Apolipoprotein E4 Domain Interaction Accelerates Diet-Induced Atherosclerosis in Hypomorphic Arg-61 Apoe Mice

Delphine Eberlé, Roy Y. Kim, Fu Sang Luk, Nabora Soledad Reyes de Mochel, Nathalie Gaudreault, Victor R. Olivas, Nikit Kumar, Jessica M. Posada, Andrew C. Birkeland, Joseph H. Rapp, Robert L. Raffai

Objectives—Apolipoprotein (apo) E4 is an established risk factor for atherosclerosis, but the structural components underlying this association remain unclear. ApoE4 is characterized by 2 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 proatherogenic 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 (ApoeT^sh or ApoeR^sh mice). On a chow diet, both mouse models were normolipidemic with similar levels of plasma apoE and lipoproteins. However, on a high-cholesterol diet, ApoeR^sh mice displayed increased levels of total plasma cholesterol and very-low-density lipoprotein as well as larger atherosclerotic plaques in the aortic root, arch, and descending aorta compared with ApoeT^sh mice. In addition, evidence of cellular dysfunction was identified in peritoneal ApoeR^sh macrophages which released lower amounts of apoE in culture medium and displayed increased expression of major histocompatibility complex class II molecules.

Conclusions—These data indicate that domain interaction mediates proatherogenic 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. (Arterioscler Thromb Vasc Biol. 2012;32:1116-1123.)

Key Words: 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 susceptibility.1 In humans, apoE exists in 3 common isoforms (apoE2, apoE3, and apoE4) that are encoded by 3 alleles (ε2, ε3, and ε4).2 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 established that the ε4 allele is associated with increased plasma cholesterol, low-density lipoprotein (LDL)–cholesterol levels, and risk of premature atherosclerosis.3,4 Approximately 20% of individuals worldwide carry at least 1 ε4 allele,5 emphasizing the major impact of this cardiovascular risk factor on the general population.

ApoE is composed of 2 globular domains which function independently;6 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 2 unique structural features: domain interaction, which causes the 2 globular domains of apoE4 to interact,6,7 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 proatherogenic 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 noncharged 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.1 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) whereas Thr-61 mouse apoE preferentially distributes to high-density lipoprotein 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 hyperlipidemia1 and atherosclerosis (Eberlé D, Raffai RL, unpublished data, 2010). However, hypomorphic versions of these mouse models are highly susceptible to diet-induced hypercholesterolemia attributable to their low plasma apoE levels (~10% of wild-type (WT) levels).12 Here, we assessed susceptibility to diet-induced atherosclerosis in hypomorphic Apoe mice expressing either Thr-61 apoE (ApoeTh/h) or Arg-61 apoE (ApoeRh/h). We show that on an atherogenic diet, both male and female ApoeRh/h mice developed increased atherosclerosis in the aortic root and the aorta, and that the presence of domain interaction in ApoeRh/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 proatherogenic effects of human apoE4 in part by modulating lipoprotein metabolism and macrophage biology.

Materials and Methods
For complete details on Materials and Methods, please refer to the online-only Data Supplement.

Mice and Diets
Hypomorphic ApoeTh/h mice were generated by homologous recombination in embryonic stem cells as previously described for ApoeRh/h mice.12 All hypomorphic Apoe mice carried the inducible Mx1-cre transgene that can be activated to repair the hypomorphic allele and restore normal Apoe expression levels.12 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 (wt/wt) (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 which only doubles their plasma cholesterol level,12 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 4-hour-fasted mice. Plasma lipoproteins were fractionated by fast performance liquid chromatography 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 apoE,12 apoA1, and apoB.12

Analysis of Atherosclerotic Lesion Size and Composition
After 9 or 15 weeks of HCD, overnight-fasted mice were euthanized and tissues were collected. Aortic root plaque and necrotic core areas were quantified in hematoxylin and eosin–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 software.

Blood Leukocyte Analysis
Leukocyte subsets were identified by flow cytometry using combinations of antibodies specific for cell surface markers detailed in the online-only Data Supplement. Analysis was performed using FlowJo software using specific gates as specified in Figure 1 in the online-only Data Supplement.

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 48 hours in Dulbecco’s modified Eagle’s medium containing 10% lipoprotein-deficient serum using a previously described anti-mouse apoE antibody.12 Foam cell formation was quantified by the accumulation of fluorescent dilaibered oxidized LDL. Relative cholesterol efflux (%) was measured in macrophages loaded with fluorescent 25-NBD-cholesterol and acetylated LDL for 24 hours, equilibrated in DMEM 0.3% BSA, and stimulated 6 hours with human apoA1 or with mouse high-density lipoprotein in Dulbecco’s modified Eagle’s medium 0.3% BSA or in Dulbecco’s modified Eagle’s medium only.

Gene Expression
Gene expression was determined by quantitative real-time polymerase chain reaction using in-house made primers (Table I in the online-only Data Supplement) or Assay-On-Demand. Expression was normalized to housekeeping genes, TATA box-binding protein (Tbp),
Table. Metabolic parameters of chow and HCD-fed hypomorphic Apoe mice

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<th>0 week</th>
<th>4 weeks</th>
<th>12 weeks</th>
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<tr>
<td></td>
<td>Th/h (n=13-15)</td>
<td>R/h (n=15-20)</td>
<td>Th/h (n=12-13)</td>
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<tr>
<td>Females</td>
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<tr>
<td>BW (g)</td>
<td>14 ± 1</td>
<td>15 ± 1</td>
<td>17 ± 1</td>
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<tr>
<td>BG (mg/dl)</td>
<td>125 ± 8</td>
<td>121 ± 21</td>
<td>95 ± 11</td>
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<tr>
<td>TC (mg/dl)</td>
<td>77 ± 21</td>
<td>78 ± 21</td>
<td>626 ± 76</td>
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<tr>
<td>TG (mg/dl)</td>
<td>32 ± 7</td>
<td>33 ± 8</td>
<td>6 ± 2</td>
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<tr>
<td>Males</td>
<td></td>
<td></td>
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<tr>
<td>BW (g)</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
<td>22 ± 2</td>
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<tr>
<td>BG (mg/dl)</td>
<td>137 ± 18</td>
<td>141 ± 18</td>
<td>94 ± 13</td>
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<tr>
<td>TC (mg/dl)</td>
<td>92 ± 25</td>
<td>85 ± 22</td>
<td>504 ± 77</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>43 ± 11</td>
<td>42 ± 10</td>
<td>12 ± 2</td>
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BW indicates body weight; BG, blood glucose; TC, plasma cholesterol; TG, triglyceride.
Mean±SD.
*P<0.05 ApoeTh/h vs ApoeR/h by Bonferroni post-ANOVA.

or peptidylprolyl isomerase A (Ppia), and levels calculated according to the 2-ΔCt cycle threshold method.

Statistical Analysis
Data are presented as means±SD or means±SEM as mentioned in legends. Data were analyzed with GraphPad Prism 5 software using 2-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 ApoeTh/h and ApoeR/h Mice
ApoeTh/h and ApoeR/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 transcript.12 When fed a chow diet, both strains of hypomorphic Apoe female mice displayed similar levels of Apoe mRNA in liver (∼13% of WT levels, Figure 1B), resulting in similar levels of plasma apoE (∼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 ApoeTh/h and ApoeR/h mice.

Domain Interaction Increases Diet-Induced Plasma VLDL-Cholesterol
When fed a chow diet, both female and male ApoeTh/h and ApoeR/h mice were normolipidemic with similar plasma lipid levels (Table) and lipoprotein cholesterol profiles, mainly containing high-density lipoprotein (Figure 2A). However, when fed HCD, both hypomorphic Apoe mice developed pronounced hypercholesterolemia (Table), at least 2-fold higher than nonhypomorphic Apoe mice (Table II in the online-only Data Supplement). After 4 and 12 weeks of HCD, female ApoeR/h mice showed a modest but significant increase in plasma cholesterol levels compared with ApoeTh/h females (12.1% and 11.6%, respectively; Table), whereas levels were not different in male mice (Table).

Analysis of plasma lipoproteins isolated by fast performance liquid chromatography or ultracentrifugation demonstrated that ApoeR/h mice consistently accumulated more cholesterol in VLDL than ApoeTh/h mice. After 4 weeks of HCD, both female and male ApoeR/h mice showed a 19% and 17% increase, respectively, in VLDL-cholesterol levels compared with ApoeTh/h counterparts (Figure 2A). Levels of all lipid classes, phospholipids, free cholesterol, cholesterol esters, and triglycerides were increased by 11% to 49% in VLDL fractions from HCD-fed ApoeR/h female and male mice (Figure 2B and 2C). Because these differences were modest, we reassessed plasma and lipoprotein cholesterol levels after only 1 week of diet initiation to investigate the kinetics of HCD response between ApoeR/h and ApoeTh/h mice. The increase in VLDL-cholesterol in ApoeR/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 sexes of ApoeR/h mice (males: 23.7%, P=0.002, n=15–20; females: 16.7%, P=0.1, n=10). Female hypomorphic Apoe mice developed diet-induced hypercholesterolemia faster than male mice in both ApoeTh/h and ApoeR/h strains (Table and Figure 2A). Compared with ApoeTh/h mice, levels of apoB-100, apoB-48, and apoE were also increased by 77%, 52%, and 51%, respectively, in VLDL fractions of male ApoeR/h mice after 1 week of HCD (Figure 2G). Similar increases were seen in fast performance liquid chromatography fractions from female mice (Figure 2F). Higher levels of apoB-100, apoB-48, and apoE in VLDL fractions of ApoeR/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 ApoeR/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 ApoeR/h and ApoeTh/h mice fed with HCD (not shown).
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 Rh/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 Rh/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 Rh/h mice was reproduced in separate cohorts of females and male mice (Figure IIA and IIB in the online-only Data Supplement).

The proatherogenic 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 Rh/h mice displayed increased lesion area within the descending aorta relative to Apoe Th/h mice (+73% and +74%, respectively), although there was no difference in the aortic arch at this time point (Figure 3B). The increased atherosclerosis in the aortic roots of Apoe Rh/h mice was reproduced in separate cohorts of females and male mice (Figure IIA and IIB in the online-only Data Supplement).

Domain Interaction Accelerates Diet-Induced Atherosclerosis

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 sex. 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 IIA in the online-only Data Supplement) between Apoe Th/h and Apoe Rh/h male mice fed 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 organs (liver, epididymal fat, and spleen) involved in metabolic and immune functions (not shown).

Figure 2. Cholesterol (A), lipid classes (B and C), and apolipoprotein (apo) distribution in lipoproteins isolated by ultracentrifugation (D and E) or fast performance liquid chromatography (F and G) from hypomorphic female and male Apoe mice (A: n=2 [0 week], n=3 [1 week of HCD], and n=3–4 [4–12 weeks of HCD] pools of plasma taken from ≥4 mice, for each genotype and sex, Mean±SEM, ***P<0.001, Bonferroni post-ANOVA; B and C: n=1 to 2 pools of plasma taken per genotype and sex [6–12 weeks of HCD]; D and E: Coomassie-stained gel [6 weeks of HCD]; F and G: Western Blot [1 week of HCD]). HCD indicates high-cholesterol diet; PL, phospholipids; TG, triglycerides; CE, cholesterol esters; FC, free cholesterol; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein. Th/h, Apoe Th/h; Rh/h, Apoe Rh/h.
CD4+ and CD8+ T-cell numbers and activation status (Figure IIIB and IIIC in the online-only Data Supplement). Similar data were obtained in female mice (Figure III in the online-only Data Supplement). Thus, the proatherogenic 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**

Although the increased atherosclerosis in ApoeR<sup>h/h</sup> 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<sup>R<sub>h/h</sub></sup> macrophages compared with Apoe<sup>T<sub>h/h</sub></sup> 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<sup>R<sub>h/h</sub></sup> mice showed a modest but detectable 30% decrease in Apoe mRNA levels compared with Apoe<sup>T<sub>h/h</sub></sup> macrophages (P<0.05). We also sought to assess Apoe mRNA expression levels in resident peritoneal macrophages freshly isolated from both strains of mice before culturing them. As shown in Figure 5C, macrophages isolated from Apoe<sup>T<sub>h/h</sub></sup> and Apoe<sup>R<sub>h/h</sub></sup> 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 sexes 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. We first assessed oxidized LDL uptake capacity and found that macrophages isolated from both...
Second, oxidized LDL (Figure IVA in the online-only Data Supplement). These results suggest that domain interaction does not affect foam cell formation and cholesterol efflux in macrophages derived from hypomorphic Apoe mice. We showed that both male and female ApoeR<sub>Rh/h</sub> 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 proatherogenic properties of human apoE4 domain interaction could reside in its propensity to promote atherosclerosis development rather than by altering plaque composition. Interestingly, the proatherogenic effect of domain interaction was most noticeable at an early lesion stage in both the aortic root and the arch of female ApoeR<sub>Rh/h</sub> mice, suggesting that domain interaction facilitates lesion initiation. Further, we found that domain interaction promoted the accumulation of proatherogenic lipoproteins in ApoeR<sub>Rb/h</sub> 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 with apoE3 individuals. However, unlike humans, ApoeR<sub>Rb/h</sub> mice fed an atherogenic diet developed increased LDL levels. Similar results were reported in studies of knock-in mice that expressed the human apoE4 isoform in place of the murine Apoe locus.
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 mice.

Hypercholesterolemia promotes blood leukocyte activation and proliferation, 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 ApoeR<sup>R<sub>h/h</sub></sup> 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 ApoeR<sup>R<sub>h/h</sub></sup> 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 lipoproteins. Although studies have reported that apoE4 binds to the LDLR with a slightly higher affinity than apoE3, 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 long-standing hypothesis derived from early lipoprotein turnover studies in humans proposed that by accelerating VLDL clearance in the liver, apoE4 could downregulate hepatic Ldr expression and thereby raise plasma LDL levels. 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. This 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 of apoE4 individuals showing increased apoB-48 lipoprotein levels in the postprandial state and increased conversion of VLDL to LDL 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<sup>R<sub>h/h</sub></sup> macrophages. Our results are consistent with the slight decrease in apoE production observed in macrophages derived from apoE4 individuals. As we did not observe a major change in Apoe expression, it is likely that domain interaction affects posttranslational regulation of apoE in macrophages. Interestingly, it has been proposed that the enhanced affinity of macrophage-derived apoE4 for cell surface proteoglycans and other apoE receptors such as the LDLR enhance the reuptake of secreted apoE4, lowering its amount released into the medium. Recently, domain interaction was found to decrease apoE secretion in astrocytes and slow down the trafficking of apoE molecules along the secretory pathway in neurons. 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 interaction in modulating macrophage cholesterol homeostasis. We observed no differences in cholesterol accumulation and efflux between Apoe<sup>T<sub>h/h</sub></sup> and Apoe<sup>R<sub>h/h</sub></sup> 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 endogeneous apoE4 and apoE3 isoforms on macrophage cholesterol efflux have either detected no differences or slight alterations with apoE4 only in passive efflux and when the LDLR was overexpressed. Although it can seem surprising to observe no variation in cholesterol efflux capacity in the presence of decreased apoE secretion, Basu et al reported many years ago that apoE secretion and cholesterol efflux can be uncoupled, suggesting that the 2 processes may function via independent pathways.

Finally, we found that domain interaction influenced macrophage activation. We show that Apoe<sup>R<sub>h/h</sub></sup> 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 reported increased MHCII levels on macrophages derived from Apoe<sup>T<sub>h/h</sub></sup> mice compared with WT macrophages. In that study, the enhanced cellular activation state of Apoe<sup>T<sub>h/h</sub></sup> macrophages resulted in increased T cell proliferation and activation. Another recent study found that T cell proliferation was increased in human apoE4 transgenic mice. Multiple studies have identified T cell–mediated immune responses in the artery wall as key components in the initiation and progression of atherosclerosis in mice. More recent studies demonstrated reduced atherosclerosis in hyperlipidemic mice in response to the suppression of T cell–mediated immunity in the artery wall. 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 disease. Domain interaction appears critical for the pathological effects of apoE4 in Alzheimer disease, causing various defects in neurons and astrocytes. Domain interaction was found to enhance ER stress and decrease apoE secretion in astrocytes, however Brodbeck et al 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 proatherogenic 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 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. Our findings support testing the
efficacy of such compounds in preventing diet-induced atherosclerosis in our models.

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Disclosures
None.

References
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Detailed methods

Mice and Diets

Hypomorphic Apoe mice expressing Thr-61 apoE (ApoeTh/h) were generated by homologous recombination in embryonic stem cells as previously described for Arg-61 apoE mice (ApoeRh/h) 1. ApoeTh/h and ApoeRh/h mice differ by a single nucleotide in exon 3 (169C/G) of the Apoe locus, coding for a mouse apoE protein containing either Thr-61 or Arg-61 respectively. Mice were genotyped using a PCR based assay allowing the detection of a DdeI restriction enzyme site (New England Biolbas, UK) introduced by the substitution of Thr-61 by Arg-61. Both hypomorphic ApoeTh/h and ApoeRh/h mice were backcrossed for 12 generations to C57Bl/6 background and SNP (Single Nucleotide Polymorphisms) analysis revealed a 99.95% C57Bl6 background. All hypomorphic Apoe mice carried the inducible Mx1-cre transgene that can be activated to repair the hypomorphic allele and restore normal Apoe expression levels 1. However, in this study, the Mx1-cre transgene was not induced and remained silent throughout the study 1,2 resulting in consistently reduced Apoe expression among all hypomorphic mice as shown in Figure 1B. The mice were weaned at 21 days of age and fed ad libitum a standard mouse chow diet (2916, Harlan Teklad, Madison, WI). At 6 weeks of age, female and male mice were fed the atherogenic high-cholesterol Paigen Diet (HCD, 16% fat, 1.25% cholesterol and 0.5% cholic acid (w/w)) (D12336, Research Diets Inc., New Brunswick, NJ) for the indicated period of time. Arg-61 apoE mice (R61) 3 and Thr-61 apoE mice (Wild-type, WT) were maintained in house. All animals were housed in a facility with a 12-h light/12-h dark cycle and were cared for according to the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Metabolic Parameters, Plasma Lipids and Lipoprotein Fractionation

Body weight, blood glucose, plasma lipids and lipoproteins were monitored in 4h-fasted mice. Blood glucose was measured with a Contour glucometer (Bayer, Mishawaka, IN) using a drop of blood collected by tail incision. Mice were anesthetized with isoflurane (3 to 5% in oxygen for 4 min) and
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Plasma and FPLC fractions were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad Laboratories, CA) and subjected to western blotting with primary antibodies directed against mouse apoE (1/2500 to 1/10 000,3), apoA1 (1/30 000, Meridian Life Science, Inc., ME) and apoB (1/10 000, 1). Signal was quantified using either IRDye680LT-conjugated anti-rabbit antibody and the Odyssey system (LI-COR Biosciences, NE) or horseradish peroxidase-conjugated anti-rabbit antibody (Vector Laboratories Inc., CA), chemiluminescent reagent (Pierce, IL) and AlphaImager Gel Documentation system (Cell Biosciences, Inc., CA).
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Mice were fasted overnight, anesthetized by intra-peritoneal injection of tribromoethanol (Avertin, 250mg/kg of body weight) and sacrificed by heart blood puncture followed by perfusion with 10 ml of ice-cold PBS containing RNase inhibitor (Sigma, MO). Hearts were embedded in tissue freezing medium (OCT) and flash frozen in isopentane bath. The entire aortic root area was serially sectioned at 10 µm intervals from the base of the aortic sinus and mounted on slides (SuperFrost Plus, Fisher). Morphometric analysis was performed on hematoxylin and eosin (H&E) stained sections where lesion area (µm²) was defined by the surface between the internal elastic lamina and the luminal boundary. The average quantification of six sections spaced by 50 µm each, starting from the base of the aortic root where 3 leaflets are clearly visible, is presented for each mouse. Sections were viewed with a Zeiss AxioObserver microscope, digitized with a Retiga-SRV CCD camera equipped with RGB color filter (Qimaging, Surrey, BC, Canada) and quantified using ImageJ software (U.S. NIH, MD). At sacrifice, aortas (from the aortic arch to the ileal bifurcation) were removed, fixed with formalin, cleaned out of adventitial fat, cut longitudinally and pinned flat on black wax under a dissecting microscope. Prerarations of en face aortas and arches were photographed at a fixed magnification and atherosclerotic lesion areas in each aortic segment (i.e. arch and descending regions) were quantified as the percentage of the area of each segment.

Analysis of Atherosclerotic Lesion Composition

Necrotic core area was quantified by measuring H&E-negative acellular areas in the intima as previously described. Adjacent sections were stained with picro-sirius red counterstained with Fast-green to reveal collagen under brightfield and polarized light as previously described. The detection of apoE and MOMA-positive macrophages within atheroma was performed by immunofluorescence using simultaneously rabbit anti-mouse apoE (1/200) and rat-anti mouse MOMA (1/200, Cedarlane, Laboratories Limited, ON, CA), followed by incubation with Alexa594 conjugated donkey anti-rabbit and Alexa488 conjugated anti-rat secondary antibodies (1/1000, Invitrogen). Nuclei were stained with Hoescht 33342 (1/5 000; Invitrogen) and slides were mounted in SlowFade Gold (Invitrogen). Sections
were viewed with a Zeiss AxiosObserver microscope as described above. Surface areas covered by apoE and MOMA were quantified with Metamorph software (Molecular Devices Inc., Sunnyvale, CA).

**Blood Leukocyte Analysis**

Blood leukocyte concentrations and percentages were assessed by flow cytometry in mice fasted overnight. Blood aliquots were mixed with EDTA and kept at room temperature for the entire staining procedure. After a pre-incubation step to block CD16/CD32 (Fc Block, BD Pharmigen), blood cell suspensions were stained with various combinations of antibodies (CD45 (30-F11), B220 (RA3-6B2), CD3 (145-2C11), Ly6G (1A8), CD11b (M170), NK1.1 (PK-136), Ly-6C (AL-21), CD62L (MEL-14) from BD Biosciences; CD115 (AFS98) from eBioscience). After red blood cell lysis (BD Red Cell Lysing Solution, BD Pharmigen) and fixation, samples were kept on ice and analyzed with a C6 Flow Cytometer (Accuri Cytometers Inc., MI) providing 4 colors detection, linear forward scatter and side scatter detections and sample volume determination. Analysis were performed with FlowJo software (Tree Star Inc., OR) using specific gates as indicated in **Figure I**.

**Analysis of Freshly Isolated Peritoneal Macrophages**

Resident peritoneal macrophages of chow or HCD-fed mice were isolated by a standard lavage procedure of the peritoneal cavity with 10 ml of Ca²⁺/Mg²⁺-free PBS. For flow cytometry analysis, peritoneal cells were pre-incubated with Fc block (BD Pharmigen) and stained with a combination of antibodies (F4/80 (BM8) from Caltag, CD11b (M170), I-Ab/I-Ed (MHC class II, 2G9), CD86 (B7-2, GL1) and CD80 (B7-1, 16-10A1) from BD Biosciences). Cell suspensions were analyzed with a C6 Flow Cytometer (Accuri Cytometers Inc., MI) and FlowJo software analysis (Tree Star Inc., OR) using a macrophage gate (F4/80⁺). For gene expression analysis, peritoneal cells were incubated 2h at 37°C in DMEM containing 24-well plates to allow macrophage adhesion to the culture vessel. Non-adherent cells were then washed away with PBS, and macrophages were resuspended in RLT buffer (Qiagen, CA) for RNA extraction. This isolation procedure routinely led to high purity preparations (97% of CD11b⁺/F480⁺ macrophages).
**In vitro Macrophage Cellular and Medium ApoE Quantification**

Resident peritoneal macrophages of chow-fed age-matched mice (10-20 weeks old, pool of 4-5 mice) were isolated by the lavage and adhesion procedure described above and cultured in complete medium (DMEM 20% L-cell conditioned medium 10% Fetal Bovin Serum (FBS)). Two days after isolation, cells were washed 3 times with warm DMEM, and switched to DMEM containing 10% lipoprotein-deficient serum (Sigma, MO) to exclude any exogenous apoE. After 48 hours of culture, medium was collected, mixed with protease inhibitors (Halt Protease Inhibitor, Thermo Fisher Scientific) and centrifuged to remove any cell debris. Macrophages were washed with PBS and lysed in RIPA buffer (50 M Tris HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Halt Protease Inhibitor, Thermo Fisher Scientific). Cell lysate protein concentrations were determined with DC Protein Assay (Bio-Rad Laboratories, Life Science Research, Hercules, CA) according to the manufacturer’s instructions. A volume of medium corresponding to 10µg of cellular protein was used for western blot analysis of apoE levels in medium. For apoE immunofluorescence detection, cells were washed 3 times with warm PBS, fixed with formalin. After quenching residual aldehydes with 100 mM Glycine, cells were permeabilized with 0.1% Triton, washed and blocked with 10% donkey serum. ApoE was detected by a rabbit anti-mouse apoE 7 (1/200) and an alexa488 conjugated donkey anti-rabbit antibody (Invitrogen). Nuclei were stained with Hoescht 33342 (Invitrogen). Samples were viewed with a Zeiss AxioObserver microscope as described above. For each well, a minimum of 4 images were taken. ApoE integrated intensity and nuclei counts were quantified with Metamorph software (Molecular Devices Inc., Sunnyvale, CA). For Apoe mRNA analysis, macrophages were re-suspended in RLT buffer (Qiagen, CA) for RNA extraction.

**In vitro foam cell formation and cholesterol efflux**

Foam cell formation was evaluated in peritoneal macrophages plated in 8 well chamber slides and cultured with 10 or 50µg/ml dil-OxLDL (BTI, Stoughton, MA). After 8 hours of incubation, cells were fixed with 3% paraformaldehyde, quenched with 100 mM Glycine, and nuclei were stained with Hoescht 33342 (Invitrogen). Samples were viewed with a Zeiss AxioObserver microscope as described above. For
each well, a minimum of 4 images were taken. Percentage of macrophages containing dilOxLDL in their cytoplasm was quantified with Metamorph software (Molecular Devices Inc., Sunnyvale, CA). For cholesterol efflux experiments, peritoneal macrophages were isolated 4 days after a peritoneal injection of concanavalin A (40mg/ml, Sigma). Cultured macrophages were loaded with fluorescent 5µg/ml 25-NBD-cholesterol (Avanti Polar Lipids, Alabaster, AL) and 50µg/ml AcLDL (BTI, Stoughton, MA) for 24h, equilibrated in DMEM 0.3% BSA overnight and efflux was assessed after 6h of incubation in DMEM containing 0.3% BSA with or without added 40µg/ml human apoA1 (Sigma-Aldrich, MO) or 100µg/ml HDL isolated from WT mice, and in DMEM with or without added 100µg/ml HDL isolated from Apoe\(^{-/-}\) mice. Efflux efficiency was calculated as fluorescence intensity in medium divided by fluorescence intensity in medium + cells, and expressed as relative efflux (%) where 100% corresponds to the passive efflux of Apoe\(^{h/h}\) macrophages.

**Gene expression**

Total RNA was isolated from liver or peritoneal macrophages using RNeasy Mini kit including a DNase step according to the manufacturer’s instruction (Qiagen, CA). A quantity of Total RNA (1µg for liver or 200ng for macrophages) was retro-transcribed into cDNA (iScript, Bio-Rad Laboratories, Life Sciences Research, Hercules, CA). Gene expression was determined by quantitative real-time PCR using an ABI Prism 7900 (Applied Biosystems, Foster City, CA) using in house made primer pairs with validated efficiency (>85%) (Table I) or Assay-On-Demand (Applied Biosystems). Two µl of diluted (1/20) cDNA template were used in each PCR reaction. Each target gene expression was normalized to the housekeeping gene, TATA box binding protein (Tbp) or peptidylprolyl isomerase A (Ppia), and levels calculated according to the 2-\(\Delta\Delta\text{Ct}\) method.

**Statistical Analysis**

Data are presented as Mean±SD or Mean±SEM as mentioned in legends. Data were analyzed with GraphPad Prism 5 software (GraphPad software inc., La Jolla, CA) using two-tailed Student t-tests unless otherwise stated. A difference with a P value < 0.05 was considered significant.
Supplemental figures legends

**Figure I.** Blood leukocyte analysis was performed by flow cytometry using an Accuri C6 Flow cytometer with 4 color detection, forward scatter (FSC-A, FSC-H) and side scatter detection (SSC-A) and volume determination. Four different antibody combinations (A: CD115-Alexa 488, Ly6G-PE, CD3-PE, B220-PerCPCy5.5, CD45-APC; B: Ly-6C-FITC, CD115-PE, CD11b-PerCPCy5.5, CD62L-APC, C: CD4-FITC, CD3-PE, CD8-APC and D: CD4-FITC, CD44-PE, CD8-PECy7, CD62L-APC) were used to identify cell populations (leukocytes, neutrophils, B cells, T cells as well as Ly-6C\text{low}, Ly-6C\text{high}, CD62L\text{+} and CD44\text{+} subsets). For each analysis, cellular debris and aggregates were first gated out. Cells were then gated as indicated on the representative dot plots and gating strategies.

**Figure II.** Increased atherosclerotic lesion area in aortic roots of additional cohorts of (A) female and (B) male hypomorphic Apoe\text{Rh/h} mice fed a HCD for 9 and 15 weeks respectively (n=7-10 per genotype and gender). Individual values and means are shown. *p<0.05 and **p<0.01

**Figure III.** (A) B cells (B220\text{+}), T cells (CD3\text{+}), neutrophil (ly6G\text{+}) and monocyte (CD115\text{+}) blood concentrations, (B) Percentages of Ly6c\text{low}, Ly6\text{high}, CD62L\text{+} monocyte subsets and (C) CD4\text{+} and CD8\text{+} T cells (%CD3\text{+}); CD44\text{+} and CD62L\text{+} T cells (%CD4\text{+} and CD8\text{+}) in HCD-fed hypomorphic Apoe male and female mice (n=7-10 per genotype and gender). Mean±SEM

**Figure IV.** (A) Foam cell formation assessed by % cells that uptake dil-OxLDL incubated for 8h either at 10 or 50µg/ml (n=2-3) or by dilOxLDL intensity per cell. AU, Arbitrary Unit. (B, C) Relative cholesterol efflux (%) of macrophages loaded with 25-NBD cholesterol and AcLDL and incubated with (B) DMEM containing 0.3%BSA with or without added apoA1 or WT HDL (n=3) and (C) DMEM with or without
added $\text{Apoe}^{+/c}$ HDL (n=6) for 6 hours. For each experiment, passive efflux of $\text{ApoeT}^{bh}$ macrophages was set to 100%. Mean±SEM. NS, not significant.
## Supplemental Tables

### Table I. Sequences of primers used for RT-PCR (Sybr Green)

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Table II. Metabolic parameters of non-hypomorphic WT and Arg-61 (R61) mice fed a chow and HCD

<table>
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<th></th>
<th>0 week</th>
<th>6 weeks</th>
<th>15 weeks</th>
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<tr>
<td></td>
<td>WT</td>
<td>R61</td>
<td>WT</td>
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<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BW (g)</td>
<td>15±1</td>
<td>14±0.3*</td>
<td>19±0.2</td>
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<td>BG (mg/dl)</td>
<td>146±9</td>
<td>148±27</td>
<td>112±16</td>
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<td>TC (mg/dl)</td>
<td>87±7</td>
<td>86±4</td>
<td>205±31</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>41±13</td>
<td>49±14</td>
<td>9±1</td>
</tr>
<tr>
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<tr>
<td>BW (g)</td>
<td>19±1</td>
<td>19±1</td>
<td>21±1</td>
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<tr>
<td>BG (mg/dl)</td>
<td>187±21</td>
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<td>TC (mg/dl)</td>
<td>74±3</td>
<td>76±5</td>
<td>236±22</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>41±9</td>
<td>49±5</td>
<td>11±2</td>
</tr>
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</table>

Body weight (BW), blood glucose (BG), plasma total cholesterol (TC) and triglycerides (TG) were measured in 4h-fasted mice at 6 weeks of age on a chow diet (0) and after 6 weeks and 15 weeks of HCD feeding. Mean±SD. * p<0.05 Apoe\textsuperscript{Th/h} vs Apoe\textsuperscript{R/h} by ANOVA and Bonferroni post-tests. n/a, non-available.
Table III. Expression levels of ER stress-related proteins in freshly isolated peritoneal macrophages from hypomorphic Apoe mice fed a chow or HCD for 4 weeks

<table>
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<th>Gene</th>
<th>Chow</th>
<th>HCD</th>
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<td>T&lt;sup&gt;h/h&lt;/sup&gt;</td>
<td>R&lt;sup&gt;h/h&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>n=5M/5F</td>
<td>n=5M/5F</td>
</tr>
<tr>
<td><strong>Atf4</strong></td>
<td>1.00 ± 0.12</td>
<td>0.90 ± 0.14</td>
</tr>
<tr>
<td><strong>Chop</strong></td>
<td>1.00 ± 0.11</td>
<td>0.87 ± 0.11 *</td>
</tr>
<tr>
<td><strong>Grp78</strong></td>
<td>1.00 ± 0.12</td>
<td>0.89 ± 0.27</td>
</tr>
<tr>
<td><strong>TRB3</strong></td>
<td>1.00 ± 0.10</td>
<td>0.82 ± 0.15 **</td>
</tr>
</tbody>
</table>

Gene expression levels are normalized to Apoe<sup>T<sup>h/h</sup></sup> mice fed a chow diet. n=Males/Females in each group. Mean±SD. * p<0.05, ** p<0.01, *** p<0.001 Apoe<sup>T<sup>h/h</sup></sup> vs Apoe<sup>R<sup>h/h</sup></sup> by t-test.
**Table IV.** Expression levels of ER stress-related proteins in cultured peritoneal macrophages from hypomorphic *Apoe* mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T^{h/h}$</td>
<td>$R^{h/h}$</td>
<td></td>
</tr>
<tr>
<td><strong>Atf4</strong></td>
<td>1.00 ± 0.11</td>
<td>0.90 ± 0.09</td>
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<tr>
<td><strong>Chop</strong></td>
<td>1.00 ± 0.11</td>
<td>0.94 ± 0.10</td>
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<tr>
<td><strong>TRB3</strong></td>
<td>1.00 ± 0.12</td>
<td>1.04 ± 0.22</td>
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</tr>
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Gene expression levels are normalized to *Apoe* $T^{h/h}$ macrophages. Two independent experiments performed in duplicate (n=6). Mean±SD.
Supplemental References


Supplemental figures
Figure II

A  
Females

B  
Males

Lesion area (μm²)

9 weeks

15 weeks
Figure IV

A

B

C
SUPPLEMENTAL MATERIAL

Detailed methods

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Resident peritoneal macrophages of chow-fed age-matched mice (10-20 weeks old, pool of 4-5 mice) were isolated by the lavage and adhesion procedure described above and cultured in complete medium (DMEM 20% L-cell conditioned medium 10% Fetal Bovin Serum (FBS)). Two days after isolation, cells were washed 3 times with warm DMEM, and switched to DMEM containing 10% lipoprotein-deficient serum (Sigma, MO) to exclude any exogenous apoE. After 48 hours of culture, medium was collected, mixed with protease inhibitors (Halt Protease Inhibitor, Thermo Fisher Scientific) and centrifuged to remove any cell debris. Macrophages were washed with PBS and lysed in RIPA buffer (50 M Tris HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Halt Protease Inhibitor, Thermo Fisher Scientific). Cell lysate protein concentrations were determined with DC Protein Assay (Bio-Rad Laboratories, Life Science Research, Hercules, CA) according to the manufacturer’s instructions. A volume of medium corresponding to 10µg of cellular protein was used for western blot analysis of apoE levels in medium. For apoE immunofluorescence detection, cells were washed 3 times with warm PBS, fixed with formalin. After quenching residual aldehydes with 100 mM Glycine, cells were permeabilized with 0.1% Triton, washed and blocked with 10% donkey serum. ApoE was detected by a rabbit anti-mouse apoE \(^7\) (1/200) and an alexa488 conjugated donkey anti-rabbit antibody (Invitrogen). Nuclei were stained with Hoescht 33342 (Invitrogen). Samples were viewed with a Zeiss AxioObserver microscope as described above. For each well, a minimum of 4 images were taken. ApoE integrated intensity and nuclei counts were quantified with Metamorph software (Molecular Devices Inc., Sunnyvale, CA). For Apoe mRNA analysis, macrophages were re-suspended in RLT buffer (Qiagen, CA) for RNA extraction.

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Supplemental figures legends

**Figure I.** Blood leukocyte analysis was performed by flow cytometry using an Accuri C6 Flow cytometer with 4 color detection, forward scatter (FSC-A, FSC-H) and side scatter detection (SSC-A) and volume determination. Four different antibody combinations (A: CD115-Alexa 488, Ly6G-PE, CD3-PE, B220-PerCPCy5.5, CD45-APC; B: Ly-6C-FITC, CD115-PE, CD11b-PerCPCy5.5, CD62L-APC, C: CD4-FITC, CD3-PE, CD8-APC and D: CD4-FITC, CD44-PE, CD62L-APC) were used to identify cell populations (leukocytes, neutrophils, B cells, T cells as well as Ly-6C\textsuperscript{low}, Ly-6C\textsuperscript{high}, CD62L\textsuperscript{+} and CD44\textsuperscript{+} subsets). For each analysis, cellular debris and aggregates were first gated out. Cells were then gated as indicated on the representative dot plots and gating strategies.

**Figure II.** Increased atherosclerotic lesion area in aortic roots of additional cohorts of (A) female and (B) male hypomorphic Apoe\textsuperscript{h/h} mice fed a HCD for 9 and 15 weeks respectively (n=7-10 per genotype and gender). Individual values and means are shown. *p<0.05 and **p<0.01

**Figure III.** (A) B cells (B220\textsuperscript{+}), T cells (CD3\textsuperscript{+}), neutrophil (ly6G\textsuperscript{+}) and monocyte (CD115\textsuperscript{+}) blood concentrations, (B) Percentages of Ly6c\textsuperscript{low}, Ly6\textsuperscript{high}, CD62L\textsuperscript{+} monocyte subsets and (C) CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (%CD3\textsuperscript{+}); CD44\textsuperscript{+} and CD62L\textsuperscript{+} T cells (%CD4\textsuperscript{+} and CD8\textsuperscript{+}) in HCD-fed hypomorphic Apoe male and female mice (n=7-10 per genotype and gender). Mean±SEM

**Figure IV.** (A) Foam cell formation assessed by % cells that uptake dil-OxLDL incubated for 8h either at 10 or 50µg/ml (n=2-3) or by dilOxLDL intensity per cell. AU, Arbitrary Unit. (B, C) Relative cholesterol efflux (%) of macrophages loaded with 25-NBD cholesterol and AcLDL and incubated with (B) DMEM containing 0.3%BSA with or without added apoA1 or WT HDL (n=3) and (C) DMEM with or without
added Apoe<sup>+/−</sup> HDL (n=6) for 6 hours. For each experiment, passive efflux of Apoe<sup>−/−</sup> macrophages was set to 100%. Mean±SEM. NS, not significant.
## Supplemental Tables

*Table I.* Sequences of primers used for RT-PCR (Sybr Green)

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td><strong>Apoe</strong></td>
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<td>tcc tcc atc agt gcc gtc agt tc</td>
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<td><strong>Atf4</strong></td>
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</tr>
<tr>
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<td>NM_007837 gac cag gtt ctg ctt tca gg</td>
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**Table II.** Metabolic parameters of non-hypomorphic WT and Arg-61 (R61) mice fed a chow and HCD

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>6 weeks</th>
<th>15 weeks</th>
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<tbody>
<tr>
<td></td>
<td><em>WT</em></td>
<td><em>R61</em></td>
<td><em>WT</em></td>
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<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
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</table>

**Females**

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>6 weeks</th>
<th>15 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>15 ± 1</td>
<td>19 ± 0.2</td>
<td>21 ± 0.3</td>
</tr>
<tr>
<td>BG (mg/dl)</td>
<td>146 ± 9</td>
<td>112 ± 16</td>
<td>81 ± 12</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>87 ± 7</td>
<td>205 ± 31</td>
<td>246 ± 61</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>41 ± 13</td>
<td>9 ± 1</td>
<td>n/a</td>
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</table>

**Males**

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>6 weeks</th>
<th>15 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>19 ± 1</td>
<td>21 ± 1</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>BG (mg/dl)</td>
<td>187 ± 21</td>
<td>93 ± 7</td>
<td>84 ± 9</td>
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<tr>
<td>TC (mg/dl)</td>
<td>74 ± 3</td>
<td>236 ± 22</td>
<td>262 ± 18</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>41 ± 9</td>
<td>11 ± 2</td>
<td>n/a</td>
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</table>

Body weight (BW), blood glucose (BG), plasma total cholesterol (TC) and triglycerides (TG) were measured in 4h-fasted mice at 6 weeks of age on a chow diet (0) and after 6 weeks and 15 weeks of HCD feeding. Mean±SD. * p<0.05 *Apoe*<sup>h/h</sup> vs *Apoe*<sup>R/h</sup> by ANOVA and Bonferroni post-tests. n/a, non-available.
Table III. Expression levels of ER stress-related proteins in freshly isolated peritoneal macrophages from hypomorphic Apoe mice fed a chow or HCD for 4 weeks

Gene expression levels are normalized to Apoe<sup>T<sub>h/h</sub></sup> mice fed a chow diet. n=Males/Females in each group. Mean±SD. * p<0.05, ** p<0.01, *** p<0.001 Apoe<sup>T<sub>h/h</sub></sup> vs Apoe<sup>R<sub>h/h</sub></sup> by t-test.
Table IV. Expression levels of ER stress-related proteins in cultured peritoneal macrophages from hypomorphic Apoe mice

Gene expression levels are normalized to Apoe^Th/h macrophages. Two independent experiments performed in duplicate (n=6). Mean±SD.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow^Th/h</th>
<th>Chow^R/h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atf4</em></td>
<td>1.00 ± 0.11</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td><em>Chop</em></td>
<td>1.00 ± 0.11</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td><em>TRB3</em></td>
<td>1.00 ± 0.12</td>
<td>1.04 ± 0.22</td>
</tr>
</tbody>
</table>
Supplemental References


Supplemental figures
Figure II

A

Females

\[ 5 \times 10^5 \]

\[ 4 \times 10^5 \]

\[ 3 \times 10^5 \]

\[ 2 \times 10^5 \]

\[ 1 \times 10^5 \]

9 weeks

\[ \] **

\[ \] T^{hh}

\[ \] R^{hh}

B

Males

\[ 5 \times 10^5 \]

\[ 4 \times 10^5 \]

\[ 3 \times 10^5 \]

\[ 2 \times 10^5 \]

\[ 1 \times 10^5 \]

15 weeks

\[ \] *

\[ \] T^{hh}

\[ \] R^{hh}
Figure III

A  
Males

B  

C  

Subsets (% Monocytes)

Subsets (% Monocytes)

Subsets (% T cells)

Subsets (% T cells)
Figure IV

A

B

C
SUPPLEMENTAL MATERIAL

Detailed methods

Mice and Diets

Hypomorphic Apoe mice expressing Thr-61 apoE (ApoeTh/h) were generated by homologous recombination in embryonic stem cells as previously described for Arg-61 apoE mice (ApoeRh/h) \(^1\). ApoeTh/h and ApoeRh/h mice differ by a single nucleotide in exon 3 (169C/G) of the Apoe locus, coding for a mouse apoE protein containing either Thr-61 or Arg-61 respectively. Mice were genotyped using a PCR based assay allowing the detection of a Dde1 restriction enzyme site (New England Biolbas, UK) introduced by the substitution of Thr-61 by Arg-61. Both hypomorphic ApoeTh/h and ApoeRh/h mice were backcrossed for 12 generations to C57Bl/6 background and SNP (Single Nucleotide Polymorphisms) analysis revealed a 99.95% C57Bl6 background. All hypomorphic Apoe mice carried the inducible Mx1-cre transgene that can be activated to repair the hypomorphic allele and restore normal Apoe expression levels \(^1\). However, in this study, the Mx1-cre transgene was not induced and remained silent throughout the study \(^1,2\) resulting in consistently reduced Apoe expression among all hypomorphic mice as shown in Figure 1B. The mice were weaned at 21 days of age and fed ad libitum a standard mouse chow diet (2916, Harlan Teklad, Madison, WI). At 6 weeks of age, female and male mice were fed the atherogenic high-cholesterol Paigen Diet (HCD, 16% fat, 1.25% cholesterol and 0.5% cholic acid (w/w)) (D12336, Research Diets Inc., New Brunswick, NJ) for the indicated period of time. Arg-61 apoE mice (R61) \(^3\) and Thr-61 apoE mice (Wild-type, WT) were maintained in house. All animals were housed in a facility with a 12-h light/12-h dark cycle and were cared for according to the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Metabolic Parameters, Plasma Lipids and Lipoprotein Fractionation

Body weight, blood glucose, plasma lipids and lipoproteins were monitored in 4h-fasted mice. Blood glucose was measured with a Contour glucometer (Bayer, Mishawaka, IN) using a drop of blood collected by tail incision. Mice were anesthetized with isoflurane (3 to 5% in oxygen for 4 min) and
plasma was obtained from blood drawn from the retro-orbital sinus (200µl) mixed with 2µl of 0.5mol/L ethylene diamine tetraacetic acid (EDTA) and 2µl protease inhibitors cocktail (Halt Protease Inhibitor, Thermo Fisher Scientific). For lipoprotein analysis, plasma was pooled from several mice (n≥4) and fractionated by fast performance liquid chromatography (FPLC) on a Superose 6 GL 10/30 column (GE Healthcare, NJ) or by sequential density ultracentrifugation using KBr gradient (d<1.006 (VLDL), 1.006<d<1.020 (IDL), 1.020<d<1.063 (LDL), 1.063<d<1.021 (HDL)) as previously described 4. Briefly, the plasma density was first adjusted to 1.006 g/ml with PBS and centrifuged at 52 000 rpm for 24 h at 8°C in a TLA 100.3 rotor in an Optima TL Ultracentrifuge (Beckman instruments, Fullerton, CA). The top fraction containing VLDL was collected and the bottom fraction was adjusted to a density of 1.020 g/ml with KBr and centrifuged for an additional 24 h as described above to isolate IDL. This step was repeated twice more to isolate LDL and HDL at the previously mentioned densities. Lipoproteins isolated by density ultracentrifugation were dialyzed against phosphate buffered saline (PBS) and resolved by 4-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for Coomassie blue staining or western blot analysis. Lipid levels in plasma and lipoprotein fractions were measured by colorimetric assays according to the manufacturer’s instructions (Total and free Cholesterol E, L-type TG M, Phospholipids C, Wako, VA) adapted for a VersaMax microplate reader (Molecular Devices, Corporation, Sunnyvale, CA).

**Western Blot Quantitation of ApoE, ApoA1 and ApoB**

Plasma and FPLC fractions were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad Laboratories, CA) and subjected to western blotting with primary antibodies directed against mouse apoE (1/2500 to 1/10 000,3), apoA1 (1/30 000, Meridian Life Science, Inc., ME) and apoB (1/10 000, 1). Signal was quantified using either IRDye680LT-conjugated anti-rabbit antibody and the Odyssey system (LI-COR Biosciences, NE) or horseradish peroxidase-conjugated anti-rabbit antibody (Vector Laboratories Inc., CA), chemiluminescent reagent (Pierce, IL) and Alphalmlager Gel Documentation system (Cell Biosciences, Inc., CA).
Quantification of Atherosclerosis Lesion Area

Mice were fasted overnight, anesthetized by intra-peritoneal injection of tribromoethanol (Avertin, 250mg/kg of body weight) and sacrificed by heart blood puncture followed by perfusion with 10 ml of ice-cold PBS containing RNase inhibitor (Sigma, MO). Hearts were embedded in tissue freezing medium (OCT) and flash frozen in isopentane bath. The entire aortic root area was serially sectioned at 10 µm intervals from the base of the aortic sinus and mounted on slides (SuperFrost Plus, Fisher). Morphometric analysis was performed on hematoxylin and eosin (H&E) stained sections where lesion area (µm$^2$) was defined by the surface between the internal elastic lamina and the luminal boundary. The average quantification of six sections spaced by 50 µm each, starting from the base of the aortic root where 3 leaflets are clearly visible, is presented for each mouse. Sections were viewed with a Zeiss AxioObserver microscope, digitized with a Retiga-SRV CCD camera equipped with RGB color filter (Qimaging, Surrey, BC, Canada) and quantified using ImageJ software (U.S. NIH, MD). At sacrifice, aortas (from the aortic arch to the ileal bifurcation) were removed, fixed with formalin, cleaned out of adventitial fat, cut longitudinally and pinned flat on black wax under a dissecting microscope. Preparations of en face aortas and arches were photographed at a fixed magnification and atherosclerotic lesion areas in each aortic segment (i.e. arch and descending regions) were quantified as the percentage of the area of each segment.

Analysis of Atherosclerotic Lesion Composition

Necrotic core area was quantified by measuring H&E-negative acellular areas in the intima as previously described 5. Adjacent sections were stained with picro-sirius red counterstained with Fast-green to reveal collagen under brightfield and polarized light as previously described 6. The detection of apoE and MOMA-positive macrophages within atheroma was performed by immunofluorescence using simultaneously rabbit anti-mouse apoE 7 (1/200) and rat-anti mouse MOMA (1/200, Cedarlane, Laboratories Limited, ON, CA), followed by incubation with Alexa594 conjugated donkey anti-rabbit and Alexa488 conjugated anti-rat secondary antibodies (1/1000, Invitrogen). Nuclei were stained with Hoescht 33342 (1/5 000; Invitrogen) and slides were mounted in SlowFade Gold (Invitrogen). Sections
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Data are presented as Mean±SD or Mean±SEM as mentioned in legends. Data were analyzed with GraphPad Prism 5 software (GraphPad software inc., La Jolla, CA) using two-tailed Student t-tests unless otherwise stated. A difference with a P value < 0.05 was considered significant.
Supplemental figures legends

**Figure I.** Blood leukocyte analysis was performed by flow cytometry using an Accuri C6 Flow cytometer with 4 color detection, forward scatter (FSC-A, FSC-H) and side scatter detection (SSC-A) and volume determination. Four different antibody combinations (A: CD115-Alexa 488, Ly6G-PE, CD3-PE, B220-PerCPcy5.5, CD45-APC; B: Ly-6C-FITC, CD115-PE, CD11b-PerCPcy5.5, CD62L-APC, C: CD4-FITC, CD3-PE, CD8-APC and D: CD4-FITC, CD44-PE, CD62L-APC) were used to identify cell populations (leukocytes, neutrophils, B cells, T cells as well as Ly-6C low, Ly-6C high, CD62L+ and CD44+ subsets). For each analysis, cellular debris and aggregates were first gated out. Cells were then gated as indicated on the representative dot plots and gating strategies.

**Figure II.** Increased atherosclerotic lesion area in aortic roots of additional cohorts of (A) female and (B) male hypomorphic Apoe R h mice fed a HCD for 9 and 15 weeks respectively (n=7-10 per genotype and gender). Individual values and means are shown. *p<0.05 and **p<0.01

**Figure III.** (A) B cells (B220+), T cells (CD3+), neutrophil (ly6G+) and monocyte (CD115+) blood concentrations, (B) Percentages of Ly6c low, Ly6 high, CD62L+ monocyte subsets and (C) CD4+ and CD8+ T cells (%CD3+); CD44+ and CD62L+ T cells (%CD4+ and CD8+) in HCD-fed hypomorphic Apoe male and female mice (n=7-10 per genotype and gender). Mean±SEM

**Figure IV.** (A) Foam cell formation assessed by % cells that uptake dil-OxLDL incubated for 8h either at 10 or 50µg/ml (n=2-3) or by dilOxLDL intensity per cell. AU, Arbitrary Unit. (B, C) Relative cholesterol efflux (%) of macrophages loaded with 25-NBD cholesterol and AcLDL and incubated with (B) DMEM containing 0.3%BSA with or without added apoA1 or WT HDL (n=3) and (C) DMEM with or without
added $Apoe^{+/−}$ HDL (n=6) for 6 hours. For each experiment, passive efflux of $Apoe^{−/−}$ macrophages was set to 100%. Mean±SEM. NS, not significant.
Supplemental Tables

Table I. Sequences of primers used for RT-PCR (Sybr Green)

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Apoe</td>
<td>NM_009696.3</td>
<td>tgt ggg ccg tgc tgt tgg tc</td>
</tr>
<tr>
<td>Atf4</td>
<td>NM_009716.2</td>
<td>ttg tcg gtt aca gca aca ctg</td>
</tr>
<tr>
<td>Chop</td>
<td>NM_007837</td>
<td>gac cag gtt ctg ctt tca gg</td>
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<td>Ppia</td>
<td>NM_008907</td>
<td>atg gca aat gct gga cca aa</td>
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<td>Grp78</td>
<td></td>
<td>gaa agg atg gtt aat gat gct gag</td>
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<td>Ldlr</td>
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<tr>
<td>Trb3</td>
<td>NM_175093.2</td>
<td>ctc tga ggc tcc agg aca ag</td>
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**Table II.** Metabolic parameters of non-hypomorphic WT and Arg-61 (R61) mice fed a chow and HCD

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>6 weeks</th>
<th>15 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>WT</strong></td>
<td><strong>R61</strong></td>
<td><strong>WT</strong></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>15±1</td>
<td>14±0.3*</td>
<td>19±0.2</td>
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<tr>
<td>BG (mg/dl)</td>
<td>146±9</td>
<td>148±27</td>
<td>112±16</td>
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<tr>
<td>TC (mg/dl)</td>
<td>87±7</td>
<td>86±4</td>
<td>205±31</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>41±13</td>
<td>49±14</td>
<td>9±1</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>19±1</td>
<td>19±1</td>
<td>21±1</td>
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<tr>
<td>BG (mg/dl)</td>
<td>187±21</td>
<td>178±9</td>
<td>93±7</td>
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<tr>
<td>TC (mg/dl)</td>
<td>74±3</td>
<td>76±5</td>
<td>236±22</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>41±9</td>
<td>49±5</td>
<td>11±2</td>
</tr>
</tbody>
</table>

Body weight (BW), blood glucose (BG), plasma total cholesterol (TC) and triglycerides (TG) were measured in 4h-fasted mice at 6 weeks of age on a chow diet (0) and after 6 weeks and 15 weeks of HCD feeding. Mean±SD. * p<0.05 Apoe<sup>T<sub>th</sub></sup> vs Apoe<sup>R<sub>th</sub></sup> by ANOVA and Bonferroni post-tests. n/a, non-available.
Table III. Expression levels of ER stress-related proteins in freshly isolated peritoneal macrophages from hypomorphic Apoe mice fed a chow or HCD for 4 weeks

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow</th>
<th>HCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( T^{h/h} )</td>
<td>( R^{h/h} )</td>
</tr>
<tr>
<td>( n=5M/5F )</td>
<td>( n=4M/5F )</td>
<td>( n=5M/5F )</td>
</tr>
<tr>
<td>( Atf4 )</td>
<td>1.00 ± 0.12</td>
<td>0.90 ± 0.14</td>
</tr>
<tr>
<td>( Chop )</td>
<td>1.00 ± 0.11</td>
<td>0.87 ± 0.11</td>
</tr>
<tr>
<td>( Grp78 )</td>
<td>1.00 ± 0.12</td>
<td>0.89 ± 0.27</td>
</tr>
<tr>
<td>( TRB3 )</td>
<td>1.00 ± 0.10</td>
<td>0.82 ± 0.15</td>
</tr>
</tbody>
</table>

Gene expression levels are normalized to \( Apoe^{Th/h} \) mice fed a chow diet. \( n=\text{Males/Females} \) in each group. Mean±SD. * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \) \( Apoe^{Th/h} \) vs \( Apoe^{Rh/h} \) by t-test.
Table IV. Expression levels of ER stress-related proteins in cultured peritoneal macrophages from hypomorphic Apoe mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow T&lt;sup&gt;h/h&lt;/sup&gt;</th>
<th>Chow R&lt;sup&gt;h/h&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atf4</td>
<td>1.00 ± 0.11</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td>Chop</td>
<td>1.00 ± 0.11</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>TRB3</td>
<td>1.00 ± 0.12</td>
<td>1.04 ± 0.22</td>
</tr>
</tbody>
</table>

Gene expression levels are normalized to Apoe<sup>T<sup>h/h</sup></sup> macrophages. Two independent experiments performed in duplicate (n=6). Mean±SD.
Supplemental References


Supplemental figures
Figure II

A  Females

B  Males

Lesion area (μm²)

9 weeks

15 weeks

T^{thh}  R^{thh}
Figure IV

A

B

C