**Conclusions**—Reduced transcription of *Mertk*, rather than differences in MERTK protein structure, determines the reduced efficiency of apoptotic cell clearance in the *Aath4aDBA/DBA* mice, which, in turn, contributes to their increased susceptibility to atherosclerosis.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vascul Biol. 2017;37:e82-e91. DOI: 10.1161/ATVBAHA.117.309522.)

**Key Words:** aorta ■ atherosclerosis ■ macrophages ■ mice ■ phagocytosis

Atherosclerosis is a complex multifactorial disease, and individual susceptibility to plaque development is influenced by many genetic factors. We previously showed that plaque size in mice is dependent on the strain and vascular location in the early stage of atherosclerosis.1-4 At the aortic root, apolipoprotein E-deficient (*Apoe*−/−) mice on a DBA/2J background (DBA/2J-*Apoe*−/−) develop larger plaques than those on a C57BL/6J background (C57BL/6J-*Apoe*−/−), whereas *Apoe*−/− mice on a DBA/2J background (129S6-*Apoe*−/−) are resistant to plaque development compared with DBA/2J-*Apoe*−/− or C57BL/6J-*Apoe*−/− mice. In the aortic arch area, however, 129S6-*Apoe*−/− and DBA/2J-*Apoe*−/− mice show larger lesion size than C57BL/6J-*Apoe*−/− mice. These observations clearly indicate that the location specificity of plaque development is genetically controlled.

Our QTL analysis using an intercross between DBA/2J-*Apoe*−/− and 129S6-*Apoe*−/− revealed *Aath4*, an atherosclerosis QTL for the aortic arch area on the distal part of chromosome 2 (Peak: 137 Mb, confidence interval: 123-148 Mb).3 The DBA/2J allele of *Aath4* confers susceptibility to atherosclerosis, whereas the 129S6 allele confers resistance. *Aath4* was not detected in a C57BL/6J-*Apoe*−/− × 129S6-*Apoe*−/− cross,2 indicating that *Aath4* sequences unique to DBA/2J are responsible for the different phenotypes. Many candidate genes are present in the chromosomal region in which *Aath4* resides, including several phagocytosis-related genes.

One of the candidates is *c-mer proto-oncogene tyrosine kinase (Mertk)* located at 128.5 Mb, which encodes a member of the TAM (Tyro3, Axl, and Mer) receptor tyrosine kinase family. MERTK is primarily expressed in monocytes as well as in epithelial and reproductive tissues,5 and it is involved in phagocytosis of apoptotic cells.6,7 It has 2 immunoglobulin-like domains and 2 fibronectin type III domains in the extracellular region, and binds to apoptotic cells via bridging molecules such as GAS6 and protein S.5,8,9 The binding promotes phosphorylation of the tyrosine kinase domain located within the intracellular region of MERTK; this phosphorylation leads to activation of downstream signaling and induces structural changes in cytoskeletons, enabling the cell to engulf its target cells.10 Macrophages from mice with an inactivated *Mertk* kinase domain (*Mertk−/−*) are deficient in the clearance of apoptotic cells (efferocytosis).6 The

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Mertk−/− mice are viable, but show retinal degeneration, because of the failure of the retinal pigment epithelium to engulf the outer segments of photoreceptors.7,11 MERTK also plays a pivotal role in atherosclerotic plaque development via its effects on efferocytosis: Mertk deficiency in Apoe−/− mice promotes accumulation of apoptotic cells and expansion of necrotic cores within plaques12; and low-density lipoprotein receptor-deficient (Ldlr −/−) mice with Mertk −/− bone marrow show accumulation of apoptotic cells and accelerated atherosclerosis.13

In this study, we have generated and studied a mouse line, Aath4aDBA/DBA, in which a 5′ region of the Aath4 from DBA/2J has been transferred onto a 129S6-Apoe−/− background. We show that these mice have elevated plaque susceptibility and reduced efferocytosis by macrophages. We also demonstrate that decreased transcription of Mertk, not DBA-unique amino acid alterations in MERTK, determines the limited efferocytosis that occurs in Aath4aDBA/DBA mice.

Materials and Methods

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

### Results

#### Generation of Aath4aDBA/DBA Mice

Aath4aDBA/DBA congenic mice were constructed by backcrossing DBA/2J-Apoe−/− mice to the 129S6-Apoe−/− strain for more than 7 generations. The backcrossed genomic segments in chromosome 2 (117–137 Mb) are shown in Figure 1A. The DBA allele of SNP (single nucleotide polymorphism) rs27446327 was used as a marker for Chr 2: 128 Mb. Heterozygous mice were mated to generate homozygotes. SNPs rs30884601, rs36935260, rs48427643, and rs30243498 were also typed at early generations to ensure that DBA alleles at other atherosclerosis QTLs (Chr 1: 155 Mb, Chr 1: 165 Mb, Chr 2: 143 Mb, and Chr 10: 86 Mb) were eliminated.

#### Aath4aDBA/DBA Male Mice Develop Larger Plaques With Increased Calcium Deposits

As summarized in Table 1, Aath4a129/129 control mice and Aath4aDBA/DBA mice showed no significant differences in body weight. In males, plasma cholesterol levels were almost the same between controls and Aath4aDBA/DBA, whereas triglycerides were significantly higher in Aath4a DBA/DBA (P=0.002). In female Aath4aDBA/DBA mice, plasma total cholesterol was lower (P=0.010) and HDL-cholesterol was higher (P=0.016) compared with Aath4a129/129 controls, but triglycerides were not significantly different.

At 5 months of age, Aath4aDBA/DBA male mice developed ≈50% larger plaques at the inner curve of the aortic arch compared with control mice (P<0.05; Figure 1B, left). Plaque size at
we selected 7 raised lesions with similar size from Aath4aDBA/DBA mice and control mice, and observed in any of these branches between Aath4aDBA/DBA and Aath4a129/129 mice. Although very few, TUNEL (terminal deoxynucleotidyl trans-ferase dUTP nick-end labeling) positive nuclei were detected within the plaques of both Aath4aDBA/DBA and Aath4a129/129 mice. In more mature plaques, calcium deposits were detected equally in both Aath4aDBA/DBA mice and Aath4a129/129 controls, and they were located deeper in the plaques, near the internal lamina (Figure IIE and IIF in the online-only Data Supplement). Enhanced cell death or reduced clearance of necrotic cores, in the raised plaques in 28 of 36 Aath4aDBA/DBA deposits were detectable, often associated with the acellular region of Aath4aDBA/DBA plaques of similar size and locations were examined histologically, the early raised lesions of Aath4aDBA/DBA mice also developed significantly larger brachiocephalic artery lesions compared with the control (P<0.05), but did not differ in the left common carotid and left subclavian arteries. In females, no significant difference was observed in any of these branches between Aath4aDBA/DBA mice and controls (Figure I in the online-only Data Supplement).

The plaques developing in the aortic roots of Aath4aDBA/DBA and control mice ranged from a monolayer of foam cells to intermediate stage plaques. Necrotic area was ≈5× larger in Aath4aDBA/DBA than in control mice (Figure 2C). Fibrous cap thickness was variable, and the difference was not statistically significant between the 2 groups (Figure 2D). However, collagen content in these lesions was less in the Aath4aDBA/DBA mice, suggesting that resolution of inflammation associated with the plaque development is likely delayed in Aath4aDBA/DBA (Figure 2E). General inflammation in these mice was low, judged by the low concentration of transforming growth factor-β1 and undetectable interleukin-10 in plasma. No significant differences in mRNA levels of interleukin-1β and transforming growth factor-β1 in the aorta were observed between control Aath4a129/129 and Aath4aDBA/DBA mice or in plasma transforming growth factor-β1 levels (Figure IV A and IVB in the online-only Data Supplement), probably because these mice were fed with normal chow.

Together these results indicate that the DBA2/J allele of Aath4 enhances atherosclerosis in both the aortic arch and the root. Increased cell death in the early stages of plaque development may be associated with this enhancement.

### Reduced Mertk Expression in Aath4aDBA/DBA Macrophages

The Aath4a QTL includes Mertk, which is important for effero-cytosis, the phagocytic removal of apoptotic cells. MERTK is localized on cell surface where it is proteolytically cleaved by ADAM metalloproteinase domain 17 (ADAM17) to produce soluble MER (sMER). sMER attenuates MERTK-triggered intracellular signaling by blocking bridging molecules. In thioglycollate-elicited peritoneal macrophages isolated from Aath4aDBA/DBA, Mertk mRNA levels were less than 50% of that in Aath4a129/129 control macrophages (Figure 3A). In parallel with the mRNA expression, the amount of MERTK protein in cultured macrophages was also reduced in Aath4aDBA/DBA (Figure 3B), as well as cell-surface expression of MERTK (Figure 3C).

sMER in the conditioned medium of Aath4aDBA/DBA macrophage and plasma sMER in Aath4aDBA/DBA mice were also reduced compared with those in controls, consistent with the reduction of protein amounts in macrophages (Figure 3B and 3D). MERTK protein was abundantly present within the atherosclerotic plaques of the Aath4a129/129 control mice, bordering the macrophage marker CD68-positive area, whereas the amount was significantly decreased in Aath4aDBA/DBA plaques (Figure V in the online-only Data Supplement).

### Reduced Phagocytosis in Aath4aDBA/DBA Macrophages

MERTK is important for the normal execution of effero-cytosis during atherosclerotic plaque development. We, therefore, examined phagocytosis of apoptotic Jurkat T cells by peritoneal macrophages of Aath4aDBA/DBA mice and found that the uptake of apoptotic cells was reduced to ≈40% of Aath4a129/129 controls (Figures 4A through 4C; Figure VI in the online-only Data Supplement).

Ghosni et al have reported that there are two types of peritoneal macrophages: large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs). LPMs predominate in the steady state peritoneal cavity but are decreased by inflammatory stimuli such as lipopolysaccharide and

### Table. Body Weights and Plasma Lipids in the Control and Aath4aDBA/DBA Mice

<table>
<thead>
<tr>
<th></th>
<th>Aath4a129/129 (n)</th>
<th>Aath4aDBA/DBA (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28.59±0.37 (33)</td>
<td>28.08±0.42 (23)</td>
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<td>Female</td>
<td>21.30±0.38 (27)</td>
<td>21.37±0.37 (20)</td>
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<tr>
<td><strong>T-Chol, mg/dL</strong></td>
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<tr>
<td>Male</td>
<td>680.9±23.0 (37)</td>
<td>738.2±29.2 (23)</td>
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<tr>
<td>Female</td>
<td>572.3±27.5 (26)</td>
<td>482.2±31.3* (20)</td>
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<tr>
<td><strong>HDL-C, mg/dL</strong></td>
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<tr>
<td>Male</td>
<td>79.4±5.0 (33)</td>
<td>77.1±9.5 (9)</td>
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<tr>
<td>Female</td>
<td>45.1±3.7 (26)</td>
<td>60.2±4.2* (20)</td>
</tr>
<tr>
<td><strong>TG, mg/dL</strong></td>
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</tr>
<tr>
<td>Male</td>
<td>79±(3)</td>
<td>125±8† (23)</td>
</tr>
<tr>
<td>Female</td>
<td>48±4 (25)</td>
<td>58±5 (19)</td>
</tr>
</tbody>
</table>

Data are shown as the mean±SE. HDL-C indicates high-density lipoprotein cholesterol; T-Chol, total cholesterol; and TG, triglyceride.

*P<0.05.
†P<0.01 vs Aath4a129/129.
Figure 2. Aath4aDBA/DBA mice develop plaques with increased calcium deposits, larger necrotic core, and less collagen content. A, Representative early but raised plaques at the aortic root of control Aath4a129/129 (129/129) and Aath4aDBA/DBA (DBA/DBA) mice at 5 mo of age. The plaques with similar sizes stained with SudanIVB and hematoxylin are shown. Aath4aDBA/DBA mouse (right) contains extensive calcium deposits (arrowheads). B, Calcium deposits detected by von Kossa stain. Numerous deposits were observed in the early plaques of Aath4aDBA/DBA mice (right), whereas rarely seen in those of control Aath4a129/129 mice (left). Bar=100 μm. C, Necrotic core size in the plaques of Aath4a129/129 (129/129) and Aath4aDBA/DBA (DBA/DBA) mice at 5 mo old. Advanced lesions that are similar in total size were selected and necrotic area was measured in the selected lesions (n=7). D, Fibrous cap thickness of advanced plaques. In the 7 advanced lesions selected in C, fibrous cap thickness was measured at 3 sites per lesion and averaged. E, Collagen content in the advanced lesions detected by trichrome staining. The Aath4aDBA/DBA (DBA/DBA) lesion contains less collagen (blue). Bar=100 μm. N.S. indicates not significant.
thioglycollate, which induce infiltration of SPMs into the peritoneal cavity from myeloid progenitor cells in the bone marrow. We found that 3 days after stimulation by thioglycollate, the percentage of LPM was significantly smaller in Aath4aDBA/DBA than in control mice (0.8% versus 3.0% in Aath4aDBA/DBA and control mice, respectively; P <0.001; Figure 5A and 5B). Both SPMs and LPMs express MERTK, although in Aath4aDBA/DBA macrophages, MERTK was markedly reduced in both SPMs and LPMs (Figure 5C). Consistent with a previous report, phagocytosis was higher in LPMs than in SPMs,17 but importantly, both LPMs and SPMs showed similarly reduced phagocytosis in Aath4aDBA/DBA macrophages (Figure 5D).

MERTK Amino Acid Differences Do Not Affect Efferocytosis

There are 9 amino acid substitutions between 129S6 and DBA/2J MERTK, 3 of which (W25G, T80E, and S479R) are predicted to potentially alter protein function (Figure 6A). The S479R substitution located adjacent to the ADAM17 cleavage site does not seem to have a significant effect on the shedding of MERTK because the proportion of sMER:MER was not altered between control and Aath4aDBA/DBA macrophages, MERTK was markedly reduced in both SPMs and LPMs (Figure 5C). Consistent with a previous report, phagocytosis was higher in LPMs than in SPMs,17 but importantly, both LPMs and SPMs showed similarly reduced phagocytosis in Aath4aDBA/DBA macrophages (Figure 5D).

We next examined the ability of the HEK293T cells expressing MERTK to phagocytose apoptotic Jurkat T cells. Overexpression of MERTK increased phagocytosis of apoptotic Jurkat T cells compared with the empty vector-transfected control cells, but no significant difference was observed between the 129S6 and the DBA/2J (empty vectors: 0.8±0.5%, 129S6-MERTK: 28.4±1.5%, and DBA/2J-MERTK: 29.5±1.5%; Figure 6C). Addition of protein S, which bridges apoptotic cells and MERTK, stimulated phagocytosis in all of them, but a genetic effect was not observed (empty vectors: 4.1±1.6%, 129S6-MERTK: 45.3±1.5%, and DBA/2J-MERTK: 48.7±2.7%; Figure 6C). Thus, the T80E substitution in the first immunoglobulin-like domain, which contains a ligand binding site, does not affect the binding to bridging protein S. These results indicate that the DBA/2J-specific amino acid substitutions in MERTK do not affect phagocytic function.

Differences in Merk Expression Levels Modulate Efferocytosis

Previous works have shown that mice lacking Merk and Apoe (Merk−−/Apoe−−) have increased accumulation of apoptotic cells and expanded necrotic cores in their plaques when compared with Merk−+/Apoe−− mice,15 and that peritoneal macrophages from Merk-deficient mice (Merk−−) phagocytose apoptotic cells at a much reduced level (<20% compared with Merk−+/ macrophages).9 This raises the question whether differences in the level of expression of MERTK affect efferocytosis in a graded manner. To test this, we examined phagocytosis of apoptotic Jurkat T cells by peritoneal macrophages isolated from wild-type (Merk−+/+) and Merk heterozygous
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Mertk heterozygous macrophages, which are supposed to express 50% of MERTK, show ≈70% phagocytosis compared with wild-type macrophages (P=0.002; Figure 6D), indicating that the amount of MERTK modulates efficiency of phagocytosis.

In agreement, when the amount of Mertk-plasmid transfected to HEK293 cells was gradually decreased, cell surface 129-MERTK and DBA-MERTK and phagocytosis were equally reduced (Figure 6E). We conclude that the amount of MERTK determines the efficiency of phagocytosis in a dose-dependent, not an all-or-none manner, and that decreased Mertk expression in Aath4aDBA/DBA macrophages is the cause of the reduced phagocytosis.

Transcription Activity of the Proximal Promoter Region of Mertk
There are a large number of nucleotide polymorphisms throughout the Mertk gene, which could affect steady state levels of mRNA by affecting its stability. Accordingly, to clarify the cause of reduced Mertk mRNA in Aath4aDBA/DBA macrophages, we asked whether the degradation of Mertk mRNA is enhanced in Aath4aDBA/DBA macrophages. We found that the estimated half-life of Mertk mRNA was 5.6 hours in the control Aath4a129/129 macrophages and 5.7 hours in the Aath4aDBA/DBA macrophages, suggesting that the lower amount of Mertk mRNA in Aath4aDBA/DBA macrophages is likely caused by reduced Mertk transcription, rather than by faster...
degradation of Mertk mRNA (Figure VII in the online-only Data Supplement).

We next searched for DBA/2J-specific differences in the promoter region of the Mertk gene that could affect the efficiency of transcription. Within the 1.1 kb immediately upstream of the Mertk transcription start site, there are 10 SNPs and 1 deletion that are unique to DBA compared with B6 and 129 sequences (Figure VIIIA in the online-only Data Supplement). The Mertk gene has a CpG island promoter and does not contain a typical TATA box or CAAT box.

To test whether the sequence differences in the proximal promoter region of Mertk affect the transcription level, we compared promoter activity of 129S6 and DBA/2J by a luciferase reporter assay. Mertk promoters of both 129S6 and DBA/2J enhanced luciferase expression in HEK293T cells ≈5× compared with empty vector, but the transcription activities of the Mertk promoter from 129S6 and DBA/2J were not significantly different (Figure VIIIB in the online-only Data Supplement). Furthermore, addition of factors that are thought to regulate Mertk expression including granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interleukin-4 did not significantly change the promoter activities (Figure VIIIC in the online-only Data Supplement). Our experiments suggest that the genomic variants affecting the expression of Mertk must reside outside of this proximal 1.1 kb promoter region.

Indeed, when we searched for eQTLs associated with the expression levels of Mertk from Hybrid Mouse Diversity Panel by UCLA (https://systems.genetics.ucla.edu/data/hmdp),19 more than 15 SNPs within the Aath4a interval were significantly associated with Mertk expression levels (Table I in the online-only Data Supplement). The SNPs that are shared by...
129 and B6 but unique in DBA, and located between 63 kb and 2.6 Mb upstream of the *Mertk* gene, seem to regulate its expression in the liver and adipose tissue, whereas SNPs located between 2.3 and 2.5 Mb downstream were broadly associated with the expression levels in macrophages (Table I in the online-only Data Supplement). Two SNPs at −325 and −202 kb upstream also showed strong association with the *Mertk* expression in macrophages, but 129 and DBA share the same variants at these positions. Together, it is likely that distant genetic variants affect the expression of *Mertk*.

**Discussion**

Each QTL identified in the crosses of inbred mice generally spans a large genomic distance, sometimes almost an entire chromosome. In complex phenotypes such as atherosclerosis, where a large number of genes are involved, transferring a target region onto an inbred background and creating congenic line is a powerful step toward identifying causative genes. Here we have analyzed the effect of the atherosclerotic QTL *Aath4* by establishing a congenic line (*Aath4* DBA/DBA), where the S′ region of DBA *Aath4* was backcrossed onto a 129S6-*Apoe*−/− background. As expected, the resulting *Aath4* DBA/DBA males had significantly larger plaques, and macrophages isolated from these mice exhibited reduced efferocytosis as a consequence of allele-specific decrease in MERTK expression. Together, our results provide strong evidence that the increased susceptibility to atherosclerosis determined by the DBA allele of *Aath4* is, at least in part, due to decreased MERTK expression.

MERTK is known to play a significant role in efferocytosis and the resolution of inflammation during atherosclerosis.2,13 In this report, we have demonstrated that 9 nonsynonymous SNPs in *Mertk*, which are uniquely different in DBA/2J compared with C57BL/6 and 129S6, are not responsible for the reduced efferocytosis observed in peritoneal macrophages from *Aath4* DBA/DBA. Notably, our experiments demonstrated that the level of MERTK expression controls phagocytosis in *Mertk*−/− and *Aath4* DBA/DBA macrophages in a dose-dependent, not an all-or-none manner. This is consistent with many published reports that disease-related SNPs identified by GWAS (genome-wide association study) are usually located in introns or intergenic regions rather than in coding sequences. Unlike diseases in which mutations in a single gene cause a drastic phenotype, the pathogenesis of atherosclerosis is more complex and involves numerous factors. SNPs or other alterations in regulatory regions typically lead to small changes in gene expression, which cumulatively influence susceptibility to disease. Our experiments show that *Mertk* expression is likely reduced at the transcription level because the stability of *Mertk* mRNA was unchanged in the *Aath4* DBA/DBA macrophages. Furthermore, our reporter assay tests show that the genetic differences that cause different *Mertk* expression in DBA/2J and 129S6 are likely to be outside the proximal 1.1 kb promoter region, and search of eQTL database suggests that the expression of *Mertk* is affected by distant genetic variants. In humans, an SNP rs869016 in intron 1 of *MERTK* is associated with decreased risk of carotid atherosclerosis, although it is unknown whether the SNP modulates *Mertk* mRNA expression.20 Further investigation of the causative variants is clearly required.

Efferocytosis, the phagocytosis of apoptotic cells by macrophages, is critical in preventing progression of atheroma.21 Consistently, it has been shown that *Mertk*−/− or other alterations in regulatory regions typically lead to small molecules fed a Western-type diet have increased numbers of apoptotic cells and expanded necrosis in advanced lesions.12 In early plaques, removal of apoptotic cells is normally efficient; although, we have observed signs of basophilic (dystrophic) calcium deposits even in the early raised plaques of *Aath4* DBA/DBA mice. Our attempt to detect apoptotic cells in vivo in plaques by TUNEL assay was not productive to make a comparison because TUNEL-positive nuclei were few per section in both *Aath4* DBA/DBA and *Aath4* DBA/DBA. This is because mice were fed with normal chow to eliminate additional effects caused by high-fat diet, and most of the lesions we observed were at their early stages. However, the calcium deposits are likely the remnants of dead/dying cells via apoptotic and necrotic processes, giving strong evidence that the normal process of removing dead cells is restricted in the *Aath4* DBA/DBA mice. Delayed apoptotic cell clearance is expected to cause acceleration of plaque development and necrotic core formation. The calcium deposits in the early plaques, however, seem to be short-lived because larger calcified areas in advanced *Aath4* DBA/DBA plaques are not different from the control plaques and are seen mostly deeper in the intima near the internal elastic lamina. Although the thickness of fibrous caps was not significantly different, the *Aath4* DBA/DBA plaques contained less interstitial matrix proteins than similar-sized plaques in the control mice. In advanced plaques, efferocytosis by macrophages becomes less effective than in early plaques, but an alteration in the balance between synthesis and degradation of matrix protein would be expected to contribute to vulnerable plaque morphology. Although we have not encountered any premature deaths associated with the *Aath4* DBA/DBA mice, a more detailed examination of later stage plaques would be worthwhile.

Despite the well-known heterogeneity of mouse peritoneal macrophages,15,16 we found that MERTK is expressed in both LPMs and SPMs, and that it is similarly reduced in both populations of *Aath4* DBA/DBA macrophages. We also observed that the LPMs, which have higher phagocytic activity than SPMs,17 were consistently fewer in *Aath4* DBA/DBA than in controls. Fewer LPMs and more SPMs could be another factor contributing to the reduced phagocytosis in *Aath4* DBA/DBA peritoneal macrophages. SPMs are induced from circulating monocytes by inflammatory stimuli, whereas LPMs are thought to be tissue-resident macrophages, maintained locally by differentiation from precursor cells. Macrophages in atherosclerotic plaques are similarly heterogeneous,22,23 and the role of circulating monocyte-derived macrophages in the development of plaques has been well-documented. Evidence is also beginning to accumulate that tissue-resident macrophages originating from the adventitial precursor cells contribute to atherogenesis. Factors that control the relative balance of these populations are not known, but a shift in the population of the *Aath4* DBA/DBA macrophages in their plaques is conceivable. Whether MERTK expression directly affects this shift or not requires further study.

Although we identified *Aath4* as a QTL specific to the arch lesion, the *Aath4* DBA/DBA mice have significantly larger plaques in both the aortic arch and the root. This is in agreement with our prediction that the reduced efferocytosis of *Aath4* DBA/DBA macrophages should equally affect both the aortic arch and the
root. Our previous studies of F2 population between 129S6-
Apoe-/- and C57BL/6-Apoe-/- indicated that 129S6 genome
carries the sequence that affects the aortic arch geometry, which
are also associated with increased aortic arch atherosclerosis.2
Therefore, the variant in MerTK expression is a risk factor that
is additional to the 129S6 sequences that determine the aortic
arch development because the Aath4aDBA/DBA strain is based
upon the 129S6 genome. Moreover, the contribution of MerTK
expression to the 50% increase in root lesion is, although sig-
nificant, relatively small, considering that the parental DBA/2J
show 10x larger plaques in the root than 129S6 mice.

The genomic region of DBA/2J carried in the Aath4aDBA/DBA
mice is large, and the effect of DNA variants in other loca-
tions in this region must be considered. For example, Siglec1
at 131 Mb, which encodes sialoadhesin (CD169), is involved
in the retention of hematopoietic stem cells in the bone mar-
row niche.24 It is also expressed in a subset of tissue-resi-
 dent macrophages, preventing excessive inflammation upon
injury.25,26 Similarly, CD169 may also play a role in phagocy-
tosis.26,27 Altered expression of Bcl2l11 at 128 Mb, encoding for a
proapoptotic protein Bim, could affect early cell death in the
Aath4aDBA/DBA plaques. In addition, as the backcrossed
region in the Aath4aDBA/DBA line is the proximal half of Aath4,
the distal half may include genes that are proatherogenic only
in the arch, or are athero-suppressive specifically in the root.

One of the candidates in distal Aath4 is Cd93 at 148 Mb, a
transmembrane glycoprotein which is expressed in endothel-
ial and myeloid cells. CD93-deficient mice show a defect in
the clearance of apoptotic cells in vivo,28 and an SNP in Cd93
gene is associated with an increased risk of coronary heart
disease.29 Analysis of a congenic line carrying the distal half
of Aath4 of DBA/2J is currently under way.

We also noted that the plaque size difference was observed
which only in Aath4aDBA/DBA males, whereas the male/female
differences in plaque size were not seen in the parental strains:
DBA/2J-Apoe-/- mice show larger root lesions compared with
129S6-Apoe-/- in both males and females;1 arch lesion
size was comparable between the 2 strains in both males and
females.4 The QTLs on Chr 2 were detected in both males
and females in the F2 population from DBA/2J-Apoe-/- and
129S6-Apoe-/- mice.3,4 Because no QTL was detected on Chr
2 for plasma lipids in the intercross between the 2 strains, the
plasma lipid differences between males and females are not
likely the strong determinant of the plaque size differences.3
The major determinants for the plaque sizes in females must,
therefore, lie outside of the Aath4a interval. The sex dimor-
phism in the Aath4aDBA/DBA mice requires further investigation.

In summary, our experiments have shown that the DBA
allele of the 5’ region of Aath4 causes inefficient effec-
tosis via lower expression of MERTK, and that difference
contributes to the enhanced plaque development observed in
Aath4aDBA/DBA mice. Because the effects of reduced MERTK
and effecotosis are seen in the both aortic arch and root
areas, further studies will be required to fully understand
the factors that cause location specificity in atherosclerosis.

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None.

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Atherosclerosis and the DBA/2J Allele of Mertk

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Highlights

• Aath4aDBA/DBA, a congenic line of an atherosclerosis QTL Aath4 on distal Chr 2, was generated by transferring DBA/2J alleles of Aath4 to the129S6-Apoe<sup>−/−</sup> strain.

• Aath4aDBA/DBA males develop larger plaques and peritoneal macrophages isolated from Aath4aDBA/DBA showed reduced phagocytosis of apoptotic cells.

• Lower Mertk transcription, rather than DBA/2J-specific amino acid substitutions, causes restricted efferocytosis in Aath4aDBA/DBA.
DBA/2J Haplotype on Distal Chromosome 2 Reduces Mertk Expression, Restricts Efferocytosis, and Increases Susceptibility to Atherosclerosis

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SUPPLEMENTAL MATERIAL.

Material and Methods

Animals
Apoe<sup>−/−</sup> mice on a 129S6/SvEvTac (129-apoE) background were generated in our laboratory as previously described.<sup>1</sup> Apoe<sup>−/−</sup> mice on a DBA/2J background were obtained from Jackson Laboratory (#007067, D2.129P2(B6)-Apoe<sup>tm1Unc/J</sup>). Aath<sub>4</sub>DBA/DBA congenic mice were generated as described in the results section. Mertk<sup>−/−</sup> mice (C57BL/6 background) were a kind gift from Dr. GK Matsushima at the University of North Carolina at Chapel Hill. Mice were fed regular mouse chow (Teklad global soy protein-free extruded rodent diet, irradiated, 2920X, Harlan Laboratories) and handled under protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill.

Phenotyping
Body weight, tissue weight, plasma concentration of total cholesterol, triglyceride, HDL-cholesterol and glucose were determined at 5 month of age after 2-4 hour fast. Mice were anesthetized by an overdose of avertin (2,2,2-tribromophenol), and perfused with 4% paraformaldehyde. Atherosclerotic plaque size at the inner curve of aortic arch and at the aortic root was measured as previously described.<sup>2</sup>

Isolation of peritoneal macrophages
Two to three month old mice were intraperitoneally injected with 1 ml of 4% thioglycollate in PBS. Three days after the injection, the elicited peritoneal cells were collected and incubated in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum for four hours. Cells were washed with PBS to remove the non-adherent cells, and then incubated at 37 °C for an overnight.

qPCR of Mertk mRNA
Total RNA was isolated from macrophages using RNeasy mini kit (QIAGEN) according to the manufacturer’s instructions. Real-time quantitative PCR was performed by the 7500 Real Time PCR system (Applied Biosystems). Forward
primer: 5'-GGACTGCTTGATGAACTGTA-3', reverse primer: 5'-AGCCTCAACACAGAAGGTG-3', probe: 5'-TGATCTCTGCTGGAGTGCTGATCCC-3' was used to detect Mertk mRNA.

Western blot
Cells were lysed in MERTK lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% v/v glycerol, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate) supplemented with Complete Mini protease inhibitor cocktail (Roche). For detection of sMER, conditioned medium was concentrated using Amicon Ultra centrifugal filter (Millipore). Samples were separated on 10% SDS-polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes (Millipore). After blocking in 2% Amersham ECL blocking regent (GE Healthcare Life Sciences), membranes were incubated with anti-Mer (1:500, R&D), followed by incubation with peroxidase-conjugated anti-goat antibody (1:5000, Santa Cruz), then developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific).

Cell surface expression of MERTK
Macrophages were stained with FITC-conjugated anti-CD11b (1:200, Clone M1/70, BD Pharmingen), APC-conjugated anti-F4/80 (1:25, Clone BM8, eBioscience) and PE-conjugated anti-MERTK (1:40, Clone DS5MMER, eBioscience) antibodies. Fluorescence was measured on LSRFortessa (BD Biosciences) and assessed by FlowJo software.

Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay
Mouse hearts were embedded in O.C.T. compound (Tissue-Tek) and frozen in liquid nitrogen. Frozen sections of the heart including aortic root (7 μm) were dried, fixed in cold acetone, and blocked with 5% normal donkey serum. Slides were incubated with primary antibodies: goat anti-Mer (1:50, R&D) and rat anti-CD68 (1:100, Clone FA-11, Thermo Fisher Scientific), followed by incubation with secondary antibodies: Alexa Fluor 488-conjugated donkey anti-goat (1:500, Thermo Fisher Scientific) and Alexa 594-conjugated donkey anti-rat (1:500, Thermo Fisher
Apoptotic cells were detected using ApopTag Red In Situ apoptosis Detection Kit (EMD Millipore) according to the manufacturer’s protocol.

**Phagocytosis assay**

Peritoneal macrophages were isolated as described above and plated on 6-well plates. HEK293T cells were plated on 6-well plates, transfected with Mertk cDNA plasmids by FuGENE HD (Promega) according to the instructions, and used for phagocytosis assay 24 hours after the transfection. Jurkat T cells (ATCC) were induced to undergo apoptosis by incubation with 1 μM staurosporine (Sigma) for 3 to 4 hours at 37 °C. Apoptosis was verified by Annexin V-FITC Apoptosis Detection Kit (eBioscience). This consistently resulted in more than 50% apoptotic cells and less than 5% necrotic cells (Figure VIA in online-only Data Supplement). Apoptotic Jurkat T cells were stained with 100 ng/ml pHrodo™ Red SE (Thermo Fisher Scientific) for 30 minutes at room temperature, washed with PBS, resuspended in DMEM and then added to the macrophages or HEK293T cells at 1×10^6 cells per well, with or without 25 nM of human Protein S (Haematologic Technologies Inc.). After the incubation for 60 minutes at 37 °C, cells were detached from the plates by Accutase (EMD Millipore), stained with anti-CD11b-FITC (1:200, Clone M1/70, BD Pharmingen), or goat anti-Mer (1:10, R&D AF591) and anti-goat-APC (1:50, R&D F108), and then resuspended in HBSS containing 2% FBS and 0.5 μM DAPI. Phagocytosis was measured on LSRFortessa (BD Biosciences) and assessed by FlowJo software.

**Mouse Mertk cDNA isolation**

Total RNA was extracted from peritoneal macrophages of 129S6 and DBA/2J mice using RNeasy mini kit (QIAGEN). After cDNA was synthesized using SuperScript III (Life Technologies), the full length of the Mertk cDNA was amplified by PCR using the forward primer: 5'-CGGCCGCTAGCATGGTTCTGGCCCACGTGCTA-3' and the reverse primer: 5'-GCTGCTCAGGATGGTTTCTGCCCCACTGCTA-3'. The PCR fragments were cloned into pCMV6-AC-IRES-GFP-Puro vector (OriGene).

**Mertk mRNA stability assay**

Peritoneal macrophages were isolated as described above and cultured at 37 °C,
5% CO₂ for 4 days. 5 μg/ml of Actinomycin D (Sigma A1410) was added to each well to block synthesis of RNA. At the indicated time point, cells were washed with PBS and lysed, and total RNA was isolated using RNeasy mini kit (QIAGEN) for qPCR assay.

**Luciferase assay**

1.1 kb fragments corresponding to –1042 to +81 from the transcription start site in the promotor region of the *Mertk* gene were amplified from 129S6 and DBA/2J genomic DNA, and were cloned into pMCS-Cypridina Luc vector (Thermo Fisher Scientific). Plasmid DNA from three independent colonies of each construct was prepared and DNA sequences were verified by sequencing. HEK293T cells were transfected with the control empty plasmid or MERTK-Luc plasmids using FuGENE HD (Promega). Twenty-four hours after transfection, luciferase activities in the media were measured using Pierce™ Cypridina Luciferase Glow Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. pTK-*Gaussia*-Dura Luc DNA was co-transfected and assayed for normalization of the transfection efficiency of each well. 100 ng/ml GM-CSF, 50 ng/ml M-CSF and 20 ng/ml IL-4 (R&D) were added to the media 6 hours after transfection and luciferase assay was performed after the additional incubation for 18 hours.

**Statistics**

Plaque size was transformed to square root normal distribution. Comparisons of control and *Aath4a*⁰⁷⁸⁶⁷⁸⁸⁹⁰⁹ were done by t-test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer’s HSD test. *P* < 0.05 was considered as statistically significant. Data were analyzed using JMP software version 9.0 (SAS Institute).
Supplemental References


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<th>SNP (Chr)</th>
<th>SNP position (bp)</th>
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SNPs associated with \( \text{Mertk} \) (Chr 2: 128.5–128.6 Mb) expression that meet the criteria of \( P < 1.00E-05 \) were selected from eQTL database of Hybrid Mouse Diversity Panel (HMDP). Data from macrophage are shown in bold. Note that rs8264884, rs33501026, rs29716658 and rs29682971 have the same genotype pattern where DBA is uniquely different from B6 and 129.
Figure I.
Comparison of plaque size at the brachiocephalic artery (BCA, top), left common carotid artery (LCCA, middle), and left subclavian artery (LSCA, bottom) between the control $Aath4a^{129/129}$ and $Aath4a^{DBA/DBA}$ mice at 5 months old. Plaque size is indicated in square root (sqrt) of area in $\mu m^2$. Box-and-whisker plots: midline, median; box, 25th and 75th percentiles; whiskers, $1.5 \times$ interquartile range. Numbers of mice are indicated below the plots.
Figure II. Atherosclerotic plaques in control Aath4a\textsuperscript{129/129} (A, C and E) and Aath4a\textsuperscript{DBA/DBA} mice (B, D and F).

Plaques similar in size were compared. A and B. Early plaques involving the aortic wall are mainly foam cell lesions. Extensive infiltration of lipids in the SM layers is seen in plaques of both genotypes. C and D. Advanced complex lesions. The plaque of a control mouse (left) has a large necrotic core with cholesterol clefts and relatively thick fibrous cap. Necrotic cores of a plaque in Aath4a\textsuperscript{DBA/DBA} mouse (right) has thinner fibrous cap and contains extensive calcium deposits (arrowheads). E and F. Advanced lesions with areas of inflammation within the core were also seen in both genotypes. Disruption of the media and an inflammatory response within the adventitia are also commonly seen. Extensive calcium deposits deeper in the plaque is illustrated in the control plaque on the left (arrowheads). Bar = 100 μm.
Figure III. Apoptotic cells in the plaques.
Apoptotic cells (arrows) were detected by TUNEL staining (red) in the root lesions of $\text{Aath4a}^{129/129}$ (129/129) and $\text{Aath4a}^{DBA/DBA}$ (DBA/DBA) mice at 5 months old. Nuclei were visualized by DAPI (blue).
Figure IV. Inflammatory responses are not prominent either in $Aath4a^{129/129}$ or $Aath4a^{DBA/DBA}$ mice.

A. Expression levels of IL-1β and TGFβ1 mRNA in the aorta were detected by quantitative RT-PCR to estimate local inflammatory responses in $Aath4a^{129/129}$ (129/129) and $Aath4a^{DBA/DBA}$ (DBA/DBA) mice at 5 months old. B. Plasma TGFβ1 concentration was measured by mouse TGFβ1 DuoSet ELISA kit (R&D) to compare systemic inflammatory responses.
Figure V. MERTK expression was reduced in the plaques of *Aath4aDBA/DBA*. Representative immunofluorescent staining of MERTK (green) and a macrophage marker CD68 (red) in the atherosclerotic lesions in *Aath4a*<sup>129/129</sup> (*129/129*) and *Aath4a*<sup>DBA/DBA</sup> (*DBA/DBA*). MERTK was decreased in the *Aath4a*<sup>DBA/DBA</sup> lesion. DIC, differential interference contrast; V, aortic valve; P, plaque; SM, smooth muscle layer.
Figure VI. Gating strategy for the phagocytosis assay.

A. Representative flow cytometry panels for the induction of apoptosis in human Jurkat T cells. Jurkat T cells were incubated with or without 1 μM of staurosporine for 4 hours. Apoptosis was detected by staining with Annexin V-FITC and propidium iodide. The Annexin V positive, PI negative population represents early apoptotic cells (Q4 area).

B. A representative flow cytometry panel showing a negative control for the phagocytosis assay. Panels of apoptotic Jurkat T cells (ACs) and non-phagocytosing peritoneal macrophages are overlaid. ACs and macrophages were labeled with pHrodo Red and anti-CD11b-FITC, respectively.

C. Representative flow cytometry panels showing phagocytosis of ACs by peritoneal macrophages isolated from the control Aath4a^129/129 (129/129) and Aath4a^DBA/DBA (DBA/DBA) mice. ACs and macrophages were labeled with pHrodo Red and anti-CD11b-FITC, respectively.
Figure VII. Stability of Mertk mRNA in peritoneal macrophages. Mertk mRNA expression in peritoneal macrophages from the control Aath4a$^{129/129}$ (129/129) and Aath4a$^{DBA/DBA}$ (DBA/DBA) mice. Synthesis of RNA was blocked by 5 μg/ml of Actinomycin D and cells were lysed at the indicated time points.
Figure VIII. Transcriptional regulation of Mertk.

A. The DNA sequence of 1.1 kb upstream of the transcription start site of Mertk of 129S6. The SbfI/SacII fragment was inserted into a luciferase vector. The recognition sites for SbfI and SacII are indicated. The boxed sequences show the position of SNPs and a deletion specific to the DBA/2J sequence. The underlined sequences indicate potential transcription factor-binding sites. +1 indicates transcription start site. Translation start site (ATG) is bold.

B. Luciferase assay using SbfI and SacII promoter fragments of Mertk from 129S6 and DBA2J. EV, empty vector. n = 3.

C. Effects of GM-CSF, M-CSF, and IL-4 on the transcriptional activity of 5′ region of Mertk (n = 3). Data are shown as the mean ± SE.