Genetic Susceptibility of the Arterial Wall Is an Important Determinant of Atherosclerosis in C57BL/6 and FVB/N Mouse Strains

Jeong Shim, Aase Handberg, Caroline Östergren, Erling Falk, Jacob F. Bentzon

Objective—How genetic variations among inbred mouse strains translate into differences in atherosclerosis susceptibility is of significant interest for the development of new therapeutic strategies. The objective of the present study was to examine whether genetically controlled arterial wall properties influence atherosclerosis susceptibility in FVB/N (FVB) and C57BL/6 (B6) apolipoprotein E knockout (apoE−/−) mouse strains.

Methods and Results—Common carotid artery segments from B6 apoE−/−, F1 apoE−/−, and FVB apoE−/− mice were transplanted to hybrid F1 apoE−/− mice, which can accept grafts from both parental strains without adaptive immune responses. The mice were fed a high-fat diet, and atherosclerosis was induced in the transplanted artery segments by placement of a perivascular constrictive collar. Artery segments from B6 apoE−/− mice developed much larger atherosclerotic lesions than artery segments from FVB or F1 apoE−/− mice. No differences in aortic arch atherosclerosis of the recipient mice were observed between groups.

Conclusion—Genetically controlled factors acting at the level of the arterial wall are important determinants of atherosclerosis susceptibility in FVB apoE−/− and B6 apoE−/− mice. (Arterioscler Thromb Vasc Biol. 2011;31:1814-1820.)

Key Words: atherosclerosis ■ genetically altered mice

The root cause of atherosclerosis is hypercholesterolemia, but some individuals do not develop atherosclerosis despite severe hypercholesterolemia, whereas others develop clinically significant atherosclerosis with low total cholesterol levels.1,2 Population studies have revealed that genetics is an important determinant of this variable atherosclerosis susceptibility, but much remains to be learned about the genes involved and the way genetic variations are translated into differences in atherosclerosis susceptibility.3,4

Apolipoprotein E knockout (apoE−/−) mice have high levels of circulating cholesterol-rich lipoproteins caused by the lack of apoE-mediated lipoprotein clearance,5 but the extent of atherosclerosis development is strongly dependent on the genetic background of the apoE−/− mouse strain. For instance, C57BL/6 (B6) apoE−/− mice develop 9-fold more atherosclerosis in the aortic root than FVB/N (FVB) apoE−/− mice despite lower levels of total cholesterol.6 Hybrid (B6xFVB)F1 (F1) apoE−/− mice develop lesions of intermediate size.

Identification of the genes that determine such differences in atherosclerosis susceptibility may direct attention to novel drug targets.7 This is an unbiased approach not restricted to building on prior knowledge and may therefore identify atherosclerosis-modifying genes with previously unknown functions. Furthermore, because functional variants of the identified genes already exist among relatively healthy inbred mouse strains, the strategy, at least to some extent, selects for proteins that may be targeted by drugs without devastating side effects.

Studies of FVB×B6 intercrosses have led to the identification of a number of quantitative trait loci (QTL) that influence atherosclerosis susceptibility.8–10 A complementary strategy to detect the causative gene variations is to identify functional differences in the complex pathophysiology of atherosclerosis between the strains. Using this approach, recent studies of other strain combinations have shown that the difference in atherosclerosis susceptibility between C3H apoE−/− and B6 apoE−/− mice is mediated, at least in part, by the reaction of vascular wall cells to hypercholesterolemia,11,12 whereas in another strain comparison, BALB/c versus B6, the difference in atherosclerosis susceptibility appeared to be mediated by other mechanisms.13

We have previously described a model in which site-directed atherosclerosis is induced in transplanted common carotid artery (CCA) segments by the application of a constrictive perivascular collar,14 and we have shown that smooth muscle cells (SMCs) and endothelial cells in this model remain of local origin, with no contributions from...
putative circulating progenitor cells. In the present study, we used this model to show that the low atherosclerosis susceptibility of the FVB apoE/−/− mouse strains is, at least partly, caused by a relative resistance of the arterial wall to atherosclerosis formation.

Methods

Animals

All procedures were approved by the Danish Animal Experiments Inspectorate. Female B6 apoE/−/− (B6.129P2-Apoetm1Unc, Taconic M&B, Ry, Denmark) and male FVB apoE/−/− mice (kindly provided by Jan L. Breslow, Rockefeller University, New York, NY) were crossed to obtain F1 apoE/−/− hybrids. For all surgical procedures, mice were anesthetized with isoflurane (induction 5%, maintenance 1.5% to 2.0%) and buprenorphine (0.1 mg/kg SC), and given postoperative analgesics (Rimadyl, 5 mg/kg SC, repeated every 24 hours) for 3 days.

CCA Transplantation

CCA transplants were performed as previously described when the mice were 8 weeks of age (see Figure 1 for schematic overview of experiments). Briefly, the anesthetized donor mouse was killed by exsanguination and flushed with 10 mL of heparin-saline (100 IU/mL) through the left ventricle. A segment of approximately 2 mm of the right CCA of the donor mouse was harvested and stored in ice-cold saline. The neck of the recipient mouse was incised through the midline and the right CCA was dissected free of surrounding tissue. Vessel clamps (ABB-11V, S&T, Neuhausen, Switzerland) were applied 2 minutes after the injection of 200 IU/kg heparin IM. A small piece of the recipient CCA was cut out, and the remaining recipient vessel ends were flushed with saline to remove blood. The donor graft was then interpositioned and anastomosed end-to-end with 5 symmetrically positioned 11-0 nylon sutures (Ethicon, Johnson & Johnson, Birkerød, Denmark). Body weights of donor and recipient mice were similar in all groups (see Supplemental Table, available online at http://atvb.ahajournals.org).

Constrictive Collar Application

At 12 weeks of age, the CCA transplanted mice were put on a high-fat diet (0.25% wt/wt cholesterol and 15% wt/wt cocoa butter, diet 4021.06, Arie Blok BV, Woerden, the Netherlands), and 2 weeks later, atherosclerotic lesions were induced by applying a constrictive perivascular collar around the distal part of the transplanted segments using a technique modified from von der Thüsen et al. A silicone tube (inner diameter 0.31 mm, Helixmark, Helix Medical Inc) was placed around the transplanted artery and secured with two 8-0 nylon ligatures. Constrictive collars were also applied to the CCA of FVB, F1, and B6 apoE/−/− mice that had not undergone prior CCA transplantation.

Atherosclerosis Quantification

At 1, 3, or 6 weeks after collar insertion, mice were fasted from the morning for 4 to 8 hours, anesthetized (5 mg of pentobarbital IP), and exsanguinated by withdrawing blood from the right ventricle into EDTA-coated tubes. The mice were then flushed with a cardioplegic solution, perfusion-fixed at 100 mm Hg for 5 minutes with 4% phosphate-buffered formaldehyde (pH 7.2) via the left ventricle, and then immersed in the fixative for 6 hours before storage in cold phosphate buffer. The right CCA with the transplanted segment was removed and either dehydrated and embedded in paraffin (6 weeks) or cryopreserved and embedded in OCT compound (1 or 3 weeks). Sections were obtained at 50-μm intervals, cutting upstream from the proximal end of the constrictive collar and through the induced lesion. After 5 levels, sections were obtained at 100-μm intervals until no more plaque was visible or until the proximal anastomosis was reached. All sections were stained with orcein, and plaque sizes were measured blindly using computer-assisted image analysis (ImageJ, National Institutes of Health). Total plaque volume was calculated according to the Cavalieri principle. Atherosclerosis in F1 apoE/−/− recipients euthanized 6 weeks after collar insertion was quantified by illumination of cleaned, unstained, and unopened aortic arches as previously described. Briefly, the arches were photographed with a dissection microscope, and the area occupied by atherosclerotic plaques, easily recognizable through the translucent wall, was measured using ImageJ.
Plaque Composition Analysis
Plaque composition was analyzed in plaques from the transplanted FVB and B6 apoE<sup>−/−</sup> CCA segments obtained at 1, 3, or 6 weeks after collar insertion. Sections at ~100 μm from the proximal end of the constrictive collar were stained with Sirius Red for quantification of collagen-rich connective tissue and with antibodies against smooth muscle α-actin (SMαA) and Mac2 for counting of plaque SMCs and macrophages, respectively.

The fraction of Sirius Red–staining plaque was analyzed using the threshold color plugin for ImageJ (http://www.dentistry.bham.ac.uk/landing/software/software.html). SMαA was detected using mouse monoclonal anti-SMαA (1:50, Clone 1A4, Dako, Glostrup, Denmark) after blocking of endogenous mouse immunoglobulins with donkey anti-mouse Fab fragments (1:10, Jackson Immunoresearch) and formaldehyde fixation (4%, 10 minutes). Mac2 was detected by rat anti-mouse Mac2 antibody (1:500, CL8942AP, Cedarlane Labs, Trichem Aps, Frederikssund, Denmark). Both primary antibodies were followed by Texas Red–conjugated secondary antibodies (1:400, Jackson Immunoresearch). Only SMCs and macrophage profiles containing nuclei (4',6-diamidino-2-phenylindole–stained) were counted.

Plasma Lipids
Cholesterol was measured in duplicates in EDTA-plasma from each mouse by a manual cholesterol kit (enzymatic end point method) from Randox (Crumlin). For profiling of lipoproteins by gel filtration, pools (100 μL) of EDTA-plasma from FVB, F1, and B6 apoE<sup>−/−</sup> mice (Figure 1A) were centrifuged through a Durapore polyvinylidene difluoride membrane (pore size 0.22 μm, Millipore-Ultrafree Amicon, Billerica, MA), loaded on a Superose 6 10/300 GL column (GE Healthcare, Bromly, Denmark) and separated at room temperature at a flow rate of 0.25 mL/minute in 50 mmol/L Tris, 0.3 mol/L NaCl and 0.1% human serum albumin, pH 7.2. Elution fractions of 120 seconds were collected, and cholesterol content was measured.

Statistical Analysis
Overall comparison between groups was performed with Kruskal-Wallis test, except for cholesterol data where overall comparison was performed with 1-way ANOVA. Test for Gaussian distribution was performed using the Kolmogorov-Smirnov test implemented in the Prism statistical software (GraphPad, San Diego, CA). If an overall significant difference was found, pairwise group comparisons were performed using the Dunn post test or the Tukey multiple comparison test as appropriate. Two-sample nonparametric comparisons were performed with Mann-Whitney tests. *P*<0.05 was considered to be statistically significant. In the first experiment, we excluded 2 mice (1 B6 and 1 FVB apoE<sup>−/−</sup> mouse not listed in Figure 1A) because morphology of the induced lesions and the fact that their sizes were 28- and 539-fold larger than the second-largest lesions in each group suggested that total occlusion had occurred.

Results
Plasma Cholesterol Analysis
Consistent with previous reports, plasma cholesterol levels or lipoprotein profiles did not explain the difference in atherosclerosis development between FVB apoE<sup>−/−</sup> and B6 apoE<sup>−/−</sup> mouse strains. To the contrary, atherosclerosis-resistant FVB apoE<sup>−/−</sup> mice exhibited significantly higher cholesterol levels in both very-low-density lipoprotein and low-density lipoprotein fractions (Figure 2).

Atherosclerosis Induced by Constrictive Collars
It is generally assumed that the pathogenesis of collar-induced atherosclerosis is similar to that of spontaneous atherosclerosis, although this tenet is difficult to prove. Like spontaneous atherosclerosis, collar-induced atherosclerosis does not form in normocholesterolemic mice but is strictly dependent on the presence of hypercholesterolemia, develops at sites with low wall shear stress, and is initiated as foam cell lesions that develop into fibroatheromas (Figure 3). This is strikingly different from neointima induced by wire injury or ligation, procedures that, curiously, lead to much larger lesions in FVB than in B6 mice. To confirm that collar-induced atherosclerosis reflected the differences previously described for spontaneous atherosclerosis in the aortic root (ie, lesion size in B6 apoE<sup>−/−</sup> is much larger than in FVB apoE<sup>−/−</sup> mice), we induced plaques in the carotids of B6, F1, and FVB apoE<sup>−/−</sup> mice. In FVB apoE<sup>−/−</sup> mice, no foam cell lesions or only very small ones were present after 6 weeks (median lesion size 0 μm<sup>3</sup>), whereas much larger and more complex lesions that included fibrous tissue and often cholesterol crystals developed in B6 apoE<sup>−/−</sup> mice (median 0.6×10<sup>6</sup> μm<sup>3</sup>) (Figures 3A to 3C and 4). Lesion sizes in F1 apoE<sup>−/−</sup> mice were intermediary (median 0.06×10<sup>6</sup> μm<sup>3</sup>). We performed a similar study in male mice and obtained similar results (data not shown).

The induction of atherosclerosis with the perivascular collar is likely dependent on the degree of vessel constriction, and because the collars were of fixed size, any systematic differences in carotid artery diameter between mouse strains might in itself have led to differences in lesion size. To exclude this possibility, we measured inner circumference of...
the nontreated left carotid artery in the B6 apoE<sup>−/−</sup> (1130±156 μm), F1 apoE<sup>−/−</sup> (1073±46 μm), and FVB apoE<sup>−/−</sup> (1156±212 μm) mice, but no significant differences were detected. This is also consistent with previous reports on vessel dimensions in these mouse strains. Furthermore, as a proxy for vessel size, body weights of the different mouse strains were not different at the time of collar placement or at euthanization (Supplemental Table).

The Genotype of the Vessel Wall Determines Susceptibility to Atherosclerosis

Atherogenesis is complex, with many participating cell types, including bone marrow-derived cells (macrophages, lymphocytes, dendritic cells, mast cells) and vessel wall-derived cells (SMCs, endothelial cells), and a plethora of potentially important systemic factors, including hormones, metabolites, and blood pressure. We hypothesized that factors inherent to the local vessel wall may control atherosclerosis susceptibility in B6 and FVB apoE<sup>−/−</sup> mice and performed transplants of carotid segments from FVB, F1, and B6 apoE<sup>−/−</sup> strains into F1 apoE<sup>−/−</sup> mice to test this idea.

Hybrid F1 apoE<sup>−/−</sup> mice can receive tissues from both parental strains without adaptive immune responses, and although there is a potential for natural killer cell reactions toward the FVB apoE<sup>−/−</sup> and B6 apoE<sup>−/−</sup> transplants in our experimental design, we did not observe signs of allograft vasculopathy either by visual inspection during surgery when collars were applied (6 weeks after transplantation) or, more directly, by histology of B6 (n=3) or FVB apoE<sup>−/−</sup> transplants (n=3) 1 week after collar placement (7 weeks after transplantation) (Supplemental Figure I).

Six weeks after collar insertion, atherosclerotic plaques had formed within the transplanted segments extending from immediate proximal to the inserted collar toward the proximal atherosclerosis. Lesions in the F1 apoE<sup>−/−</sup> arterial segments transplanted into F1 apoE<sup>−/−</sup> mice were larger than lesions induced in F1 apoE<sup>−/−</sup> mice without preceding transplantation (P=0.06 by Mann-Whitney test). Also, lesions in both B6 apoE<sup>−/−</sup> and FVB apoE<sup>−/−</sup> arterial segments transplanted into F1 apoE<sup>−/−</sup> mice were significantly larger than similar lesions induced in B6 apoE<sup>−/−</sup> and FVB apoE<sup>−/−</sup> mice (P=0.0007 and P=0.0003, respectively, by Mann-Whitney tests).

Interestingly, when segments transplanted into F1 apoE<sup>−/−</sup> mice were compared, plaques were dramatically larger in B6 apoE<sup>−/−</sup> segments (median 55.8×10<sup>6</sup> μm<sup>3</sup>) compared with both FVB (median 1.7×10<sup>6</sup> μm<sup>3</sup>) and F1 apoE<sup>−/−</sup> (median 0.6×10<sup>6</sup> μm<sup>3</sup>) segments (Figures 3D to 3F and 5A), indicating that proatherogenic gene variants acting recessively with respect to the B6 allele affect the susceptibility of the vessel wall to atherosclerosis. No differences in recipient atherosclerosis in F1 apoE<sup>−/−</sup> mice were seen (Figure 5B and Supplemental Figure II).

Differentially Regulated Mechanisms

To investigate whether plaque composition was influenced by the genotype of the arterial wall, we analyzed the development and composition of plaques in transplanted FVB and B6 apoE<sup>−/−</sup> vessel segments at a fixed distance (100 μm) upstream from the collar. The vast difference in plaque growth induced by collar insertion (Figure 6A) biases the comparison of plaque composition because of the association...
between plaque size and composition during atherogenesis in mice. Early and small lesions contain mostly foam cell macrophages, whereas more mature and bigger plaques contain more SMCs and fibrous tissue. Instead of comparing plaque composition at single time points, where plaque size is a tremendous source of bias, we therefore pooled observations and studied the relationship between plaque size and plaque composition. No apparent differences in plaque composition of similar-sized lesions between FVB and B6 apoE<sup>−/−</sup> vessel segments were noted (Figure 6B to 6G).

**Discussion**

The mechanisms mediating the difference in atherosclerosis development between B6 apoE<sup>−/−</sup> and FVB apoE<sup>−/−</sup> mice are not understood. Even though the FVB apoE<sup>−/−</sup> strain has even higher levels of both very-low-density lipoprotein and low-density lipoprotein cholesterol, it develops little atherosclerosis. Our present study indicates that genetically controlled properties of the arterial wall make FVB apoE<sup>−/−</sup> mice relatively less prone to atherosclerosis.

**Strategies to Identify Susceptibility Genes**

Several studies have searched for gene variants that influence atherosclerosis susceptibility in inbred mouse strains using QTL analysis. Several susceptibility genes have been identified in this way, including genes not previously known to be involved in atherosclerosis, but a number of challenges limit progress. One difficulty is the identification of causative genes among a large number of candidates within QTL regions. Novel steps, including expression QTL and more sophisticated crosses, have been taken to reduce this problem but it remains a formidable effort. A more fundamental limitation is the complex genetic architecture of many traits, including atherosclerosis susceptibility. Rather than being caused by few large-effect gene variations, atherosclerosis susceptibility in inbred mice and humans result from the integration of numerous small-effect gene variations, most of them undetectable by QTL analysis using conventional sample sizes. For example, in an F2 intercross of FVB apoE<sup>−/−</sup> and B6 apoE<sup>−/−</sup> mice, Dansky et al found only 1 genome-wide significant QTL, and in fact, genetic variation at this locus does not contribute to the relative atherosclerosis resistance of the FVB apoE<sup>−/−</sup> strain because F2 mice with the FVB allele develop more atherosclerosis than F2 mice with the B6 allele.

**Strategies to Identify Biological Pathways**

Even though the genetics of atherosclerosis is complex, many genetic variations with subtle individual effects may converge on a limited set of pathways that may be targets for efficient antiatherogenic intervention. Complementary approaches, such as the one taken in the present study, may be needed to identify these pathways when no large-effect gene variations, detectable by genetic techniques, reveal their nature. The present study indicates that genetically controlled properties of the arterial wall renders FVB arteries dramatically less prone to plaque development compared with B6 arteries. These properties may include (1) differential regulation or coding-sequence differences of genes that are expressed in SMCs or endothelial cells and involved in the atherosclerotic process or (2) genes that control the structure of the normal arterial wall, eg, extracellular matrix composition, making it more or less susceptible to atherosclerosis in the face of hypercholesterolemia. Our investigation of 3 candidate vessel wall-controlled processes (macrophage recruitment, SMC recruitment, and fibrous tissue production) did not reveal obvious candidates for the underlying mechanism, but our rather coarse approach does not, on the other hand, rule out that these processes may be involved.

**Candidate Genes**

The present study suggests that genes that are expressed in SMCs or endothelial cells and have cis-expression QTL in these cell types are particularly interesting candidates for causative susceptibility genes in QTL regions identified previously in B6 and FVB crosses. One such gene may be *Adam17*. In a recent study, Teupser et al identified a QTL...
region on chromosome 12 in an intercross between FVB and B6 low-density lipoprotein receptor–knockout mice.9 Congenic mice revealed that B6/B6 alleles at this locus increased lesion development by approximately 50% compared with the B6/FVB genotype, and subsequent studies showed that the Adam17 gene, which is located in the region, is differentially expressed between B6 and FVB aortas and had a cis-expression QTL in liver tissue.10 Although this gene cannot alone explain the vast difference in arterial susceptibility observed between B6 and FVB mice, it may direct attention to vascular cell-exerted control of the local cytokine environment as a candidate mechanism.

Limitations
Atherosclerotic lesions develop at branch sites and in curvatures of the vascular tree where special flow fields characterized by low wall shear stress prevail.27,28 Consistently, the straight segment of the CCA in apoE−/− mice is normally resistant to atherosclerosis but can be turned into atherosclerosis-susceptible region by placement of a constrictive collar that lowers wall shear stress and causes other changes to the flow field immediate proximal to the collar.19 Although this process bears much resemblance to spontaneous atherosclerosis in terms of both etiology and pathogenesis, there might be significant differences, and proper caution should thus be exerted when extrapolating from this experimental model.

Another important aspect of our findings is that is that lesions induced by constrictive collars in normal carotid arteries were smaller than those induced in transplanted segments despite the fact that the collars were applied at the same age and for the same period of time. One contributing factor may be that transplanted CCA segments were more difficult to dissect free of periadventitial tissue, and it is plausible that placement of the collar therefore caused a relatively higher degree of flow disturbance, leading to a greater atherogenic stimulus. Less than perfect anastomoses may also in itself have contributed to reduced blood flow. Furthermore, even though transplantation of parental solid tissue into hybrid mice does not elicit B or T cell–mediated immunity, it may trigger natural killer cells as recently shown for B6 hearts that developed significant allograft vasculopathy by week 5 to 8 after transplantation into (B6xB/c)F1 hybrid mice.22 In our study, lesions clearly developed as a result of collar placement (Figure 6A), and no allograft vasculopathy was present 7 weeks after transplantation. It remains possible, however, that more subtle influences of natural killer cells, eg, the secretion of proinflammatory cytokines, may have accelerated lesion formation after atherosclerosis induction and, if this were to be the case, that potential differences in the magnitude of such activity might have influenced the results of the present and similar studies in the field.12

Conclusions
Our experiments indicate that genetically controlled properties of the arterial wall mediate a major part of the difference in atherosclerosis susceptibility between FVB apoE−/− and B6 apoE−/− inbred mice.

Figure 6. Composition of plaques in transplanted FVB and B6 apoE−/− CCA segments. A, Lesion growth. B to D, The number of macrophages and SMCs, and the area of fibrous plaques were measured in adjacent sections at ~100 μm proximal to the collar and plotted against plaque size. FVB data points are shown in filled symbols and comprise plaques harvested 1 week ( ), 3 weeks ( ), and 6 weeks ( ) after collar placement. FVB data points are shown in filled symbols ( ) for 1 week, ( ) for 3 weeks, and ( ) for 6 weeks after collar placement). Plaques in FVB apoE−/− CCA segments contained fewer macrophages, SMCs, and collagen-rich tissue but were also much smaller, and when plaques of similar sizes were compared, no obvious compositional differences were noted. E, Immunofluorescence detection of Mac2 to identify macrophages. F, Immunofluorescence detection of SMαA to identify SMCs. G, Sirius Red–stained sections as examined with polarized microscopy to analyze the amount and distribution of collagen-rich tissue in plaques. The lumen, the internal elastic lamina, and the external elastic lamina are traced (gray dotted line). The adventitia of the transplanted segments was particularly collagen-rich. L indicates lumen; P, plaque; M, media; A, adventitia. Scale bars = 50 μm (E and F) or 200 μm (G).
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Disclosures

None.

References


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Supplemental Material.

Supplementary Table. Body weights of mice

<table>
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<tr>
<th></th>
<th>Weight at collar application (g)</th>
<th>Weight at euthanization (g)</th>
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<tr>
<td><strong>A. Collar-Induced Lesions</strong></td>
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<td>FVB apoE⁻/⁻ (n=7)</td>
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<td><strong>B. CCA Transplants</strong></td>
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Values are median (interquartile range) for weight. CCA, common carotid artery

Supplementary Figure I

Supplementary Figure 1. Cross-sections of FVB apoE⁻/⁻ (A) and B6 apoE⁻/⁻ (B) carotid segments 7 weeks after transplantation into F1 apoE⁻/⁻ mice and 1 week after collar application. Sections are taken 500 µm upstream to the collar. No allograft vasculopathy lesions are seen. Scale bar 100 µm.
Supplementary Figure II

Supplementary Figure 2. Unstained and unopened aortic arch and major branches from an F1 apoE<sup>−/−</sup> recipient mouse. The non-pellucid atherosclerotic plaques are easily visible through the translucent arterial wall. The area occupied by plaque in the aortic arch (from above the aortic leaflets to 1 mm below the left subclavian artery) and in the first 1 mm of each of the branches was measured. Scale bar 1 mm.