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BAFF over-expression reduces atherosclerosis via TACI-dependent B cell activation

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Abstract

Patients with SLE exhibit accelerated atherosclerosis, a chronic inflammatory disease of the arterial wall. The impact of B cells in atherosclerosis is controversial, with both protective and pathogenic roles described. For example, natural IgM binding conserved oxidized lipid epitopes protect against atherosclerosis, while anti-oxidized low-density lipoprotein (oxLDL) IgG likely promotes disease. Since B cell activating factor of the TNF family (BAFF) promotes B cell class-switch recombination and humoral autoimmunity, we hypothesized that excess BAFF would accelerate atherosclerosis. In contrast, BAFF overexpression markedly reduced hypercholesterolemia and atherosclerosis in hyperlipidemic mice. BAFF-mediated atheroprotection required B cells and was associated with increased protective anti-oxLDL IgM. Surprisingly, high-titer anti-oxLDL IgM production and reduced atherosclerosis was dependent on the BAFF family receptor transmembrane activator and CAML interactor (TACI). In summary, we identified a novel role for B cell-specific, BAFF-dependent TACI signals in atherosclerosis pathogenesis, of particular relevance to the use of BAFF-targeted therapies in SLE.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of the vascular intima modulated by both the innate and adaptive arms of the immune system (1). Patients with autoimmune disease, in particular systemic lupus erythematosus (SLE) and rheumatoid arthritis, have a markedly increased risk of atherosclerotic cardiovascular disease suggesting additional immunologic factors impact atherogenesis in the setting of autoimmunity (2). In SLE, elevated cardiovascular risk cannot be fully explained by either Framingham risk factors, accumulated organ damage or exposure to immunosuppressive therapy (3, 4), and a large randomized trial of statins in SLE failed to identify any cardioprotective effects (4, 5). These data emphasize the urgent need for improved understanding of the underlying immune

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mechanisms impacting atherosclerosis with a goal of targeted therapies able to modulate this life-threatening disease process.

Although B cells clearly impact the pathogenesis of systemic autoimmunity, the role of B cells in atherosclerosis has been controversial, with both atheroprotective and atherogenic effects described. For example, initial studies using splenectomized apolipoprotein E-null (*Apoe*$^{-/-}$) and B cell-deficient (μMT) low-density lipoprotein receptor-null (*Ldlr*$^{-/-}$) mice demonstrated accelerated atherosclerosis in the absence of B cells (6, 7). These atheroprotective roles for B cells have been attributed to the production of protective natural IgM antibody (Ab) binding phosphorylcholine (PC) on *Streptococcus pneumoniae* surface capsule, apoptotic cells and oxidized low-density lipoprotein (oxLDL) (8). Supporting this idea, *Ldlr*$^{-/-}$ mice unable to secrete IgM (*sIgM*$^{-/-}$) develop accelerated atherosclerosis (9), while both passive immunization with anti-PC IgM and active *S. pneumoniae* immunization attenuates atherosclerosis (10, 11). In addition, adoptive transfer of WT, but not *sIgM*$^{-/-}$, peritoneal B1a B cells reduces atherosclerosis in splenectomized *Apoe*$^{-/-}$ mice (12), consistent with B1 B cells being the predominant source of natural IgM (13).

In contrast to these protective functions, B cells can actively promote atherosclerosis, particularly in autoimmune settings. Deletion of FcγRIIb (an inhibitory Fc-receptor expressed on B cells and other cells types) promotes the development of class-switched IgG2c Ab to oxidized LDL and leads to enhanced atherogenesis in *Ldlr*−/− and *Apoe*$^{-/-}$ mice (14, 15). Further, B cell depletion with anti-CD20 Ab decreases the development of atherosclerosis in both *Ldlr*−/− and *ApoE*−/− murine models of atherosclerosis (16, 17). Protection in this setting correlates with a decline in anti-oxLDL IgG and coordinate preservation of anti-oxLDL IgM titers (17), consistent with the resistance of natural Ab producing B1 B cells to anti-CD20 depletion (18). In addition to these animals studies, consistent findings are noted in patients with SLE, in whom those with the lowest titers of anti-PC IgM have the highest risk of atherosclerosis (19, 20), while lupus patients with a history of cardiovascular disease have elevated IgG anti-oxLDL Ab titers compared with other SLE patients and population controls (21).

Together, these human and animal studies support the paradigm whereby preformed natural IgM Ab protect against the development of atherosclerosis while class-switched autoantibodies against related oxidized lipid epitopes promote atherogenesis. However, the specific B cell-intrinsic signals required for class-switch recombination to pathogenic Ab isotypes in atherosclerosis have not been fully addressed.

B cell survival cytokine BAFF (B cell activating factor of the TNF Family) is a crucial B cytokine that has been closely linked to lupus pathogenesis. In addition to the development of lupus-like disease in BAFF transgenic (BAFF-Tg) mice (22, 23), BAFF levels are elevated in a subset of SLE patients (24) and a BAFF-inhibiting therapeutic antibody, Belimumab, demonstrated clinical efficacy in human lupus (25). Since atherosclerotic cardiovascular disease is accelerated in humans with autoimmune disease, we hypothesized that the over-expression of BAFF in hyperlipidemic mice would promote atherogenesis by promoting pathogenic class-switched IgG anti-oxLDL Ab. In contrast to this idea, BAFF over-expression markedly reduced hypercholesterolemia and atherosclerosis in *Apoe*$^{-/-}$

mice. Despite BAFF family receptor expression on non-B cell lineages, these protective effects of BAFF required the presence of peripheral B cells. Further, BAFF overexpression correlated with a marked increase in atheroprotective anti-oxLDL IgM Ab titers, which was dependent on the B cell surface receptor transmembrane activator and CAML interactor (TACI). Together, our findings uncover a novel role for BAFF-dependent TACI activation in promoting protective B cell functions in atherosclerosis, of relevance to the pathogenesis of atherosclerotic cardiovascular disease in patients with and without systemic autoimmunity.

MATERIALS AND METHODS

Mice

Apoe$^{-/-}$ (26), BAFF-Tg (23), μMT (27), and *Taci*$^{-/-}$ (28) mice on C57BL/6 background and relevant murine crosses were bred and maintained in the specific pathogen-free (SPF) animal facility of Seattle Children's Research Institute (Seattle, WA). To control for potential genetic differences between strains, all studies were performed on littermate controls. In addition, mean percentage C57BL/6J genetic background based on single nucleotide polymorphism (SNP) analysis of representative experimental animals was 97.2% and 98.4% in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.BAFF-Tg mice, respectively (https://www.jax.org/jax-mice-and-services/breeding-and-rederivation-services/genome-scanning). All animal studies were conducted in accordance with Seattle Children's Research Institute IACUC approved protocols.

Induction and quantification of atherosclerosis

6 week old female mice of indicated genotypes were placed on high-fat, high-cholesterol Western diet (21% fat, 0.2% cholesterol; Harlan Teklad, TD88137) for 8 or 12 weeks. Serum was obtained by cardiac puncture from animals fasted for 6 hours prior to sacrifice. The heart and proximal aorta were fixed with 10% formalin, and frozen at −80°C in OCT compound. Serial frozen sections were cut from the aortic sinus, counterstained with Oil Red O, and average lesion area quantified with ImagePro-Plus software (Media Cybernetics), as described (29).

Serum cholesterol analysis

Fasted serum cholesterol was assayed using colorimetric assay kits (Stanbio Laboratory; 1010-430). Plasma lipoprotein profiles were analyzed by fast protein liquid chromatography (FPLC) using sera pooled from 5 to 6 individual mice, as described (29).

ELISA

Ab ELISAs were performed on 96 well Immuno plates (Nunc) were coated with: MDA-LDL (Academy Bio-Medical; 20P-MD-L110) or phosphorylcholine PC(10)–BSA (10 μg/ml; Biosearch Technologies PC-1011-10), as described (30)

In vivo BAFF inhibition

Western diet-fed *Apoe*$^{-/-}$.μMT mice were injected I.P. with hamster anti-BAFF monoclonal Ab (10F4 clone; (31)) or hamster IgG1 isotype control on days 0, 5, then every 14 days for

duration of study. Trough serum was analyzed for 10F4 Ab titer and free serum BAFF level by ELISA, as described (31).

Statistical Evaluation

P-values were calculated using the two-tailed Student's t test, and the one-way ANOVA followed by Tukey's multiple comparison test (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

Excess BAFF limits hyperlipidemia and atherosclerosis in *Apoe*$^{-/-}$ mice fed a high-fat, high-cholesterol diet

To test the impact of elevated BAFF levels on murine atherosclerosis, we crossed *Apoe*$^{-/-}$ with transgenic mice over-expressing BAFF in myeloid cells using the human CD68 promoter (BAFF-Tg) (23). Independent cohorts of 6-week-old *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.BAFF-Tg female littermate mice were placed on a high-fat, high-cholesterol Western diet (WD) for 8 weeks to rapidly induce hyperlipidemia and atherosclerosis. As predicted, WD-fed *Apoe*$^{-/-}$ mice developed IgM Ab against PC and the oxidized lipid malondialdehyde conjugated with low-density lipoprotein (MDA-LDL) (8), with titers of these IgM Ab markedly increased by BAFF overexpression (Fig. 1 A, C). In addition, *Apoe*$^{-/-}$.BAFF-Tg mice developed elevated class-switched IgG Ab against PC and MDA-LDL, with the pro-inflammatory IgG subclasses IgG2b and IgG2c exhibiting the greatest increase (Fig. 1 B, C). Since IgG2c Ab bind pro-inflammatory Fc-receptors on myeloid cells (32), we predicted that increased titers of anti-oxLDL IgG2c Ab would promote atherogenesis in WD-fed *Apoe*$^{-/-}$ mice. Surprisingly, BAFF overexpression resulted in ~50% reduction in total serum cholesterol levels after 8 weeks on WD without impacting overall weight gain (Fig. 1 D, E). Analysis of FPLC-separated lipoprotein fractions demonstrated a prominent increase in VLDL in WD-fed *Apoe*$^{-/-}$ mice. Notably, BAFF overexpression resulted in a marked decrease in the VLDL peak without significantly impacting HDL levels (Fig. 1 F). We next analyzed the impact of increased serum BAFF levels on atherogenesis by quantification of Oil Red O^{+} area on serial aortic sinus sections from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.BAFF-Tg mice after 8 weeks on WD. Strikingly, *Apoe*$^{-/-}$.BAFF-Tg mice exhibited a ~80% reduction in aortic root atheroma (Fig. 1 G, H). Together, these findings highlight the unexpected observation that increased serum BAFF protects against hyperlipidemia and atheroma formation in an established murine model of atherosclerosis.

BAFF-mediated atheroprotection requires B cells

BAFF exerts biologic activity by binding the cell surface receptors BAFF receptor (BAFF-R), TACI and B cell maturation antigen (BCMA), while the related cytokine APRIL (a proliferation-inducing ligand) binds TACI and the BCMA (33). Although BAFF primarily impacts B cell function, additional immune lineages express BAFF-family receptors, including: BAFF-R expression by activated CD4^{+} T cells and regulatory T cells (34); and TACI expression on monocytes (35) and dendritic cells (36). In addition, BAFF is produced by adipocytes, with levels of adipose tissue BAFF expression correlating with obesity in murine models (37, 38). Moreover, expression of all 3 BAFF-family receptors (BAFF-R, TACI, BCMA) has been observed on adipocytes, with surface levels modulated by pro-

inflammatory stimuli (37, 39). Thus, decreased hyperlipidemia and atherosclerosis in *Apoe*$^{-/-}$.BAFF-Tg mice may occur independently of B cells.

For this reason, we examined whether BAFF-mediated atheroprotection required B cells via two parallel strategies. First, we generated atherosclerosis-prone mice lacking B cells by crossing *Apoe*$^{-/-}$ and μMT strains. Importantly, μMT mice have elevated serum BAFF levels because of loss of surface BAFF binding by B cells (Serum BAFF: 13+/−1.5ng/mL (WT) vs. 166+/−38ng/mL (μMT); P<0.0001, by two-tailed Student's t test). To test the B cell-independent impact of BAFF on atherosclerosis progression, we treated WD-fed *Apoe*$^{-/-}$.μMT mice with anti-BAFF monoclonal Ab (10F4 clone; (31)) or hamster IgG1 isotype control (Fig. 2 A). This dosing strategy was associated with therapeutic trough serum 10F4 levels (31) and resulted in complete binding of free serum BAFF (Fig. 2 B, C). In contrast to our findings in Figure 1, the atheroprotective effect of BAFF was not observed in the absence of B cells as the extent of aortic root atheroma was equivalent in 10F4- vs. isotype-treated *Apoe*$^{-/-}$.μMT mice (Fig. 2 D, E).

As a second model to test whether BAFF impacted the progression of atherosclerosis via a B cell-dependent vs. -independent mechanism, we crossed *Apoe*$^{-/-}$.BAFF-Tg with μMT mice. Notably, increased serum BAFF was not associated with a reduction in serum cholesterol in B cell-deficient *Apoe*$^{-/-}$.BAFF-Tg animals after 8 weeks on WD (Fig. 2 F). In parallel with total cholesterol levels, we observed no difference in serum lipoprotein profiles, based on FPLC analysis of WD-fed *Apoe*$^{-/-}$.μMT vs. *Apoe*$^{-/-}$.μMT.BAFF-Tg mice (Fig. 2 G). Finally, the severity of aortic root atherosclerosis was not impacted by BAFF overexpression in the absence of B cells (Fig. 2 H, I). Although μMT mice exhibit altered lymphoid architecture that may impact atherosclerosis progression, these combined data demonstrate that, despite BAFF-family receptor expression on myeloid lineages and adipocytes, excess BAFF limits hypercholesterolemia and decreases atherogenesis via B cell-dependent mechanisms.

TACI is required for BAFF-mediated atheroprotection

Having documented that BAFF acts on B cells to decrease atherosclerosis, we next asked which BAFF-family receptor (BAFF-R, TACI or BCMA) is the relevant BAFF target in atherosclerosis. BAFF-R signals are required for peripheral B cell survival beyond the transitional stage, and activation of this receptor likely explains the prominent B cell hyperplasia in BAFF-Tg mice (40). However, previous work has demonstrated that that BAFF-R-deficient *Apoe*$^{-/-}$ mice develop reduced atherosclerosis, implying that BAFF-R is unlikely to mediate an atheroprotective effect (41). In addition, BCMA promotes plasma cell survival, but basal serum IgM and IgG titers are not altered in BCMA-deficient mice (42, 43). For these reasons, we hypothesized that TACI-dependent signals might be required to limit hypercholesterolemia and atherosclerosis progression in *Apoe*$^{-/-}$.BAFF-Tg mice. In support of this idea, *Taci*$^{-/-}$ mice have lower serum IgM levels suggesting that lack of TACI may decrease the formation of atheroprotective IgM against oxidized lipid epitopes. Further, while TACI was initially hypothesized to act as a negative regulator of BAFF-mediated signals, recent independent studies, including one from our laboratory, demonstrate that

TACI signals are required for class-switched autoantibody formation in BAFF-Tg mice (30, 44).

To test whether TACI signals are required for BAFF-mediated atheroprotection, **we generated *Apoe*$^{-/-}$.BAFF-Tg.*Taci*$^{-/-}$ mice. Notably, the increase in serum PC and anti-MDA-LDL IgM titers in WD-fed *Apoe*$^{-/-}$.BAFF-Tg mice was not observed in the setting of *Taci* deficiency (Fig. 3 A). In addition, WD-fed *Apoe*$^{-/-}$.BAFF-Tg.*Taci*$^{-/-}$ mice developed marked hypercholesterolemia equivalent to *Apoe*$^{-/-}$ animals, based on both FPLC lipoprotein profile analysis and total serum cholesterol levels (Fig. 3 B, C). Finally, *Apoe*$^{-/-}$.BAFF-Tg.*Taci*$^{-/-}$ mice developed large atheromatous lesions within the aortic root that were equal in size to WD-fed *Apoe*$^{-/-}$ controls (Fig. 3 D, E). Although BAFF-mediated activation of BAFF-R and/or BCMA might also impact atherosclerosis progression in this model, these findings demonstrate that TACI is required for the BAFF-mediated decrease in hypercholesterolemia and attenuation of atherosclerosis in BAFF-Tg mice.

Taken together, our findings highlight an unanticipated role for BAFF in lipid metabolism and the progression of atherosclerosis. We show that excess BAFF results in markedly reduced aortic atherosclerosis, via a B cell- and TACI-dependent mechanism. While TACI signals might exert additional impacts on atheroma formation via B cell-independent mechanisms, our combined observations suggest a novel role for TACI-driven B cell activation in atherosclerotic cardiovascular disease. Although B cell antigen-presentation and cytokine production also impact immune responses independently of Ab formation, prior animal and human studies have strongly implicated Ab binding oxidized lipid epitopes in the pathogenesis of atherosclerosis. Consistent with these data, BAFF-mediated atheroprotection correlated with a prominent TACI-dependent increase in natural IgM Ab titers, further emphasizing the importance of IgM Ab against oxidized lipid epitopes in limiting atherosclerosis. In this context, promoting anti-oxLDL Ab by vaccination has been proposed as an immunomodulatory strategy in atherosclerotic cardiovascular disease (45). Our study suggests that activating B cell TACI signals may significantly enhance the therapeutic efficacy of this approach.

While BAFF has been implicated in the pathogenesis of human SLE, lupus patients also exhibit increased cardiovascular disease (2). Therefore, our findings raise the concern that therapeutic BAFF inhibition may exert unanticipated impacts on cardiovascular risk in SLE. Although anti-CD20-mediated B cell depletion reduced atherosclerosis in murine models (16, 17), BAFF inhibition may have distinct impacts on the relative balance of atheroprotective and pro-atherogenic Ab titers. Further, TACI-Ig (Atacicept), a dual inhibitor of both APRIL and BAFF currently in clinical development for treatment of SLE, is associated with prominent ~70% reduction in total serum IgM and ~40% decrease serum IgG titers (46). Given the critical importance of TACI signals in B cell-mediated atheroprotection, inhibition of both known TACI ligands using TACI-Ig may additionally impact atherosclerosis progression compared with isolated BAFF blockade. Alternatively, BAFF and/or APRIL blockade may be associated with a relative preservation of protective IgM Ab relative to class-switched pathogenic Ab. In this regard, the impact of therapeutic BAFF family inhibition on atherosclerosis progression has not yet been assessed in hyperlipidemic murine models. In summary, our study highlights a novel role for TACI-

dependent BAFF signals in protection against atherosclerosis progression, findings suggesting that lupus patients undergoing therapeutic BAFF inhibition should be closely monitored for increased cardiovascular events.

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Abbreviations

μMT	B cell-deficient
Ab	antibody
ApoE	apolipoprotein E
BAFF	B cell activating factor of the TNF family
BAFF-R	BAFF receptor
BAFF-Tg	BAFF transgenic
BCMA	B cell maturation antigen
FPLC	fast protein liquid chromatography
Ldlr	low-density lipoprotein receptor
MDA-LDL	malondialdehyde-modified low density lipoprotein
oxLDL	oxidized low density lipoprotein
PC	phosphorylcholine
SLE	systemic lupus erythematosus
TACI	transmembrane activator and CAML interactor
VLDL	very low density lipoprotein
WD	Western diet.

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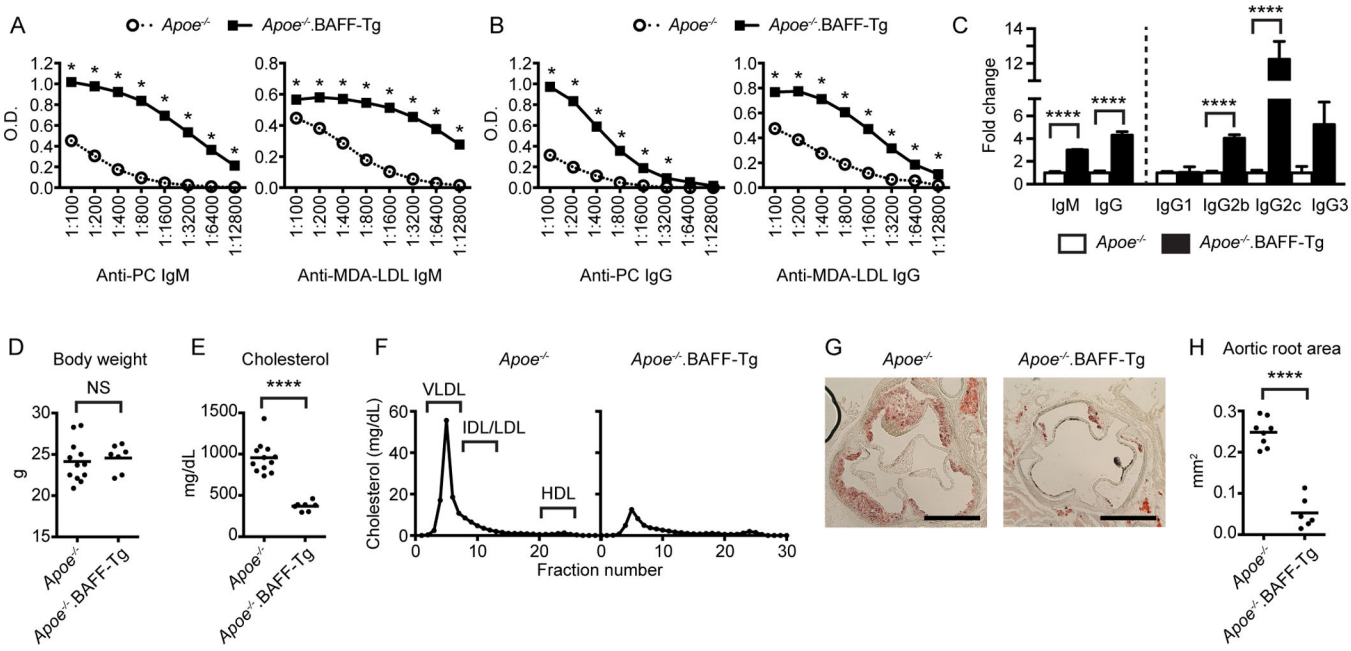

Figure 1. Excess BAFF limits hypercholesterolemia and atherosclerosis in *Apoe*$^{-/-}$ mice

(A, B) Anti-PC and anti-MDA-LDL IgM (A) and IgG (B) Ab titers from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.BAFF-Tg mice after 8 weeks on WD. *, $P<0.01$ by two-tailed Student's t test. (C) Fold change in anti-MDA-LDL IgM, IgG, and IgG subclass specific Ab in *Apoe*$^{-/-}$ vs. *Apoe*$^{-/-}$.BAFF-Tg mice. Error bars indicate SEM. (D, E) Body weight (D) and fasting serum cholesterol level (E) in represented genotypes after 8 weeks on WD. (F) Lipoprotein distribution (in mg/dL) by FPLC analysis of pooled sera from fasted *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.BAFF-Tg mice after 8 weeks on WD showing very low-density (VLDL), intermediate/low-density (IDL/LDL) and high-density (HDL) lipoprotein peaks, respectively. (G) Representative images of Oil Red O-stained aortic root sections. Bars, 500μM. (H) Atheroma lesion area in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.BAFF-Tg mice after 8 weeks on WD. (C, D, E, H) ****, $P<0.0001$; NS, not significant, by two-tailed Student's t test.

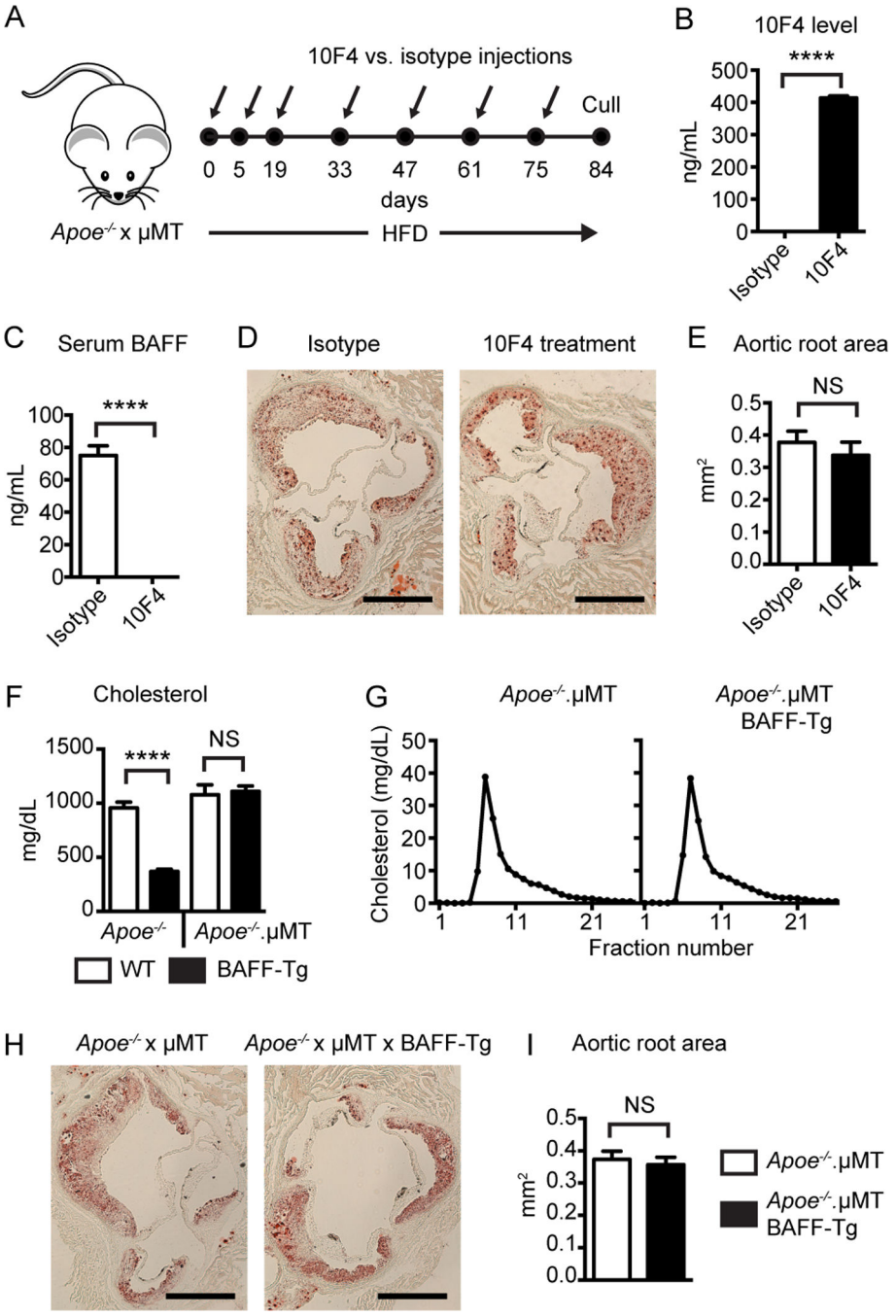

Figure 2. BAFF-mediated atheroprotection requires B cells

(A) Experimental design of BAFF inhibition in WD-fed *Apoe*$^{-/-}$.µMT mice. (B, C) Trough 10F4 (B) and free serum BAFF (C) levels in 10F4- and isotype-treated *Apoe*$^{-/-}$.µMT mice (D, E). Representative images of Oil Red O-stained aortic root sections (D, Bars, 500µM), and atheroma lesion area (E) in 10F4- and isotype-treated *Apoe*$^{-/-}$.µMT mice after 12 weeks on WD. (F, G) Total serum cholesterol (F) and lipoprotein distribution (G) in represented genotypes after 8 weeks on WD. (H, I) Representative images of Oil Red O-stained aortic root sections (H, Bars, 500µM), and atheroma lesion area (I) in *Apoe*$^{-/-}$.µMT and

Apoe$^{-/-}$.μMT.BAFF-Tg mice after 8 weeks on WD. (B, C, E, F, I) Error bars indicate SEM. ****, $P<0.0001$; NS, not significant, by two-tailed Student's t test.

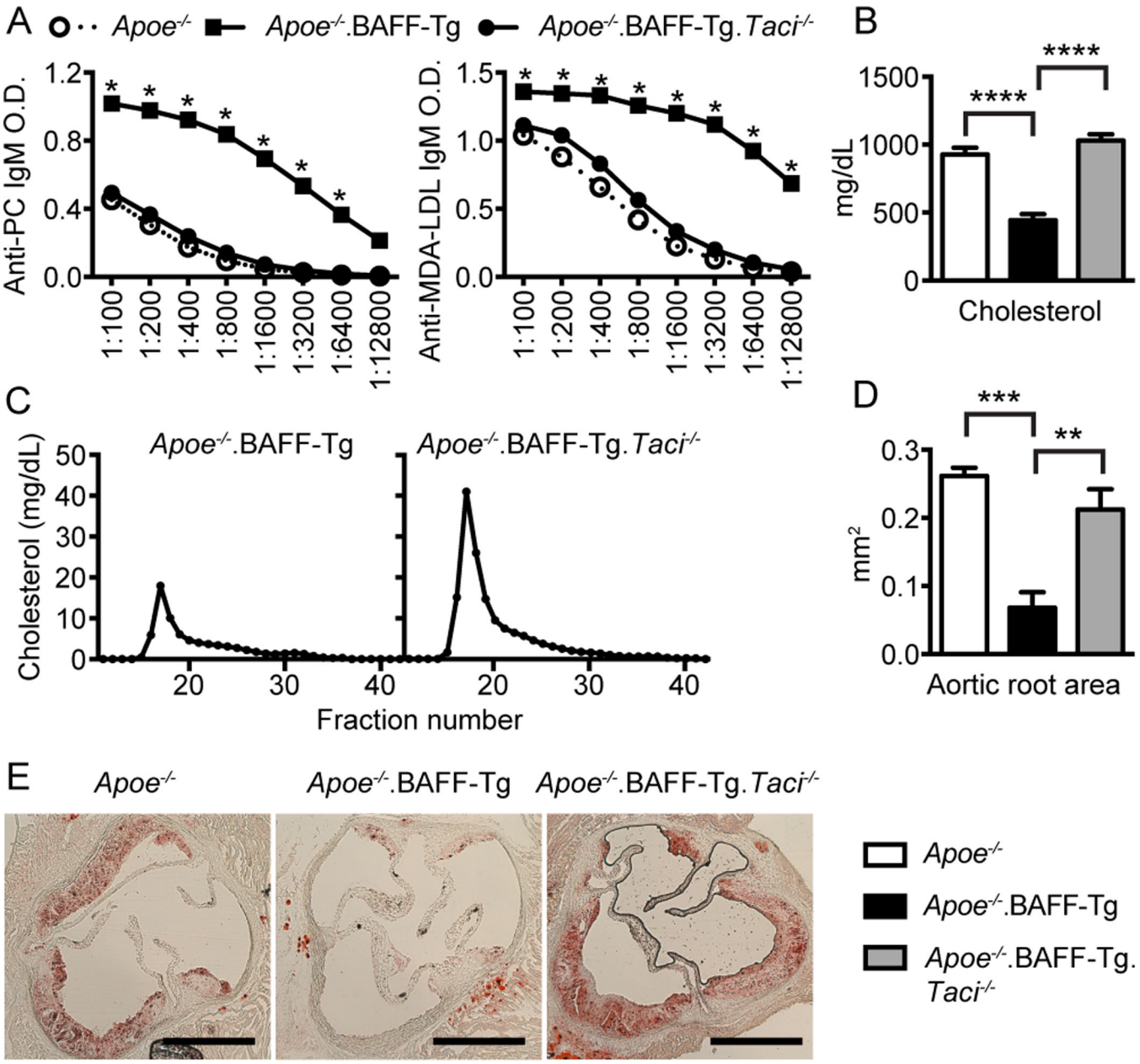

Figure 3. TACI signals promote BAFF-mediated atheroprotection

(A) Anti-PC and anti-MDA-LDL IgM Ab in *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.BAFF-Tg and *Apoe*$^{-/-}$.BAFF-Tg.*Taci*$^{-/-}$ mice after 8 weeks on WD. *, P<0.0001 in *Apoe*$^{-/-}$.BAFF-Tg vs. *Apoe*$^{-/-}$.BAFF-Tg.*Taci*$^{-/-}$ Ab titers, by two-tailed Student's t test. (B, C) Total serum cholesterol (B) and serum lipoprotein distribution (C) in indicated genotypes after 8 weeks on WD. (D, E) Atheroma lesion area (D) and representative images of Oil Red O-stained aortic root sections (E, Bars, 500μM) in *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.BAFF-Tg and *Apoe*$^{-/-}$.BAFF-Tg.*Taci*$^{-/-}$ mice after 8 weeks on WD. (B, D) Error bars indicate SEM; **, P<0.01; ***, P<0.001; ****, P<0.0001; by one-way ANOVA, followed by Tukey's multiple comparison test