

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2012 May ; 32(5): 1116–1123. doi:10.1161/ATVBAHA.112.246389.

Apolipoprotein E4 domain interaction accelerates diet-induced atherosclerosis in hypomorphic Arg-61 *ApoE* mice

Delphine Eberlé¹, Roy Y. Kim¹, Fu Sang Luk¹, Nabora Soledad Reyes de Mochel¹, Nathalie Gaudreault¹, Victor R. Olivas¹, Nikit Kumar¹, Jessica M. Posada¹, Andrew C. Birkeland¹, Joseph H. Rapp¹, and Robert L. Raffai¹

¹Department of Surgery, University of California San Francisco & VA Medical Center, San Francisco, CA

Abstract

Objective—Apolipoprotein (apo) E4 is an established risk factor for atherosclerosis, but the structural components underlying this association remain unclear. ApoE4 is characterized by two biophysical properties: domain interaction and molten globule state. Substituting Arg-61 for Thr-61 in mouse apoE introduces domain interaction without molten globule state, allowing us to delineate potential pro-atherogenic effects of domain interaction in vivo.

Methods and Results—We studied atherosclerosis susceptibility of hypomorphic *ApoE* mice expressing either Thr-61 or Arg-61 apoE (*ApoE*^{Th/h} or *ApoE*^{R/h} mice). On a chow diet, both mouse models were normo-lipidemic with similar levels of plasma apoE and lipoproteins. However, on a high cholesterol diet, *ApoE*^{R/h} mice displayed increased levels of total plasma cholesterol and VLDL as well as larger atherosclerotic plaques in the aortic root, arch and descending aorta compared to *ApoE*^{Th/h} mice. In addition, evidence of cellular dysfunction was identified in peritoneal *ApoE*^{R/h} macrophages which released lower amounts of apoE in culture medium and displayed increased expression of MHC class II molecules.

Conclusions—These data indicate that domain interaction mediates pro-atherogenic effects of apoE4 in part by modulating lipoprotein metabolism and macrophage biology. Pharmaceutical targeting of domain interaction could lead to new treatments for atherosclerosis in apoE4 individuals.

Keywords

Apolipoprotein E4; Domain interaction; Atherosclerosis; Lipoproteins; Macrophages

As a central modulator of plasma lipoprotein clearance, apolipoprotein (apo) E is a recognized determinant of cardiovascular disease susceptibility¹. In humans, apoE exists in three common isoforms (apoE2, apoE3 and apoE4) that are encoded by three alleles ($\epsilon 2$, $\epsilon 3$ and $\epsilon 4$)¹. ApoE3 (Cys-112, Arg-158) is regarded as the normal isoform whereas apoE4 (Arg-112, Arg-158) and apoE2 (Cys-112, Cys-158) are variants. Numerous reports have

Correspondence to Robert L. Raffai, Ph.D, Department of Surgery, University of California San Francisco & VA Medical Center, 4150 Clement Street, San Francisco, CA 94121, Telephone: (415)-221-4810 ext 2541, robert.raffai@ucsfmedctr.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure
None

established that the $\epsilon 4$ allele is associated with increased plasma cholesterol, LDL-cholesterol levels and risk of premature atherosclerosis ^{2,3}. Approximately 20% of individuals worldwide carry at least one $\epsilon 4$ allele ³, emphasizing the major impact of this cardiovascular risk factor on the general population.

ApoE is composed of two globular domains which function independently ⁴: The amino-terminal domain contains the binding site for the LDL receptor (LDLR), and the carboxyl-terminal domain contains the major lipoprotein-binding region. A series of biochemical and x-ray crystallographic studies revealed that apoE4 differs from apoE3 by at least two unique structural features: domain interaction which causes the two globular domains of apoE4 to interact ^{5,6}, and the molten globule state which predisposes apoE4 to instability and unfolding ⁷. Previous studies using knock-in and transgenic mouse models of human apoE4 examined mechanisms by which apoE4 promotes atherosclerosis ⁸⁻¹⁰. However, these studies could not determine the potential individual pro-atherogenic contributions of either domain interaction or molten globule state respectively. Understanding how the biophysical properties of apoE4 impact atherosclerosis is critical to gain new mechanistic insights into apoE4 biology as well as to develop targeted therapeutics.

Domain interaction results from the Cys-112 to Arg-112 mutation specific to the apoE4 isoform. This modification causes Arg-61 in the amino-terminal domain to change conformation and interact with Glu-255 in the carboxyl-terminal domain through the formation of a salt bridge, leading to a more compact structure of the protein ^{5,6}. Changing Arg-61 to the non-charged amino acid Thr-61 prevents the formation of domain interaction in human apoE4, highlighting the critical importance of Arg-61 in causing this structural property ⁵. Like human apoE4, mouse apoE contains the equivalent of Arg-112 and Glu-255; however, it lacks the critical Arg-61 equivalent (it contains Thr-61). Substituting Arg-61 for Thr-61 into the mouse *ApoE* locus introduced domain interaction without molten globule state ⁶, and created the Arg-61 apoE mouse model ¹¹, allowing us to study the pathological properties of domain interaction *in vivo*. Like human apoE4, Arg-61 mouse apoE preferentially distributes to very-low-density lipoprotein (VLDL) while Thr-61 mouse apoE preferentially distributes to high-density lipoprotein (HDL) similar to human apoE3 ¹¹.

In this study, our goal was to determine the potential contribution of domain interaction in atherosclerosis susceptibility. Mice expressing normal levels of Thr-61 and Arg-61 apoE are resistant to diet-induced hyperlipidemia ¹¹ and atherosclerosis (unpublished data, 2010). However, hypomorphic versions of these mouse models are highly susceptible to diet-induced hypercholesterolemia due to their low plasma apoE levels ($\approx 10\%$ of WT levels) ¹². Here, we assessed susceptibility to diet-induced atherosclerosis in hypomorphic *ApoE* mice expressing either Thr-61 apoE (*ApoE^{T^{h/h}}*) or Arg-61 apoE (*ApoE^{R^{h/h}}*). We show that on an atherogenic diet, both male and female *ApoE^{R^{h/h}}* mice developed increased atherosclerosis in the aortic root and the aorta, and that the presence of domain interaction in *ApoE^{R^{h/h}}* mice was associated with increased plasma VLDL-cholesterol, decreased macrophage-derived apoE secretion and increased macrophage activation. Our study provides evidence that domain interaction mediates pro-atherogenic effects of human apoE4 in part by modulating lipoprotein metabolism and macrophage biology.

Methods

For complete details on methods, please refer to the supplemental materials (available online at <http://atvb.ahajournals.org>).

Mice and Diets

Hypomorphic *ApoE*^{T^{h/h}} mice were generated by homologous recombination in embryonic stem cells as previously described for *ApoE*^{R^{h/h}} mice¹². All hypomorphic *ApoE* mice carried the inducible Mx1-cre transgene that can be activated to repair the hypomorphic allele and restore normal *ApoE* expression levels¹². However, in this study, the Mx1-cre transgene was not induced and remained silent throughout the study^{12, 13} resulting in consistently reduced *ApoE* expression among all hypomorphic mice as shown in Figure 1B. Hypomorphic *ApoE* mice were backcrossed for 12 generations to C57Bl/6 mice. Mice were fed a chow diet (2916, Harlan Teklad, Madison, WI) or an atherogenic high cholesterol diet (HCD) (16% fat, 1.25% cholesterol and 0.5% cholic acid (w/w) (D12336, Research Diets Inc., New Brunswick, NJ). This atherogenic diet provokes very high plasma cholesterol levels in hypomorphic *ApoE* mice, unlike the Western diet without cholate that only doubles their plasma cholesterol level¹², which would likely induce small atherosclerotic lesions only after 6 to 9 months. Animal protocols were approved by the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Plasma Lipid and Lipoprotein Analysis

Metabolic parameters were monitored in 4h-fasted mice. Plasma lipoproteins were fractionated by fast performance liquid chromatography (FPLC) or by sequential density ultracentrifugation using a pool of plasma taken from at least 4 mice. Lipid levels were measured by colorimetric assays. Plasma and lipoproteins were resolved by SDS-PAGE, subjected to Coomassie blue staining or western blotting with antibodies directed against mouse apoE¹², apoA1, and apoB¹².

Analysis of Atherosclerotic Lesion Size and Composition

After 9 or 15 weeks of HCD, overnight-fasted mice were sacrificed and tissues were collected. Aortic root plaque and necrotic core areas were quantified in hematoxylin and eosin (H&E) stained sections. Collagen was revealed by picro-sirius red staining. Detections of apoE and MOMA-2 positive macrophages were performed by immunofluorescence. Surface areas in aortic root or en face aorta were quantified with ImageJ or Metamorph softwares.

Blood Leukocyte Analysis

Leukocyte subsets were identified by flow cytometry using combinations of antibodies specific for cell surface markers detailed in the supplemental materials (available online at <http://atvb.ahajournals.org>). Analysis was performed using FlowJo software using specific gates as specified in Figure I (available online).

Peritoneal Macrophages Analysis

Macrophages isolated by peritoneal lavage were either analyzed directly by flow cytometry or after separation from other cells by their adhesion to culture vessels. Medium and cellular apoE levels were assessed in macrophages cultured for 48h in DMEM containing 10% lipoprotein-deficient serum using a previously described anti-mouse apoE antibody¹². Foam cell formation was quantified by the accumulation of fluorescent dil-OxLDL. Relative cholesterol efflux (%) was measured in macrophages loaded with fluorescent 25-NBD-cholesterol and AcLDL for 24h, equilibrated in DMEM 0.3% BSA and stimulated 6h with human apoA1 or with mouse HDL in DMEM 0.3% BSA or in DMEM only.

Gene expression

Gene expression was determined by quantitative real-time PCR using in house made primers (Table I, available online) or Assay-On-Demand. Expression was normalized to

housekeeping genes, TATA box binding protein (*Tbp*) or peptidylprolyl isomerase A (*Ppia*), and levels calculated according to the $2^{-\Delta\text{Ct}}$ method.

Statistical Analysis

Data are presented as Mean \pm SD or Mean \pm SEM as mentioned in legends. Data were analyzed with GraphPad Prism 5 software using two-tailed Student t-tests unless otherwise stated. A difference with a P value < 0.05 was considered significant.

Results

Hepatic and plasma apoE levels in chow-fed *ApoE*^{T^h/h} and *ApoE*^{R^h/h} mice

ApoE^{T^h/h} and *ApoE*^{R^h/h} mice were genetically identical except for a single nucleotide change in exon 3 (169C/G) coding for the Thr-61 to Arg-61 substitution (Figure 1A). Both hypomorphic *ApoE* alleles contained a LoxP-flanked neomycin cassette in intron 3 responsible for reduced apoE expression, presumably by interfering with the splicing efficiency of the primary *ApoE* mRNA transcript¹². When fed a chow diet, both strains of hypomorphic *ApoE* female mice displayed similar levels of *ApoE* mRNA in liver (\approx 13% of WT levels, Figure 1B), resulting in similar levels of plasma apoE (\approx 14% of WT levels, Figure 1C). Plasma apoA1 levels were also comparable in both strains of hypomorphic *ApoE* mice and similar to those of WT mice (Figure 1C). Similar results were obtained in male mice (**not shown**). These results demonstrate that hepatic and plasma apoE levels were similarly reduced in both chow-fed *ApoE*^{T^h/h} and *ApoE*^{R^h/h} mice.

Domain interaction increases diet-induced plasma VLDL-cholesterol

When fed a chow diet, both female and male *ApoE*^{T^h/h} and *ApoE*^{R^h/h} mice were normolipidemic with similar plasma lipid levels (Table 1) and lipoprotein cholesterol profiles, mainly containing HDL (Figure 2A). However, when fed a high cholesterol diet (HCD), both hypomorphic *ApoE* mice developed pronounced hypercholesterolemia (Table 1), at least two-fold higher than non-hypomorphic *ApoE* mice (Table II, available online). After 4 and 12 weeks of HCD, female *ApoE*^{R^h/h} mice showed a modest but significant increase in plasma cholesterol levels compared to *ApoE*^{T^h/h} females (12.1% and 11.6% respectively, Table 1) while levels were not different in male mice (Table 1).

Analysis of plasma lipoproteins isolated by FPLC or ultracentrifugation demonstrated that *ApoE*^{R^h/h} mice consistently accumulated more cholesterol in VLDL than *ApoE*^{T^h/h} mice. After 4 weeks of HCD, both female and male *ApoE*^{R^h/h} mice showed a 19% and 17% increase respectively in VLDL-cholesterol levels compared to *ApoE*^{T^h/h} counterparts (Figure 2A). Levels of all lipid classes, phospholipids (PL), free cholesterol (FC), cholesterol esters (CE) and triglycerides (TG) were increased by 11% to 49% in VLDL fractions from HCD-fed *ApoE*^{R^h/h} female and male mice (Figure 2B and 2C). Because these differences were modest, we re-assessed plasma and lipoprotein cholesterol levels after only 1 week of diet initiation to investigate the kinetics of HCD-response between *ApoE*^{R^h/h} and *ApoE*^{T^h/h} mice. The increase in VLDL-cholesterol in *ApoE*^{R^h/h} mice was even more pronounced at this time point averaging 47% and 28% for male and female mice respectively (Figure 2A), and resulted in increased total plasma cholesterol levels in both genders of *ApoE*^{R^h/h} mice (males: 23.7%, $p=0.002$, $n=15-20$ and females: 16.7%, $p=0.1$, $n=10$). Female hypomorphic *ApoE* mice developed diet-induced hypercholesterolemia faster than male mice in both *ApoE*^{T^h/h} and *ApoE*^{R^h/h} strains (Table 1 and Figure 2A). Compared to *ApoE*^{T^h/h} mice, levels of apoB-100, apoB-48 and apoE were also increased by 77%, 52% and 51% respectively in VLDL fractions of male *ApoE*^{R^h/h} mice after 1 week of HCD (Figure 2G). Similar increases were seen in FPLC fractions from female mice (Figure 2F). Higher levels of apoB-100, apoB-48 and apoE in VLDL fractions of *ApoE*^{R^h/h} female and

male mice were also observed using density ultracentrifugation isolation after 6 weeks of HCD (Figure 2D and 2E). Taken together, these data demonstrate that domain interaction caused a small but significant accumulation of apoB-containing VLDL particles in plasma of both female and male *ApoE*^{R^{h/h}} mice when fed a HCD. This accumulation of VLDL occurred despite similar expression levels of *ApoE* and the LDL receptor (*Ldlr*) in the livers of female *ApoE*^{R^{h/h}} and *ApoE*^{T^{h/h}} mice fed with HCD (**not shown**).

Domain interaction accelerates diet-induced atherosclerosis

We first assessed atherosclerotic lesion formation in aortic roots of female hypomorphic *ApoE* mice fed a HCD for 9 and 15 weeks, and of male mice fed a HCD for 15 weeks. As shown in Figure 3A, female *ApoE*^{R^{h/h}} mice displayed a 45% increase in atherosclerotic lesion area after 9 weeks of HCD and trended to an increase after 15 weeks of HCD, indicating that the increase in lesion size was more pronounced at an early stage of atherosclerosis. Male *ApoE*^{R^{h/h}} mice showed a 62% increase in atherosclerotic lesion area after 15 weeks of HCD (Figure 3B). The increased atherosclerosis in the aortic roots of *ApoE*^{R^{h/h}} mice was reproduced in separate cohorts of females and male mice (Figure IIA and IIB, available online). The pro-atherogenic effect of domain interaction was also examined in en face aorta preparations of female and male hypomorphic *ApoE* mice fed the HCD for 15 weeks. Using this methodology, both female and male *ApoE*^{R^{h/h}} mice displayed increased lesion area within the descending aorta relative to *ApoE*^{T^{h/h}} mice (+73% and +74% respectively), although there was no difference in the aortic arch at this time point (Figure 3C and 3D). Finally, female *ApoE*^{R^{h/h}} mice fed the HCD for 9 weeks displayed a 136% increase in lesion area in the aortic arch (Figure 3C). Our results indicate that domain interaction accelerated diet-induced atherosclerosis in both genders of mice. This effect was independent of any change in the weight of organs (liver, epididymal fat and spleen) involved in metabolic and immune functions (**not shown**).

The composition of aortic root atheromas was further investigated by immunohistological analysis (Figure 4A). The relative proportions of apoE (Figure 4B), lesional macrophages (Figure 4C), necrotic core (Figure 4D) and collagen (Figure 4E) in aortic root sections varied significantly with the lesion stage (9 versus 15 weeks) and gender. However, lesion composition was not significantly different between mice of either genotype, suggesting that apoE4 domain interaction did not significantly change atheroma composition. Overall, our data suggest that atherosclerosis developed in a similar manner in mice expressing either apoE isoform but that the process occurred more rapidly in mice expressing Arg-61 apoE.

Domain interaction does not alter blood leukocyte levels

Because atherosclerosis develops in part through leukocyte infiltration into the artery wall, we assessed whether domain interaction affected blood monocyte, neutrophil and lymphocyte counts. We did not observe any differences in circulating leukocyte counts (Figure IIIA, available online) between *ApoE*^{T^{h/h}} and *ApoE*^{R^{h/h}} male mice fed a HCD for 4 weeks. The percentages of Ly6C^{low} and Ly6C^{high} monocyte subsets as well as the percentages of CD62L⁺ monocytes were not different between both strains of HCD-fed hypomorphic *ApoE* mice nor were there differences in CD4⁺ and CD8⁺ T cell numbers and activation status (Figure IIIB and IIIC, available online). Similar data were obtained in female mice (Figure III). Thus, the pro-atherogenic effects of domain interaction do not extend to alter circulating leukocyte populations in HCD-fed hypomorphic *ApoE* mice.

Domain interaction reduces the amount of apoE released into the medium of cultured macrophages

Since the increased atherosclerosis in *ApoE*^{R^{h/h}} mice occurred with a slight but significant increase in plasma VLDL levels and total cholesterol, we asked whether domain interaction

caused cellular dysfunction, particularly in macrophages. ApoE is expressed by macrophages and was detected in lesional macrophages within the plaque of both strains of hypomorphic mice (Figure 4A). First, we tested whether domain interaction affected macrophage apoE levels. Using resident peritoneal macrophages isolated from hypomorphic *ApoE* mice, we observed a 52% decrease in the amount of apoE accumulating in the culture medium of *ApoE^{R^{h/h}}* macrophages compared to *ApoE^{T^{h/h}}* macrophages (Figure 5A). However, there was no change in cellular apoE levels in macrophages as detected by immunofluorescence (Figure 5B). In this condition, cultured peritoneal macrophages isolated from *ApoE^{R^{h/h}}* mice showed a modest but detectable 30% decrease in *ApoE* mRNA levels compared to *ApoE^{T^{h/h}}* macrophages ($p < 0.05$). We also sought to assess *ApoE* mRNA expression levels in resident peritoneal macrophages freshly isolated from both strains of mice prior to culturing them. As shown in Figure 5C, macrophages isolated from *ApoE^{T^{h/h}}* and *ApoE^{R^{h/h}}* mice fed a chow or HCD for 4 weeks displayed similar levels of *ApoE* mRNA (Figure 5C). HCD-feeding significantly increased *ApoE* mRNA expression in macrophages isolated from both strains and genders of mice (≈ 1.5 and 2.5 fold in females and males respectively) (Figure 5C). Taken together, these results demonstrate that domain interaction reduces macrophage-derived apoE secretion.

Domain interaction and macrophage lipid homeostasis

To assess possible functional defects in macrophages caused by domain interaction, we focused on assessing its impact on lipid, stress and immune homeostasis, previously described to be regulated by macrophage apoE^{14, 15}. We first assessed oxidized LDL (oxLDL) uptake capacity and found that macrophages isolated from both *ApoE^{T^{h/h}}* and *ApoE^{R^{h/h}}* mice accumulated similar amounts of oxLDL (Figure IVA, available online). Second, *ApoE^{T^{h/h}}* and *ApoE^{R^{h/h}}* macrophages showed a similar cholesterol efflux capacity both in a non-stimulated condition (passive efflux) or by active acceptors such as apoA1 and HDL (Figure IVB and C, available online). These results suggest that domain interaction does not affect foam cell formation and cholesterol efflux in macrophages derived from hypomorphic *ApoE* mice.

Domain interaction and macrophage ER stress

A recent study documented that astrocytes derived from Arg-61 apoE mice displayed features of endoplasmic reticulum (ER) stress¹⁶. Thus, we assessed the expression levels of ER stress-related proteins in resident peritoneal macrophages. Expression levels of ER stress indicators *Atf4*, *Chop* and *Trb3* were generally slightly lower in macrophages freshly isolated from *ApoE^{R^{h/h}}* mice fed either a chow or HCD (Table III, available online). When macrophages were cultured in basal medium for 48h, no significant differences were observed in the expression levels of these ER stress-related proteins (Table IV, available online). Overall, our results suggest that the presence of domain interaction in Arg-61 apoE does not induce major changes in ER stress pathways at the gene expression level in macrophages derived from hypomorphic *ApoE* mice.

Domain interaction enhances MHC class II expression in macrophages

Finally, apoE has been shown to modulate antigen presenting capacity in macrophages by reducing the expression of major histocompatibility complex class II (MHCII) and some co-stimulatory molecules such as CD80 and CD40¹⁵. Thus, we tested whether domain interaction impacted macrophage activation in response to HCD. Resident peritoneal macrophages were identified using flow cytometry by their expression of the specific cell surface marker F4/80 (Figure 5D). We observed a 2 fold increase in the proportion of macrophages positive for MHCII isolated from *ApoE^{R^{h/h}}* mice fed a HCD for 4 weeks (18.5% vs 38.1% for *ApoE^{T^{h/h}}* mice) (Figure 5D). This was not accompanied by an increase in the frequency of macrophages positive for co-stimulatory molecules, CD80 and CD86.

Instead, the frequency of CD80⁺ macrophages was not different while the frequency of CD86⁺ macrophages was slightly decreased (Figure 5D). All together, these data demonstrate that apoE4 domain interaction increases the proportion of activated macrophages positive for MHCII.

Discussion

Human apoE4 differs from apoE3 by at least 2 structural features: the domain interaction that causes the two globular domains of apoE4 to interact⁶, and the molten globule state that predisposes apoE4 to instability and unfolding⁷. To delineate the role of domain interaction in atherosclerosis, we studied the atherosclerosis susceptibility of hypomorphic *ApoE* mice expressing either Arg-61 apoE (*ApoE^{R^{h/h}}*) or Thr-61 apoE (*ApoE^{T^{h/h}}*). Substituting Arg-61 for Thr-61 in mouse apoE introduced domain interaction without molten globule state^{6, 11} allowing us to identify pathological effects specifically due to domain interaction. In this study, we found that domain interaction accelerated atherosclerosis in hypomorphic *ApoE* mice. We showed that both male and female *ApoE^{R^{h/h}}* mice developed increased atherosclerosis in the aortic root and aorta when fed an atherogenic diet. However domain interaction did not cause measurable differences in atheroma composition in terms of macrophage, collagen and necrotic core content. These results suggest that the pro-atherogenic properties of human apoE4 domain interaction could reside in its propensity to promote atherosclerosis development rather than by altering plaque composition. Interestingly, the pro-atherogenic effect of domain interaction was most noticeable at an early lesion stage in both the aortic root and the arch of female *ApoE^{R^{h/h}}* mice, suggesting that domain interaction facilitates lesion initiation.

Further, we found that domain interaction promoted the accumulation of pro-atherogenic lipoproteins in *ApoE^{R^{h/h}}* mouse plasma. Our results are consistent with observations made in human apoE4 individuals who display modest increases in total plasma cholesterol and LDL levels compared to apoE3 individuals³. However, unlike humans, *ApoE^{R^{h/h}}* mice fed an atherogenic diet developed increased VLDL levels. Similar results were reported in studies of knock-in mice that expressed the human apoE4 isoform in place of the murine *ApoE* locus^{8, 17}. In humans, the liver produces exclusively apoB-100 containing VLDL that are converted to LDL by lipolytic catabolism in the circulation. In contrast, the mouse liver secretes both apoB-100 and apoB-48 containing VLDL. Consequently, unlike humans, hyperlipidemic mice accumulate small quantities of apoB-100 LDL and larger amounts of apoB-48 VLDL remnants. Interestingly, a recent study showed that VLDL level is a better predictor of atherosclerosis than LDL level in mice¹⁸.

Hypercholesterolemia promotes blood leukocyte activation and proliferation¹⁹, important driving forces of atherosclerosis progression. In this study, we found that populations of blood monocytes, lymphocytes and neutrophils were similar in both strains of hypomorphic *ApoE* mice. It is possible that the increase in blood cholesterol levels observed in *ApoE^{R^{h/h}}* mice may be too subtle to translate into detectable changes in circulating leukocyte populations, thus obscuring potential effects of domain interaction.

The mechanism by which domain interaction causes accumulation of VLDL in *ApoE^{R^{h/h}}* mice is unclear. As a high affinity ligand for members of the LDLR gene family, apoE plays a critical role in receptor-mediated clearance of plasma remnant lipoproteins¹. Although studies have reported that apoE4 binds to the LDLR with a slightly higher affinity than apoE3^{8, 20, 21}, the presence of apoE4 is paradoxically associated with higher plasma levels of apoB lipoproteins in both humans and mice. Several mechanisms have been proposed to account for these observations. A longstanding hypothesis derived from early lipoprotein turnover studies in humans proposed that by accelerating VLDL clearance in the liver,

apoE4 could down-regulate hepatic *Ldlr* expression and thereby raise plasma LDL levels^{22, 23}. However, recent studies support an alternate mechanism by which the enhanced affinity of apoE4 for the LDLR would enhance the sequestration of VLDL on hepatocyte cell surfaces but delay their internalization and clearance^{8, 9, 17}. This effect would enhance the lipolytic conversion of VLDL to cholesterol-enriched remnants and favor their accumulation in plasma after being released from the surface of hepatocytes, leading to elevated apoB-100 LDL in humans and elevated apoB-48 VLDL remnants in mice. Studies in apoE4 individuals showing increased apoB-48 lipoproteins levels in the postprandial state²⁴ and increased conversion of VLDL to LDL²⁵ provide support for this hypothesis.

A second major finding of this study is that domain interaction affected macrophage biology. We show that domain interaction reduced the amount of apoE released into the culture medium of *ApoE*^{R^{h/h} macrophages. Our results are consistent with the slight decrease in apoE production observed in macrophages derived from apoE4 individuals²⁶. As we did not observe a major change in *ApoE* expression, it is likely that domain interaction affects post-translational regulation of apoE in macrophages. Interestingly, it has been proposed that the enhanced affinity of macrophage-derived apoE4 for cell surface proteoglycans^{26, 27} and/or other apoE receptors such as the LDLR²⁸ enhance the reuptake of secreted apoE4, lowering its amount released into the medium. Recently, domain interaction was found to decrease apoE secretion in astrocytes¹⁶ and slow down the trafficking of apoE molecules along the secretory pathway in neurons²⁹. Overall, our findings and those of others suggest that domain interaction is an important modulator of cell-derived apoE4 production.}

Our study also addressed the potential role of domain of interaction in modulating macrophage cholesterol homeostasis. We observed no differences in cholesterol accumulation and efflux between *ApoE*^{T^{h/h} and *ApoE*^{R^{h/h} macrophages, suggesting that endogenously expressed Arg-61 apoE does not impact cholesterol homeostasis in hypomorphic *ApoE* macrophages. Studies performed to assess the differential effects of endogenous apoE4 and apoE3 isoforms on macrophage cholesterol efflux have either detected no differences^{26, 27, 30, 31} or slight alterations with apoE4 only in passive efflux and when the LDLR was overexpressed³¹. While it can seem surprising to observe no variation in cholesterol efflux capacity in the presence of decreased apoE secretion, Basu *et al.*³² reported many years ago that apoE secretion and cholesterol efflux can be uncoupled, suggesting that the two processes may function via independent pathways.}}

Lastly, we found that domain interaction influenced macrophage activation. We show that *ApoE*^{R^{h/h} mice fed an atherogenic diet displayed a higher percentage of peritoneal macrophages positive for MHCII, a molecule critical for antigen presentation and activation of T cells. In fact, Tenger *et al.*¹⁵ reported increased MHCII levels on macrophages derived from *ApoE*^{-/-} mice compared to WT macrophages. In that study, the enhanced cellular activation state of *ApoE*^{-/-} macrophages resulted in increased T cell proliferation and activation¹⁵. Another recent study found that T cell proliferation was increased in human apoE4 transgenic mice³³. Multiple studies have identified T cell-mediated immune responses in the artery wall as key components in the initiation and progression of atherosclerosis in mice³⁴. More recent studies demonstrated reduced atherosclerosis in hyperlipidemic mice in response to the suppression of T cell-mediated immunity in the artery wall³⁵. These findings, together with results reported here, suggest that domain interaction accelerates atherosclerosis in part by enhancing macrophage antigen presentation capacity to T cells and their potential activation in the artery wall. Further studies are required to test this hypothesis.}

Beyond its association with atherosclerosis, apoE4 is also a major risk factor for Alzheimer's disease³⁶. Domain interaction appears critical for apoE4's pathological effects in Alzheimer's disease³⁷, causing various defects in neurons^{29,38} and astrocytes¹⁶. Domain interaction was found to enhance ER stress and decrease apoE secretion in astrocytes¹⁶, however Brodbeck *et al.*²⁹ did not observe enhanced ER stress in neurons. In this study, we found that domain interaction caused reduced macrophage-derived apoE secretion, but did not result in enhanced ER stress. It is possible that domain interaction causes cell-specific defects that do not require coupling of enhanced ER stress with decreased apoE secretion.

In conclusion, our study shows that domain interaction accelerates diet-induced atherosclerosis and enhances several pro-atherogenic factors including plasma VLDL accumulation, decreased macrophage-derived apoE production and enhanced macrophage activation. Our results have important implications for the design of future treatments to prevent and/or treat atherosclerosis in apoE4 individuals. In this regard, small-molecule structure correctors of apoE4 domain interaction were recently shown to rescue neuronal defects in mice expressing human apoE4^{29,38}. Our findings support testing the efficacy of such compounds in preventing diet-induced atherosclerosis in our models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Sandra Erickson, Karl Weisgraber, Michael Conte, and Kang Li, as well as Joel Haas for critical review of the manuscript. We thank Susie Wong for excellent technical assistance.

Sources of Funding

This work was supported by grants from the American Heart Association Western States affiliate (0565117Y) and from the National Institutes of Health (HL089871) to R.L.R., both of which were administered by the Northern California Institute for Research and Education; a Merit Review award, 5101BX000532, from the Department of Veterans Affairs to RLR and by funds from the Foundation for Accelerated Vascular Research and the Department of Surgery, University of California, San Francisco, both to RLR. The work was performed at the Veterans Affairs Medical Center, San Francisco, California.

References

1. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*. 1988; 240:622–630. [PubMed: 3283935]
2. Davignon J, Gregg RE, Sing CF. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis*. 1988; 8:1–21. [PubMed: 3277611]
3. Bennet AM, Di Angelantonio E, Ye Z, Wensley F, Dahlin A, Ahlbom A, Keavney B, Collins R, Wiman B, de Faire U, Danesh J. Association of apolipoprotein E genotypes with lipid levels and coronary risk. *Jama*. 2007; 298:1300–1311. [PubMed: 17878422]
4. Weisgraber KH. Apolipoprotein E: structure-function relationships. *Adv Protein Chem*. 1994; 45:249–302. [PubMed: 8154371]
5. Dong LM, Weisgraber KH. Human apolipoprotein E4 domain interaction. Arginine 61 and glutamic acid 255 interact to direct the preference for very low density lipoproteins. *J Biol Chem*. 1996; 271:19053–19057. [PubMed: 8702576]
6. Hatters DM, Budamagunta MS, Voss JC, Weisgraber KH. Modulation of apolipoprotein E structure by domain interaction: differences in lipid-bound and lipid-free forms. *J Biol Chem*. 2005; 280:34288–34295. [PubMed: 16076841]

7. Morrow JA, Hatters DM, Lu B, Hochtl P, Oberg KA, Rupp B, Weisgraber KH. Apolipoprotein E4 forms a molten globule. A potential basis for its association with disease. *J Biol Chem.* 2002; 277:50380–50385. [PubMed: 12393895]
8. Knouff C, Hinsdale ME, Mezdour H, Altenburg MK, Watanabe M, Quarfordt SH, Sullivan PM, Maeda N. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *J Clin Invest.* 1999; 103:1579–1586. [PubMed: 10359567]
9. Altenburg M, Arbones-Mainar J, Johnson L, Wilder J, Maeda N. Human LDL receptor enhances sequestration of ApoE4 and VLDL remnants on the surface of hepatocytes but not their internalization in mice. *Arterioscler Thromb Vasc Biol.* 2008; 28:1104–1110. [PubMed: 18369154]
10. Hopkins PC, Huang Y, McGuire JG, Pitas RE. Evidence for differential effects of apoE3 and apoE4 on HDL metabolism. *J Lipid Res.* 2002; 43:1881–1889. [PubMed: 12401887]
11. Raffai RL, Dong LM, Farese RV Jr, Weisgraber KH. Introduction of human apolipoprotein E4 "domain interaction" into mouse apolipoprotein E. *Proc Natl Acad Sci U S A.* 2001; 98:11587–11591. [PubMed: 11553788]
12. Raffai RL, Weisgraber KH. Hypomorphic apolipoprotein E mice: a new model of conditional gene repair to examine apolipoprotein E-mediated metabolism. *J Biol Chem.* 2002; 277:11064–11068. [PubMed: 11792702]
13. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science.* 1995; 269:1427–1429. [PubMed: 7660125]
14. Huang ZH, Fitzgerald ML, Mazzone T. Distinct cellular loci for the ABCA1-dependent and ABCA1-independent lipid efflux mediated by endogenous apolipoprotein E expression. *Arterioscler Thromb Vasc Biol.* 2006; 26:157–162. [PubMed: 16254198]
15. Tenger C, Zhou X. Apolipoprotein E modulates immune activation by acting on the antigen-presenting cell. *Immunology.* 2003; 109:392–397. [PubMed: 12807485]
16. Zhong N, Ramaswamy G, Weisgraber KH. Apolipoprotein E4 domain interaction induces endoplasmic reticulum stress and impairs astrocyte function. *J Biol Chem.* 2009; 284:27273–27280. [PubMed: 19666463]
17. Malloy SI, Altenburg MK, Knouff C, Lanningham-Foster L, Parks JS, Maeda N. Harmful effects of increased LDLR expression in mice with human APOE*4 but not APOE*3. *Arterioscler Thromb Vasc Biol.* 2004; 24:91–97. [PubMed: 12969990]
18. VanderLaan PA, Reardon CA, Thisted RA, Getz GS. VLDL best predicts aortic root atherosclerosis in LDL receptor deficient mice. *J Lipid Res.* 2009; 50:376–385. [PubMed: 18957695]
19. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytois and give rise to macrophages in atheromata. *J Clin Invest.* 2007; 117:195–205. [PubMed: 17200719]
20. Mamotte CD, Sturm M, Foo JI, van Bockxmeer FM, Taylor RR. Comparison of the LDL-receptor binding of VLDL and LDL from apoE4 and apoE3 homozygotes. *Am J Physiol.* 1999; 276:E553–E557. [PubMed: 10070023]
21. Chou CY, Jen WP, Hsieh YH, Shiao MS, Chang GG. Structural and functional variations in human apolipoprotein E3 and E4. *J Biol Chem.* 2006; 281:13333–13344. [PubMed: 16540478]
22. Gregg RE, Zech LA, Schaefer EJ, Stark D, Wilson D, Brewer HB Jr. Abnormal in vivo metabolism of apolipoprotein E4 in humans. *J Clin Invest.* 1986; 78:815–821. [PubMed: 3745440]
23. Weintraub MS, Eisenberg S, Breslow JL. Dietary fat clearance in normal subjects is regulated by genetic variation in apolipoprotein E. *J Clin Invest.* 1987; 80:1571–1577. [PubMed: 3479440]
24. Bergeron N, Havel RJ. Prolonged postprandial responses of lipids and apolipoproteins in triglyceride-rich lipoproteins of individuals expressing an apolipoprotein epsilon 4 allele. *J Clin Invest.* 1996; 97:65–72. [PubMed: 8550852]
25. Demant T, Bedford D, Packard CJ, Shepherd J. Influence of apolipoprotein E polymorphism on apolipoprotein B-100 metabolism in normolipemic subjects. *J Clin Invest.* 1991; 88:1490–1501. [PubMed: 1939641]
26. Cullen P, Cignarella A, Brennhause B, Mohr S, Assmann G, von Eckardstein A. Phenotype-dependent differences in apolipoprotein E metabolism and in cholesterol homeostasis in human monocyte-derived macrophages. *J Clin Invest.* 1998; 101:1670–1677. [PubMed: 9541497]

27. Hara M, Matsushima T, Satoh H, Iso-o N, Noto H, Togo M, Kimura S, Hashimoto Y, Tsukamoto K. Isoform-dependent cholesterol efflux from macrophages by apolipoprotein E is modulated by cell surface proteoglycans. *Arterioscler Thromb Vasc Biol.* 2003; 23:269–274. [PubMed: 12588770]
28. Lucic D, Huang ZH, Gu de S, Altenburg MK, Maeda N, Mazzone T. Regulation of macrophage apoE secretion and sterol efflux by the LDL receptor. *J Lipid Res.* 2007; 48:366–372. [PubMed: 17079794]
29. Brodbeck J, McGuire J, Liu Z, Meyer-Franke A, Balestra ME, Jeong DE, Pleiss M, McComas C, Hess F, Witter D, Peterson S, Childers M, Goulet M, Liverton N, Hargreaves R, Freedman S, Weisgraber KH, Mahley RW, Huang Y. Structure-dependent Impairment of Intracellular Apolipoprotein E4 Trafficking and Its Detrimental Effects Are Rescued by Small-molecule Structure Correctors. *J Biol Chem.* 2011; 286:17217–17226. [PubMed: 21454574]
30. Smith JD, Miyata M, Ginsberg M, Grigaux C, Shmookler E, Plump AS. Cyclic AMP induces apolipoprotein E binding activity and promotes cholesterol efflux from a macrophage cell line to apolipoprotein acceptors. *J Biol Chem.* 1996; 271:30647–30655. [PubMed: 8940040]
31. Altenburg M, Johnson L, Wilder J, Maeda N. Apolipoprotein E4 in macrophages enhances atherogenesis in a low density lipoprotein receptor-dependent manner. *J Biol Chem.* 2007; 282:7817–7824. [PubMed: 17234631]
32. Basu SK, Goldstein JL, Brown MS. Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science.* 1983; 219:871–873. [PubMed: 6823554]
33. Zhang HL, Mao XJ, Zhang XM, Li HF, Zheng XY, Adem A, Mix E, Zhu J. APOE epsilon3 attenuates experimental autoimmune neuritis by modulating T cell, macrophage and Schwann cell functions. *Exp Neurol.* 2011; 230:197–206. [PubMed: 21550340]
34. Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol.* 2011; 12:204–212. [PubMed: 21321594]
35. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med.* 2006; 12:178–180. [PubMed: 16462800]
36. Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E: structure determines function, from atherosclerosis to Alzheimer's disease to AIDS. *J Lipid Res.* 2009; 50(Suppl):S183–S188. [PubMed: 19106071]
37. Zhong N, Scarce-Levie K, Ramaswamy G, Weisgraber KH. Apolipoprotein E4 domain interaction: synaptic and cognitive deficits in mice. *Alzheimers Dement.* 2008; 4:179–192. [PubMed: 18631967]
38. Chen HK, Ji ZS, Dodson SE, Miranda RD, Rosenblum CI, Reynolds IJ, Freedman SB, Weisgraber KH, Huang Y, Mahley RW. Apolipoprotein E4 domain interaction mediates detrimental effects on mitochondria and is a potential therapeutic target for Alzheimer disease. *J Biol Chem.* 2011; 286:5215–5221. [PubMed: 21118811]

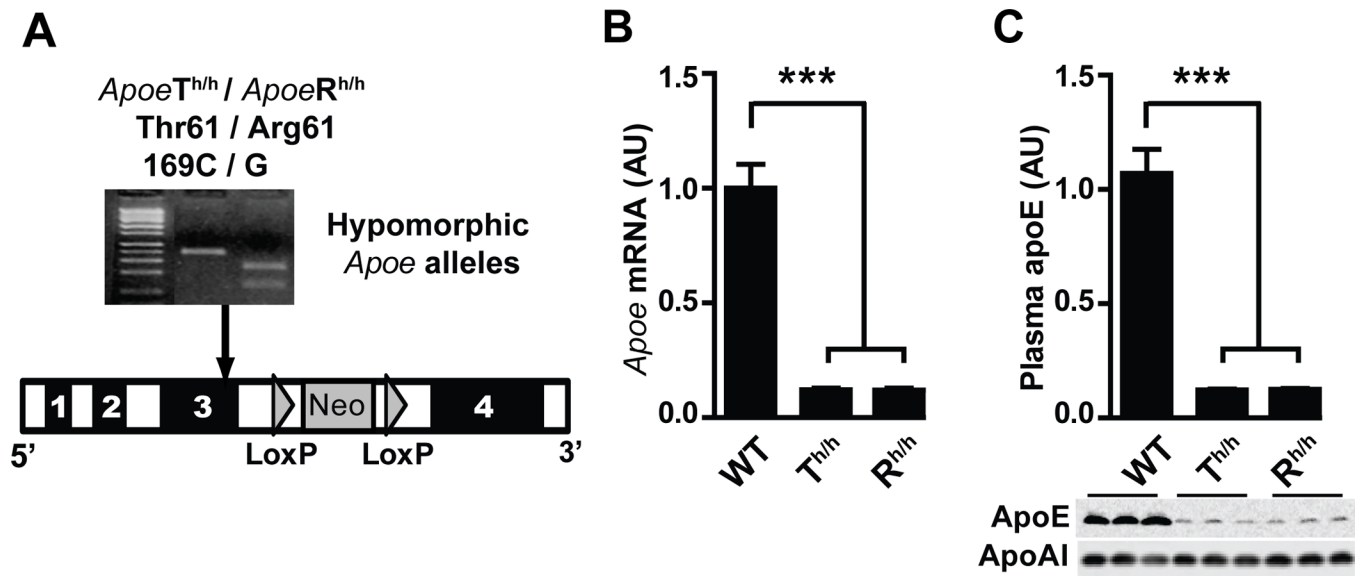


Figure 1.

(A) Hypomorphic *ApoE* alleles differ by a single nucleotide change in exon 3 (169C/G) coding for either Thr-61 apoE (*ApoE*^{T^{h/h}}) or Arg-61 apoE (*ApoE*^{R^{h/h}}). (B) Hepatic *ApoE* mRNA and (C) plasma apoE levels in chow-fed female mice of indicated genotypes (n=3–7). AU, Arbitrary Unit. Mean±SEM, ***p<0.001

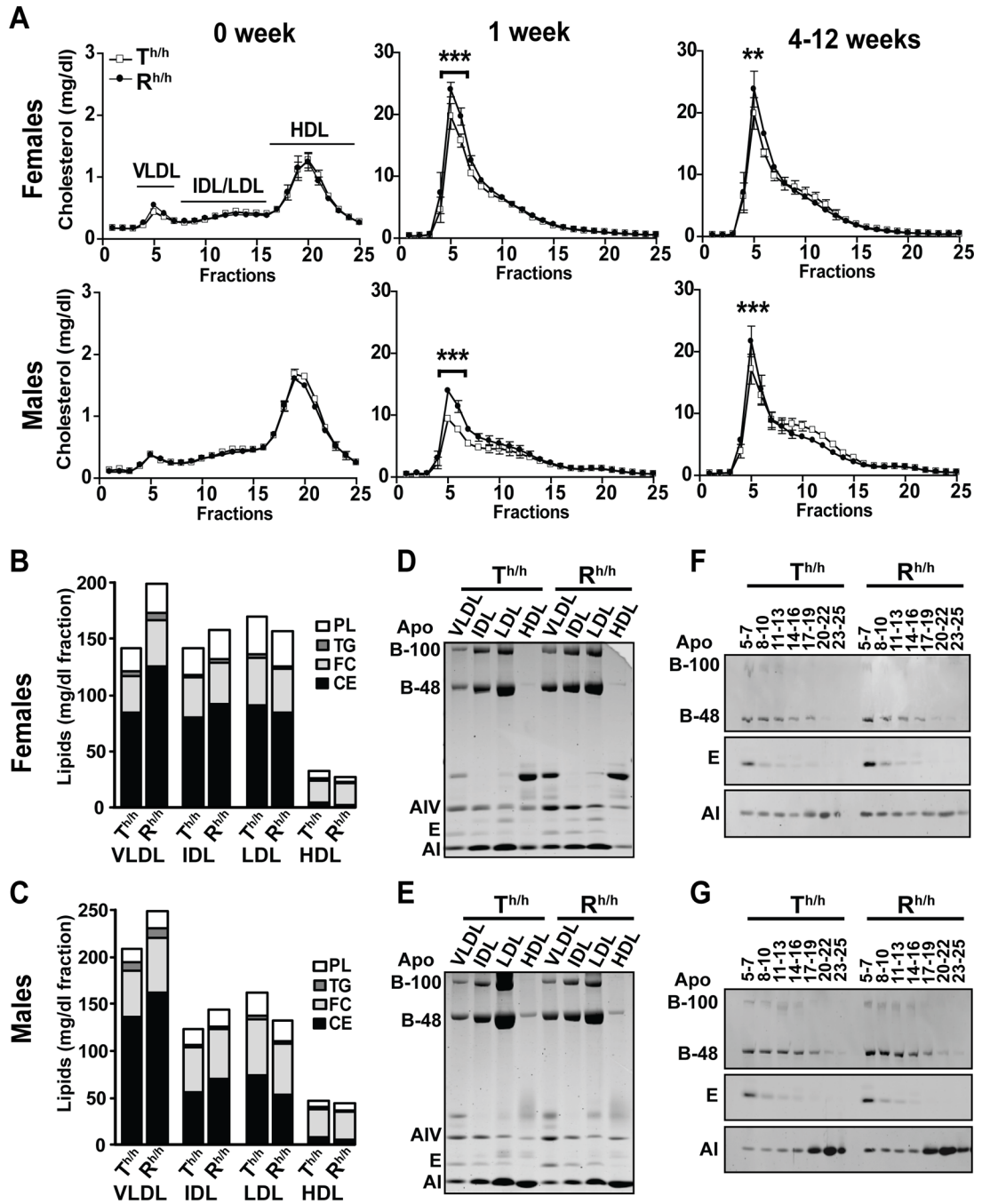


Figure 2. Cholesterol (A), lipid classes (B, C) and apolipoprotein distribution in lipoproteins isolated by ultracentrifugation (D, E) or FPLC (F, G) from hypomorphic female and male *ApoE* mice (A: n=2 (0 week), n=3 (1 week HCD) and n=3-4 (4-12 weeks HCD) pools of plasma taken from 4 mice, for each genotype and gender, Mean±SEM, **p<0.01, ***p<0.001, Bonferroni post-ANOVA; B, C: n=1-2 pools of plasma taken per genotype and gender (6-12 weeks HCD); D, E: Coomassie stained gel (6 weeks HCD); F, G: Western Blot (1 week HCD)).

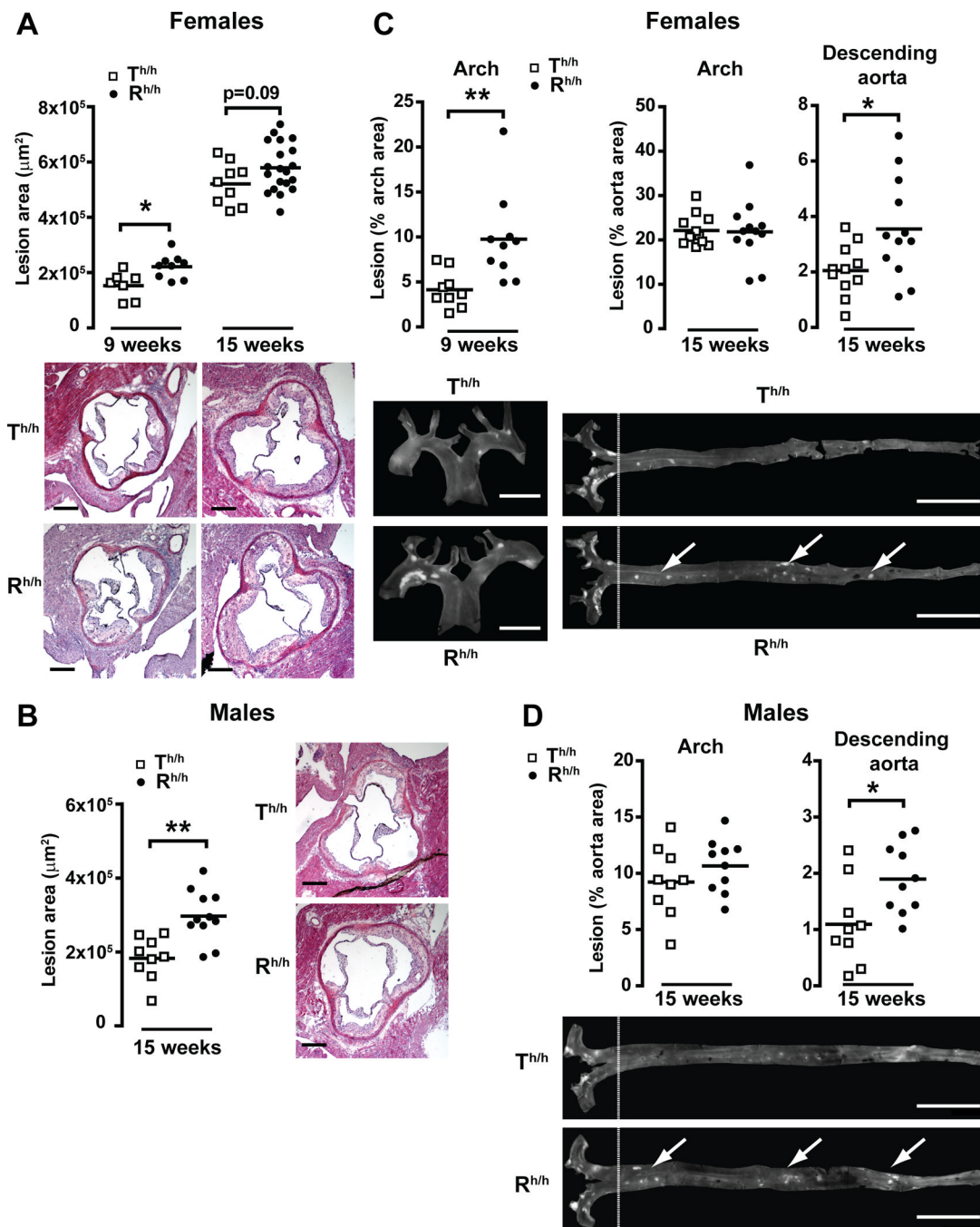


Figure 3.

Atherosclerotic lesion area in (A, B) aortic roots (n=7–20, representative H&E-stained sections, scale bar=300 μm) and (C) en face aorta and arch (% each segment) (n=9–12, representative picture, scale bar=0.5cm for aorta and 0.2cm for arch) of HCD-fed hypomorphic female and male *ApoE* mice. Individual values and means are shown. * $p<0.05$ and ** $p<0.01$

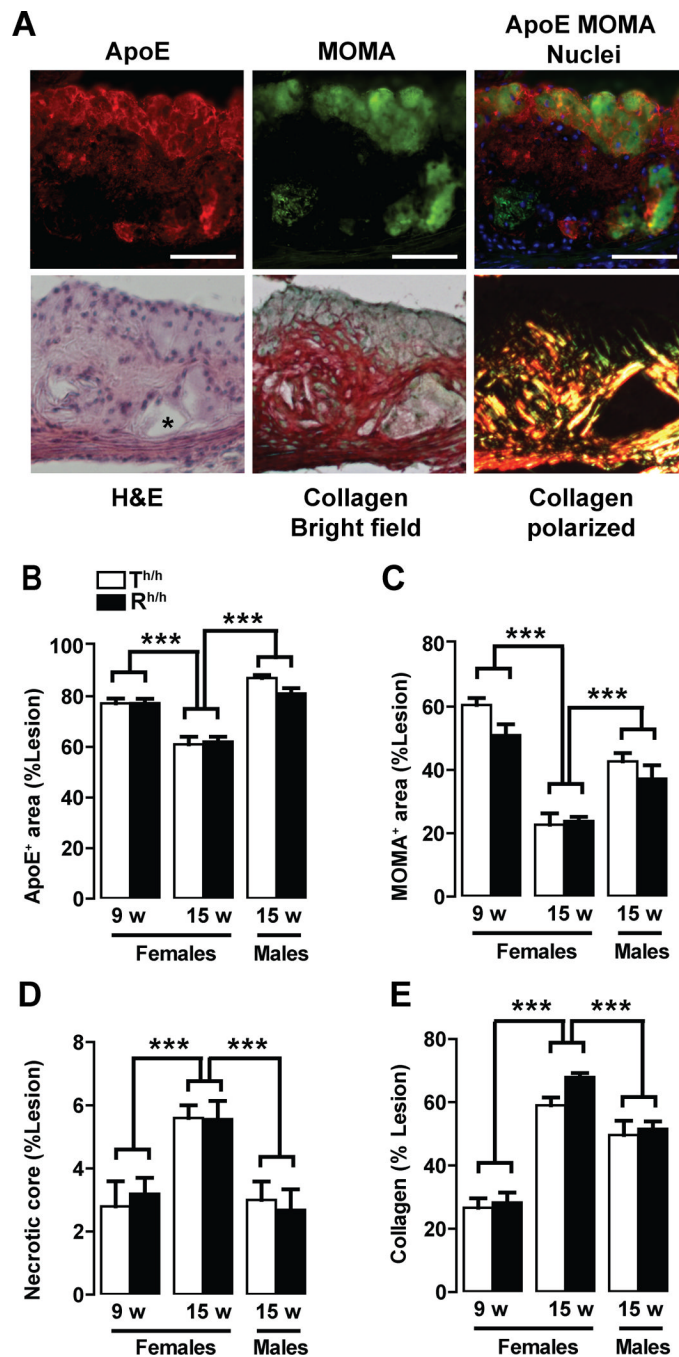
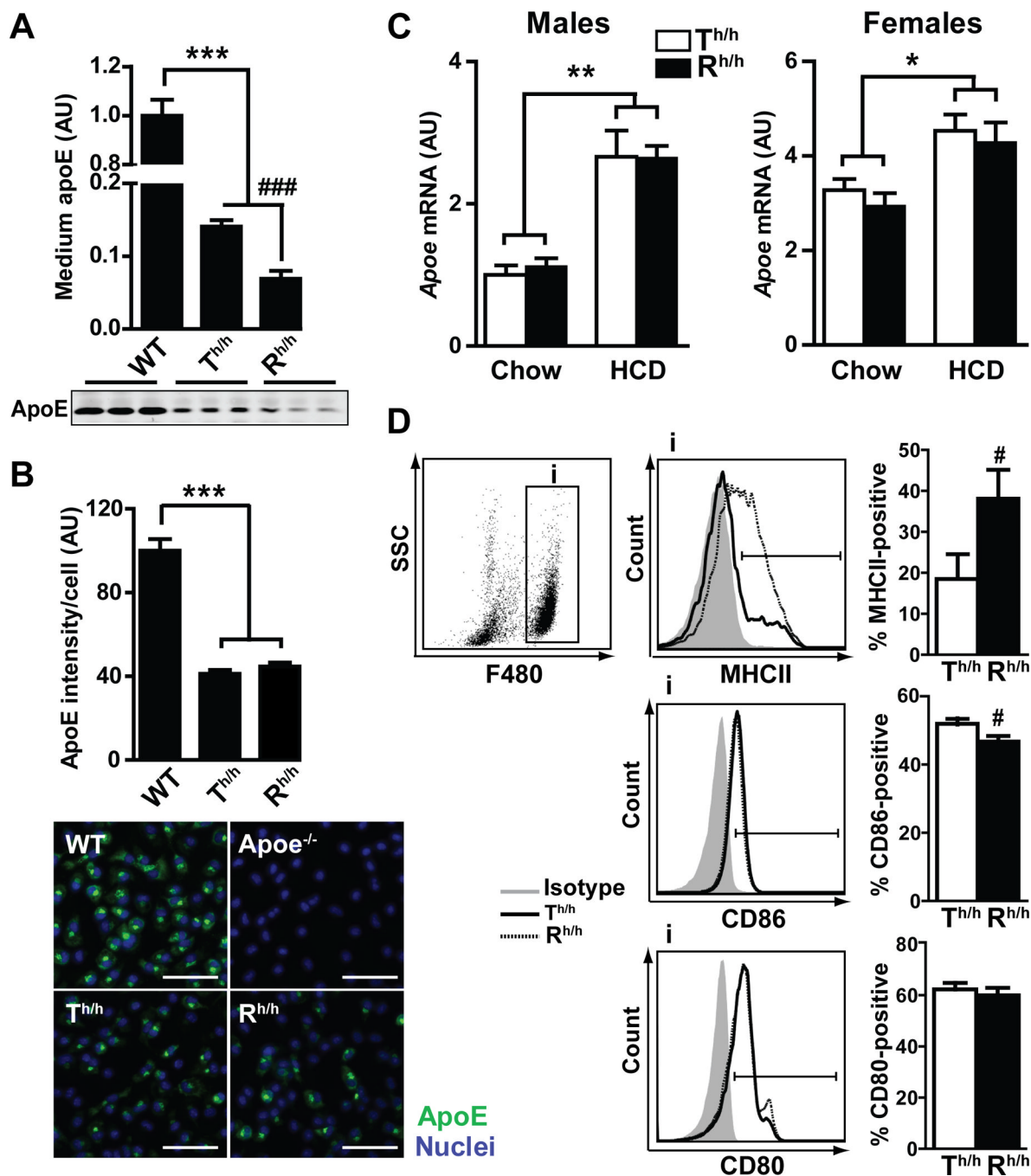


Figure 4.

(A) Consecutive aortic root sections showing apoE, MOMA-positive macrophages, nuclei; H&E-negative necrotic core (asterisk); and Sirius red-stained collagen viewed by bright field or polarized light (Scale bar=100 μ m) and (B, C, D, E) relative quantification (% lesion area) (n=6–20). Mean \pm SEM, ***p<0.001 two-way ANOVA testing lesion stage or gender effect

**Figure 5.**

ApoE levels in (A) medium and (B) cultured macrophages (average of 2 independent experiments, each performed in triplicate, representative western blot and immunostain, scale bar=50 μ m). (C) *Apoe* mRNA (n=4–8) and (D) activated populations of macrophages isolated from mice fed a HCD for 4 weeks (n=9–10 females and males grouped). Mean \pm SEM, ***p<0.001 versus WT; #p<0.05, ###p<0.001 *ApoE*^{T^{h/h}} versus *ApoE*^{R^{h/h}}

Table 1

Metabolic parameters of chow and HCD-fed hypomorphic *ApoE* mice

	0 week		4 weeks		12 weeks	
	T ^{b/h}	R ^{b/h}	T ^{b/h}	R ^{b/h}	T ^{b/h}	R ^{b/h}
	n=13-15	n=15-20	n=12-13	n=13-18	n=13-14	n=16-20
<i>Females</i>						
BW (g)	14 ± 1	15 ± 1	17 ± 1	18 ± 1	20 ± 1	20 ± 1
BG (mg/dl)	125 ± 8	121 ± 21	95 ± 11	101 ± 10	112 ± 21	110 ± 19
TC (mg/dl)	77 ± 21	78 ± 21	626 ± 76	701 ± 75*	634 ± 75	707 ± 80*
TG (mg/dl)	32 ± 7	33 ± 8	6 ± 2	11 ± 4	5 ± 1	10 ± 5*
<i>Males</i>						
BW (g)	19 ± 1	18 ± 1	22 ± 2	22 ± 1	25 ± 1	24 ± 1
BG (mg/dl)	137 ± 18	141 ± 18	94 ± 13	94 ± 13	98 ± 10	98 ± 15
TC (mg/dl)	92 ± 25	85 ± 22	504 ± 77	515 ± 74	686 ± 138	656 ± 124
TG (mg/dl)	43 ± 11	42 ± 10	12 ± 2	11 ± 2	13 ± 5	11 ± 3

Body weight (BW), blood glucose (BG), plasma total cholesterol (TC) and triglycerides (TG). Mean ± SD.

* p<0.05 *ApoE^{fl/h}* vs *ApoE^{fl/h}* by Bonferroni post-ANOVA.