described previously\textsuperscript{18}. Sequential 6 \(\mu\)m sections of the aortic root were cut as previously described\textsuperscript{18, 19}. After sectioning, slides were stained with Oil Red O and the percent lesion area for 10 serial sections at 60 \(\mu\)m intervals for each aorta was determined using ImageJ software. The percent lesion area was determined by dividing the area of the root occupied by atherosclerotic lesions by the total area of the root\textsuperscript{20}.

**Tissue RT-PCR**

Before extraction of total RNA with Trizol, whole mouse aortas were perfused with saline to remove blood. In addition, fat and adventitia were also quickly removed, as described previously\textsuperscript{1}. RT-PCR for mouse IL-1\(\beta\) mRNA was performed as described previously\textsuperscript{1}.

**Statistical Analysis**

Values are presented as the mean ± SD. All groups were compared for all possible combinations using a student’s t test, with \(p<0.05\) considered as statistically significant.
References


Supplemental Table I

Mean Lipoprotein Particle Diameters in LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> and LDLr<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Lipoprotein Fraction</th>
<th>LDLr&lt;sup&gt;−/−&lt;/sup&gt;, ApoA-I&lt;sup&gt;−/−&lt;/sup&gt; (nm)</th>
<th>LDLr&lt;sup&gt;−/−&lt;/sup&gt; (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>54.5 ± 6.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.6 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL</td>
<td>21.7 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.7 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL</td>
<td>15.4 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.7 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Lipoprotein samples were prepared by ultracentrifugation from plasma taken from 8 individual mice per genotype, as described in “Materials and Methods”. The table shows population means for lipoprotein particle diameter (nm) ± SEM. Unlike letters indicate a significant difference at p <0.05. Mice were fed atherogenic diets as described in “Materials and Methods”. Plasma cholesterol levels were 736 ± 58 and 1048 ± 55 for LDLr<sup>−/−</sup>, ApoA-I<sup>−/−</sup> and LDLr<sup>−/−</sup> mice, respectively.
**Supplemental Figure I. Coat appearance of diet-fed LDLr⁻, ApoA-I⁻ and LDLr⁻ mice.**

These photographs show that representative mice from each genotype fed the atherogenic diet for 10 wks have similar coat appearances and body weights.

**Supplemental Figure II. Splenic cell populations.** Panels A-D, show dot plots for 4 specific lymphocyte populations; Panels A’-D’, show total cell numbers for each of the corresponding populations from 10 wk chow and diet-fed mice. Panels A and A’, double negative T cells (CD3⁺CD4⁻CD8⁻B220⁺); Panels B and B’, naïve B cells (IgM⁺B220⁺); Panels C and C’, DCs or dendritic cells (CD3⁻CD11c⁺); and Panels D and D’, macrophages (CD11c⁻CD11b⁻F4/80⁺).

Chow and diet-fed Fas¹⁻⁻ mice, a well characterized model of human autoimmunity, showing enlarged lymph nodes and spleen were also studied for comparison purposes. In Panel A, dot plots were gated on the CD3⁺B220⁺ cell population; in Panels B and C, dot plots were gated on viable cells; and in Panel D, dot plots were gated on the CD11c⁻ population. All values represent the mean ± SD of three independent experiments with 4 -5 mice per genotype in each experiment. Unlike letters indicate a statistically significant difference at p<0.05.

**Supplemental Figure III: Relative difference in immune cell populations from lymph nodes and spleen.** Panel A-C, show relative splenic immune cell populations from chow- and diet-fed mice; Panels D-F, shows relative changes in lymph node cell populations from chow- and diet-fed mice. Panels A and D, show relative changes in spleen and lymph nodes cell populations from mice fed 10 wk chow, respectively. Panels B and E, show relative changes in spleen and lymph nodes, from selected cell populations from mice fed the atherogenic diet for 4 wks, respectively. Panes C and E, show relative changes in selected cell populations from mice fed the atherogenic diet for 10 wks, from spleen and lymph nodes, respectively. The data are expressed as the total number of cells in DKO mice relative to number of cells from SKO mice.
for the indicated cell population. A fold difference of 1.0 indicates equivalence between the two genotypes. Asterisks* indicates significance at p<0.05. For LNs and spleen, one representative cell population is shown for each category: effector/effectort memory T cells (CD4⁺CD62L⁻) denoted here as effector/memory T cells, activated T cells (CD4⁺CD69⁺), double negative T cells (CD3⁻CD4⁻CD8⁻), dendritic cells (CD3⁻CD11b⁺CD11c⁻), naïve B cells (IgM⁻B220⁺), and macrophages (CD11c⁻CD11b⁻F4/80⁺). All values represent the mean ± SD of three independent experiments with 4 -5 mice per genotype in each experiment. Asterisks indicate a statistically significant difference at p<0.05.

**Supplemental Figure IV: Activated T cells.** Panels A and B, show the percent of CD69⁺ activated T cells in CD4⁺ and CD8⁺ populations in LNs, respectively; Panels C and D, show the percent of CD69⁺ activated T cells in CD4⁺ and CD8⁺ populations in spleen, respectively. Values represent the mean ± SD for three independent experiments with 4 -5 mice per genotype in each experiment, fed chow or atherogenic diet for 10 wks. Unlike letters indicate a statistically significance difference at p<0.05.

**Supplemental Figure V: T cell proliferation and BrdU incorporation.** Proliferation of effector CD4⁺CD44⁺ cells is shown in the Top and Bottom Panels A-C and CD8⁺CD44⁺ in the Top and Bottom Panels D-F. BrdU incorporation in LN T cells, is shown in the Top Panels A-F and spleen in the Bottom Panels A-F, from 10 wk diet-fed DKO and SKO mice. The viable cell population is plotted on the left as either CD4 or CD8 versus CD44 and the gated responses are shown on the right for CD4⁺CD44⁺ or CD8⁺CD44⁺ cells, respectively. Control mice did not receive BrdU, Top and Bottom, Panels C and F, to show background levels were <0.2%. Top Panels A-C show that LN CD4⁺CD44⁺ cells from DKO mice incorporated 52% BrdU into effector T cells while SKO mice had 35% incorporation. Bottom Panels A-C show that splenic
CD4⁺CD44<sup>high</sup> cells from DKO mice had incorporated 42% BrdU while SKO had 35% incorporation. Top Panels D to F show that LN CD8⁺CD44<sup>high</sup> cells from both DKO and SKO mice had ~25% incorporation while Bottom Panels D to F show that splenic CD8⁻CD44<sup>high</sup> cells from both DKO and SKO mice had 39%. The results represent 2 independent experiments with 2 mice from each genotype for each experiment.

**Supplemental Figure VI. Dietary cholesterol induction of plasma autoantibodies.** Relative levels of anti-β2-GPI, and anti-oxLDL, are shown in Panels A and B, respectively. Autoantibodies were measured in the plasma from 10 wk chow and diet-fed DKO, SKO and Fas<sup>lpr/lpr</sup> mice. The values represent the mean ± SD of 4 to 12 mice per genotype. Unlike letters indicate statistical significance at p<0.05.

**Supplemental Figure VII: Atherosclerosis and Inflammation.** Panel A, shows a representative Oil Red O stained sections of the aortic root from a 10 wk diet-fed DKO and a representative SKO mouse. The mean percent lesion area for all the mice was 43.8 ± 7.2 for DKO and 33.8 ± 5.7 for SKO mice, mean ± SD of n=5 mice per genotype, respectively; Panel B, shows the mass of total aortic cholesterol measured by GC from 10 wk diet-fed DKO and SKO mice described in “Materials and Methods”. Panel C, shows the relative increase in aortic IL-1β mRNA, as measured by RT-PCR, described in “Materials and Methods”. Unlike letters indicate statistical significance at p<0.05. All values represent the mean ± SD of n=5-10 female and male mice per genotype.
Supplemental Figure 1
Supplemental Figure II

A. Double Negative T Cells
CD3+CD4-CD8-B220

A'. Cell Number

B. B Cells
IgM+B220

B'. Cell Number

C. Dendritic Cells
CD3+CD11c+

C'. Cell Number

D. Macrophages
CD11c+CD11b+F4/80+

D'. Cell Number

Legend:
- Red: DKO (LDLr<sup>-/-</sup>, ApoA-1<sup>-/-</sup>)
- Blue: SKO (LDLr<sup>-/-</sup>)
- White: Fas<sup>lpr/lpr</sup>

Supplemental Figure II
Supplemental Figure IV

- **A** Lymph Node CD4^+ CD69^{high}:
  - Chow: Control group
  - Diet: Experimental group

- **B** Lymph Node CD8^+ CD69^{high}:
  - Chow: Control group
  - Diet: Experimental group

- **C** Spleen CD4^+ CD69^{high}:
  - Chow: Control group
  - Diet: Experimental group

- **D** Spleen CD8^+ CD69^{high}:
  - Chow: Control group
  - Diet: Experimental group