Atherosclerotic vascular disease (ASVD) is the leading cause of death in Western societies. Complications of the disease are usually caused by rupture or erosion of an unstable atherosclerotic plaque, resulting in thrombus formation and arterial occlusion. The complex etiology involves both genetic and environmental factors. Although genetic factors underlying traditional risk factors are well known, new data emerging from genome-wide association studies are revealing novel loci mediating disease susceptibility independent of traditional factors. In particular, a risk locus on chromosome (chr) 9p21.3 is strongly associated with ASVD (including myocardial infarction, stroke, and aortic aneurysm) but independent of plasma cholesterol levels and hypertension. Although the association with ASVD is robust and has been widely replicated in different ethnic groups, the underlying pathogenic mechanism remains unknown.

The association at 9p21 appears to be the most robust common (minor allele frequency >10%) genetic determinant for ASVD in the human genome. The single-nucleotide polymorphisms most strongly associated with disease risk map to a 58-kb region containing a long, noncoding RNA (ANRIL) and lying ≈100 kb centromeric to the CDKN2A/B tumor suppressor locus encoding inhibitors of cellular proliferation. Multiple ANRIL splice variants exist, complicating genetic association studies. Decreased expression of the tumor suppressors p16INK4a, p19ARF, and p15INK4b has been observed among carriers of the ASVD risk allele in some studies but not others. Targeted deletion of the homologous region in mice resulted in markedly decreased expression of Cdkn2ab transcripts and increased proliferation of vascular smooth muscle cells (vSMCs) in vitro. However, no effect on atherosclerosis was observed in the Western diet–fed wild-type background mice, a highly atheroresistant model. Thus, the potential role of these transcripts and the pathogenic mechanism leading to increased atherosclerotic risk at the 9p21 locus remains unclear.

We have used a murine genetic approach to map atherosclerosis susceptibility loci, including a locus on mouse chr 4 that was confirmed in a congenic strain. The effect on...
disease susceptibility was independent of plasma cholesterol levels, body weight, and plasma glucose levels.\(^\text{15}\) We now report refined genetic mapping indicating that the introgressed interval contains at least 2 loci, 1 overlapping the \textit{Athsq1} QTL and 1 covering the region of homology with the human 9p21 ASVD locus. Gene expression studies revealed decreased mRNA levels of \textit{p16\(^{\text{NKd4}}\)} and \textit{p19\(^{\text{ARF}}\)}, but not \textit{p15\(^{\text{NKb4}}\)}, in macrophages derived from susceptible congenic mice. Mechanistically, we investigated the hypothesis that increased proliferation of macrophages (or mixed populations of monocyte/macrophages) due to reduced expression of \textit{Cdkn2a} cell proliferation inhibitor transcripts in myeloid lineage cells might be responsible, at least in part, for the accelerated atherosclerosis phenotype.

**Methods**

An expanded Methods section is available in the Supplemental Methods, available online at http://atvb.ahajournals.org. B6.MOLF-\textit{Athsq1} congenic mice were bred as described.\(^\text{15}\) Cdkn2a\(^{-/-}\) mice\(^\text{16}\) backcrossed into B6\(^\text{17}\) were kindly provided by Dr Sean Morrison (University of Michigan). Gene expression profiling or real-time polymerase chain reaction was carried out using peritoneal or splenic monocyte/macrophages. Raw profiling data are available in the National Center for Biotechnology Information GEO database (GSE 24342). Atherosclerotic lesions were quantified using morphometric analysis as described.\(^\text{14,15}\) Bone marrow (BM) transplantation experiments were performed as described.\(^\text{18}\) Monocyte subsets were analyzed by standard flow cytometry analysis.\(^\text{19}\) Two-factor ANOVA and \(t\)-tests were performed using Statview 5.0 (Abacus Concepts, Inc). The threshold for significance was \(P<0.05\). Data shown are mean±SEM.

**Results**

**Genetic Complexity of an Atherosclerosis Susceptibility Locus Containing \textit{Athsq1}, as Well as a 5.4- to 9-Mb Interval Containing \textit{Cdkn2a/b}**

A series of subcongenic strains was generated to narrow a previously reported\(^\text{15}\) 54-Mb interval on chr 4 harboring an atherosclerosis susceptibility gene(s). Mice were bred to homozygosity, fed 9-week WTD, and analyzed for atherosclerotic lesion development. Mice carrying a 17- or 11-Mb proximal subinterval but not the 4-Mb proximal tip exhibited approximately 2-fold greater mean lesion area than noncongenic controls (\(P<0.0001\)) (Figure 1A and 1B). The effect was observed in both males and females. The negative lesion phenotype of the 4-Mb subcongenic was confirmed in a second cohort (Supplemental Figure I). These data narrow the proximal interval to a 5.4- to 9-Mb region containing 8 to 21 known protein coding genes, including \textit{Cdkn2a/b} (Figure 1C). Of note, this locus did not segregate with versican accumulation (data not shown), a phenotype originally observed in the full congenic strain.\(^\text{15}\) An additional distal locus was identified in a subcongenic strain carrying a nonoverlapping donor interval (Supplemental Figure 1B and IC). The distal locus overlaps the linkage peak from the original \textit{Athsq1} mapping cross, but detailed mapping and phenotypic assessment have not been carried out. Together, the data indicate genetic complexity of the full 54-Mb interval, with refined mapping of the proximal locus leading to identification of a critical region containing 8 to 21 genes.

Having previously demonstrated a role for BM-derived cells in determining the proatherosclerotic phenotype of \textit{Athsq1} congenics,\(^\text{15}\) we now performed a reverse BM transplantation to determine whether vSMCs or endothelial cells contributed to the phenotype as well. B6-\textit{Ldlr}\(^{-/-}\) BM was injected into lethally irradiated 17-Mb subcongens and noncongenic controls. Following repopulation of the BM, mice were fed 11-week WTD and analyzed for atherosclerotic lesion development. No difference in mean lesion area was observed between the groups (Supplemental Figure II). Similar results were obtained for males and females. Importantly, these data indicate that resident cells of the vessel wall are not involved in the accelerated atherosclerosis phenotype associated with the proximal 17-Mb locus. Thus, we focused further mechanistic studies of this strain in BM-derived monocyte/macrophages.

**Microarray and Transcript-Specific Gene Expression Analyses Reveal Decreased Macrophage Expression of \textit{p16\(^{\text{NKd4}}\)} and \textit{p19\(^{\text{ARF}}\)}, but Not \textit{p15\(^{\text{NKb4}}\)}, in \textit{Athsq1} Congenic Mice**

As an independent approach to narrow the list of candidate genes (>600 genes in the full 54-Mb congenic interval and ≈160 genes in the 17-Mb interval), we performed gene expression analysis using microarrays. Elicited peritoneal macrophages derived from full (54-Mb) congenics and noncongenic controls were collected after 6-week WTD feeding. Differentially expressed genes were defined as exhibiting ≥20% difference in expression level, with a significance threshold corrected for multiple testing.\(^\text{20}\) Three to 6 of the differentially expressed transcripts reside within the narrowed 5.4- to 9-Mb proximal interval and could be considered causal candidate genes (Table). Strikingly, the most significant difference was a ≈6-fold decrease in \textit{Cdkn2a} levels in congenic compared with control macrophages. \textit{Cdkn2a} encodes 2 transcripts involved in cell proliferation regulation: \textit{p16\(^{\text{NKd4}}\)} and \textit{p19\(^{\text{ARF}}\)}.\(^\text{21}\) The transcripts use different promoters and alternative reading frames, resulting in proteins with no amino acid homology.\(^\text{22}\) Because of the \textit{Cdkn2a} exonic structure, the microarray could not discern \textit{p16\(^{\text{NKd4}}\)} versus \textit{p19\(^{\text{ARF}}\)} transcripts. Transcript-specific real-time polymerase chain reaction confirmed the results in the 17-Mb subcongenic strain and showed reduced expression of both \textit{p16\(^{\text{NKd4}}\)} and \textit{p19\(^{\text{ARF}}\)}, but not the adjacent \textit{p15\(^{\text{NKb4}}\)} transcript, in macrophages from the atherosclerosis-prone 54- and 17-Mb congenics compared with noncongenic controls (Figure 2A). Similar results were observed for both transcripts in resident peritoneal macrophages, and for \textit{p16\(^{\text{NKd4}}\)} in splenic monocyte/macrophages, from 54- and 17-Mb congenic mice compared with controls (Figure 2B and 2C). Of note, gene expression in peripheral blood monocytes was low (with \textit{Ct} values >38), and we could not reliably detect a difference between congenics and controls. Thus, an atherosclerosis-prone murine strain exhibits decreased expression of the \textit{p16\(^{\text{NKd4}}\)} and \textit{p19\(^{\text{ARF}}\)} tumor suppressor genes in macrophages and mixed monocyte/macrophage populations.
BM-Specific Cdkn2a Deficiency Results in Accelerated Atherosclerosis, Increased Ly6Chi Monocytes, and Increased 5-Bromo-2’-Deoxyuridine Incorporation Into Monocytes and Macrophages

To directly test the hypothesis that decreased Cdkn2a expression in BM-derived cells is proatherogenic, we performed a BM transplantation study using a previously described Cdkn2a-deficient mouse. The targeted mutation replaces exons 2 and 3 with a neo cassette, knocking out both p16INK4a and p19ARF expression, and has been crossed into a uniform B6 background. In an effort to simulate the modest reduction of p16INK4a and p19ARF expression observed in congenics.

Figure 1. Refined mapping of an atherosclerosis susceptibility locus to a 5.4- to 9-Mb region including the region of homology with a human atherosclerotic vascular disease (ASVD) risk interval on 9p21. A, Physical map of mouse chromosome 4 and MOLF donor intervals (white boxes) carried by congenic strains in the B6-Ldr<sup>−/−</sup> background. Mb indicates megabases; b/b, homozygosity for B6 alleles; m/m, homozygosity for MOLF alleles. The rectangle indicates the narrowed interval defined by data in B. B, Mean lesion areas in control (b/b) and congenic strains fed 9-week WTD. Two-factor ANOVA was performed with square root transformation. Horizontal lines indicate group means for males (circles) and females (triangles). NS indicates not significant. C, Microsatellite markers delineating proximal (D4Mit349/D4Mit27) and distal (D4Mit350/D4Mit154) recombination breakpoints of the refined risk interval. The interval includes the region of homology with a human risk interval on 9p21.

BM-Specific Cdkn2a Deficiency Results in Accelerated Atherosclerosis, Increased Ly6<sup>Hi</sup> Monocytes, and Increased 5-Bromo-2’-Deoxyuridine Incorporation Into Monocytes and Macrophages

To directly test the hypothesis that decreased Cdkn2a expression in BM-derived cells is proatherogenic, we performed a
Table. Cdkn2a Is the Most Differentially Expressed Macrophage-Derived Gene Residing in the 5.4- to 9-Mb Mapped Susceptibility Interval

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Map Location (Mb)</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change (Relative to b/b)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>100093087_TGL_at</td>
<td>88.3</td>
<td>Klh9</td>
<td>Kelch-like 9</td>
<td>1.29</td>
<td>0.0001</td>
</tr>
<tr>
<td>100103804_TGL_at</td>
<td>88.0</td>
<td>Ptpiad2</td>
<td>Protein tyrosine phosphatase-like A domain containing 2</td>
<td>1.24</td>
<td>0.0065</td>
</tr>
<tr>
<td>100102382_TGL_at</td>
<td>88.8</td>
<td>Mtap</td>
<td>Methylthioadenosine phosphorylase</td>
<td>−1.26</td>
<td>7.77×10^−5</td>
</tr>
<tr>
<td>100116877_TGL_at</td>
<td>88.0</td>
<td>Ptpiad2</td>
<td>Protein tyrosine phosphatase-like A domain containing 2</td>
<td>−1.30</td>
<td>0.0071</td>
</tr>
<tr>
<td>100097525_TGL_at</td>
<td>94.7</td>
<td>Jun</td>
<td>Jun oncogene</td>
<td>−1.39</td>
<td>0.0028</td>
</tr>
<tr>
<td>100113480_TGL_at</td>
<td>93.0</td>
<td>Tusc1</td>
<td>Tumor suppressor candidate 1</td>
<td>−1.88</td>
<td>5.36×10^−7</td>
</tr>
<tr>
<td>100099086_TGL_at</td>
<td>88.9</td>
<td>Cdkn2a</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>−5.98</td>
<td>1.25×10^−6</td>
</tr>
</tbody>
</table>

Fold change is listed for all transcripts encoded within the 5.4- to 9-Mb interval and exhibiting a ≥20% difference in expression level in peritoneal macrophages derived from Athsq1 54-Mb full congenic or noncongenic (b/b) mice fed 6-wk WTD. n=10 mice/group. Note that Ptpiad2 is listed twice (+1.24-fold and −1.30-fold changes), with known splice variants exhibiting different expression patterns.

compared with controls, we used donor B6-Ldlr+/+ mice heterozygous for the Cdkn2a null allele. B6-Ldlr−/− recipients were injected with B6-Ldlr+/+ or B6-Ldlr−−/−, Cdkn2a+/−/− BM and fed 10-week WTD following repopulation of the BM. No differences were observed in body weight, plasma total cholesterol, high-density lipoprotein, or triglycerides (Supplemental Table 1). Resident peritoneal macrophages exhibited a 2.5-fold decrease in expression of p16^INK4a but not p19^ARF or p19^Kip4 (another INK4-class gene encoded by a distant region of mouse chr 4) in B6-Ldlr−/−, Cdkn2a+/−/− recipients compared with controls (Figure 3A). Similarly, splenic CD11b+ macrophages/macrophages exhibited a 4-fold decrease in both p16^INK4a and p19^ARF but not p18^Kip4 (Figure 3B). Importantly, mean atherosclerotic lesion area was increased in B6-Ldlr−/−, Cdkn2a+/−/− recipients compared with controls (P=0.04, 2-factor ANOVA) (Figure 3C), with nonsignificant single-sex increases of 34% and 17% for males and females, respectively. Thus, heterozygous BM-specific deficiency of Cdkn2a is sufficient to confer a modestly accelerated atherosclerosis phenotype in mice.

To test potential atherogenic mechanisms consistent with decreased p16^INK4a or p19^ARF expression in monocyte/macrophages, we assessed apoptosis and measures of monocyteosis in the BM transplanted mice. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining of atherosclerotic lesions revealed few positive cells and no difference between B6-Ldlr−/−, Cdkn2a+/−/− recipients and controls (data not shown). No differences were observed in circulating white blood cell counts or total monocytes in blood derived from B6-Ldlr−/−, Cdkn2a+/−/− recipients and controls (data not shown). However, flow cytometry analysis of blood monocytes revealed increased Ly6C^hi monocytes after chow (0-week time point) and WTD feeding (Figure 4A and 4B). Increased percentages of Ly6C^hi and decreased percentages of Ly6C^lo subsets (Figure 4B) resulted in a significant increase in the ratio of Ly6C^hi/Ly6C^lo monocytes in the circulation of B6-Ldlr−/−, Cdkn2a+/−/− recipients compared with controls (Supplemental Figure IIIA). Note that the 0- and 2-week time points are prelesional, indicating that increased Ly6C^hi monocytes are not secondary to accelerated atherosclerosis.15 Similar results were observed for the 17-Mb congenic strain compared with noncongenic controls (Supplemental Figure IIIIB). Moreover, an increased ratio of Ly6C^hi/Ly6C^lo monocytes was observed in splenic monocytes derived from B6-Ldlr−/−, Cdkn2a+/−/− mice compared with B6-Ldlr−/− controls (P=0.003) (Supplemental Figure IVA and IVB).

To test for a direct effect of heterozygous Cdkn2a deficiency on monocyte proliferation, we injected mice with 5-bromo-2′-deoxyuridine (BrdU) before euthanization at the 10-week time point. B6-Ldlr−/−, Cdkn2a+/−/− recipients exhibited an increased percentage of BrdU+ monocytes compared with controls, indicating increased monocyte proliferation (Figure 4C and 4D). Moreover, the increase in BrdU incorporation occurs mainly in the Ly6C^hi subset (Figure 4C, left panel).

To test for an effect of BM-derived Cdkn2a deficiency on tissue macrophage proliferation, we assayed elicited peritoneal macrophages by flow cytometry following intraperitoneal BrdU injection. Although there was no difference in the total number of CD45−CD11b+F4/80+ macrophages (Figure 5A), there was a significant increase in the percentage of BrdU+ macrophages from B6-Ldlr−/−, Cdkn2a+/−/− mice compared with B6-Ldlr−/− controls (P<0.04) (Figure 5B and 5C). We also tested for increased BrdU incorporation within lesions of a small cohort of B6-Ldlr−/−, Cdkn2a+/−/− mice compared with controls using immunohistochemistry. Although ample numbers of cells (mean=42–47 cells) stained positively following a 7-day pulse, the within-group variation was high, and power calculations indicated that we would need to study 50 mice/group to have an 80% chance of detecting a significant difference (P<0.05) comparable in magnitude to the difference in lesion area (ie, ≈30%).

Together, these data provide direct evidence for a suppressive effect of BM-derived p16^INK4a or p19^ARF expression on inflammatory monocyte/macrophage proliferation.

**Discussion**

Before the identification of 9p21 as a risk locus for human ASVD, we used a murine genetic approach to identify the homologous region of mouse chr 4 as a modifier of athero-
sclerosis susceptibility. In the current study, we provided refined genetic mapping and transcriptional evidence that Cdkn2a mediates at least some of the altered atherosclerosis susceptibility of this region through altered expression in macrophages. In support of this model, heterozygous deficiency of Cdkn2a transcripts in BM-derived cells was found to be sufficient to confer accelerated atherogenesis in the B6-Ldlr/+/H11002 background. The moderate (2–4-fold) reduction in p16INK4a and p19ARF expression associated with increased atherosclerosis in these murine models is consistent with the reduction of p16INK4a and p19ARF expression in T cells of carriers of the 9p21 risk allele. Moreover, our study shows for the first time that the underlying pathogenic mechanism may involve increased proliferation/expansion of the Ly6Chi inflammatory monocyte population in the circulation, as well as increased proliferation of tissue macrophages. This provides a plausible mechanism to account, in part, for accelerated atherogenesis in the chr

Figure 2. Decreased expression of p16INK4a and p19ARF, but not p15INK4b, cell proliferation inhibitor transcripts in bone marrow–derived cells from chromosome 4 congenic mice compared with controls. Real-time polymerase chain reaction results in cells derived from B6-Ldlr/+/H11002 (b/b) and congenic (54- or 17-Mb) mice. A. Concanavalin A–elicited peritoneal macrophages, 6-week WTD, n = 10 mice/group. B. Resident peritoneal macrophages, 9-week WTD, n = 8 to 12 mice/group. C. Splenic CD11b+ monocyte/macrophages, 15-week WTD, n = 7 to 9 mice/group. ANOVA (A) or t test (B and C) was performed with log transformation. *P < 0.05, **P < 0.005, †P < 0.0005 compared with b/b controls.
4 congenic mouse, with possible relevance to the mechanism of increased atherogenesis in humans bearing the 9p21 risk allele.

Consistent with our BM transplantation study, whole-body deletion of $p19^{ARF}$ was recently shown to be proatherosclerotic in the B6-ApoE$^{-/-}$ background. The mechanism of action suggested in the Apoe$^{-/-}$ model was decreased apoptosis, although the culprit cell type was not identified. We did not observe differences in plaque apoptosis in either the Athsq1 congenic model or mice carrying BM deficiency of Cdkn2a compared with the respective controls. The discrepancy between the 2 studies could be due to different experimental designs (BM versus whole-body deficiency), different targeted alleles ($p16^{INK4a}$/$p19^{ARF}$ versus $p19^{ARF}$ alone), different mouse models (Ldlr$^{-/-}$ versus Apoe$^{-/-}$), or a nontarget effect of a carrier gene in 1 of the mutant models.

**Figure 3.** Bone marrow (BM)-specific Cdkn2a deficiency is sufficient to promote atherosclerosis in B6-Ldlr$^{-/-}$ mice. A and B, Transcript-specific real-time polymerase chain reaction results for Cdkn2a ($p16^{INK4a}$ and $p19^{ARF}$) and $p18^{INK4c}$ (another INK4-class gene encoded by a distant region of mouse chromosome 4). n = 7 mice/group. Unpaired $t$ test was performed with log transformation. C, Mean lesion areas from B6-Ldlr$^{-/-}$ mice transplanted with B6-Ldr$^{-/-}$ or B6-Ldr$^{-/-}$, Cdkn2a$^{-/-}$ BM and fed 10-week WTD. Two-factor ANOVA was performed with square root transformation. Horizontal lines indicate group means for males (circles) and females (triangles). *$P$ < 0.05.
However, although decreased apoptosis may accelerate early lesion formation, increased apoptosis likely contributes to lesion progression to advanced plaques with clinically significant consequences, such as myocardial infarction associated with 9p21.

Chr 4 mouse deletion mutants with decreased expression of all 3 Cdkn2a/b transcripts exhibited increased proliferation of vSMCs. However, vSMC proliferation does not readily explain the human atherosclerotic phenotype because it generally leads to cap thickening and plaque stabilization. The results of a reverse BM transplantation experiment reported herein were inconsistent with a role for vSMCs in the atherosclerosis phenotype of Aths<sup>17-Mb</sup> subcongenic mice (Supplemental Figure II). In line with this, Folkersen et al<sup>27</sup> failed to demonstrate an association between vascular gene expression of CDKN2A/B and 9p21 risk genotype.

Evidence for an association between elevated white blood cell counts and ASVD risk has been documented in more than a dozen prospective epidemiological studies. There is also substantial evidence in animal models for a causal relationship between monocytosis and atherogenesis. In mouse models of diet-induced atherosclerosis, the subset of Ly6C<sup>hi</sup>-expressing monocytes expands with hypercholesterolemia and selectively enters sites of inflammation, including atherosclerotic lesions, compared with the Ly6C<sup>lo</sup> subset. In addition, single gene mutations resulting in reduced blood monocytes also reduced atherosclerosis, independently of plasma cholesterol levels. Although the relationship between monocytosis and atherosclerosis has been well documented, detailed mechanisms responsible for monocytosis are lacking. Consistent with a recent finding, our study suggests that the control of myeloid cell proliferation during hypercholesterolemia is an important factor determining magnitude of leukocytosis and the atherogenic response.

A variety of mechanisms for the involvement of CDKN2A/B, ANRIL, or both in the 9p21 ASVD locus have been proposed. Regulation of CDKN2A/B gene expression has been suggested to occur via a cis or trans mechanism(s) involving either the structurally overlapping ANRIL<sup>39</sup> or other regulatory motifs residing within the 58-kb risk locus.<sup>11,40</sup> It is also possible that ANRIL may mediate effects at 9p21 without involvement of

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**Figure 4.** Increased inflammatory Ly6C<sup>hi</sup> monocytes, mediated by increased cell proliferation, in the circulation of B6-Ldlr<sup>−/−</sup> mice transplanted with Ldlr<sup>−/−</sup>, Cdkn2a<sup>−/−</sup> bone marrow (BM) compared with controls. A, Flow cytometry analysis of blood monocytes from Ldlr<sup>−/−</sup> or Ldlr<sup>−/−</sup>, Cdkn2a<sup>−/−</sup> BM recipients fed chow or WTD. Monocytes were gated as CD45<sup>+</sup> CD115<sup>+</sup> Ly6C<sup>hi</sup> or CD45<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>lo</sup>. B, Quantification of Ly6C<sup>hi</sup> Ly6C<sup>lo</sup> cells among total CD45<sup>+</sup>CD115<sup>+</sup> cells. C, Analysis of proliferating CD45<sup>+</sup>Ly6C<sup>hi</sup> cells among total CD45<sup>+</sup>CD115<sup>+</sup>monocytes. n=11 mice/group. *P<0.05, **P<0.005, †P<0.0005.
A 

\[
\begin{array}{c|c|c}
\text{CD115} & \text{Cdkn2a}^{+/+} & \text{Cdkn2a}^{-/-} \\
\hline
0 & 0 & 0 \\
0.05 & 0.12 & 0.21 \\
0.1 & 0.15 & 0.25 \\
0.15 & 0.18 & 0.3 \\
0.2 & 0.22 & 0.35 \\
0.25 & 0.26 & 0.4 \\
0.3 & 0.3 & 0.45 \\
0.35 & 0.35 & 0.5 \\
0.4 & 0.4 & 0.55 \\
0.45 & 0.45 & 0.6 \\
0.5 & 0.5 & 0.65 \\
\end{array}
\]

B

Counts

\[
\begin{array}{c|c|c}
\text{BrdU} & \text{Cdkn2a}^{+/+} & \text{Cdkn2a}^{-/-} \\
\hline
0 & 0 & 0 \\
0.05 & 0.12 & 0.21 \\
0.1 & 0.15 & 0.25 \\
0.15 & 0.18 & 0.3 \\
0.2 & 0.22 & 0.35 \\
0.25 & 0.26 & 0.4 \\
0.3 & 0.3 & 0.45 \\
0.35 & 0.35 & 0.5 \\
0.4 & 0.4 & 0.55 \\
0.45 & 0.45 & 0.6 \\
0.5 & 0.5 & 0.65 \\
\end{array}
\]

C

\[
\begin{array}{c|c|c}
\text{BrdU} & \text{Cdkn2a}^{+/+} & \text{Cdkn2a}^{-/-} \\
\hline
0 & 0 & 0 \\
0.05 & 0.12 & 0.21 \\
0.1 & 0.15 & 0.25 \\
0.15 & 0.18 & 0.3 \\
0.2 & 0.22 & 0.35 \\
0.25 & 0.26 & 0.4 \\
0.3 & 0.3 & 0.45 \\
0.35 & 0.35 & 0.5 \\
0.4 & 0.4 & 0.55 \\
0.45 & 0.45 & 0.6 \\
0.5 & 0.5 & 0.65 \\
\end{array}
\]

**Figure 5.** Increased proliferation of tissue macrophages derived from B6-Ldlr+/+ mice transplanted with Ldlr-/-, Cdkn2a-/- bone marrow compared with controls. A, Flow cytometry analysis of concanavalin A–elicited peritoneal macrophages from B6-Ldlr+/+ or B6-Ldlr-/-, Cdkn2a-/- mice fed 4 to 5 weeks WTD. Macrophages were gated as CD45°CD115°F4/80°. C, Quantification of BrdU incorporation at the 4- to 5-week time point. Cells were gated as 5-bromo-2’-deoxyuridine (BrdU)°. C, Quantification of BrdU° cells among total CD45°CD115°F4/80° macrophages. n=6 to 7 mice/group. *P<0.05.

**CDKN2A/B, through regulation of gene expression at an unlinked locus.** The mouse genome does not contain a contiguous sequence with homology to ANRIL. However, markedly decreased expression of Cdkn2a/b transcripts in the 70-kb deletion mutant suggests the existence of a cis-acting enhancer.13 In our Athsq1 congenic mouse, there is a loss of coordinate regulation with decreased expression of Cdkn2a (p16INK4a and p15INK4b) transcripts but not Cdkn2b (p15INK4b). Thus, in our model, the causal variant is likely to be cis-acting and specific to the Cdkn2a locus.

The 9p21 locus has been associated with multiple vascular phenotypes. These include myocardial infarction, abdominal aortic and intracranial aneurysms, and peripheral artery disease. Interestingly, monocyte/macrophage recruitment is an important process in cerebral aneurysm formation, and Ccl2 deficiency has been shown to inhibit macrophage accumulation in aneurysmal walls and significantly decrease aneurysm formation in an experimentally induced mouse model. In another study, increased immunostaining of CD68 antigen was observed in intracranial aneurysms compared with control tissue. In ischemic stroke, macrophage accumulation has been associated with severity of brain injury. Thus, alterations in monocyte/macrophage proliferation could potentially provide a common underlying mechanism for vascular phenotypes associated with the 9p21 locus.

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**Disclosures**

Dr Puig works at Merck Research Laboratories.

**References**


Cdkn2a Is an Atherosclerosis Modifier Locus That Regulates Monocyte/Macrophage Proliferation

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SUPPLEMENT MATERIAL
Supplemental Methods

Mice
MOLF/Ei (MOLF) and B6.129S7-Ldlr<sup>em1Her</sup> (B6-Ldlr<sup>−/−</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.MOLF-Athsq1 congenic mice were generated by introgression of a 54 megabase (Mb) MOLF donor interval into the B6-Ldlr<sup>−/−</sup> background, followed by intercrossing to produce homozygotes, as described. Characterization of the lesion phenotype in N6-derived congenic mice (previously described) compared to N14-derived mice (reported herein) revealed no differences (data not shown). Subcongenic mice (17-, 11-, and 4-Mb) were derived from the 54-Mb strain; recombinants were identified and intercrossed at N13-N17 generations. Cdkn2a<sup>−/−</sup> mice<sup>2</sup> backcrossed into B6<sup>3</sup> were kindly provided by Dr. Sean Morrison (University of Michigan) and crossbred to B6-Ldlr<sup>−/−</sup> mice in our colony. Mice were fed Western-type diet (WTD) containing 21% milk fat and 0.15% cholesterol (TD 88137, Harlan Teklad) for the indicated time periods before sacrifice. All procedures were in accordance with institutional guidelines.

DNA extraction and genotyping
Microsatellite markers and Ldlr alleles were typed by polymerase chain reaction (PCR) as described.<sup>1,4</sup> Cdkn2a alleles were commercially typed by PCR (Gene Typer LLC).

Gene expression profiling
Peritoneal monocyte/macrophages were isolated from B6-Ldlr<sup>−/−</sup> or 54-Mb congenic mice three days after no treatment (resident) or intraperitoneal injection of concanavalin A. Cells were plated and washed one hour later to remove non-adherent cells, followed by overnight incubation in DMEM (25 mM glucose), 10% FBS, and 1% penicillin/streptomycin. Splenic monocyte/macrophages were isolated to >90% purity by Magnetic Activated Cell Sorting (MACS) using an anti-CD11b antibody (Miltenyi).<sup>5,6</sup> RNA was isolated using an RNA micro kit (Qiagen) according to the manufacturer’s protocol. Microarray analysis was performed as described.<sup>7</sup> Merck/Affymetrix mouse 1.0 custom arrays monitoring 38384 individual transcripts (25,846 Entrez genes) were used. A Custom Definition File is available in GEO database (http://www.ncbi.nlm.nih.gov/geo/GPL9734). NCBI build 37 mapping definitions matched probesets in the array with transcripts. Total RNA from 10 mice were analyzed in each group. Raw intensity was normalized using the RMA algorithm. Prefiltering removed transcripts not detected (marked as "absent" by using the MAS5 algorithm with p>0.05) in 50% or more of replicates in all treatment groups, which were not considered further. Two-sided t-test (p<0.05) between congenic and control identified genes differentially expressed. The level of false positive genes was kept under 10% and assessed by permuting 100 times the labels in each dataset prior to any gene filtering, calculating the size of the signature in each permutation, and averaging the results to determine the level of noise. Raw data are available in the NCBI gene expression and hybridization array data repository, GEO (GSE 24342).
Atherosclerotic Lesion Measurements
Paraffin-embedded serial sections were prepared from the aortic root as described.\textsuperscript{1,4} Lesion area was quantified by morphometric analysis of H&E stained sections, and average lesion size determined from six sections per mouse.

BM Transplantation
Irradiated recipient B6-\textit{Ldlr}\textsuperscript{+/−} mice or 17-Mb congenic mice were injected with B6-\textit{Ldlr}\textsuperscript{+/−} BM as described.\textsuperscript{8} An additional study was performed with recipient B6-\textit{Ldlr}\textsuperscript{+/−} mice injected with either B6-\textit{Ldlr}\textsuperscript{+/+}, \textit{Cdkn2a}\textsuperscript{+/+} or B6-\textit{Ldlr}\textsuperscript{+/+}, \textit{Cdkn2a}\textsuperscript{−/−} BM. Reconstitution of the BM with donor cells was confirmed at six weeks (wks) post injection with microsatellite markers for the congenic interval or allele-specific \textit{Cdkn2a} and \textit{Ldlr} primers, respectively. Mice were then fed 10-11 wk WTD before euthanasia.

Bromodeoxyuridine (BrdU) labeling
Mice were injected intraperitoneally with 200 ul of 2 mg BrdU. The pulse was 18 hrs for the analysis of circulating monocytes. For assessment of peritoneal macrophage proliferation, cells were harvested after three consecutive injections of BrdU over 72 hrs following a 24-hr concanavalin A injection.

Flow cytometry analysis
Flow cytometry was performed as described.\textsuperscript{9} Cells were stained using an antibody cocktail including CD45-APC-Cy7, CD115-APC, Ly6C-PerCP, and BrdU-FITC (BD Pharmingen). Monocytes were identified as CD45\textsuperscript{+} CD115\textsuperscript{+} cells and further gated as Ly6Chi or Ly6Clo. Peritoneal monocyte/macrophages were freshly collected and stained with CD45, CD115 and F4/80-PE. Multiparameter analyses were performed using a BD LSR II flow cytometer (Becton Dickinson) with DiVa software. Data were analyzed using FlowJo software (Tree Star, Inc.).

Statistical analysis
Two-factor ANOVA and \textit{t} tests were performed using STATVIEW 5.0 (Abacus Concepts, Inc). Both males and females were included in lesion analyses using both genotype and sex as factors in the ANOVA. The post-test was Fisher’s PLSD and the threshold for significance was \( p = 0.05 \). Data shown are mean ± SEM.
**Supplemental Table I**

Body weight and plasma lipoprotein profiles of B6-Ldlr−/− mice receiving Ldlr+/− or Ldlr+/−, Cdkn2a +/− bone marrow. Mice were fed 10-wk WTD. Data are mean ±SD, n=17-19 mice/group.

<table>
<thead>
<tr>
<th>BM donor genotype</th>
<th>BWT (g)</th>
<th>TC (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>Non-HDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ldlr+/−</td>
<td>26.5±3.1</td>
<td>1230±339</td>
<td>83±22</td>
<td>445±12</td>
<td>1147±338</td>
</tr>
<tr>
<td>Ldlr−/−, Cdkn2a+/−</td>
<td>26.4±4.4</td>
<td>1363±229</td>
<td>82±14</td>
<td>424±11</td>
<td>1276±232</td>
</tr>
</tbody>
</table>

Abbreviations: BWT, body weight; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; Non-HDL-C, non-HDL cholesterol.
Supplemental Figure I. Confirmed localization of an atherosclerosis susceptibility locus to a 5.4-9 Mb region of mouse chromosome 4, and identification of an additional distal locus. A, Mean lesion areas from control (b/b), full congenic (54-Mb) and subcongenic (4-Mb) mice fed 6-wk WTD. No difference in mean lesion area between the control and 4-Mb subcongenic strain indicates that the 4-Mb interval is excluded as a causal interval. B, Comparative map of the MOLF-derived intervals (white boxes) carried by the proximal subcongenic strains and a newly identified distal strain, subB. All strains in the B6-Ldlr<sup>−/−</sup> background. Mb, megabase; b/b, homozygosity for B6 alleles; m/m, homozygosity for MOLF alleles. C, Mean lesion areas from control (b/b) and the distal subcongeneric strain (m/m, subB). Two factor ANOVA was performed with square root transformation (A and C). Horizontal bars indicate group means for males (blue) and females (red). NS, not significant.
Supplemental Figure II. Vascular cells from 17-Mb subcongenic mice do not contribute to the pro-atherosclerotic phenotype. Mean lesion areas from a reverse BM transplantation study in which both control (b/b) and subcongenic (17-Mb) recipients received BM from B6-Ldlr⁻/⁻ mice. Following repopulation of the BM, mice were fed 11-wk WTD. Two-factor ANOVA was performed with square root transformation. Horizontal bars indicate group means for males (blue) and females (red). NS, not significant.
Supplemental Figure III. Increased ratio of Ly6C^hi:Ly6C^lo monocytes observed in the circulation of B6-Ldlr^-/- mice injected with B6-Ldr^+/-, Cdkn2a^+/+ BM, and 17-Mb congenics, compared to respective controls. A, Ratio of Ly6C^hi:Ly6C^lo monocyte subsets in the circulation of B6-Ldlr^-/- mice receiving either B6-Ldr^+/+ or B6-Ldr^+/+ Cdkn2a^+/+ BM. Blood was collected following 0-10 weeks of WTD feeding. B, Blood was collected from the 17-Mb congenics and b/b controls after 3-, 6- and 9-wk WTD feeding. Data shown are mean ± SEM. *p ≤ 0.05. **p ≤ 0.005, † p < 0.0005. N=8-11/group.
Supplemental Figure IV

**A**

Flow cytometry analysis of spleen monocytes from B6-Ldlr⁻/⁻, Cdkn2a⁺⁻ mice compared to B6-Ldlr⁻/⁻ controls. Monocytes were gated as CD45⁺CD115⁺ Ly6C⁺ or CD45⁺CD115⁺ Ly6C⁻ and quantification of the percentages of Ly6C⁺ and Ly6C⁻ cells among total CD45⁺CD115⁺ cells was performed.

**B**

Increased ratio of Ly6C⁺: Ly6C⁻ monocytes in spleens derived from Ldlr⁻/⁻, Cdkn2a⁺⁻ mice compared to the controls. Splenic monocytes were collected after 9-10 weeks of WTD feeding. N=7-9/group.
Supplemental References


