Title

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Authors

Chait, Alan
Wang, Shari
Goodspeed, Leela
et al.

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Sexually dimorphic relationships among serum amyloid A3, inflammation, and cholesterol metabolism modulate atherosclerosis in mice

A Chait[1,2], **S Wang**[1,2], **L Goodspeed**[1,2], **D Gomes**[1,2], **KE Turk**[1,2], **T Wietecha**[2,3], **J Tang**[1,2], **C Storey**[1,2], **KD O'Brien**[2,3], **KB Rubinow**[1,2], **C Tang**[1,2], **T Vaisar**[1,2], **SA Gharib**[4], **AJ Lusis**[5], **LJ den Hartigh**[1,2]

[1]University of Washington, Department of Medicine, Division of Metabolism, Endocrinology, and Nutrition, Seattle, WA

[2]University of Washington, Diabetes Institute, Seattle, WA

[3]University of Washington, Department of Medicine, Division of Cardiology, Seattle, WA

[4]University of Washington, Department of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, Computational Medicine Core, Center for Lung Biology, Seattle, WA

[5]University of California, Los Angeles, Department of Human Genetics, Los Angeles, CA

Abstract

Objective: Expression of the extrahepatic acute-phase protein serum amyloid A3 (Saa3) increases in response to acute and chronic inflammatory stimuli and is elevated in adipose tissue and macrophages in obese mice. A recent report suggested that Saa3 is pro-atherogenic in male *ApoE*$^{-/-}$ mice. Due to our previous observation that female but not male Saa3-deficient mice are protected from obesity, adipose inflammation, and hyperlipidemia, we sought to determine whether Saa3 differentially modulates atherosclerosis in mice of both sexes.

Approach and Results: To promote atherosclerosis, *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ male and female mice were crossed with *Ldlr*$^{-/-}$ mice. All mice consumed a diet high in saturated fat and sucrose with 0.15% added cholesterol for 16 weeks. Plasma lipids and atherosclerosis levels were assessed. Female *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ mice exhibited elevated cholesterol levels relative to *Saa3*$^{+/+}$*Ldlr*$^{-/-}$ controls, and exhibited increased atherosclerosis, while male *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ mice were protected from atherosclerosis. Data from the Hybrid Mouse Diversity Panel revealed that Saa3 associates strongly with inflammatory, Trem2-associated, and tissue remodeling genes and pathways in males but not females, an effect confirmed in liver tissue, atherosclerotic lesions, and cultured macrophages. Macrophages isolated from male and female mice showed differential inflammatory effects of Saa3 deficiency, an effect linked with sex steroid signaling. Cholesterol efflux capacity was increased in *Saa3*$^{-/-}$ males only.

Corresponding author: Laura den Hartigh, PhD, University of Washington, 750 Republican Street, Box 358062, Seattle, WA 98109, lauradh@u.washington.edu.

Disclosures

The authors have no disclosures.

Conclusions: Saa3 is pro-atherogenic in male but atheroprotective in female mice, effects that may be related to sex-specific relationships between Saa3, cholesterol metabolism, inflammatory genes, and Trem2 macrophages.

Keywords

Macrophages; triggering receptor expressed on myeloid cells 2 (Trem2); low-density lipoprotein receptor; hybrid mouse diversity panel; cholesterol efflux

Introduction

Atherosclerotic cardiovascular disease (CVD) is a major cause of morbidity and mortality in developed countries, with men having more heart attacks and at an earlier age than women. Systemic and local chronic inflammation contributes to the development and progression of atherosclerosis, characterized by the accumulation of lipids and inflammatory cells in the arterial wall, which can become progressively less stable, leading to rupture and subsequent ischemic events[1]. The recent Canakinumab Antiinflammatory Thrombosis Outcome Study trial showed that reducing systemic inflammation with an anti-IL-1β monoclonal antibody did not affect plasma lipid levels but reduced the risk of cardiovascular events in subjects with a history of previous myocardial infarctions[2], providing proof-of-concept validation of the role of inflammation in CVD. Notably, 70% of the study participants were men. However, the Cardiovascular Inflammation Reduction Trial failed to show a clinical benefit of the anti-inflammatory drug methotrexate against the risk of CVD[3]. Thus, the precise contribution of inflammation to atherosclerotic CVD in men and women remains to be fully elucidated, necessitating a more complete understanding of specific inflammatory mediators and mechanisms.

Members of the serum amyloid A (Saa) family are proteins released in response to inflammation[4], and have been implicated in chronic inflammatory diseases such as atherosclerosis[5–7] and obesity[8–10]. Of the four Saa subtypes, Saa1–3 exhibit the strongest response to inflammatory stimuli, with Saa1 and Saa2 predominantly expressed by the liver and Saa3 produced more ubiquitously by extra-hepatic tissues such as adipose and lung, as well as to a small extent by the liver[11]. Saa3 is expressed in mice, but is a pseudogene in humans due to a premature stop codon[12]. Thus, humans express SAA1 and SAA2 extrahepatically instead of SAA3, making it difficult to identify a clear role for extrahepatic SAA. In mice, Saa derived from the liver circulates associated with high density lipoproteins (HDL), and in some situations also with apoB-containing lipoproteins[13], whereas extrahepatic Saa (i.e., Saa3) has only been shown to circulate under extreme and specific inflammatory conditions[14] and is more loosely associated with lipoproteins. Since hepatic and extra-hepatic Saa could be functionally distinct, we have taken advantage of the fortuitous difference in Saa subtypes produced by the liver and extrahepatic tissue in mice to specifically study the role of Saa3 as a model of extrahepatic Saa in atherosclerosis.

There is some consensus suggesting that Saa subtypes are pro-atherogenic. SAA is detectable in human atherosclerotic lesions and is expressed in several cell types associated with atherosclerosis, including monocytes/macrophages, endothelial cells, and smooth

muscle cells[15]. This observation is supported by several studies in mice. One study showed a transient improvement in atherosclerosis in female *Saa1*$^{-/-}$*Saa2*$^{-/-}$ mice fed a Western-type diet for 6 weeks[16]; another showed improvements in atherosclerosis with deletion of the major Saa receptor, FPR2/ALX, in low-density lipoprotein receptor (LDLR) deficient mice[17]; and a third study in *ApoE*$^{-/-}$ mice showing that overexpressing Saa1/2 worsened atherosclerosis[18]. Despite the evidence supporting an atherogenic role of Saa, another study did not show reductions in atherosclerosis in male and female *Saa1*$^{-/-}$*Saa2*$^{-/-}$ mice on the *ApoE*$^{-/-}$ background fed a standard laboratory diet for 52 weeks[19], highlighting the need for further study in this area. Recently, Thompson et al. have suggested that Saa3 in particular can be pro-atherogenic in *ApoE*$^{-/-}$ mice, as evidenced by worsened atherosclerosis when Saa3 was overexpressed, and by improved atherosclerosis when Saa3 was silenced[20]. However, these effects were only reported in male mice. Given that our previous work showcased the sexually dimorphic nature of Saa3-mediated effects on obesity-associated phenotypes, in which only female mice deficient in Saa3 were protected from diet-induced obesity, inflammation, and hyperlipidemia[21], we hypothesized that there would also be a sexual dimorphic effect of Saa3 on atherosclerosis. To test this hypothesis, both male and female mice either sufficient or globally deficient in Saa3 were crossed onto the low-density lipoprotein receptor (Ldlr)-deficient background (*Saa3*$^{+/+}$*Ldlr*$^{-/-}$, *Saa3*$^{-/-}$*Ldlr*$^{-/-}$), and fed a high fat high sucrose (HFHS) diet with added cholesterol (0.15%) for 16 weeks. Lipoprotein profiles, aortic atherosclerosis, and hepatic inflammation were assessed and compared with hepatic data from the Atherosclerosis Hybrid Mouse Diversity panel (Ath-HMDP)[22], in which transcriptomics was performed on liver samples from over 100 inbred strains of mice prone to atherosclerosis, to explore Saa3 associations. Further *in vitro* studies using macrophages, a major cell type that expresses Saa3 and is intimately involved in atherosclerosis, were performed to identify potential atherosclerotic pathways influenced by Saa3. Our current results suggest that Saa3 is atherogenic in male mice, consistent with the findings of Tannock et al.[20], but atheroprotective in female mice. This effect could be due to the strong associations between Saa3 and inflammatory and lipid metabolism genes observed in male mice in the Ath-HMDP dataset, and to a sexual dimorphic relationship between Saa3, inflammation, and Trem2 in macrophages.

Materials and Methods

The data that support the findings in this study are available from the corresponding author upon request. An expanded version of the Material and Methods is available in the online Supplemental Materials.

Mouse study design

To promote atherosclerosis, mice either sufficient or globally deficient in *Saa3* (previously described[21]) were crossed with Ldlr-deficient mice (The Jackson Laboratory #002207), yielding *Saa3*$^{+/+}$*Ldlr*$^{-/-}$ single knock out and *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ double knock out mice. Group-housed 10-week-old *Saa3*$^{+/+}$*Ldlr*$^{-/-}$ (12 males, 15 females) and *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ (16 males, 16 females) mice were fed a diet high in saturated fat and sucrose (HFHS) for 16 weeks to promote atherosclerosis. All experimental procedures were undertaken with approval from the Institution Animal Care and Use Committee of the University of Washington, and

followed the guidelines of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Plasma analyses

Triglycerides and cholesterol were measured from fasting plasma. Pooled fasting plasma was subjected to fast-phase liquid chromatography (FPLC) and cholesterol quantified from the resulting fractions using colorimetric assays as previously described[23]. We used every mouse in each group for FPLC analyses, such that each group contains representation from each animal. Saa and Il6 were quantified from plasma using ELISA[21]. Plasma Saa likely depicts Saa1 and Saa2 because Saa3 does not circulate in similar models[24]. HDL proteomics analyses are described in the online Supplemental Materials.

Atherosclerosis

Atherosclerosis from the aortic arch was quantified using the *en face* method using Sudan IV staining as described previously[25]. Aortic sinus lesion area was quantified from paraffin-embedded sections of the heart. Hearts were horizontally serially-sectioned at the level of the aortic sinus (described in more detail in the online Supplemental Materials).

Association analysis: Hybrid Mouse Diversity Panel for atherosclerosis

A systems genetics approach was utilized to determine which genes associate with *Saa3* in the Ath-HMDP[22], described in more detail in the online Supplemental Materials.

Macrophage experiments

To propagate bone marrow-derived macrophages (BMDM), bone marrow was isolated from donor *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ male or female mice (n=5–8 mice/group) and differentiated into BMDMs. Non-polarized BMDMs were then treated with media (control), lipopolysaccharide (LPS, 10 ng/mL for 4 hours), 17β-estradiol (100 nM for 16 hours), or testosterone (1 μM for 16 hours). For ex vivo experiments, thioglycolate-elicited macrophages (TG-Ms) were collected from *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ male or female mice (n=4–6 mice/group). One million TG-M were used for surface Trem2 flow cytometry, and the rest for gene expression analysis.

Gene and protein expression analyses

Liver tissue was harvested and snap frozen at −80^{0}C until further analysis. Bone marrow was extracted from femur and tibia and differentiated into bone marrow-derived macrophages (BMDMs) as described below and previously[25]. For gene expression, total RNA was extracted from ~100 mg of liver tissue, from 1×10^{6} BMDMs or TG-Ms using RNeasy Mini Kits (Qiagen, Valencia CA, USA). Total RNA was also extracted from the formalin-fixed aortic arches used for en face analysis using an RNeasy FFPE Kit (Qiagen, Valencia, CA USA). 0.5–2 μg of RNA was reverse-transcribed, and the cDNA thus obtained was analyzed by real-time quantitative PCR by standard protocols using an ABI 7900HT instrument. For protein analyses, BMDMs were homogenized in cold RIPA buffer with protease inhibitors, SDS-PAGE was performed using equal amounts of protein, and Western blots were probed for Triggering receptor expressed on myeloid cells 2 (Trem2, Proteintech

Group, Rosemont, IL, USA) and beta-actin (MilliporeSigma, St. Louis, MO, USA). Western blots were imaged using a LI-COR Odyssey Fc system and analyzed using Image Studio Software (Lincoln, NE, USA).

Cholesterol efflux assay

To determine if Saa3 deficiency influences cholesterol efflux, two different assays were utilized. First, to determine if Saa3 deficiency of macrophages influenced cholesterol efflux capacity, BMDMs from *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ mice were labeled with 1 μCi/ml of [^{3}H]cholesterol (PerkinElmer Life Sciences) for cholesterol efflux in DMEM/BSA medium overnight. Washed cells were then equilibrated for 18 hours, followed by incubation with DMEM/BSA +/− 10 μg/ml apoA-I (MilliporeSigma, St. Louis, MO, USA) for 5 hours. Culture medium and cells were then assayed for [^{3}H] counts. ApoA-I-mediated cholesterol efflux was calculated as the percent total [^{3}H] released into medium after subtraction of values obtained in the absence of apoA-I. Second, to determine if HDL from *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ mice differentially impacted cholesterol efflux, HDL was isolated by ultracentrifugation of mouse plasma and utilized in a cholesterol efflux assay (16 μg/mL) using J774 macrophages that had been pre-treated with cAMP, as described previously[26].

Statistical analyses

Data were analyzed using GraphPad Prism 6 software and are represented as means +/−standard errors. For plasma analyses, the effect of time and differences between treatment groups were evaluated using repeated measures analysis of variance. Significant time-by-group interactions were followed by within- and between-group post-hoc Bonferroni tests. One-way ANOVA with Tukey's test for multiple comparisons was used to compare differences between genotypes for all other data.. Equal variance was confirmed using the Brown-Forsythe test and normality was determined using the Shapiro-Wilk normality test. Non-normally distributed variables (i.e., plasma SAA) were log-transformed for analysis and back-transformed for presentation. To examine associations between *Saa3* and *Trem2* expression, Pearson coefficients were calculated from linear regression of plotted correlation data. A p value < 0.05 was considered statistically significant.

Results

Hypercholesterolemia is exacerbated in female Saa3$^{-/-}$Ldlr$^{-/-}$ mice.

Due to our previously observed sexually dimorphic effect of Saa3 on obesity characteristics[21], we sought to examine atherosclerosis in both male and female mice deficient in Saa3. To facilitate atherosclerosis, *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ male and female mice were crossed with *Ldlr*$^{-/-}$ mice and fed a HFHS diet to promote hypercholesterolemia (Fig. 1A). Female mice required Ldlr deficiency to develop hypercholesterolemia, but exhibited lower plasma cholesterol levels than males. No effects of Saa3 deletion on the development of hypercholesterolemia were observed in male mice. However, female mice lacking both Saa3 and Ldlr displayed *higher* levels of hypercholesterolemia than mice that were Saa3-sufficient (Fig. 1A). FPLC plasma fractionation revealed that while there were no apparent differences between *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ male mice, female *Saa3*$^{-/-}$ mice displayed modestly elevated levels of very low-density lipoprotein (VLDL)- and LDL-cholesterol (Fig.

1B). While Ldlr deficiency also increased plasma triglyceride levels, there were no differences between *Saa3*$^{+/+}$ and *Saa3*$^{-/-}$ mice (Fig. 1C). As we have previously reported, mice with Saa3 deficiency displayed reduced plasma Saa levels for reasons that remain unknown[21], and female *Ldlr*$^{-/-}$ mice had lower Saa levels than male *Ldlr*$^{-/-}$ mice (Fig. 1D). Male *Saa3*$^{+/+}$*Ldlr*$^{-/-}$ mice had elevated plasma Il6 levels compared to all other groups, but were barely above the limit of detection (Fig. 1E). Collectively, hypercholesterolemia induced by Ldlr deficiency was enhanced in female, but not male, *Saa3*$^{-/-}$ mice.

Atherosclerosis is reduced in Saa3$^{-/-}$Ldlr$^{-/-}$ male mice but exacerbated in Saa3$^{-/-}$Ldlr$^{-/-}$ female mice.

The next question was whether worsened hypercholesterolemia in female Saa3-deficient mice altered atherosclerosis susceptibility. Despite no differences in plasma cholesterol levels, male *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ mice developed less aortic atherosclerosis than their wild-type counterparts, with reduced aortic sinus atherosclerosis and significantly smaller necrotic core size (Fig. 2A, C). This supports a previous study suggesting that Saa3 is pro-atherogenic in male mice[20]. Strikingly, in parallel with enhanced plasma cholesterol levels, female *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ mice developed *more* aortic atherosclerosis than their wild type counterparts (Fig. 2B), an effect that was not observed in the aortic sinus (Fig. 2D). However, aortic sinus lesions from female *Saa3*$^{-/-}$*Ldlr*$^{-/-}$ mice exhibited larger necrotic cores and elevated macrophage staining than wild-type mice (Fig. 2D), suggesting that their lesions are less stable. The extent of atherosclerosis did not significantly correlate with plasma cholesterol or Saa levels in males or females (not shown). There was no appreciable atherosclerosis detected in either sex in the absence of Ldlr deficiency. Collectively, these results suggest that Saa3 deficiency is atheroprotective in males, but atherogenic in female mice.

Saa3 associates more strongly with genes and pathways associated with inflammation, tissue remodeling, and lipid metabolism in males than in females.

To elucidate a mechanism by which Saa3 differentially influences atherogenicity in males and females, we utilized available data from the Ath-HMDP[22] to evaluate hepatic genes and pathways that associate with *Saa3* in over 100 mouse strains prone to atherosclerosis. We used hepatic data due to the known influence of the liver on systemic lipid metabolism and inflammation[27]. Figure 3 depicts genes that are either exclusively or jointly positively or negatively associated with *Saa3* in males and females. Strikingly, many more genes were positively or negatively correlated with *Saa3* expression in males compared to females, indicating a much broader association between *Saa3* and the liver transcriptome in males (Fig. 3A). Of all the genes positively associated with *Saa3*, 68% of these were exclusive to males, with only 9% exclusive to females (Fig. 3A). Similarly, of all the genes negatively associated with *Saa3*, 72% were exclusive to males, with only 11.5% exclusive to females (Fig. 3A). A complete list of genes associated with *Saa3* exclusively for each sex and common to both sexes is provided in Table S1. We next performed functional enrichment analysis on the positively and negatively correlated genes to biologically categorize these gene associations. Again, the majority of pathways positively associated with *Saa3* were exclusive to males (55%), with only 3% exclusive to females (Fig. 3B). Moreover, 52% of pathways depicting negative associations with Saa3 were exclusive to males, with only 7%

exclusive to females (Fig. 3B). A complete list of gene ontology (GO) biological processes enriched exclusively in each sex and common to both sexes is provided in Table S2. In male mice, there was a profound enrichment in positively associated immune/inflammatory and tissue remodeling pathways, and negatively associated lipid, carbohydrate, and mitochondrial metabolism pathways (Fig. 3C). Such enrichment in immune/inflammatory pathways in males was evident by the association between *Saa3* and many macrophage-associated genes, including *Trem2*, *Lgals3*, *Mpeg1*, *Cd14*, *Itgax*, *Cx3cr1*, *Ccl2*, *Ccr1*, *Mertk*, *Il1b*, *Itgam*, *Spp1*, *Tnf*, and *Cd9* (Table S1). No such macrophage associations were found in females. In support of this sexually dimorphic relationship between *Saa3* and inflammatory genes, hepatic expression of inflammatory genes *Lgals3*, *Cd68*, *Il6*, and *Ccl2* was significantly higher in female mice deficient in *Saa3* compared with their wild-type counterparts, an effect that was absent in males (Fig. 4A). Expression levels of *Saa3* did not differ between males and females (not shown), but were confirmed to be absent from $Saa3^{-/-}$ mice (Fig. 4A). Collectively, these results indicate that in mouse models of atherosclerosis, hepatic *Saa3* positively associates more strongly with inflammatory genes and in particular macrophage-associated genes, and exhibits a stronger negative association with lipid/carbohydrate metabolism and mitochondrial genes in male mice compared to females.

Sexually dimorphic effect of Saa3 deficiency on macrophage inflammation is modulated by sex steroids.

We next examined the inflammatory potential of naïve (M0) BMDMs isolated from male and female $Saa3^{+/+}$ or $Saa3^{-/-}$ mice. Wild-type murine macrophages express basal levels of *Saa3*, which was strongly induced by LPS (Fig. 4B). Basal expression of *Saa3* did not differ between BMDMs isolated from male or female mice (not shown). As expected, BMDMs from $Saa3^{-/-}$ mice of both sexes did not express *Saa3*. In the basal state, there were no differences in expression of the cytokines *Tnf* and *Il1b* or the chemokine *Ccl2* between BMDMs from male $Saa3^{+/+}$ and $Saa3^{-/-}$ mice. Upon stimulation with LPS, expression levels of *Tnf*, *Il1b*, and *Ccl2* were elevated in BMDMs from male $Saa3^{+/+}$ mice, an effect that was blunted in BMDMs from male $Saa3^{-/-}$ mice (Fig. 4B). In sharp contrast to the males, BMDMs from female $Saa3^{-/-}$ mice had *elevated* basal expression levels of *Il1b*, *Tnf*, and *Ccl2*, an effect that was exacerbated following LPS induction (Fig. 4B). To determine if sex hormones play a role in the sexually dimorphic effects of Saa3 expression, BMDMs from $Saa3^{+/+}$ and $Saa3^{-/-}$ male mice were treated with 17β-estradiol (E) or testosterone (T). In the presence of 17β-estradiol, male $Saa3^{-/-}$ BMDMs exhibited an inflammatory gene expression profile that resembled female $Saa3^{-/-}$ BMDMs, with elevated expression of *Tnf*, *Il1b*, and *Ccl2* (Fig. 4C), while treatment with testosterone had little effect. By contrast, female $Saa3^{-/-}$ BMDMs responded to testosterone with blunted inflammatory gene expression, with little effect of estradiol treatment (Fig. 4C). To determine if *ex vivo* macrophages exhibited similar patterns, inflammatory gene expression was measured from thioglycolate-elicited peritoneal macrophages (TG-M). While no effect of Saa3 deletion was observed in TG-M isolated from male mice, female TG-M exhibited elevated levels of *Il1b, Tnf*, and *Ccl2* expression (Fig. 4D). Expression levels of estrogen receptor alpha (*Esr1*), while comparable between male and female $Saa3^{+/+}$ BMDMs and TG-Ms, were significantly increased in male $Saa3^{-/-}$ BMDM and decreased in female $Saa3^{-/-}$ BMDM

and TG-M (Fig. 4E), suggesting that Saa3 deficiency may augment and blunt estrogen signaling in males and females, respectively. These results indicate that Saa3 deficiency protects BMDMs against LPS-induced inflammation in males, but exacerbates inflammatory gene expression in females, suggesting a diametrically opposed role of Saa3 in males and females that may be related to sex steroid signaling.

Saa3 deficiency is accompanied by increased hepatic, aortic, and macrophage Trem2 expression in males.

A large proportion of the macrophage genes found to positively associate with Saa3 in males are representative of Trem2-expressing macrophages. This emerging class of macrophages has been characterized by high expression levels of *Trem2*, *Lgals3*, *Abcg1*, *Spp1*, *Hvcn1*, *Timp2*, and *Cd9*[28], and may play important roles in cholesterol metabolism within atherosclerotic lesions as well as plaque resolution[29]. Indeed, Ath-HMDP data indicate that these genes were highly correlated with *Saa3* expression in livers of males (Table S1). We now show that deletion of Saa3 increases hepatic expression of *Trem2* in males but not females (Fig. 5A), an effect that is exacerbated by hypercholesterolemia (*Ldlr* deficiency). Hepatic *Trem2* expression correlated negatively with *Saa3* expression in males, but exhibited a positive correlation with *Saa3* expression in females (Fig. 5A). Importantly, *Trem2* expression levels were also increased in male $Saa3^{-/-}Ldlr^{-/-}$ atherosclerotic aortic tissue, and aortic *Trem2* expression correlated negatively with the extent of aortic atherosclerosis in males, with no correlation noted in females (Fig. 5B). Moreover, male $Saa3^{-/-}$ mice exhibited increased Trem2 immunostaining in aortic sinus lesions, suggesting increased Trem2 content of atherosclerotic lesions, an effect that was not observed in females (Fig. 5C–D). Thioglycolate-elicited macrophages (TG-Ms) exhibited a trend towards increased Trem2 mRNA expression in male Saa3-deficient mice, with no such trend observed in female TG-Ms (Fig. 5E). Surface expression of Trem2 on TG-Ms did not change with Saa3-deficiency (Fig. 5E), which may be due to the inherent inflammatory nature of TG-Ms. BMDMs isolated from $Saa3^{-/-}$ male mice, but not females, exhibited increased *Trem2* gene expression, with the same Trem2 protein expression pattern confirmed by immunoblot (densitometry of 0.75±0.04 and 1.2±0.06 for male WT and KO, respectively, and 0.85±0.03 and 0.58±0.04 for female WT and KO, respectively) (Fig. 5F). Collectively, these results suggest that male mice deficient in Saa3 display increased Trem2 expression levels in the liver, atherosclerotic lesions and BMDMs, an effect that is absent in females.

Macrophages from male mice deficient in Saa3 exhibit increased cholesterol efflux capacity.

Trem2-expressing macrophages have been suggested to play a role in cholesterol efflux pathways[30]. Coupled with the strong associations between Saa3 and lipid metabolism pathways, we next examined expression of genes that are important for cholesterol efflux from macrophages. Ath-HMDP data showed that several cholesterol transporters, including *Abca1*, *Abcg1*, and *Scarb1*, are positively associated with Saa3 (Table S1). BMDMs from male $Saa3^{-/-}$ mice showed elevated basal expression levels of the cholesterol transporters *Abca1*, *Abcg1*, and *Scarb1*, an effect that was blunted in the presence of 17β-estradiol for *Abca1* and *Abcg1* only, but not affected by testosterone treatment for *Abcg1* (Fig. 6A). TG-Ms also exhibited increased *Abca1* and *Abcg1* expression levels in male BMDMs that were

deficient in Saa3 (Fig. 6B). Basal expression levels of cholesterol transporters were not altered by Saa3 deficiency in BMDMs from female mice (Fig. 6A). While 17β-estradiol had no effect on cholesterol transporter expression in female cells, testosterone dramatically increased expression of both *Abca1* and *Abcg1*, with Saa3-deficient BMDMs exhibiting a further increase in *Abcg1* expression. In agreement with this, cholesterol efflux was higher from BMDMs isolated from male $Saa3^{-/-}$ mice than their $Saa3^{+/+}$ counterparts, with no differences in BMDMs from females (Fig. 6C). We did not observe any differences in cholesterol efflux capacity between J774 macrophages when HDL from $Saa3^{+/+}$, or $Saa3^{-/-}$ male or female mice was used as a cholesterol acceptor (Fig. 6C), suggesting that the HDL cholesterol efflux capacity is not affected by Saa3 deficiency. Proteomics analysis on HDL isolated from $Saa3^{+/+}$, or $Saa3^{-/-}$ male or female mice did not show major differences that could explain the sexual dimorphic effect of Saa3 deficiency on atherosclerosis (Table S3). Collectively, these results suggest that Saa3 deficiency increases the cholesterol efflux capacity of BMDMs from male but not female mice.

Discussion

While previous studies have suggested that SAA associates with increased CVD risk in humans[6], that Saa subtypes may play a role in the development of atherosclerosis in mice[23, 31], and that Saa3 itself is pro-atherogenic[20], a solid consensus is lacking[32]. The current study confirms that Saa3 is atherogenic in male mice and adds evidence suggesting that Saa3 is atheroprotective in female mice. The mechanism by which Saa3 is pro-atherogenic in males may relate to strong positive associations between Saa3 and inflammatory genes, an effect that is weaker in females. This is supported by liver inflammatory gene expression patterns and *in vitro* experiments with macrophages, which suggest that Saa3 promotes a pro- and anti-inflammatory phenotype in male and female macrophages, respectively. Moreover, expression of Saa3 may blunt cholesterol efflux capacity of macrophages in males. This study also illuminates a potential sexually dimorphic link between Saa3 and Trem2. Thus, the current study supports the previously described pro-atherogenic role of Saa3 in male mice[20] and now provides potential mechanistic clues implicating prominent roles for inflammation and cholesterol metabolism. In addition, the results from this study highlight the underappreciated sexually dimorphic nature of Saa3.

In humans and rodents, SAA subtypes can be expressed in the liver, adipocytes, macrophages, and other extrahepatic tissues[11, 33–36]. In mice, Saa1 and Saa2 are expressed primarily in the liver in response to inflammatory stimuli, whereas Saa3 is expressed mainly by extrahepatic tissue such as adipocytes and macrophages[11, 35]. Because Saa3 protein is not expressed in humans, the major inducible forms of human SAA, i.e., SAA1 and SAA2, are expressed both by liver *and* extrahepatic tissues[12, 14, 34]. Our recent study showed that different inflammatory stimuli elicit expression of different Saa subtypes from different tissues[14]. Therefore, it is difficult to determine the tissue of origin of the chronically elevated levels of SAA in humans with CVD. Thus, mouse models provide a unique tool to distinguish between liver-derived Saa and adipose- or macrophage-derived Saa. By knocking out Saa3 in mice, we can study the impact of extra-hepatic Saa on atherosclerosis.

To our knowledge, only one previous study has examined Saa3 in the context of atherosclerosis. Thompson et al. recently observed that Saa3 is pro-atherogenic in male ApoE$^{-/-}$ mice, having shown that blunting Saa3 expression using an Saa3-antisense oligonucleotide (ASO) reduced atherosclerosis, while overexpressing Saa3 using an adeno-associated virus (AAV) approach worsened atherosclerosis[20]. In agreement with that study, we now show that while Saa3 appears to be pro-atherogenic in male mice, it may actually be anti-atherogenic in female mice. This notion is supported by a study by Krishack et al., in which female mice deficient in Saa1, Saa2 and Ldlr had no apparent differences in atherosclerosis after 12 weeks of atherogenic diet feeding compared to control mice[16]. While Saa3 was not explicitly examined in that study, it indicates that Saa is not pro-atherogenic in female mice.

Several groups have recently identified a synergistic relationship between macrophage expression and secretion of Saa3 and local inflammation. While the major finding from a study by Sanada et al. was that macrophages promoted adipocyte-derived Saa3 expression, that study also provided evidence that macrophage-derived Saa3 is important for maintaining a local inflammatory milieu[37]. This concept could readily apply to Saa3-containing atherosclerotic lesions, surrounding perivascular adipose tissue, and the liver, highlighting the potential for macrophage-derived Saa3 to propagate a pro-atherogenic environment. Indeed, data from Song et al. suggest that human atherosclerotic lesions can directly release SAA into the coronary circulation[38], while data from Meek et al. showed that Saa3 is expressed and secreted from macrophages[35], suggesting that atherosclerotic lesion macrophages are a significant source of Saa3. Similarly, male mice transplanted with bone marrow from donor mice deficient in the formyl peptide receptor, a receptor that recognizes Saa, exhibited reduced atherosclerosis levels[17]. Taken together, our results are in agreement with others to suggest that local production of Saa by macrophages can influence atherosclerosis development.

The observation that macrophage-associated genes strongly and positively correlate with Saa3 only in male liver is intriguing and could help explain the sexually dimorphic atherosclerotic phenotype. A macrophage subset, termed Trem2^{hi} or “foamy” macrophages, has recently been identified in atherosclerotic lesions, typically found within the intima or necrotic core[28, 29]. Trem2^{hi} macrophages express high levels of *Lgal3*, *Spp1*, *Cd9*, *Mertk*, and genes associated with lipid metabolism[29], are thought to be important for cholesterol efflux and lesion remodeling[29, 39], and may exert anti-inflammatory functions[40]. Strikingly, *our findings using the Ath-HMDP dataset suggest that these Trem2^{hi} macrophage genes are all strongly associated with Saa3 in the liver from male mice only*. Since our gene association data are from the liver, it is noteworthy that hepatic macrophage subsets including those rich in Trem2 have recently been identified[41], where they also associate with a remodeling phenotype[42]. Our observation that cholesterol efflux capacity is impaired by Saa3 in male macrophages, coupled with our data suggesting that aortic Trem2 is increased in Saa3-deficient male mice, lead to the hypothesis that Saa3 promotes atherosclerosis by modulating Trem2-expressing macrophages in males. Future studies could define a precise role for Saa3 in the enrichment of atherosclerotic lesions with Trem2^{hi} macrophages, including whether Saa3 is expressed from or recruits Trem2^{hi} macrophages, whether there are particular subsets of Trem2^{Hi} macrophages within atherosclerotic lesions, or if Saa3 is

important for cholesterol efflux from this macrophage population. Future studies should also decipher the sexually dimorphic relationship between Saa3 and Trem2.

It is well established that local and systemic inflammation as well as macrophage cholesterol efflux modulate the severity of atherosclerosis[1, 43]. Acute inflammation is known to reduce reverse cholesterol transport of macrophage cholesterol to the feces[44]. Such acute inflammation also increases the proportion of liver-derived Saa1/2 on HDL particles, initially shown to limit cholesterol efflux capacity from macrophages[45]. However, Saa1/2 appears to play a minor role in limiting cholesterol efflux, with only a small improvement in macrophage cholesterol efflux capacity and reverse cholesterol transport in mice deficient in Saa1/2[45, 46]. It also is known that Saa1/2 directly propagates inflammation by stimulating the release of Il-1β, MCP-1, and IL-8 from monocytes, macrophages, and neutrophils[36, 47–49], and plays an indirect role by "trapping" Saa-containing HDL particles on extracellular proteoglycans, thereby blunting HDL's access to the plasma membrane and limiting cholesterol efflux[50]. To date, no one has previously examined a role for *macrophage-derived* Saa3 on inflammation and cholesterol efflux capacity. It is possible that macrophage Saa3 contributes to the local inflammatory milieu and/or cholesterol efflux capacity by as yet unknown mechanisms. An important future study would be to assess the specific contribution of macrophage-derived Saa3 to atherosclerosis.

We have previously reported that Saa3 mediates sexually dimorphic effects in a mouse model of diet-induced obesity[21]. In our previous study, female mice deficient in Saa3 exhibited lower plasma cholesterol and triglyceride levels after 16 weeks of HFHS diet feeding and were protected from obesity-associated adipose tissue inflammation, effects that were not observed in male mice[21]. In the present study, we report that in the setting of hypercholesterolemia, female mice deficient in Saa3 exhibit *increased* plasma cholesterol levels, *worsened* atherosclerosis, and *increased* susceptibility for macrophage inflammation. The reason(s) that Saa3 may be obesogenic in female mice, but atheroprotective in the setting of hypercholesterolemia, are unclear and require further investigation. It is tempting to speculate that Saa3 modulates inflammation via pathways that may be tissue-specific. In the obese state, Saa3 is expressed primarily from hypertrophic adipocytes, with some Saa3 also expressed from adipose tissue macrophages[8]. In the setting of hyperlipidemic atherosclerosis, Saa3 expression likely derives primarily from aortic and/or hepatic macrophages. Thus, it is possible that in our two models of metabolic disease, Saa3 deficiency yields divergent results on metabolism in males and females.

In the current work, we have identified macrophages as potential players in the sexually dimorphic nature of Saa3 related to atherosclerosis using isolated TG-macs and BMDMs. The differential effect of Saa3 deletion on inflammatory gene expression in males and females is striking, with male *Saa3*$^{-/-}$ macrophages exhibiting protection from inflammatory insult and female *Saa3*$^{-/-}$ macrophages experiencing worsened inflammation. A recent study by Poynter et al. showed that BMDMs isolated from Saa3-deficient mice exhibited elevated LPS-mediated cytokine expression, in a similar manner to our female BMDM model[51]. However, it is not clear what sex was used for that experiment. Moreover, evidence of a sexually dimorphic relationship between SAA and inflammatory status exists in humans. Women with rheumatoid arthritis display higher SAA levels than men, with no evidence of

sexual dimorphism in other cytokines[52]. Logistic regression using sex as the dependent outcome variable confirmed that SAA independently predicted sex. In addition, when the ratio of estradiol to testosterone (E2/T) was included in the linear regression model, the E2/T ratio and sex were highly significant independent predictors of SAA levels, suggesting that estradiol and/or testosterone status correlates with SAA levels. It is known that 17β-estradiol blunts inflammatory responses in macrophages[53–55]. Recent work suggests that BMDMs isolated from female mice and treated with pro-inflammatory fatty acids such as palmitate or LPS exhibit lower levels of cytokine expression than male BMDMs[56]. Moreover, there appears to be a cell-autonomous nature to BMDMs that determines their inflammatory potential, as revealed by the ability of transplanted male bone marrow to promote a phenotypic “male” pattern of obesity-associated adipose tissue inflammation in recipients, even when the recipients are female[56]. What is particularly interesting about the current work is that Saa3 deficiency appears to limit this 17β-estradiol anti-inflammatory effect in female macrophages, while male Saa3-deficient macrophages treated with 17β-estradiol phenocopies this effect. Further, the differential expression of *Esr1* between male and female macrophages deficient in Saa3 suggests a potentially unknown but important interaction between the two genes. While testosterone had little effect in BMDMs from male mice, it negated the pro-inflammatory effect of Saa3 deficiency in BMDMs from female mice. Similarly, testosterone had little effect in male BMDMs, but strongly increased expression of cholesterol transporters in female BMDMs. Very few studies have examined direct testosterone effects on macrophages, but the general consensus is that testosterone promotes anti-inflammatory signaling and may reduce foam cell formation[57, 58], both of which could explain the anti-atherogenic effect of testosterone observed clinically[59]. A specific interaction between Saa3 and sex steroid signaling, should one exist, remains to be elucidated, and future studies should examine the atherogenicity of Saa3 in ovariectomized or castrated mice.

One limitation from this study was that we could not use Ath-HMDP sequencing data from aortic tissue due to the absence of available data in male mice[22]. We therefore utilized liver samples that had been isolated from *both* male and female mice to enable us to study sex-specific Saa3 gene associations. Despite the absence of aortic data, hepatic inflammatory and lipid metabolism signatures may associate with the extent of atherosclerosis in humans and mice[60]; thus, our results could inform future studies. In addition, it is unclear why the sequencing results from the Ath-HMDP suggested that Saa3 positively correlates with liver Trem2 macrophage-associated genes, while our data from BMDMs and liver suggest a negative correlation. Another limitation is that while we have presented multiple confirmatory data sets to suggest that Saa3 impairs cholesterol efflux capacity herein, it should be noted that the Abca1-mediated cholesterol efflux levels were lower than what is usually reported[61]. In our experiments we utilized naïve BMDMs instead of the more commonly used J774 or BHK cells that can express high levels of Abca1, which could explain the discrepancy. Moreover, there could have been trace amounts of sex hormones present in the FBS used for the BMDM experiments, which could confound our results. Another possibility that requires acknowledgement is that particular as yet unknown cytokines could be modulated by Saa3 deficiency, and indirectly explain the sexually dimorphic effect on atherosclerosis. Given the strong association between Saa3 and

inflammatory pathways in males, a deep dive into such accessory inflammatory pathways is warranted. Finally, while it has been previously shown that plasma SAA levels in humans correlate positively with indices of systemic inflammation, including CRP and IL-6[62, 63], this association has been reported to be stronger in women than in men[62]. This is in contrast to our results in mice which suggest that hepatic Saa3 more strongly associates with inflammatory genes in males than in females. This discrepancy could be due to differences in SAA subtypes present in humans and in mice, in which circulating SAAs could exert different functions from local SAAs, such as Saa3. Nevertheless, our results suggest that the strong association between Saa3 and inflammation in males may lead to less inflammation and more atheroprotection when Saa3 is absent in male mice.

In summary, we have presented results that support previous findings by others to suggest that Saa3 is pro-atherogenic in male mice and now add evidence that this is not the case in female mice. The sexual dimorphism in atherosclerosis related to Saa3 may be due to a stronger association between Saa3 and inflammatory pathways and genes associated with Trem2 macrophages in male mice. This is reflective of a strong anti-inflammatory effect of Saa3 deletion in male macrophages, while deletion of Saa3 from female macrophages results in an augmented inflammatory response. The sexually dimorphic nature of macrophage-derived Saa3 is also manifested by differential cholesterol efflux potential. Deciphering the precise molecular mechanism(s) by which Saa3 exhibits this sexual dimorphism in the setting of atherosclerosis requires further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAV	adeno-associated virus
Ac-LDL	acetylated low-density lipoprotein
ASO	antisense oligonucleotide
Ath-HMDP	atherosclerosis hybrid mouse diversity panel
BMDM	bone marrow-derived macrophage

CVD	cardiovascular disease
ERα	estrogen receptor alpha
FDR	false discovery rate
FPLC	fast-phase liquid chromatography
GO	gene ontology
HDL	high density lipoprotein
HFHS	high fat high sucrose
KO	knock out
LDLR	low-density lipoprotein receptor
LPS	lipopolysaccharide
LXR	liver X receptor
SAA	serum amyloid A
SSC	side scattered light
TG-M	thioglycolate-elicited macrophages
Trem2	triggering receptor expressed on myeloid cells 2
VLDL	very low-density lipoprotein
WGCNA	weighted gene co-expression network analysis
WT	wild type

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Highlights

- We report a previously unknown sexually dimorphic effect of Saa3 on atherosclerosis in mice. Using Saa3 knock out mice on an atherogenic Ldlr$^{-/-}$ background, we show that Saa3 is pro-atherogenic in male mice, but anti-atherogenic in female mice.
- Saa3 may promote atherosclerosis in male mice due to strong associations between Saa3 and inflammatory and macrophage-associated genes, an effect that is absent in female mice. This sexually dimorphic association between Saa3 and inflammatory genes has never been reported previously, with potential implications for human inflammatory sexual dimorphisms associated with CVD.
- We now reveal potential mechanisms to explain the sexually dimorphic nature of Saa3 related to atherosclerosis, including hepatic and macrophage inflammation, differential Trem2 macrophage content and phenotype, and differences in cholesterol efflux.

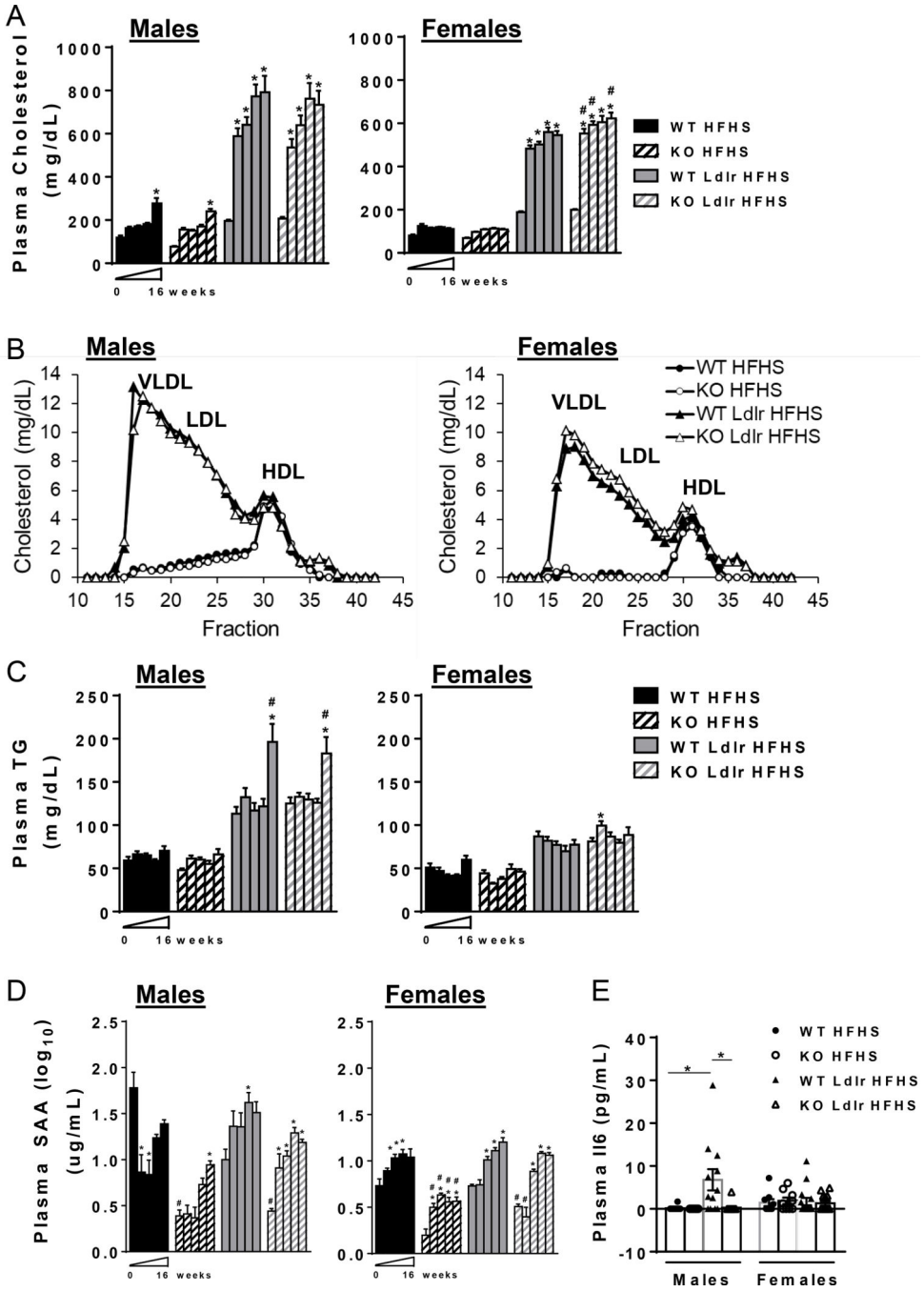

Figure 1. Mouse plasma analyses.

(**A-C**) Plasma cholesterol (**A**) and triglycerides (TG, **C**) were measured from fasted male and female *Saa3*$^{+/+}$ (WT) or *Saa3*$^{-/-}$ (KO) mice with and without Ldlr deficiency fed a high fat high sucrose (HFHS) diet at time 0 (10 weeks of age), 4, 8, 12, and 16 weeks. (**B**) Fast-phase liquid chromatography (FPLC) was performed on pooled plasma samples from each group, and cholesterol quantified from each fraction. (**D-E**) Plasma SAA (**D**) and Il6 (**E**) levels from mice (n=8–16 mice/group). Data were analyzed by two-way ANOVA with

Bonferroni post hoc test, presented as mean ± SEM. *p<0.05 from baseline (time 0); #p<0.05 from *Saa3*-sufficient mice.

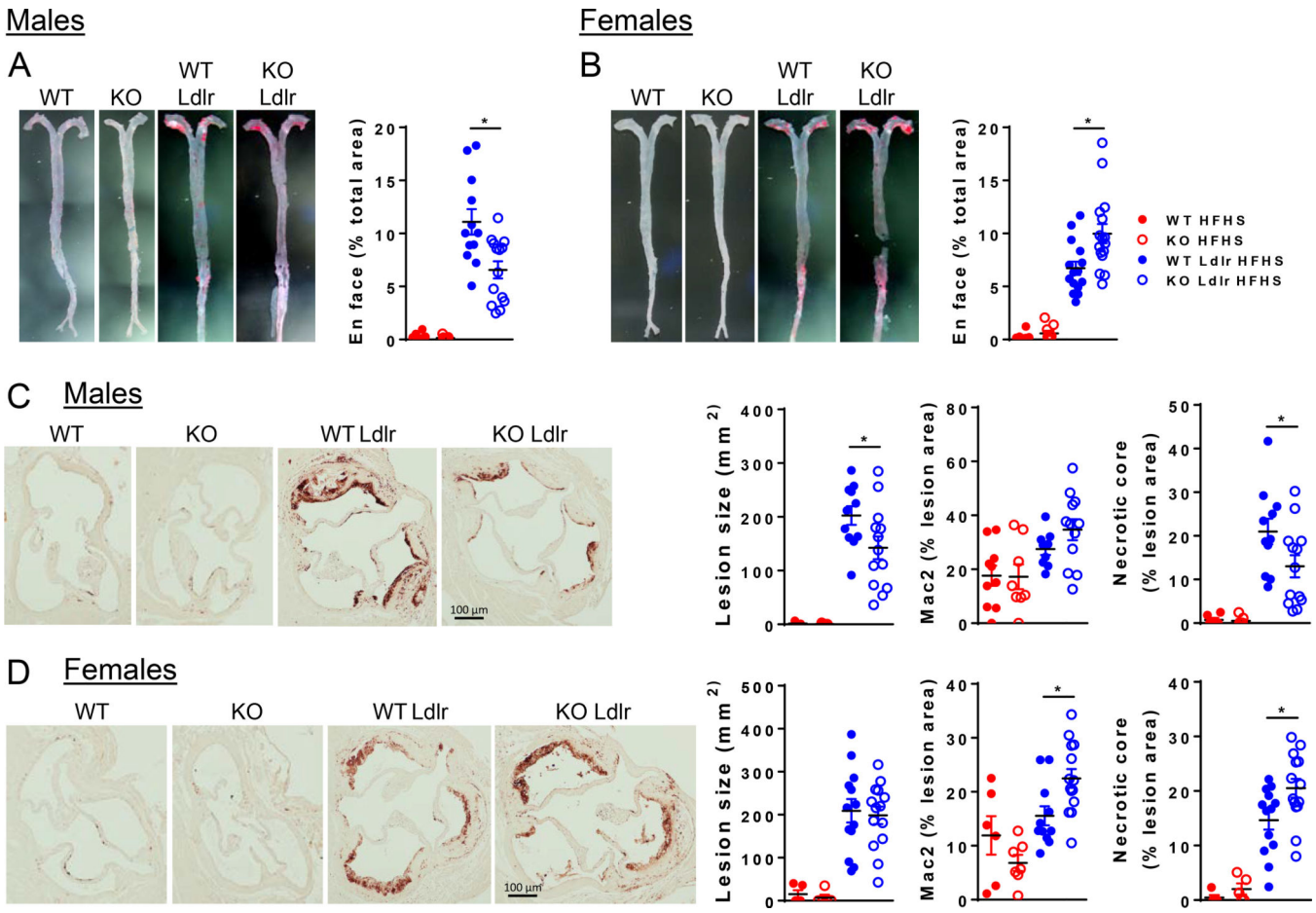

Figure 2. Quantification of atherosclerosis.

(**A-B**) Representative images of aortas prepared *en face* and stained with Sudan IV. Atherosclerotic area was calculated using Image J software and presented as a percentage of the total aortic area. (**C-D**) Representative images of aortic sinuses stained with a Mac-2 antibody. Atherosclerotic area was calculated using Image Pro Plus software and presented as total lesion size. Necrotic core area was quantified using Image J and presented as a percentage of total lesion area. Data were analyzed using one-way ANOVA with Tukey's post hoc test, presented as mean ± SEM. *p<0.05 from WT.

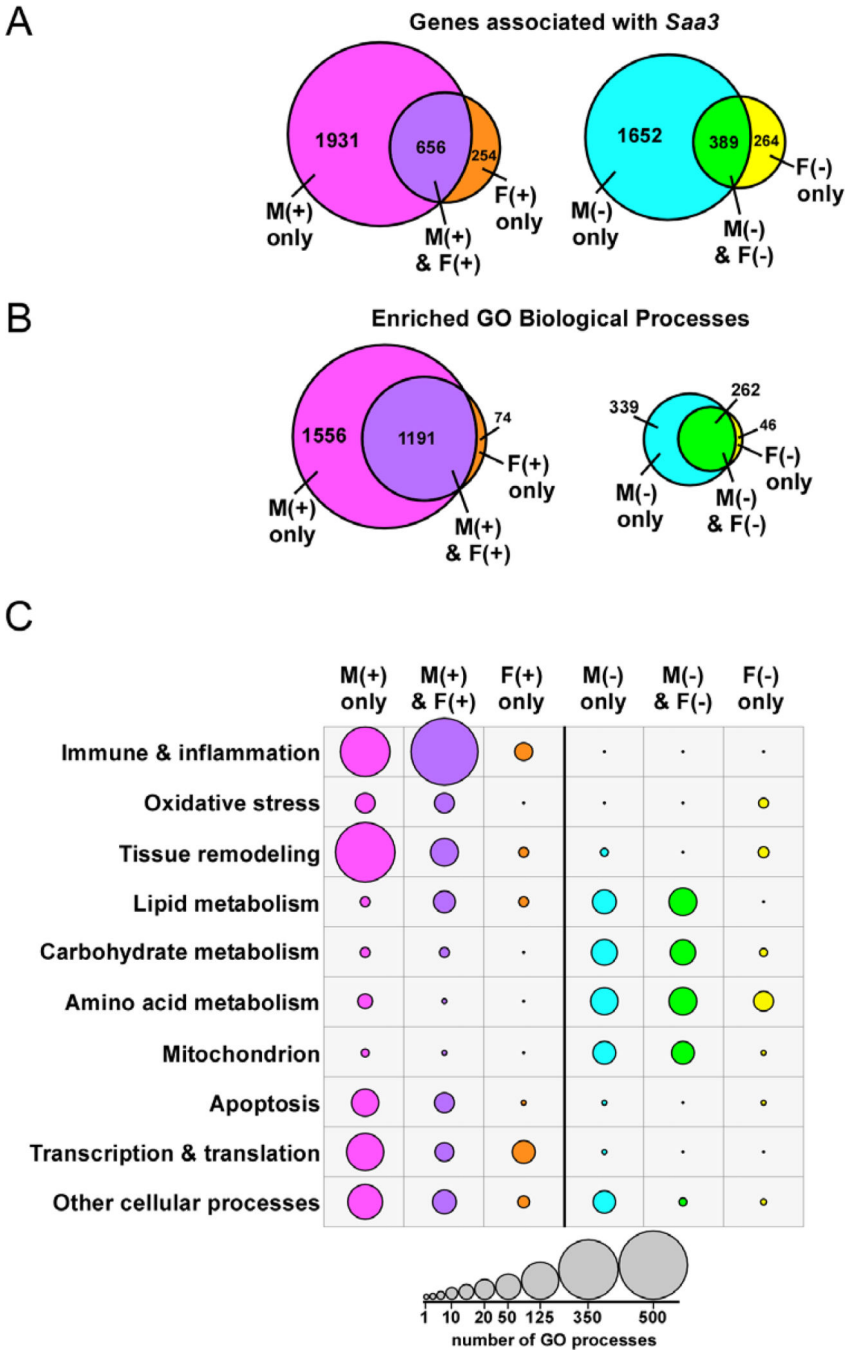

Figure 3. Representation of hepatic genes and pathways positively or negatively associated with *Saa3* in males and females.

(**A**) Venn diagram summarizing the number of unique and overlapping liver genes positively (+) or negatively (−) associated with Saa3 for males and females prone to the development of atherosclerosis. (**B**) A similar Venn representation of enriched GO biological processes based on genes positively or negatively associated with Saa3 expression. (**C**) Significantly over-represented pathways (FDR < 0.05) were grouped into functionally similar modules and are depicted based on their unique or overlapping enrichment in male and female mice.

The size of the circles corresponds to the number of genes in each module M=males; F=females.

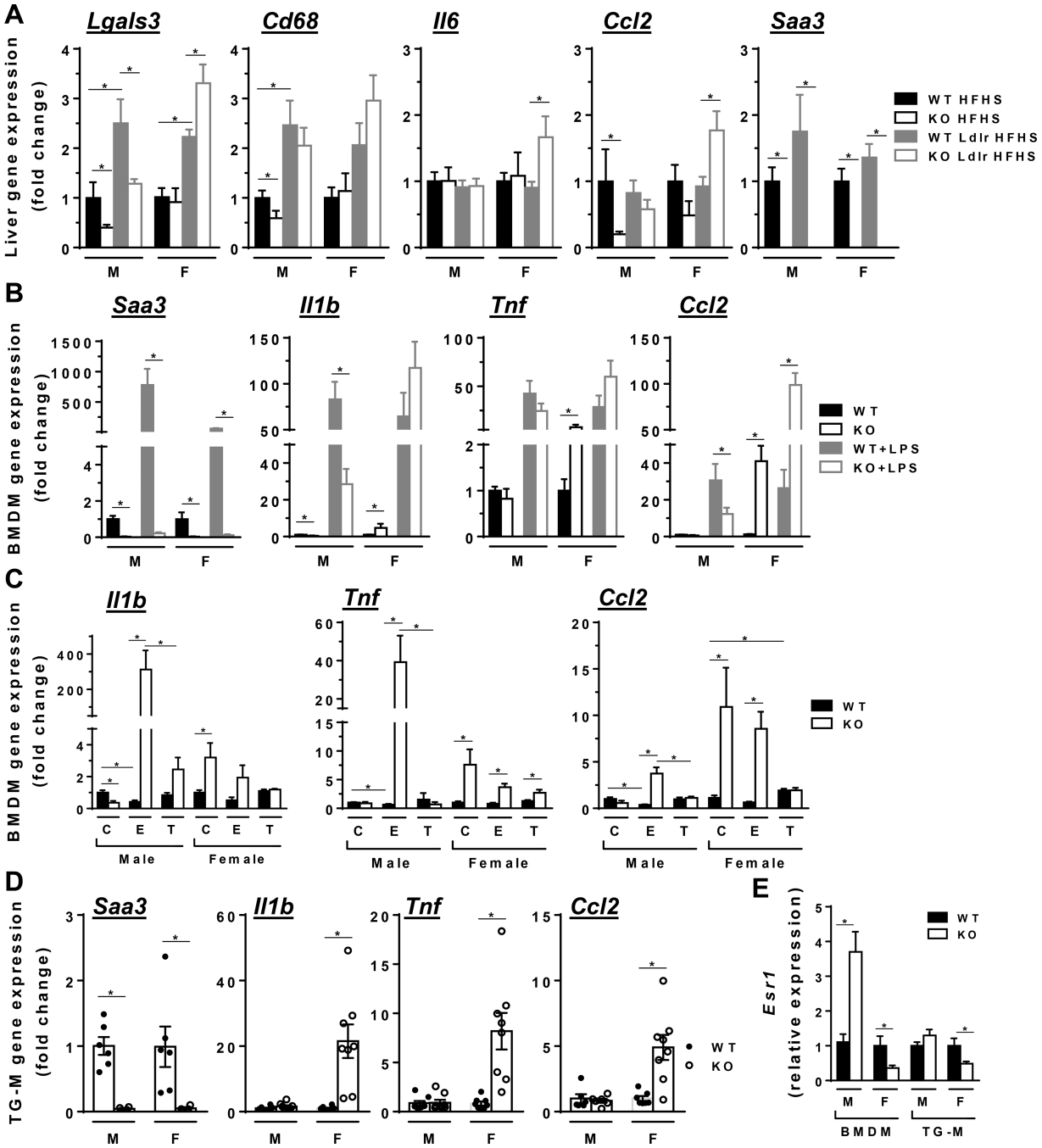

Figure 4. Sexually dimorphic inflammatory gene expression from liver and macrophages.

(**A**) Livers were isolated from wild type (WT) or *Saa3* KO (KO) HFHS-fed male (M) and female (F) mice with or without Ldlr deficiency. Expression of inflammatory genes were assessed (n=8–16). (**B-C**) BMDMs were isolated from WT or KO mice. Expression of inflammatory genes from male (M) and female (F) BMDMs treated with or without LPS (10 ng/mL for 16 hours) (**B**), 17β-estradiol (100 nM for 16 hours, E) (**C**), or testosterone (1 μM for 16 hours, T) (**C**) (n=3 in triplicate) (C=control). (**D**) mRNA expression levels in thioglycolate-elicited peritoneal macrophages (TG-M) from WT or KO mice (n=4–6). (**E**)

Esr1 mRNA expression from BMDMs (n=3 in triplicate) and TG-M (n=4–6). Data were analyzed using one-way ANOVA with Tukey's post hoc test, presented as mean ± SEM, *$p<0.05$.

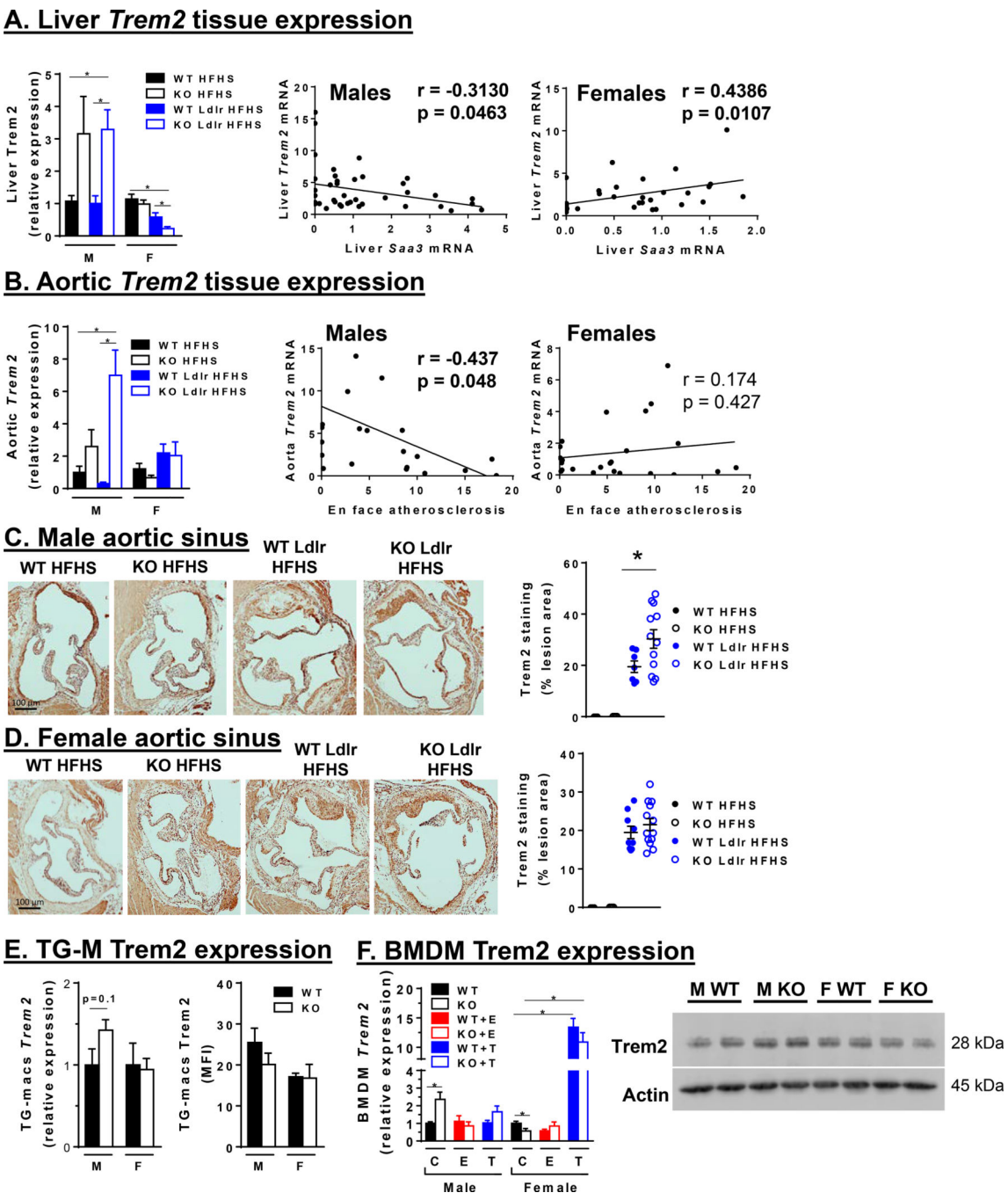

Figure 5. Trem2 expression is increased in male livers, aortas, and macrophages with *Saa3* deficiency.

(**A-B**) *Trem2* expression was assessed from: (**A**) liver (n=4–16), and (**B**) aortas (n=4–9). (**A-B**) *Trem2* mRNA expression correlations with liver Saa3 mRNA or en face atherosclerosis levels. (**C-D**) Representative images of male (**C**) and female (**D**) aortic sinus sections stained with a Trem2 antibody. Trem2 staining was quantified and expressed as a percentage of stained lesion area. (**E**) Trem2 mRNA expression was measured from TG-Ms, and mean fluorescence intensity (MFI) was assessed from flow cytometry-sorted TG-Ms gated on

Trem2^{+} F4/80^{+} cells (n=4–6/group). (**F**) BMDMs were pre-treated with media (C), 17β-estradiol (100 nM for 16 hours, E) or testosterone (1 μM for 16 hours, T) (n=3 in triplicate). Trem2 protein expression levels were quantified using densitometry from Western blots of BMDM from male or female WT or Saa3 KO mice (n=3 in duplicate). Densitometry (arbitrary units): 0.75±0.04 and 1.2±0.06 for male WT and KO, respectively, and 0.85±0.03 and 0.58±0.04 for female WT and KO, respectively. Data were analyzed using one-way ANOVA with Tukey's post hoc test, presented as mean ± SEM. *p<0.05.

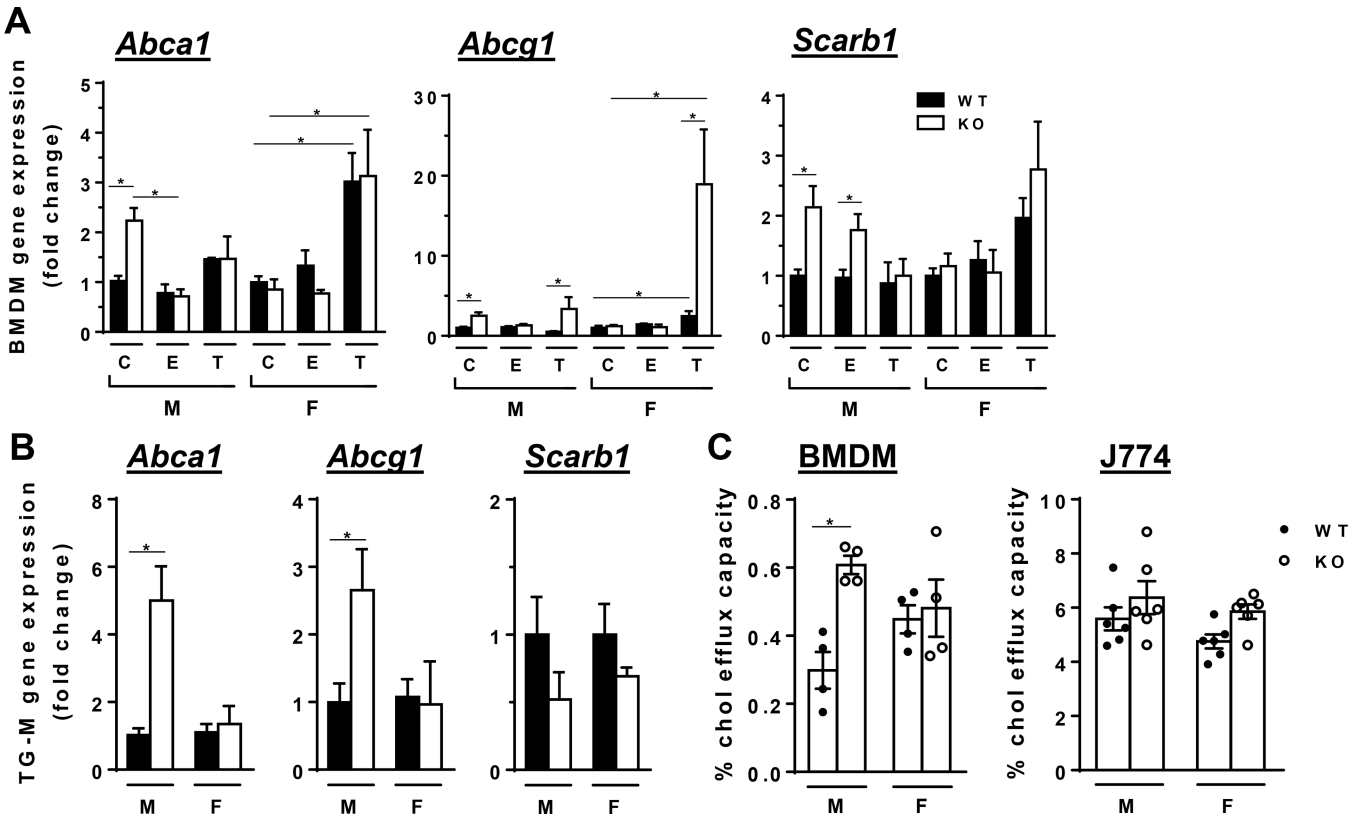

Figure 6. Cholesterol efflux in BMDMs and TG-macs.

BMDMs or thioglycolate-elicited macrophages (TG-M) were isolated from wild type (WT) or *Saa3* KO (KO) male (M) and female (F) mice. (**A**) Expression of cholesterol transporters *Abca1*, *Abcg1*, and *Scarb1* was quantified from BMDM that had been pre-treated with media (C), 17β-estradiol (100 nM for 16 hours, E), or testosterone (1 μM for 16 hours, T) (n=3 in triplicate). (**B**) Expression of cholesterol transporters was quantified from TG-M (n=4–6). (**C**) Cholesterol efflux capacity was measured from BMDM (using HDL from lean mice) or from J774 cells (using HDL from $Saa3^{+/+}$ or $Saa3^{-/-}$ mouse plasma), presented as a percentage of total efflux capacity. Data were analyzed using one-way ANOVA with Tukey's post hoc test, presented as mean ± SEM. *p<0.05.