Apolipoprotein A-I Protection Against Atherosclerosis Is Dependent on Genetic Background

Timothy J. Sontag, Paulette A. Krishack, John R. Lukens, Clarissa V. Bhanvadia, Godfrey S. Getz, Catherine A. Reardon

Objective—Inbred mouse strains have different susceptibilities to experimental atherosclerosis. The C57BL/6 strain is among the most sensitive and has, therefore, been the most widely used in atherosclerosis studies, whereas many strains are resistant. The FVB/N strain is highly resistant to atherosclerosis on the apolipoprotein E (apoE)– and low-density lipoprotein (LDL) receptor–deficient backgrounds. High-density lipoprotein and its major apoprotein, apoA-I, have been shown to be protective against atherogenesis on the C57BL/6 background. We here examine the influence of genetic background on the atheroprotective nature of apoA-I.

Approach and Results—ApoE-deficient/apoA-I-deficient mice were generated in the C57BL/6 and FVB/N strains from apoE-deficient mice. After 6 to 10 weeks on a Western-type diet, plasma lipids and atherosclerotic lesion size were assessed. Macrophage recruitment, cholesterol regulation, and blood monocyte levels were examined as potential mechanisms driving lesion size differences. FVB/N knockout mice had higher plasma very-LDL/LDL cholesterol than their C57BL/6 counterparts. ApoA-I deficiency decreased very-LDL/LDL cholesterol in C57BL/6 mice but not in FVB/N mice. FVB/N single and double knockout mice had less lesion than C57BL/6 6 to 10 weeks on diet. ApoA-I deficiency augmented lesion development only in C57BL/6 mice. Macrophage recruitment to thioglycollate-treated peritoneum and diet-induced blood monocyte levels reflected the pattern of lesion development among the 4 genotypes. ApoA-I deficiency increased macrophage cholesterol content only in C57BL/6. FVB/N plasma was a better acceptor for macrophage cholesterol efflux than C57BL/6.

Conclusions—ApoA-I is atheroprotective only in certain genetic contexts. In the C57BL/6 context, but not FVB/N, apoA-I decreases inflammatory macrophage recruitment and monocytopsis, contributors to lesion formation. (Arterioscler Thromb Vasc Biol. 2014;34:262-269.)

Key Words: cholesterol • cholesterol, HDL • macrophages • mice, inbred strains • monocytes

Atherosclerosis accounts for a large proportion of the cardiovascular disease burden in most well-fed human populations. Epidemiological studies have pinpointed low plasma high-density lipoprotein (HDL) content as a risk factor for cardiovascular disease.1–3 Experimental data in the atherosusceptible mouse strain, C57BL/6 (C57), have pointed in the same direction. Thus, overexpression of apoliprotein A-I (apoA-I), the major apoprotein of HDL, reduces atherosclerosis in mouse and rabbit models.4–5 Conversely, the deficiency of apoA-I augments atherosclerosis in some studies6–8 while showing no effect in others.9 The absence of apoA-I in the low-density lipoprotein (LDL) receptor–deficient mouse also results in a profound proinflammatory phenotype.10 This evidence has led to a variety of strategies aimed at raising plasma HDL levels in clinical trials with associated studies of cardiovascular outcome. However, the results of these studies have been ambiguous at best, with many of these trials showing no atheroprotective function for HDL, but with some concerns that the HDL levels were not sufficiently elevated.11 This has occasioned a stronger focus on the function rather than the mass of HDL. It is clear that further studies are required to probe these issues.

Inbred mouse strains have widely varying susceptibilities to experimental atherosclerosis.12 The most sensitive strain is C57 (and perhaps DBA/2), whereas many strains are resistant. Among the pairs of sensitive and resistant strains that have been studied are C57 and FVB/N (FVB), the latter being highly resistant.13,14 In genetic crosses of these 2 strains, several polymorphic genes have been identified as possible contributors to and mediators of the differences in atherosusceptibility. The recent publication of the whole FVB genome sequence makes the comparison of this strain with C57...
particularly appropriate at this time.\textsuperscript{15} We have shown that HDL differs between these 2 strains in cholesterol and protein content. FVB have higher HDL levels, with apoA-II showing the major difference in HDL content.\textsuperscript{16} Both apoA-I and apoA-II exhibit sequence polymorphisms. The 240-aa mouse apoA-I protein has 2 amino acid differences between the C57 and FVB strains (Q225K, V226A; C57 to FVB). The 79-aa apoA-II exhibit sequence polymorphisms. The 240-aa mouse apoA-I–deficient mice and crossing the heterozygotes. For WTD administration. FVB Apoe\textsuperscript{−/−} (FE) mice had significantly higher plasma cholesterol levels than did the C57 Apoe\textsuperscript{−/−} mice (CE) both on chow and on WTD. As with the SKO mice, DKO FVB (FE) had significantly higher plasma cholesterol levels than the corresponding DKO C57 (CEA) mice, especially on WTD. In addition, at all 3 time points, DKO mice had lower cholesterol levels than SKO knockout mice in both genetic backgrounds, indicating that in both backgrounds the absence of apoA-I affects plasma lipid metabolism. On Chow and after 6 weeks of WTD, the difference in plasma cholesterol between DKO and SKO mice was greater in the FVB background than in the C57 background. Similar trends were observed for plasma triglycerides. Given that most of the plasma lipids are in non-HDL lipoproteins in Apoe\textsuperscript{−/−} mice, especially when fed WTD, we examined the plasma lipoprotein levels at 6 weeks on WTD. As expected, there was little HDL in all genotypes, especially in the DKO mice. Very-LDL and LDL levels were

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

We have derived double knockout (DKO) mice in each strain, starting with crosses of the available C57 and FVB apoE-deficient (Apoe\textsuperscript{−/−}) single knockout (SKO)\textsuperscript{15} with C57 apoA1-deficient mice and crossing the heterozygotes. For the FVB strain, the Apoa1\textsuperscript{−/−} heterozygotes were backcrossed 10 generations with the FVB Apoe\textsuperscript{−/−} background and then crossed to yield the DKO. For the atherosclerosis experiments, all animals were fed the Western-type diet (WTD) because apoA-I-deficient mice have low plasma cholesterol levels, perhaps attributable to reduced cholesterol absorption.\textsuperscript{17}

In Tables 1 and 2, we report body weight, plasma cholesterol, and triglyceride in each of the 4 genotypes at baseline or 6 and 10 weeks after the initiation of the WTD. The mice in the FVB background weighed more than the mice in the C57 background on chow, but this difference was attenuated by

### Table 1. Total Body Weight, Total Plasma Cholesterol, and Total Plasma Triglyceride at 0, 6, and 10 Weeks on WTD

<table>
<thead>
<tr>
<th>Western-Type Diet</th>
<th>CE</th>
<th>CEA</th>
<th>FE</th>
<th>FEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 wk</td>
<td>16.3±1.5</td>
<td>16.3±1.6</td>
<td>22.2±2.0E</td>
<td>21.8±1.7E</td>
</tr>
<tr>
<td>6 wk</td>
<td>23.8±2.1</td>
<td>21.6±1.9</td>
<td>22.4±2.1</td>
<td>23.0±2.3</td>
</tr>
<tr>
<td>10 wk</td>
<td>23.3±2.0</td>
<td>23.5±2.1</td>
<td>24.6±2.7E</td>
<td>23.4±2.7</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>407±49</td>
<td>290±9b</td>
<td>559±34E</td>
<td>337±36E</td>
</tr>
<tr>
<td>0 wk</td>
<td>1233±230</td>
<td>753±117e</td>
<td>2051±464E</td>
<td>1782±192E</td>
</tr>
<tr>
<td>6 wk</td>
<td>1163±254</td>
<td>982±176a</td>
<td>1947±191E</td>
<td>1509±234E</td>
</tr>
<tr>
<td>10 wk</td>
<td>112±45</td>
<td>17±12e</td>
<td>105±39</td>
<td>85±73D</td>
</tr>
</tbody>
</table>

Table 2. Plasma Lipoprotein Cholesterol After 6 Weeks on WTD After FPLC Separation

<table>
<thead>
<tr>
<th>Western-Type Diet</th>
<th>CE</th>
<th>CEA</th>
<th>FE</th>
<th>FEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL cholesterol, mg/dL</td>
<td>790±160</td>
<td>475±96.3b</td>
<td>1159±302C</td>
<td>932±154E</td>
</tr>
<tr>
<td>6 wk</td>
<td>478±114</td>
<td>250±38.7c</td>
<td>859±218E</td>
<td>832±99.4E</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>79±42</td>
<td>38±30a</td>
<td>172±79E</td>
<td>96±390b</td>
</tr>
</tbody>
</table>

Data represent mean and SD of 3 to 12 mice. CE indicates C57 apolipoprotein E-deficient mice; CEA, double knockout C57 mice; FE, FVB apolipoprotein E-deficient mice; FEA, double knockout FVB mice; and WTD, Western-type diet. C57 vs FVB P value comparisons are upper-case letter, E vs EA P value comparisons are lower-case letter. A,a=P<0.05; B,b=P<0.01; C,c=P<0.005; D,d=P<0.0005; E,e=P<0.0001.
significantly higher in the FVB mice, regardless of the apoA-I status. Similar results were noted by Dansky et al in the SKO Apoe−/− mice in C57 and FVB backgrounds. In C57 mice only, the absence of apoA-I resulted in lower very-LDL and LDL than in the SKO Apoe−/− mice. Similar results have been reported in SKO C57 apoA1-deficient mice and apoA1-deficient/low-density lipoprotein receptor-deficient (Ldlr−/−) mice. Lipid component analysis of isolated lipoproteins from each genetic background indicates similar compositions, suggesting that greater lipoprotein particle numbers are driving the observed cholesterol and triglyceride differences rather than larger lipid-rich lipoproteins (data not shown).

We compared the effect of apoA-I deficiency in the 2 genetic backgrounds on lesions in 3 vascular sites (aortic root, ascending aortic arch, and innominate artery) by cross-sectional analysis at 6 and 10 weeks after WTD feeding (Figure 1). In C57 mice, the absence of apoA-I led to an increase in atherosclerosis that was significant in the ascending aorta after 6 weeks on diet and at all 3 sites after 10 weeks. In contrast, the absence of apoA-I in the FVB background had little effect on lesion size. Except for the ascending aorta at 6 weeks of WTD, the lesions in the C57 mice were larger than in the FVB mice. The lesions in the FVB mice were small, consisting essentially of foam cells (Figure I in the online-only Data Supplement). Because of the small FVB lesion size, lesions in each strain are plotted separately in Figure II in the online-only Data Supplement. The difference in apoA-I effect between the 2 strains is not solely attributable to the smaller FVB lesion size as the apoA-I effect is present in the 6-week C57 ascending aorta but not in the FVB, despite similar lesion size in the SKOs at that time point. These studies indicate that the effects of apoA-I deficiency on atherosclerotic lesion progression differ depending on genetic background.

In an attempt to understand the basis for the differential response to apoA-I deficiency on atherosclerosis in the 2 genetic backgrounds, we have explored aspects of innate immune functioning, particularly of the monocyte/macrophage pool. First, we elicited sterile peritoneal inflammation with thioglycollate injection in mice fed WTD for 6 weeks. Much lower numbers of total cells were recovered in the peritoneal cavity of FVB mice than in the case of C57 mice (Figure 2A). This is in accordance with previous reported results in wild-type mice of the 2 backgrounds. In addition, in C57, the absence of apoA-I resulted in a significant increase in the number of elicited cells. Fluorescence-activated cell sorter analysis revealed that the peritoneal cells were mostly monocytes and macrophages, with some cells staining positive for both macrophage (F4/80) and monocyte (CD115) cell surface markers (Figure 2B). The peritoneal macrophages were also assessed for cholesterol mass per cell protein. The absence of apoA-I increased macrophage cholesterol only in the C57 background (Figure 2C).

The differences in macrophage cholesterol content could well be a reflection of the efficacy of cholesterol efflux between the genotypes. To explore this, we further isolated peritoneal macrophages from each of the genotypes on chow or 6-week WTD and measured gene expression of the ATP-binding cassette transporters (ABC) ABCA1 and ABCG1 (Figure 3). On chow, the C57 ABCA1 levels were higher than FVB, with no major differences in ABCG1 levels. At 6 weeks on WTD, ABCA1 tends to be higher in macrophages from the FVB strains. In both strains, the absence of apoA-I resulted in a trend toward lower expression of ABCA1. The presence of apoA-I in FVB mice has the largest effect on ABCG1 expression, with levels in the macrophages from FE mice being significantly higher than the FEA and the CE macrophages. There was no effect of diet on ABCG1 expression in C57 but a modest increase in the expression in the FVB mice. The effect of diet on ABCA1 expression was striking, being greater in the FVB mice (>100-fold increase) compared with C57 (10- to 20-fold increase).

The influence of macrophage genotype on cholesterol uptake and efflux was measured using thioglycollate-elicted peritoneal macrophages from each mouse strain on chow diet in the presence or absence of the liver X receptor agonist T0901317, which upregulates the ABC cholesterol transporters (Figure 4A and 4B). Surprisingly, the C57 macrophages tend to efflux more cholesterol in the presence or absence of the liver X receptor agonist. No differences were seen in the uptake of cholesterol from acetylated LDL into the macrophages of each strain in the presence or absence of liver X receptor agonist.
The net efflux from macrophages depends on the activity of transporters and also the availability of extracellular acceptors. Accordingly, this was assessed using plasma derived from chow- and 10-week WTD-fed mice of each of the genotypes as acceptors of cholesterol from cholesterol-loaded J774 mouse macrophages (Figure 4C). This was performed with/without cAMP as an inducer of ABC transporter expression. Without cAMP, efflux was greater to FVB plasma than to C57 plasma, and this was not influenced by the absence of apoA-I, although the efflux to FE plasma tended to be lower than to FE. No differences were observed in efflux to plasma from chow-fed mice in the presence of cAMP, whereas the efflux was greater to FVB genotypes and SKO plasma when it was from 10-week WTD-fed mice. The cholesterol loading of J774 macrophages by plasma from the 4 mouse genotypes on the 2 diets was also examined (Figure 4D). Plasma from each mouse on chow and 10-week WTD was added to J774 cells, and the cell-associated cholesterol was measured after 24 hours. Because of the higher plasma cholesterol in the FVB background (Tables 1 and 2), on an uptake per plasma cholesterol basis, the uptake from the FVB plasmas is lower than from C57 (data not shown). In summary, although our studies provide evidence of differences in the cholesterol uptake/efflux capacity of the plasma between the mouse genotypes, they do not fully explain the specifically elevated cholesterol content seen in the CEA macrophages (Figure 2C). A more complex balance may be taking place in vivo than we see in culture.

Monocytosis is induced by high-fat feeding and is associated with increased atherosclerosis.20 In addition, recent work has implicated cholesterol efflux efficacy in the dynamics of hematopoietic stem cells, such that cholesterol-enriched stem cells proliferate and differentiate into monocytes more rapidly than is the case for stem cells maintaining a normal sterol homeostasis.21,22 To explore the possibility that monocytosis differences in the 2 strains might be a major contributor to the observed atherosclerosis differences, we enumerated total monocytes and monocyte subclasses in the blood of the 4 genotypes before and 6 weeks after WTD feeding (Figure 5A and 5B). In both diet cases, total monocytes and Ly6chi and Ly6clo monocytes were significantly lower as a proportion of CD45+ cells for FE and FEA blood than for the corresponding CE and CEA. On chow diet, the absence of apoA-I had little effect on the monocytes. However, after 6 weeks of WTD, the CEA mice had a higher proportion of total monocytes and Ly6clo monocytes than did the CE mice, with no effect in the FVB genotypes. These changes are specific for monocytes because there were no differences in

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**Figure 2.** Peritoneal cells after thioglycollate elicitation. Peritoneal cells were isolated from thioglycollate-injected mice, and total peritoneal cells were counted (A) and analyzed by fluorescence-activated cell sorter for total monocytes (CD115+), Ly6c+ monocytes, Ly6c+ monocytes, macrophages (CD11b+, CD11c+, F4/80+, CD45+), and neutrophils (CD11b+, Gr1+, CD11bhi, CD11b+, CD11bhi, CD45+). B, C, Peritoneal macrophage cellular cholesterol levels. Macrophages were isolated from the peritoneal cavity of thioglycollate-injected mice fed Western-type diet for 6 weeks by plating on petri dishes. Cellular cholesterol per mg cell protein was measured. C57 vs FVB P value comparisons are upper-case letter; E vs EA P value comparisons are underlined lower-case letter. Data represent mean and SD of 3 to 5 mice per genotype. B,b=P<0.01, C,c=P<0.005, E,e=P<0.0001.

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**Figure 3.** Macrophage ATP-binding cassette transporters (ABC) A1 and ABCG1 expression levels on chow and after 6 weeks on Western-type diet (WTD). RNA from thioglycollate-elicited macrophages was isolated by Trizol extraction, and cDNA was analyzed by real-time polymerase chain reaction using primers for ABCA1, ABCG1, and hypoxanthine-guanine phosphoribosyltransferase (HPRT). Gene expression is shown per HPRT expression levels relative to a single chow-fed CE mouse for each gene. Note the y-axis break between 4 and 14. Each data point represents an individual mouse. Also shown are mean and SE of a minimum of 12 mice per genotype. CE indicates C57 apolipoprotein E-deficient mice; CEA, double knockout C57 mice; FE, FVB apolipoprotein E-deficient mice; and FEA, double knockout FVB mice. C57 vs FVB P value comparisons are upper-case letter; E vs EA P value comparisons are underlined lower-case letter. A,a=P<0.05, B,b=P<0.01, C,c=P<0.005, D,d=P<0.0005, E,e=P<0.0001.

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**Figure 4.** Macrophage ATP-binding cassette transporters (ABC) A1 and ABCG1 expression levels on chow and after 6 weeks on Western-type diet (WTD). RNA from thioglycollate-elicited macrophages was isolated by Trizol extraction, and cDNA was analyzed by real-time polymerase chain reaction using primers for ABCA1, ABCG1, and hypoxanthine-guanine phosphoribosyltransferase (HPRT). Gene expression is shown per HPRT expression levels relative to a single chow-fed CE mouse for each gene. Note the y-axis break between 4 and 14. Each data point represents an individual mouse. Also shown are mean and SE of a minimum of 12 mice per genotype. CE indicates C57 apolipoprotein E-deficient mice; CEA, double knockout C57 mice; FE, FVB apolipoprotein E-deficient mice; and FEA, double knockout FVB mice. C57 vs FVB P value comparisons are upper-case letter; E vs EA P value comparisons are underlined lower-case letter. A,a=P<0.05, B,b=P<0.01, C,c=P<0.005, D,d=P<0.0005, E,e=P<0.0001.
neutrophils in the fat-fed mice. In the chow-fed mice, both FVB genotypes had significantly higher neutrophil counts than did C57. Overall, after 6 weeks on WTD, the relative monocyte levels in the blood closely reflect the relative lesion areas between the mouse genotypes. We additionally examined the bone marrow of 6-week WTD-fed mice to determine the effect of genetic background on the stem cells from which these monocytes are derived (Figure 5C).23 Both CE and CEA bone marrow tended to have higher numbers of common myeloid progenitor cells and significantly higher numbers of macrophage and dendritic cell progenitors and megakaryocyte/erythroid progenitor cells. Granulocyte/macrophage progenitors and Lin−/Sca1+/c-kit primitive hematopoietic cells were significantly higher in the FVB background. ApoA-I deficiency only affected Lin−/Sca1+/c-kit primitive hematopoietic cells on the FVB background. Therefore, although our results indicate that the strain differences in monocyte levels may largely be arising from hematopoietic differences, the effect of apoA-I on plasma monocyte levels seems to be arising from poststem cell differentiation.

**Discussion**

In this work, we have examined the possibility that HDL function may play a role in conferring resistance to the development of atherosclerosis in the FVB mouse strain. We have explored this by comparing mice lacking both apoproteins E and A-I in the C57BL/6 and FVB/N genetic background with their respective Apoe−/− mice as controls. Apoe−/− C57 mice are a widely used model of atherosclerosis. Not surprising in light of multiple published works, Apoe−/− FVB mice display much smaller atherosclerotic lesions in all 3 vascular sites: aortic root, innominate artery, and ascending aortic arch. The novel finding presented here is that apoA-I deficiency does not influence the development of atherosclerosis at any of these vascular sites through 10 weeks of diet. This occurs in the face of much higher non-HDL plasma lipoproteins in these mice compared with C57. Non-HDL lipoproteins (very-LDL and LDL) are regarded as strong risk factors for the development of atherosclerosis, suggesting a high degree of resistance to atherogenesis in FVB mice. However, larger lesions are found in the absence of apoA-I in C57 mice, especially after 10 weeks of diet. The larger lesions in the absence of apoA-I occur in the face of lower levels of non-HDL lipoproteins, particularly very-LDL, which would under most circumstances be expected to reduce the atherogenic drive. We should, however, add the caveat that the relationship between plasma lipoproteins and atherosclerosis is complex, especially in the Apoe−/− background.24 Other
studies have indicated a possible role for HDL cholesterol and apoA-I in various inbred mouse strain-specific differences in atherosclerosis.25,26 However, this is the first study that indicates apoA-II in various inbred mouse strain-specific differences in atherosclerosis.

Among the functions attributed to HDL and apoA-I are their ability to promote cholesterol efflux and decrease inflammatory responses. Both of these may contribute to the increased atherosclerosis in the C57 mice lacking apoA-I. We observed an increase in peritoneal macrophage cholesterol content, as well as an increase in the influx of immune cells into the peritoneal cavity, in response to thioglycollate in the CEA mice compared with the CE mice. ApoA-I interactions with the ABCA1 transporter on macrophages have been shown to influence the expression of toll-like receptors on the cell surface, as well as regulating both janus kinase 2 (JAK) and signal transducer and activator of transcription 3 (STAT3) indicating a role for apoA-I in macrophage inflammatory response.27-29 CEA mice also have increased blood monocytes. Recent work has argued strongly that the level of blood monocytes, the precursors of atherosclerotic foam cells, is regulated, in part, by cholesterol homeostasis in the bone marrow precursors.21,22 This concept is derived from studies of animals lacking the cholesterol transporters ABCA1 and ABCG1. In our studies, the DKO animals are lacking 2 of the most important acceptors of the cholesterol that is effluxed from the cell by these transporters. However, we did not identify an effect of the absence of apoA-I on cholesterol uptake or efflux using peritoneal-derived macrophages or whole plasma from the mice nor did analysis of bone marrow monocyte precursors identify an effect of apoA-I deletion, suggesting that other factors in vivo may also operate.

The resistance of FVB to atherosclerosis development in the absence of apoe is consistent with other studies reported in the literature, although this is the first single study of such mice examining 3 separate vascular sites.13,14 In concordance with the atherosclerosis results is the lower level of blood monocytes and dramatically lower elicitation of peritoneal macrophages by thioglycollate in the FVB strains. This may be attributable to much lower monocyte chemoattractant protein-1–based chemotraction into the FVB peritoneal cavity.18 Parenthetically, this is not the basis of atheroresistance in other inbred strains because some resistant strains have high levels of this chemokine.18 Whether the high plasma levels of MCP-1 in these strains also apply to the arterial wall is yet to be explicitly determined. None of the genetic studies hitherto reporting on the resistance loci in FVB mice have identified the MCP-1 gene as an atherosclerosis resistance locus. However, this does not exclude an important role for MCP-1, especially if some of the identified loci play a role in the regulation of MCP-1 activity.

It is surprising that, at least in the time frame of these experiments, the small atherosclerotic lesions in FVB Apoe−/− mice appear to be almost totally indifferent to the presence of apoA-I. There are several possible explanations for this apparent indifference. Most important is the fact that this resistance is seen in the face of a significantly higher level of atherogenic plasma lipoproteins in FVB mice. It is possible that any atheroprotective functions of apoA-I are not evident in the context of a substantially reduced atherogenic drive in these mice that is determined by several HDL-independent genetic mechanisms that override the proatherogenic drive mediated by the high levels of apoB-containing lipoproteins. Although this
may be an important contributing factor to our observations, as we suggest below, we do not think this is the whole explanation. In addition, in FVB Apoe−/− mice with higher lesion burden (ie, longer times on WTD), the lack of apoA-I may have an effect on lesion size. This merits future investigation.

Another possibility is that FVB apoA-I, which differs in sequence from that of C57 mice, is relatively inactive in macrophage cholesterol homeostasis. This is consistent with the lack of an effect of the absence of apoA-I on the cellular cholesterol content of the macrophages in the FVB mice. However, we have demonstrated that its efflux potential from cholesterol-loaded macrophage cell lines is at least as good as is the activity of C57 apoA-I.16 We have also shown that the ABC transporter expression level is as high or higher in the peritoneal macrophages of FVB mice compared with C57. We, therefore, do not think, on the basis of the information available to us, that an inactive apoA-I–dependent cholesterol efflux is a realistic explanation for the indistinguishability in FVB mice to the absence of apoA-I. It is, however, possible that FVB mice have efflux mechanisms that compensate for the absence of apoA-I. This could account for the lower levels of blood monocytes in the FVB mice in the presence or absence of apoA-I than is present in the blood of C57 Apoe−/− mice. To the extent that these monocyte precursor cholesterol homeostatic mechanisms operate in both genetic backgrounds, in the FVB bone marrow the homeostasis seems again to be less dependent on apoA-I. That is, alternate cholesterol homeostatic mechanisms may be playing a more important role in the FVB strain. Consistent with this hypothesis is our observation that FVB plasma has higher basal cholesterol efflux than C57 plasma. ApoA-I in efflux experiments accounts for only 30% to 50% of total efflux activity. Khara et al10 have shown that apoB lipoprotein-free plasma efflux activity is a good marker of cardiovascular risk. What might be these efflux acceptors for cellular cholesterol? These acceptors have not been identified, but there are several possible candidates. FVB plasma HDL contains twice as much apoA-II and apoC-III.16 Preliminary data in our laboratory indicate that FVB apoA-II is a better cholesterol acceptor than C57 apoA-II and that FEA mice have much more plasma apoA-II than their CEA counterparts. Also, FVB intestine expresses higher levels of apoA-IV, apoC-II, and apoC-III.17 There may be other putative acceptors. Further explorations are required to elucidate the detailed cholesterol homeostatic mechanisms in these mice.

In summary, the apparent insensitivity of FVB atherosclerosis to the presence of apoA-I seems to rest on 2 major contributors: the inherently lower atherogenic drive in the FVB background in which many genetic factors, some identified and some unidentified, operate independent of apoA-I; and the likely presence of redundant cholesterol homeostatic mechanisms that compensate for apoA-I absence.

The evidence for the atheroprotective effects of apoA-I and HDL rests, in part, on the preclinical studies with animal models either deficient in apoA-I or overexpressing apoA-I. Not unexpectedly, the most atherosensitive C57BL/6 mice have been the almost exclusive mouse models from which this evidence is gathered. However, the results reported here might lead to a more circumspect evaluation of this evidence.

It is clear from our results that the action of apoA-I as an antiatherogenic agent may be complex and depend on the precise context of genetics, development, and hemodynamics. Further work along these lines is clearly required. Of course, there are mechanisms not studied here that could account for the different sensitivities to atherogenesis as a function of HDL status.11 Perhaps the most important conclusion of this work is that the atheroprotective effect of apoA-I, at least in preclinical situations, is much dependent on context. It may be that the varying context in individual patients could be contributing to the lack of a clear correlation between HDL cholesterol and cardiovascular disease in human subjects.11

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**Disclosures**

None.

**References**


**Significance**

In this study, we have examined the effect of apolipoprotein A-I, the major protein on high-density lipoprotein, on atherosclerotic lesion development under conditions of genetic sensitivity or resistance to atherosclerosis in inbred mouse strains. Studies of apolipoprotein A-I have yielded conflicting results on its role in lesion development. Our findings show that the atheroprotective effects of apolipoprotein A-I are dependent on the context of lesion formation. We additionally present a role for monocyctosis in the varying murine atherosensitivities. The majority of murine atherosclerosis studies use the highly atherosensitive C57BL/6 mouse. The results presented here indicate that conclusions on the role of apolipoprotein A-I and high-density lipoprotein drawn from such studies must bear in mind the genetic background on which they are acting. These findings may ultimately be important in understanding how varying context in individual patients could be contributing to the lack of a clear correlation between high-density lipoprotein cholesterol and cardiovascular disease in human subjects.
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Materials and Methods

All chemicals were purchase from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Generation of Mouse Genotypes

Female C57BL/6J Apoe\(^{-/-}\) (CE) and Apoa1\(^{-/-}\) mice were purchased from Jackson Laboratory, Bar Harbor, ME and crossed to yield homozygous Apoe\(^{-/-}\)/Apoa1\(^{-/-}\) double knockout mice on the C57 background (CEA). FVBN/J Apoe\(^{-/-}\) mice (FE) were a generous gift from Dr. Jan Breslow. FE mice were crossed with CEA mice and the Apoa1\(^{+/-}\) heterozygous progeny were backcrossed 10 generations into the FE background at which point the Apoa1\(^{+/-}\) heterozygous mice were crossed to yield Apoe\(^{-/-}\)/Apoa1\(^{-/-}\) double knockout mice on the FVB background (FEA). The mice were bred in a specific pathogen free facility and maintained on chow diet #7913 from Harlan Labs (Indianapolis, IN) until switched to the atherogenic diet. Female mice at 8-10 weeks of age were used for all experiments. All procedures performed on the mice were in accordance with National Institute of Health and institutional guidelines.

Plasma Lipids

Total cholesterol and total triglyceride in plasma obtained from mice fasted for 4 hours were analyzed by colorimetric enzyme assays (Roche Diagnostics, Indianapolis, IN). Plasma lipoprotein distribution was analyzed by fast protein liquid chromatography (FPLC) as described.

Atherosclerosis

Female 8 week old mice of each genotype (CE, CEA, FE, and FEA) were placed on a western-type diet (WTD; Harlan Teklad TD88137) for either 6 or 10 weeks. At the end of each time point, atherosclerotic lesions in the innominate artery, ascending aorta, and aortic root was assessed using our laboratory’s standard protocol. Anesthetized mice were exsanguinated and perfused transcardially under physiological pressure with PBS for 2 min followed by a 10-min perfusion with buffered formalin solution (4% paraformaldehyde/5% sucrose in PBS). The upper arterial vasculatures were dissected from the mouse, mounted in Optimum Cutting Temperature (OCT) compound (Sakura Finetek, Torrence, CA, USA), and serial 10 \(\mu\)m frozen sections collected on a Leica cryostat (Leica Microsystems, Bannockburn, IL, USA). Atherosclerotic lesion size in the innominate artery was assessed using 4 sections between 150 to 450 \(\mu\)m above the junction of the innominate artery with the greater curvature of the aortic arch. Atherosclerotic lesion size in the ascending aorta was assessed using 3 sections at the apex of the lesser curvature of the aortic arch. Atherosclerotic lesion size in the aortic root was assessed using 3 sections beginning at the appearance of a coronary artery and the aortic valve leaflets. The sections in all three sites were separated by 100 \(\mu\)m from each other. Those sections were stained with Oil Red-O and Fast Green. Digital images were captured, and the atherosclerotic lesion size was quantified using OpenLab 3.1.5 software (PerkinElmer, Waltham, MA, USA).

Peritoneal Macrophage Elicitation

8 week old female mice of each genotype on chow or fed WTD for 6 weeks were injected intraperitoneal with 3 ml of 4% Thioglycollate. After 3 days peritoneal cells were obtained by lavage with PBS/2% fetal calf serum (FCS). An aliquot of 1 x 10\(^6\) cells was used for flow cytometry. After blocking with anti-Fc\(\gamma\)R antibody (clone 2.4G2)) cells were stained for total,
Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo} monocytes (CD115\textsuperscript{hi}, Ly6C\textsuperscript{hi} or \textsuperscript{lo} /CD45+), macrophages (CD11b\textsuperscript{hi}, CD11c\textsuperscript{hi}, F4/80\textsuperscript{hi}/CD45+), and neutrophils (CD11b\textsuperscript{hi}, Gr1\textsuperscript{hi}, CD115\textsuperscript{lo}, SSC\textsuperscript{int}/CD45+). CD45.1 was used for FVB mice and CD45.2 was used for C57 mice due to the strain variations in CD45 isoforms. All cell surface marker labels were purchased from eBioscience (San Diego, CA). Flow cytometry was performed using a BD LSRRII Flow Cytometer (BD Biosciences, San Jose, CA) with subsequent data analysis using FlowJo 10.0 software.

A separate aliquot of 5 x 10\textsuperscript{6} cells was plated on 100mm petri dishes and incubated at 37°C in 5% CO\textsubscript{2} in RPMI + 1% FCS. After 3 hrs the cells were washed with PBS and extracted with 3:2 Hexane:Isopropanol. Samples were dried under air, resuspended in saturated (9:1) Isopropanol:Tergitol. Cholesterol was measured using a colorimetric enzyme assay kit (Roche Diagnostics, Indianapolis, IN). Extracted cells were solubilized with 0.1N NaOH and protein measured using a Pierce microBCA protein kit (Thermo Scientific, Rockford, IL).

**Quantitative PCR**

RNA was prepared from isolated macrophages using Trizol extraction method (Invitrogen) and cDNA was prepared from 4ug RNA using Superscript III (Invitrogen) according to the manufacturer’s protocol. 2µl of 1:100 diluted cDNA was subjected to real-time PCR using SYBR green mix (SA Biosciences) and primer mixes (SA Biosciences). Gene expression was normalized to HPRT with the expression level of each gene expressed relative to macrophages from a single CE mouse on chow diet.

**Blood monocyte analysis**

Mice on chow diet were bled retro-orbitally and red blood cells lysed with RBC multi-species lysis buffer (eBioscience, San Diego, CA) and total cells counted. The cells were labeled and analyzed by FACS for monocytes (total, Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo}) and neutrophils as described above. The same mice were then placed on WTD for 6 weeks and blood monocyte and neutrophil levels again assessed by FACS.

**Bone marrow monocyte precursor analysis**

Bone marrow was isolated from both hind leg femur and tibia of mice fed WTD for 6 weeks by flushing with cold PBS. Red blood cells were lysed with RBC multi-species lysis buffer (eBioscience, San Diego, CA) and total cells counted. The cells were labeled and analyzed by FACS for monocyte precursor cells using a cocktail mix for lineage markers Gr1, B220, CD19, CD3, CD4, CD8, NK1.1, CD11c, CD11b (eBioscience, San Diego, CA): LSK (lineage marker negative/Sca-1-+/c-kit+), MDP (Macrophage and Dendritic-cell Progenitor; lineage marker negative/Sca-1-/c-kit+/CD115+/Flt-3+), CMP (Common Myeloid Progenitor; lineage marker negative/Sca-1-/c-kit+/CD34+/FcgRII/IIIhi), GMP (Granulocyte/Macrophage Progenitor; lineage marker negative/Sca-1-/c-kit+/CD34+/FcgRII/IIIlo), MEP (Megakaryocyte/Erythroid Progenitor; lineage marker negative/Sca-1-/c-kit+/CD34-/FcgRII/III-) \textsuperscript{5,6}.

**Cholesterol uptake and efflux by peritoneal macrophages**

Thioglycollate-elicited macrophages were prepared as described above. 1.5 x 10\textsuperscript{6} peritoneal cells were plated in each well of 12-well plates for 4 hours in DMEM (high glucose) + 10% FCS. Acetylated human LDL (AcLDL) was labeled by rotating for 4 hrs at 4°C with [\textsuperscript{3}H]-Cholesterol at 40 µCi/mg protein. The media was replaced with DMEM+1%FCS at 1 ml/well containing 25 µg/ml labeled acetylated human LDL (1 µCi/ml [\textsuperscript{3}H]-Cholesterol) +/- 10 µM
TO901317 as an LXR agonist. After 18 hr, cells were washed with PBS and 1 ml DMEM+0.25% wild type FVB plasma was added to each well. After 4 hours the media was removed from the cells and media and cells were extracted and counted as described previously.

Extracted cells were solubilized with 0.1N NaOH and protein measured using a Pierce microBCA protein kit.

**J774 Cholesterol uptake/efflux using mouse plasma as donor/acceptor**

The efflux capacity of the plasma from each mouse genotype was measured. Confluent J774 cells in 12-well plates were incubated 24 hrs with 25 µg/ml acetylated human LDL + 1µCi/ml [³H]-Cholesterol + 2µg/ml Sandoz ACAT inhibitor in the presence or absence of 0.3mM CPT-cAMP in DMEM (high glucose) + 10% FCS. Cells were washed with PBS and incubated 4 hrs with 1% plasma from chow or 10 week WTD fed CE, CEA, FE, or FEA mice. After 4 hours the media was removed from the cells and media and cells were extracted and counted as described previously.

Cholesterol uptake from the plasma of each genotype was measured. Confluent J774 cells in 12-well plates were incubated in DMEM (high glucose) + 3% mouse plasma from CE, CEA, FE, or FEA on chow or 10 week WTD. After 24 hrs cells were washed 3X with PBS and extracted 2X with 1 ml 3:2 Hexane:Isopropanol. The extracts were combined, dried under air, and resuspended in 9:1 Isopropanol:Tergitol. Cholesterol was analyzed by colorimetric enzyme assay kits (Roche Diagnostics, Indianapolis, IN) using cholesterol standards in 9:1 Isopropanol:Tergitol. Extracted cells were solubilized with 0.1N NaOH and protein measured using a Pierce microBCA protein kit.

**Statistical Analysis**

Statistical analysis was done using StatView software. Each of the 4 genotypes were compared by ANOVA followed by Bonferroni/Dunn post-hoc analysis. A p-value was <0.05 was considered significant.

**References**

2. Wool GD, Vaisar T, Reardon CA, Getz GS. An apoa-i mimetic peptide containing a prolidine residue has greater in vivo hdl binding and anti-inflammatory ability than the 4f peptide. *J Lipid Res*. 2009;50:1889-1900


Supplemental Figure I. Representative lesions in the Ascending Aorta from mice of each of the 4 genotypes at 6 and 10 weeks on western-type diet (WTD).
**Supplemental Figure II.** Atherosclerotic lesion size after 6 or 10 weeks on WTD. Atherosclerosis was quantitated in the innominate artery, ascending aorta, and aortic root in sections stained with Oil Red-O and Fast Green. Left y-axis: innominate artery and ascending aorta, right y-axis: aortic root. (A) CE, CEA 6 week WTD. (B) FE, FEA 6 week WTD. (C) CE, CEA 10 week WTD. (D) FE, FEA 10 week WTD. A,a = p<0.05, C,c = p<0.005, E,e = p<0.0001. C57 vs FVB p-value comparisons are upper-case letter, E vs. EA p-value comparisons are underlined lower-case letter. Each data point represents an individual mouse. Also shown are mean and standard error of a minimum of 10 mice per genotype.