X-box Binding Protein-1 dependent Plasma Cell Responses Limit the Development of Atherosclerosis

Sage A.P.[1*] PhD., Nus M.[1] PhD., Bagchi Chakraborty J.[1] PhD, Tsiantoulas D.[2,3], Newland S.A.[1] PhD, Finigan A.J.[1] MSc, Masters L.[1] MSc, Binder C.J.[2,3], Mallat Z[1,4] MD PhD.

[1]Division of Cardiovascular Medicine, Department of Medicine, University of Cambridge, Cambridge, UK. [2] Department of Laboratory Medicine; Medical University of Vienna, Vienna, Austria; [3]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [4]INSERM U970, Paris Cardiovascular Research Center, Paris, France; Université Paris Descartes, Sorbonne Paris Cité, Paris, France.

Short Title: T cell-dependent antibodies limit atherosclerosis

***Address for correspondence:** Dr Andrew P Sage, West Forvie building, Robinson Way, Cambridge, CB2 0SZ, UK. aps63@cam.ac.uk

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Abstract

Rationale–Diverse B cell responses and functions may be involved in atherosclerosis. Protective antibody responses, such as those against oxidized lipid epitopes, are thought to mainly derive from T cell-independent innate B cell subsets. In contrast, both pathogenic and protective roles have been associated with T cell-dependent antibodies and their importance in both humans and mouse models is still unclear.

Objective– To specifically target antibody production by plasma cells and determine the impact on atherosclerotic plaque development in mice with and without CD4+ T cells.

Methods and Results– We combined a model of specific antibody deficiency, B cell-specific CD79a-Cre x X-box binding protein-1 (XBP1) floxed mice (XBP1-cKO), with antibody mediated depletion of CD4+ T cells. Ldlr knockout mice transplanted with XBP1-cKO (or WT control littermate) bone marrow were fed western diet for 8 weeks with or without anti-CD4 depletion. All groups had similar levels of serum cholesterol. In Ldlr/ XBP1-cKO mice, serum levels of IgG, IgE and IgM were significantly attenuated, and local antibody deposition in atherosclerotic plaque was absent. Antibody deficiency significantly accelerated atherosclerosis at both the aortic root and aortic arch. T cell and monocyte responses were not modulated, but necrotic core size was greater, even when adjusting for plaque size, and collagen deposition significantly lower. Anti-CD4 depletion in Ldlr/WT mice led to a decrease of serum IgG1 and IgG2c but not IgG3, as well as decreased IgM, associated with increased atherosclerosis and necrotic cores, and a decrease in plaque collagen. The combination of antibody deficiency and anti-CD4 depletion has no additive effects on aortic root atherosclerosis.

Conclusions– The endogenous T cell-dependent humoral response can be protective. This has important implications for novel vaccine strategies for atherosclerosis and in understanding the impacts of immunotherapies used in patients at high risk for cardiovascular disease.

Keywords: Antibodies, B cells, Atherosclerosis

Non-standard Abbreviations and Acronyms: BSA – bovine serum albumin; CGG – chicken gamma globulin; cKO – conditional knockout; DC – dendritic cell; GC – Germinal centre; LDL – low density lipoprotein; MDA – malondialdehyde; MAA – malondialdehyde acetaldehyde; NP – nitrophenyl; OSE - oxidation-specific epitope; PC – phoshorylcholine; sIgM – soluble IgM; sma – smooth muscle actin; Teff – effector T cells; Tfh – follicular helper T cells; Treg – regulatory T cells, TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling; XBP1 – Xbox-binding protein-1.

Introduction

The subendothelial accumulation of low density lipoprotein (LDL) is central to the initiation and progression of atherosclerotic plaques. Modification of LDL, and a consequent shift in recognition, uptake and intracellular processing (or lack of) by phagocytes play a key role in foam cell formation, sustaining inflammation and necrotic/lipid core formation. In contrast to early phagocytosis of apoptotic cells, which is anti-inflammatory, secondary necrosis can be highly pro-inflammatory[1], recruiting and activating immune cells. A current paradigm for atherosclerosis proposes that the combination of extracellular lipid accumulation, foam cell formation and defective efferocytosis leads to a non-resolving loop of inflammation, debris accumulation and response to injury.

Oxidative modifications of LDL and cellular membranes create immunogenic epitopes recognized by antibodies[2]. B cell antibody responses can be divided broadly into 3 categories. Innate-like IgM production, primarily from B1 cells but also marginal zone B cells, occurs against common molecular pattern antigens such as malondialdehyde (MDA) and phosphorylcholine (PC) and is present perinatally and even in germ-free mice[3]. Responses to other antigens also initially result in IgM production from short-lived plasmablasts and plasma cells but then undergo various forms of adaptation. T cell-independent responses can adapt via class-switching of IgM to other isotypes such as IgA). T cell help from antigen-specific $CD4^+$ T cells leads to both class-switching and multiple rounds of affinity maturation within germinal centers of lymphoid organs, resulting in high affinity antibodies and memory B cells[4]. Antibodies to modified LDL in atherosclerosis are produced through each different mode. The origins of responses to other antigens in atherosclerosis is not well understood.

Many studies have assessed levels of antibodies against oxidized forms of LDL and more recently to the specific epitopes MDA and PC. High levels of IgM type antibodies to MDA and PC reduce the risk for cardiovascular disease[5–7], although not in all studies[8,9]. In contrast, IgG antibodies of similar specificity can in some cases be positively associated with disease[10] but more commonly show no association[5,8,9]. Recently, total serum IgG and IgM, in addition to oxLDL-specific, was associated with reduced risk of cardiovascular events[9].

In mice, many studies suggest IgM antibodies provide an important atheroprotective layer [11–15]. The specific effect of deleting IgM antibodies (rather than B cells in general) was first addressed by Lewis et al[16]. Mice lacking soluble IgM (but not B cell surface IgM or other isotypes of antibodies) have increased atherosclerosis[16]. However, in a more recent study using $sIgM^{-/-}$/*Ldlr*$^{-/-}$ mice, we demonstrated that the enhanced atherosclerosis was a result of IgE accumulation in the circulation since anti-IgE injections completely reversed the phenotype[17]. IgE accumulates because $sIgM^{-/-}$ mice have a significant reduction in IgE receptor (CD23)-expressing B cells, leading to mast cell activation and pro-atherogenic inflammation. This raises important questions about the direct role of IgM or related downstream biological events in mediating atheroprotection. IgG antibodies against modified LDL may also be protective, for example passive transfer of a human IgG1 against an MDA-modified apoB100 peptide reduces mouse atherosclerosis[18]. Recombinant Fab' and single chain forms of anti-MDA-LDL antibodies, that lack the normal Fc-mediated effects of antibodies, also reduce foam cell formation and atherosclerosis[19]. However, several studies also implicate a pathogenic role for IgG in atherosclerosis, albeit indirectly[20–22], and vascular pathologies such as chronic cardiac rejection and large vessel vasculitis are thought to be driven by pathogenic autoantibodies[23,24].

Emerging paradigms reveal many non-antibody dependent functions of B cells that could play important roles in atherosclerosis[25–27]. We and others have shown follicular B2 cell interactions with $CD4^+$ T cells, antagonized by marginal zone B2 cells, promote pathogenic T

cell responses in the atherosclerotic setting[28–33]. Few models have therefore addressed the specific role of antibodies in isolation without impacting on other B cell functions. Altogether, it is hard to predict the effects of total antibody deficiency on atherosclerosis. We designed experiments to target plasma cell antibody production specifically. Plasma cells undergo a profound transcriptional reprogramming during differentiation from activated B cells; a prominent set of genes induced in plasma cells are regulators of the endoplasmic reticulum stress pathway, which expands antibody secretion capacity[34]. A key transcription factor is Xbox-binding protein-1 (Xbp1), and previous studies have characterized in detail that mice with B cell-specific *Xbp1* genetic deletion display a specific defect in antibody production[35–37]. Using this model under the atherosclerotic *Ldlr*$^{-/-}$ background, we demonstrate that Xbp1-dependent plasma cell responses have a prominent anti-atherosclerotic influence.

Methods

Mice

CD79a^{Cre} mice were original created in the Reth lab[38] and were obtained from Jackson labs (020505). *Xbp1*$^{fl/fl}$ mice were originally created in the Glimcher lab[39] and were a gift of Prof. A. Kaser, University of Cambridge. *Ldlr*$^{-/-}$ mice were originally obtained from Jackson labs (002207). A total of 53 male *Ldlr*$^{-/-}$ mice were used in the study. Antigen-specific antibody responses were induced by immunization with nitrophenyl-chicken gamma globulin (NP-CGG; 100µg; Biosearch) in alum. Creation of bone marrow chimeric mice, atherosclerosis experiments and anti-CD4 depletion have been previously described[40]. Mice from different experimental groups were co-housed for atherosclerosis experiments. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Plaque and serum analysis

To assess plaque size and composition, aortic root cryosections were stained with Oil red O (Sigma), CD3 (Dako), MOMA2 (Biorad), α-smooth muscle actin (Sigma), IgM (Biolegend), IgG_1 (Bethyl Labs), picrosirius red (Sigma), and by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche). Oil red O stained aortic arches were dissected and imaged en face. Images were analyzed using ImageJ (NIH). Serum antibodies were analyzed using kits from Bethyl labs and total cholesterol using a cholesterol RTU kit (Biomerieux). Triglyceride levels were measured using LabAssay kit (Wako). Antibodies against oxidation-specific epitopes (OSE) were measured as previously described[15].

Immune cell phenotyping

Flow cytometry of single cell suspensions was performed as previously described[40]. Flow cytometry antibodies are detailed in supplementary table 1 and gating strategies are outlined in supplemental table 2. For ELISpot assays, multiscreen 96-well filter plates (Millipore) were coated with anti-mouse total IgM or IgG (Bethyl labs) overnight, blocked with PBS/1% bovine serum albumin (BSA), then incubated with 10^4-10^6 bone marrow or spleen cells overnight. For NP-specific antibody detection, plates were coated with NP-BSA (Biosearch). Staining was revealed with anti-mouse IgG-horseradish peroxidase (Southern Biotech) or anti-mouse IgM- horseradish peroxidase (Bethyl labs) and 3,3',5,5'-tetramethylbenzidine substrate (MABtech). Plates were analyzed using an Immunospot reader (CTL Technologies). Regulatory T cell suppression assays were conducted using magnetically purified cells (Miltenyi) as previously described[29].

Statistics

Data was analyzed in GraphPad Prism (La Jolla, CA, USA) using unpaired T test (or Mann Whitney U test for data sets not passing normality), one-way ANOVA or two-way ANOVA, as appropriate. A p value <0.05 was considered significant.

Results

Xbp1 deficiency in B cells attenuates plasma cell antibody production

To obtain a mouse model with a specific defect in antibody secretion, we recreated a previously reported B cell-specific deletion of the transcription factor XBP1, which is required for terminal plasma cell differentiation but not antecedent stages of B cell development, maintenance of mature B cells, germinal center (GC) B cell or memory B cell formation [35–37]. Mice carrying a floxed allele of the *Xbp1* gene were crossed to *Cd79a*$^{Cre/+}$ mice that express Cre only in B cells[38]. To confirm the phenotype of these mice, we performed ELISpot analysis of total IgM and IgG-producing bone marrow plasma cells (supplemental figure 1A). Plasma cells from *Cd79a*$^{+/+}$ *x Xbp1*$^{fl/fl}$ mice (wildtype; WT) formed large spots of IgM and IgG. In contrast, plasma cells from *Cd79a*$^{Cre/+}$ *x Xbp1*$^{fl/fl}$ mice (referred to hereafter as *Xbp1* B cell-conditional knockout; *Xbp1*$^{B\text{-}cKO}$) formed only small IgM producing spots with very few IgG producing spots detected (supplemental figure 1A). In addition to this baseline phenotype, we also analyzed the antigen-specific response. *Xbp1*$^{B\text{-}cKO}$ mice and WT littermates were immunized with NP-CGG and after 7 days bone marrow NP-specific plasma cells and serum NP-specific IgG were analyzed. Compared to PBS-immunized controls in which NP-specific responses were undetectable, we detected NP-specific IgG1 secreting plasma cells in WT mice. In *Xbp1*$^{B\text{-}cKO}$ mice, NP-specific plasma cells were present but reduced around 2-fold (supplemental figure 1B). NP-specific IgG1 levels in the serum from the same mice were around 16-fold less in *Xbp1*$^{B\text{-}cKO}$ than WT mice, with no detectable antibodies in PBS-immunized mice (supplemental figure 1C). The difference in antibody titer was therefore more severely affected than the difference in antibody producing plasma cells, consistent with the idea that plasma cells can form in the absence of XBP1, but their antibody production is severely impaired. As previously reported[35], total numbers of CD138^{+} B220^{-} plasma cells were normal in bone marrow by flow cytometry (supplemental figure 1D).

B cell deletion of Xbp1 in atherosclerotic mice specifically attenuates antibody production

To determine the impact of antibody deficiency on atherosclerosis, we then used *Xbp1*$^{B\text{-}cKO}$ and WT littermates as bone marrow donors to irradiated *Ldlr*$^{-/-}$ mice that develop atherosclerosis. After 4 weeks recovery, *Ldlr*$^{-/-}$ recipients were fed a western diet for 8 weeks. Since atherosclerosis may be affected by both T cell-dependent and independent antibody responses, but the role of T cell-dependent responses is much less clear, we treated subgroups of *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ chimeras with depleting anti-CD4 antibodies for the duration of the western diet feeding[40]. Littermate (and co-housed) *Ldlr*$^{-/-}$ recipients were split evenly among the 4 groups. In both groups of mice treated with anti-CD4 antibodies, depletion was maintained to a high level between injections, with only minor levels of CD4^{+} T cell recovery detected immediately before the subsequent injection (supplemental figure 1E).

To confirm that defective antibody production seen in *Xbp1*$^{B\text{-}cKO}$ mice was reproduced in atherosclerotic *Ldlr*$^{-/-}$ chimeras, we analyzed total IgG, IgE, IgA and IgM in serum using specific ELISAs. We also measured subtypes of IgG including T cell-dependent total IgG2c and T cell-independent IgG3. All isotypes were significantly reduced in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice compared to *Ldlr*$^{-/-}$/WT controls, except for IgA (figure 1A-H). Anti-CD4 depletion significantly reduced IgG2c but not IgG3 as expected (figure 1G+H). Total IgG, IgE and IgA was not affected by anti-CD4 depletion, whereas total IgM was significantly reduced, almost to levels in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (figure 1C). This suggested that a significant proportion of IgM production in atherosclerotic *Ldlr*$^{-/-}$/WT mice was CD4^{+} T cell-dependent.

We then analyzed the status of immune cells and tissues in *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice by flow cytometry. Total cell numbers in spleen, lymph nodes, bone marrow and peritoneum were not different between *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ and *Ldlr*$^{-/-}$/WT controls (supplemental figure 2). XBP1 is primarily expressed during plasma cell differentiation and is not reported to have major functions in B cells. However, the lack of antibodies may have feedback influences on the B cell compartment. We therefore performed detailed flow cytometry phenotyping of B cell subsets in bone marrow, spleen and peritoneum, including peritoneal B1 cells and splenic marginal zone B cells that are known to regulate atherosclerosis[13,33,41]. There were no differences in bone marrow B cell fractions in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice or after anti-CD4 depletion (supplemental figure 3A). In the spleen, there was an expansion of marginal zone B cells (figure 1I), similar to the phenotype of IgM-deficient *sIgM*$^{-/-}$ *Ldlr*$^{-/-}$ mice[17]. Splenic B1 cells tended to expand in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice, but this difference did not reach significance (supplemental figure 3B). This expansion of innate-like B cells was specific to the spleen as no differences in B1 or B2 cells were detected in the peritoneum (supplemental figure 3C). *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice did not display significant differences in follicular B2 cells, whereas anti-CD4 depletion led to an expansion of splenic follicular B2 cells in WT but not cKO chimeras (figure 1I). *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice displayed no changes in spleen GC B cells or in plasma cell numbers in bone marrow (figure 1J+K). In contrast, GC B cells were absent from both anti-CD4 treated groups (figure 1J), providing functional evidence of successful CD4 depletion. This is also consistent with a severe reduction in GC-dependent IgG1 and IgG2c antibody levels (figure 1G). Anti-CD4 treated mice tended to have less plasma cells, which was significant in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ (figure 1K). There was also a trend towards an increased proportion of IgM^{+} plasma cells in *Ldlr*$^{-/-}$/WT mice treated with anti-CD4 compared to Ldlr/WT controls, consistent with less class-switched responses (supplemental figure 3D).

Xbp1 deficiency in B cells does not modulate systemic T cell immune responses

We and others have previously shown that global B2 cell or specific marginal zone B cell depletion can have significant impacts on CD4^{+} T cell effector responses that promote atherosclerosis[28–30,33]. Total CD4^{+} and CD8^{+} T cells numbers were not different between *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (supplemental figure 3E). In contrast to B cell depletion models, pro-atherogenic effector T cells (T_{em}; CD62L^{-} CD44^{hi}) (figure 2A and supplemental figure 3F), proportions of CD4^{+} T cells secreting IFN-γ (figure 2B) and follicular helper T cells (T_{fh}; figure 2C) were not different between *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice. Spleen dendritic cell (DC) numbers were not different between *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ groups (figure 2D), however we observed a significant reduction in MHCII levels on both CD8α^{+} and CD11b^{+} cDCs, but not plasmacytoid DCs (figure 2E). This difference was not observed in anti-CD4 depleted mice (figure 2E) and MHCII is not expected to have any significant function in those mice. The modest reduction of MHCII in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice does not appear to have impacted on effector T cell responses (figure 2A-C), but we hypothesized that differences in DC antigen presentation could have compromised regulatory T cell function[42]. However, the levels of spleen T_{reg} were not different in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (T_{reg}; figure 2F). To assess the function of T_{reg} cells, we magnetically sorted naïve/effector T cells (T_{eff}; CD4^{+} CD25^{-}), T_{reg} (CD4^{+} CD25^{+}) and cDCs (CD11c^{+}). The proliferation of T_{eff} was induced by anti-CD3 in the presence of cDCs. The suppressive capacity of regulatory T cells was titrated by adding CD4^{+} CD25^{+} cells at different ratios from 1:1 (T_{reg}: T_{eff}) to 1:8 and comparing proliferation, measured by ^{3}H-thymidine incorporation, to T_{eff} cells in the absence of Treg. The suppressive capacity of *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ Treg was comparable to *Ldlr*$^{-/-}$/WT Treg at all ratios, suggesting an intact regulatory T cell system (figure 2G). The levels of circulating monocytes or neutrophils did not significantly differ between any groups (supplemental figure 4A+B). Overall, we concluded that *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice had a specific

deficiency in antibody production without impacting systemically on cellular immune responses.

Xbp1 deficiency in B cells enhances atherosclerosis

The development of atherosclerosis was quantified in the aortic root and the aortic arch by oil red O staining (figure 3A). Analysis of serial aortic root cryosections revealed enhanced atherosclerosis in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (figure 3B; p<0.001). Similarly, atherosclerosis in the aortic arch analyzed *en face* had advanced more rapidly in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice than in *Ldlr*$^{-/-}$/WT controls (figure 3C). In the aortic root, anti-CD4 depletion resulted in increased atherosclerosis to a similar level as that seen in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice. There was no further increase in atherosclerosis in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice treated with anti-CD4 (figure 3B). The same pattern was seen in the aortic arch, although both CD4-depleted groups had higher levels of atherosclerosis (figure 3C). The levels of circulating total cholesterol or triglycerides were not different between any groups (supplemental figure 4C+D). The body weight of *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice was higher than other groups, an effect not observed in anti-CD4 treated groups (supplemental figure 4E).

Xbp1 deficiency in B cells and anti-CD4 depletion reduce oxidized epitope-specific antibodies

Based on increased atherosclerosis in both *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ and anti-CD4 treated mice, and decreased levels of IgM, we determined the levels of OSE-specific antibodies. IgM levels to MDA, malondialdehyde-acetaldehyde (MAA) and PC epitopes (conjugated to BSA) are presented in Figure 4A-C and showed a similar trend to total antibody levels (Figure 1). A prototypic B1 cell natural antibody, T15, is detected using anti-idiotypic AB1-2 antibodies. Again, as expected, serum T15 titers were significantly reduced in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (Figure 4D). Interestingly, anti-CD4 depletion in *Ldlr*$^{-/-}$/WT mice also resulted in significantly reduced levels (Figure 4D). Serum IgG antibody titers to MDA, MAA and PC were severely reduced by B cell XBP1 deficiency and anti-CD4 depletion (figure 4E-G). We also analyzed IgG subtype antibodies to MDA-LDL (Figure 4H). All isotypes except IgG3 were significantly reduced in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice. Anti-CD4 depletion had a larger effect on IgG2c than IgG1 or IgG2b, consistent with effects on total IgG levels (figure 1). Thus, all treatment groups with enhanced atherosclerosis displayed an attenuation of circulating anti-OSE antibody levels.

XBP1 deficiency in B cells increases apoptotic cell accumulation and necrotic core

We next analyzed the plaque composition in *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice to investigate mechanisms involved in enhanced atherosclerosis. Although serum antibody levels were significantly reduced in experimental groups (figure 1), residual IgM and IgG may still infiltrate atherosclerotic plaques. However, plaque IgM staining was virtually absent in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (figure 5A). In anti-CD4 depleted mice, IgM was also significantly reduced (figure 5A), consistent with results from serum ELISAs (figure 1). IgG_1 staining (previously shown to be a predominant IgG isotype in plaques[43]) was also significantly reduced in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice and both anti-CD4 treated groups compared to *Ldlr*$^{-/-}$/WT (figure 5B). Plaque sections were also analyzed by immunostaining for foam cells/macrophages (MOMA2$^+$), smooth muscle cells (α-smooth muscle actin$^+$) and T cells (CD3$^+$). There were no differences in the relative proportions of MOMA-2 or α-SMA stained areas between any groups (supplemental figure 5A-C). Plaques from *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice tended to have increased T cell infiltration (supplemental figure 5D; p=0.053). Most plaque infiltrating T cells are CD4$^+$ and consistent with this, the number of plaque CD3$^+$ cells in anti-CD4 treated mice was very low (data not shown). Despite increased atherosclerotic plaque size (suggesting enhanced disease progression towards more complex plaques), *Ldlr*$^{-/-}$

/*Xbp1*$^{B\text{-}cKO}$ and both anti-CD4 treated groups had significantly reduced collagen deposition (figure 5C). A potential protective function for antibodies suggested to regulate atherosclerosis is the binding and removal of apoptotic or (secondary) necrotic cells[2]. In support of this, the necrotic core area was significantly increased in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$, as was the level of TUNEL staining within plaques (figure 5D+E). Plaque necrotic core and TUNEL staining were also increased in anti-CD4 depleted *Ldlr*$^{-/-}$/WT mice (figure 5D+E).

Since normal plaque progression can heavily influence plaque composition, we determined to what extent overall plaque size in each mouse contributed to the difference in plaque composition we observed. Thus, we normalized plaque area data of each mouse (data in figure 3A) by dividing by the average plaque size for that mouse. Plaque size appeared to account for the majority of differences in CD3$^+$ T cell content between *Ldlr*$^{-/-}$/WT and *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (supplemental figure 5E). However, the levels of necrotic core and TUNEL$^+$ staining was still significantly increased in both *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ (Supplemental Figure 5F+G). The reduction in plaque collagen was even more apparent after adjustment for plaque size (Supplemental Figure 5H). As an alternative analysis, we also plotted mouse plaque size against percentage necrotic core and percentage collagen area using only mice with plaques of overlapping levels of atherosclerosis (supplemental figure 6). Plaques of *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice displayed higher relative necrotic core area and lower collagen levels than *Ldlr*$^{-/-}$/WT mouse plaques even though levels still varied with plaque size. This supports the hypothesis that reduction in systemic antibodies led to reduced antibody binding within atherosclerotic plaques, leading to enhanced accumulation of necrotic cells.

Discussion

One of the hallmarks of atherosclerosis is the accumulation and failed clearance of oxidized LDL and apoptotic cell debris within the atherosclerotic plaque. Oxidized LDL and apoptotic debris are recognized by specific antibodies present in both plasma and atherosclerotic plaque. Most recently, protective antibody responses have been specifically associated with innate (T cell-independent) pathways such as natural IgM[13,44]. Our study is the first to specifically target plasma cell antibody secretion and to determine the effects of antibody deficiency in the presence of all B cell subsets and lymphoid organs. Our results suggest that protective antibody responses depend on XBP1-driven plasma cell maturation to a highly secretory state and also on CD4$^+$ T cells. We propose that a T cell-driven expansion of antibody production is a key protective pathway in response to hyperlipidemia. Both cognate and non-cognate T-cell help, which has been implicated in natural IgM expansion, may contribute to these responses.

The B cell receptor senses antigen, which in conjunction with secondary signals, induces the activation of antigen-specific B cells, leading to proliferation and subsequent differentiation into plasma cells. Plasma cells undergo a profound transcriptional reprogramming, develop extensive rough endoplasmic reticulum and Golgi systems, and upregulate both protein synthesis and folding machinery, allowing huge levels of antibody secretion[34]. The loss of XBP1 prevents the final stages of this differentiation/maturation pathway, leading to plasma cells with severely attenuated secretory capacity[35,36]. We took advantage of this phenotype to specifically interrogate the impacts on atherosclerosis, driven by the chronic and myriad accumulation of oxidized lipid antigens. It is reasonable to presume that the antigen burden is high in western diet-fed *Ldlr*$^{-/-}$ mice, and thus antibody consumption would be correspondingly rapid. Although low levels of antibodies are produced in *Ldlr*$^{-/-}$/*Xbp1*$^{B\text{-}cKO}$ mice (figure 1+4 and supplemental figure 1), our results demonstrate this severe reduction in production is sufficient to significantly accelerate plaque growth (figure 3) and promote a more unstable plaque status (figure 5). Although we cannot completely exclude an

alternative impact of XBP1 deficiency on B cell functions independent of plasma cell antibody secretion, we believe that together with previous papers, our data strongly indicate that other direct effects on B cell functions are minor. A previous report using transgenic MD4 hen egg lysozyme-specific B cells suggested a defect in plasma cell homing driven by CXCR4[36], however this was not reproduced in mice with normal B cell repertoires[37].

Although no other studies have previously addressed the effects of a specific defect in plasma cells, two previous papers reported enhanced atherosclerosis in mice lacking the secreted form of IgM[16,17]. Indirect but compelling evidence for the importance of IgM is also provided by studies showing enhanced atherosclerosis in IL5-deficient mice that have reduced IgM[11], and reduced atherosclerosis in SiglecG-deficient mice that have increased IgM[44]. In addition, passive transfer of polyclonal IgM reduces atherosclerosis in *ApoE*$^{-/-}$ mice[12]. However, in *sIgM*$^{-/-}$ *Ldlr*$^{-/-}$ mice, impaired clearance of IgE promotes atherosclerosis, rather than direct local effects of IgM deficiency[17]. Compared to *sIgM*$^{-/-}$ mice, *Ldlr*$^{-/-}$*Xbp1*$^{B\text{-}cKO}$ mice have a further deficiency, both in IgE and in all other isotypes of antibodies (figure 1A-E). This suggests the possibility that IgG antibodies of the same specificity, presumably for oxidized epitopes such as MDA or PC, may also make a significant anti-atherosclerotic contribution. Studies showing that pneumoccocal immunization of *Ldlr*$^{-/-}$ mice lead to a selective expansion of PC-specific IgM antibodies and reduced atherosclerosis initially suggested an important role for IgM antibodies[15]. However, PC-based and MDA-based vaccination strategies that demonstrated an induction of both specific IgM and IgG antibodies also resulted in robust lesion reduction[43,45]. A previous study demonstrated that IgG anti-PC binds distinct oxidized epitope containing-determinants on apoptotic cells[15]. Therefore, one explanation could be that the combination of IgM and IgG is most protective.

The complete lack of all B cell subsets (and therefore antibodies) was reported to increase atherosclerosis, both in µMT/*Apoe*$^{-/-}$ mice[46] and after bone marrow transfer of µMT bone marrow into *Ldlr*$^{-/-}$ recipients[47]. In contrast, a recent study showed reduced atherosclerosis in µMT/*Apoe*$^{-/-}$ mice[48]. An alternative strategy resulting in attenuated B cell responses is splenectomy. Splenectomy enhanced atherosclerosis[13,46] and the plaque phenotype was similar to *Ldlr*$^{-/-}$*Xbp1*$^{B\text{-}cKO}$ mice (figure 5). The effect of splenectomy can be reversed by transfer of splenic B cells from *Apoe*$^{-/-}$ mice[46] or by peritoneal B1a cell transplant[13]. Given these discrepancies, and the influence on non-antibody functions of B cells (µMT mice, splenectomy) and remaining adaptive immune responses (*sIgM*$^{-/-}$ mice) in those studies, our model adds important new insight into the influence of endogenous antibody responses. Novel advantages of the model used here include the lack of impact on B cell development and B cell activation (figure 1 and supplemental figure 3) or on cellular T cell responses (figure 2 and supplemental figure 4), which are greatly altered or removed entirely in previous models.

The influence of CD4$^+$ T cells on atherosclerosis is well known to depend on the opposing influences of different subsets of effector T cells. Th1-polarized CD4$^+$ T cells producing cytokines such as IFN-γ, IL-12 and IL-18 promote atherosclerosis, whereas regulatory T cells producing TGF-β and IL-10 dampen plaque growth[49,50]. Here, we also suggest that CD4$^+$ T cells influence atherosclerosis through regulation of antibody responses. Anti-CD4 depletion reduced IgM, IgG1 and IgG2c antibody levels but neither total IgG nor IgG3 (figure 1). Anti-CD4 depletion enhanced atherosclerosis, but the combination of anti-CD4 depletion and antibody deficiency had no additive effect on atherosclerosis development (figure 3). Anti-OSE antibodies in WT mice are primarily produced by B1 cells but adaptive B2 cell clones with similar specificity are induced by immunization[51], and based on the presence of class-switched IgG antibodies, also expand and become plasma cells in atherosclerotic mice[52]. In our study, antibodies against OSE were significantly reduced in a similar way to

total antibody levels by both XBP1 deficiency and anti-CD4 depletion. Previously, MDA-LDL immunization induced a prominent Th2-type $CD4^+$ T cell response and an IL-5 dependent increase in anti-oxLDL IgM production[11], which *in vitro* was solely produced in response to IL-5 by B1a cells. Our study suggests that endogenous protective responses to oxidized LDL epitopes may also be enhanced (in a non-cognate manner) by $CD4^+$ T cells. In a similar way to immunization, western diet feeding (as used in this study) can switch the T cell response in atherosclerotic mice from Th1 to Th2[53], and a recent study with human B cells showed that $CD4^+$ T cells greatly enhance anti-MDA and anti-PC antibody production *in vitro*[54]. We therefore conclude that $CD4^+$ T cells may play an important and significant role in anti-atherogenic antibody production. Given the previously demonstrated importance of regulatory T cells[55], it is possible that part of the pro-atherogenic effect of anti-CD4 depletion may be due to the loss of this protective influence, known to be more prominent during the time course of this experiment[56]. Indeed, in the aortic arch, but not the aortic root, there was still a (less prominent) increase in atherosclerosis after in CD4 depletion in *Ldlr*$^{-/-}$*/Xbp1*^{cKO} mice (figure 4C). However, antibody deficiency did not appear to influence the regulatory T cell system (figure 3F), and anti-CD4 depletion did not further increase lesion size or lesion composition in the aortic root of *Ldlr*$^{-/-}$*/Xbp1*^{cKO} mice (figure 5 and supplemental figure 5), suggesting a prominent role for T cell-dependent antibody responses in regulating atherosclerotic development.

A major protective influence of antibodies on atherosclerosis may derive from the clearance of oxLDL and/or secondary necrotic cellular debris. Consistent with this, the major observed difference in plaque composition in Ldlr$^{-/-}$/*Xbp1*^{cKO} mice was a significant increase in TUNEL staining and necrotic core area (figure 5). As plaques progress, smooth muscle cells become increasingly synthetic and produce a fibrous cap of collagen important for plaque stability[57]. Here, the increased lesion size in the absence of antibodies was associated with decreased proportions of collagen. This further supports the hypothesis that a greater rate of lipid/dead cell accumulation led to enhanced atherosclerosis. IgM antibodies may only be important for efferocytosis in certain contexts[58], for example in atherosclerosis where pathways mediating rapid and early apoptotic cell uptake may be defective. Importantly, OSE-specific IgM have been shown to enhance the clearance of apoptotic cells by macrophages *in vivo*[59,60]. Recently, increased anti-phagocytic CD47 on the surface of plaque cells and loss of surface Mer tyrosine kinase on plaque phagocytes have been shown to play important roles in mouse and human atherosclerosis[61,62]. These defects lead to secondary necrosis, making cells and oxLDL debris recognizable to anti-MDA and anti-PC antibodies. These antibodies provide an alternate protective layer that is nevertheless insufficient to prevent atherosclerosis progression, however the lack of such antibodies greatly accelerates plaque growth.

The use of the bone marrow transfer model has both advantages and disadvantages, and was reported to significantly modulate plaque development[63]. A developmental lack of antibodies and or B cells could have significant influence in lymphoid system development and, for example, alternative pathways that regulate apoptotic cell clearance may be upregulated. Such changes would be avoided using bone marrow transfer. However, bone marrow transfer also induces some changes in the B cell compartment, for example in the balance between B1a and B1b cells in the periphery. Nevertheless, similar and corroborating findings from BMT and non-BMT studies involving B cells[29,30] suggest this model continues to provide valuable information. Future studies are required to understand the specific contribution of CD4+ T cells and the relative atheroprotective contributions of antibody binding to oxLDL versus secondary necrotic cells.

In summary, the T cell-dependent humoral response can be protective. Our knowledge of the close reciprocal relationship between adaptive B and T cells, and links with innate responses, is rapidly evolving. This will enable not only novel and more advanced vaccine strategies for atherosclerosis, but also help us to understand the impacts of the growing number of immunotherapies used in patients at high risk for cardiovascular disease.

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Disclosures

None

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Figure 1

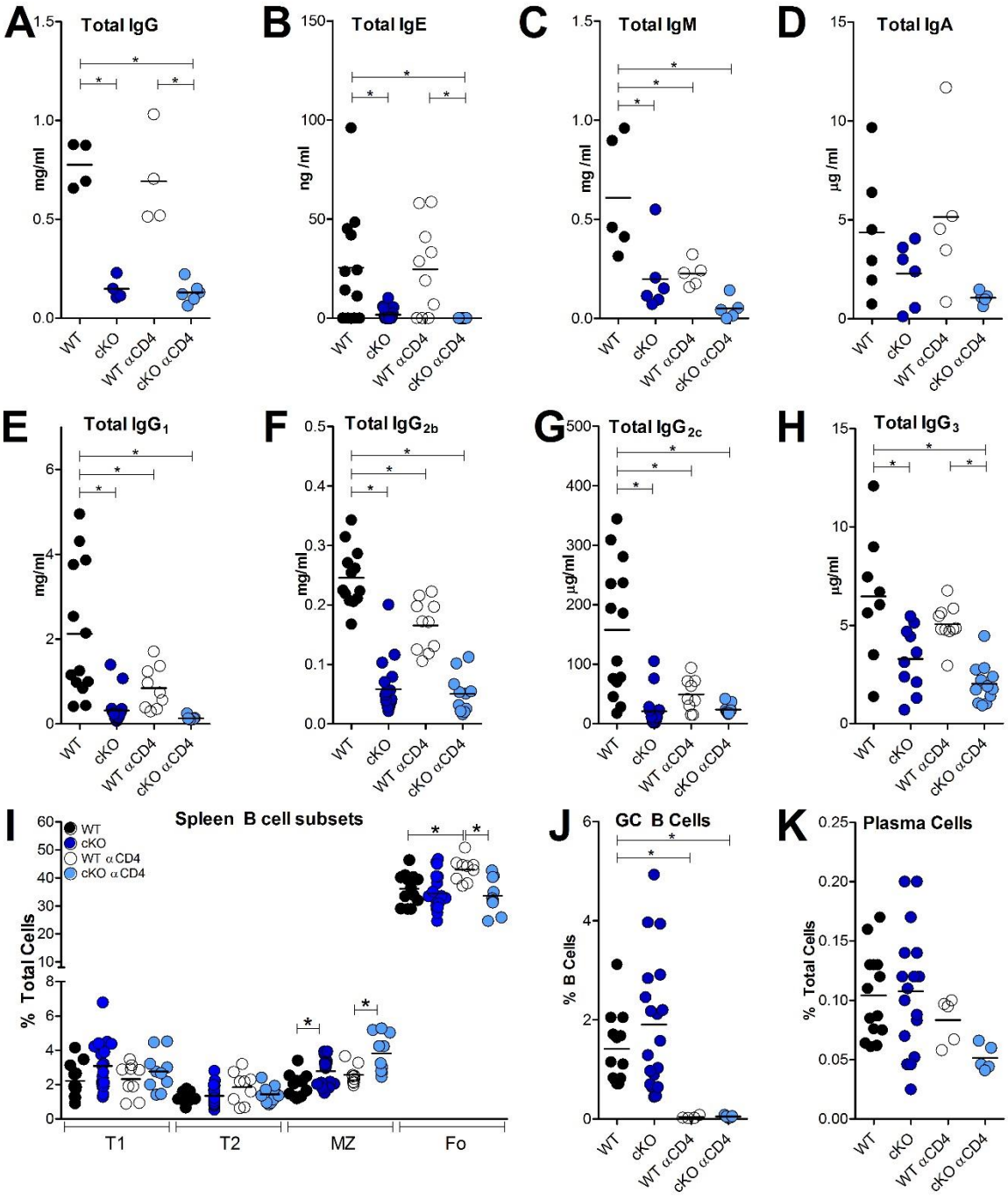

Figure 1. B cell deletion of Xbp1 in atherosclerotic mice specifically attenuates antibody production.

Antibodies and B cell system in Ldlr/WT (WT) or Ldlr/XBP $^{B\text{-}cKO}$ mice tread with (αCD4) or without anti-CD4 antibodies. **A-H.** Serum levels of specific antibody isotypes. **I**. B cell subsets as defined by flow cytometry (supplemental table 2) in spleen. **J**. Spleen germinal center B cells. **K**. Bone marrow plasma cells. *$p<0.05$.

Figure 2

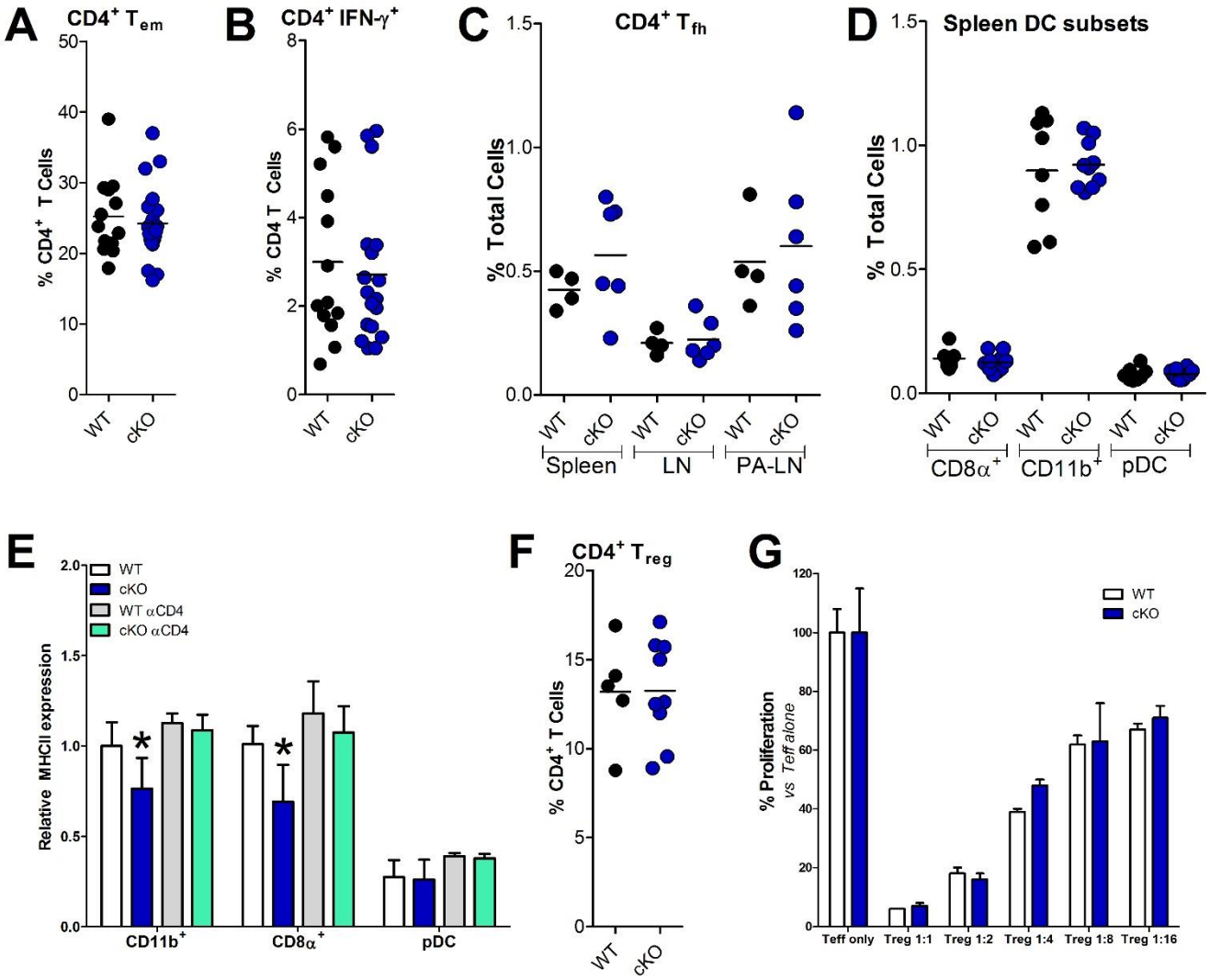

Figure 2. B Cell XBP1 deficiency does not modulate systemic T cell immune responses.

T cell activation in Ldlr/WT (WT) or Ldlr/XBP1$^{B\text{-}cKO}$ mice tread with (αCD4) or without anti-CD4 antibodies. **A**. Spleen effector (CD62L^{-} CD44hi) CD4 T cells. **B**. Spleen CD4 T cells producing IFN-γ, as detected with intracellular flow cytometry (see methods). **C**. Spleen, lymph node (LN) and para-aortic lymph node (PA-LN) follicular helper CD4 T cells (CXCR5^{+} PD-1^{hi}). D. Spleen dendritic cell subsets. **E**. Relative mean fluorescence intensity (MFI) staining for MHCII on splenic dendritic cells (data from 3 independent experiments). **F**. Splenic regulatory T cells (CD25^{+} Foxp3^{+}). **G**. Proliferation of purified splenic effector T cells in the presence of different ratios of regulatory T cells (expressed as % proliferation of Teff only). *$p<0.05$.

Figure 3

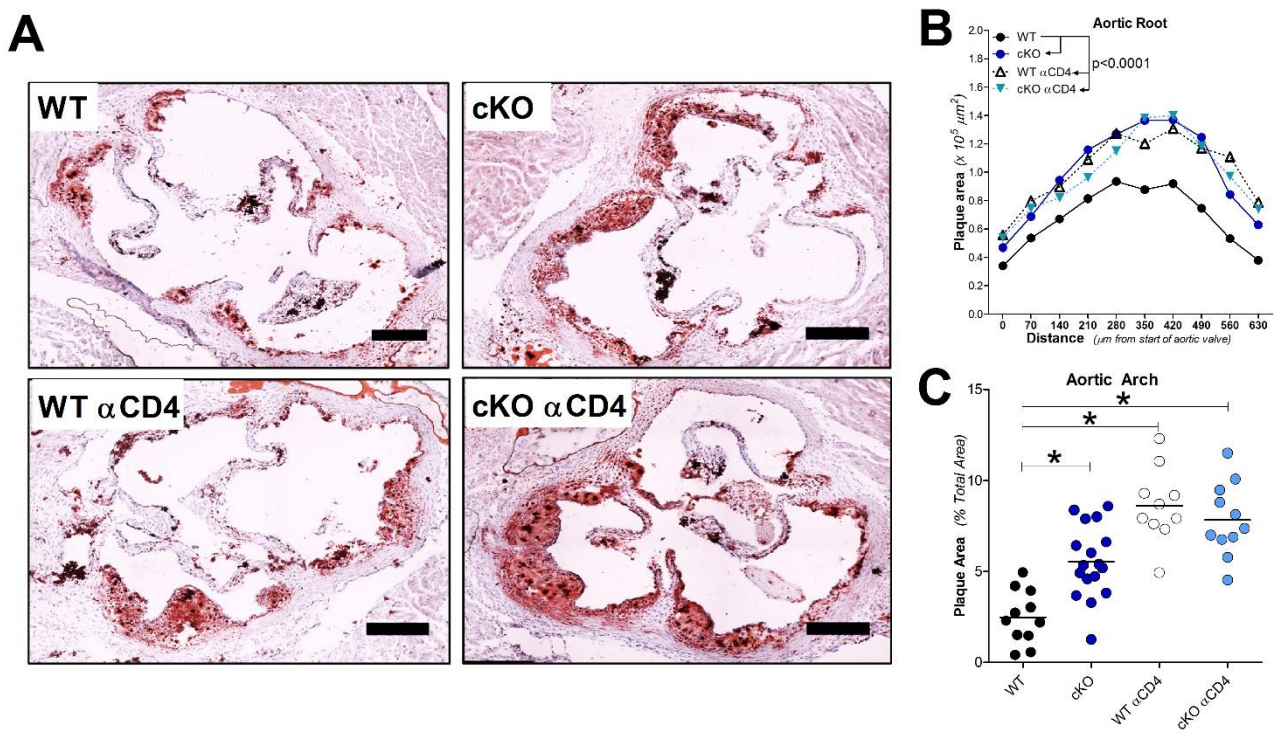

Figure 3. Xbp1 deficiency in B cells enhances atherosclerosis

A. Oil Red O-stained cryosections of aortic root plaques from Ldlr/WT (WT) or Ldlr/XBP1$^{B\text{-}cKO}$ mice tread with (αCD4) or without anti-CD4 antibodies. **B**. Quantification of aortic root atherosclerosis. Data represents group average for each of 10 sections beginning at the aortic valves. N numbers: WT: 12, cKO: 19, WT/αCD4: 10, cKOαCD4: 12. **C**. Quantification of aortic arch atherosclerosis analysed by en face oil red O staining (*$p<0.05$).

Figure 4

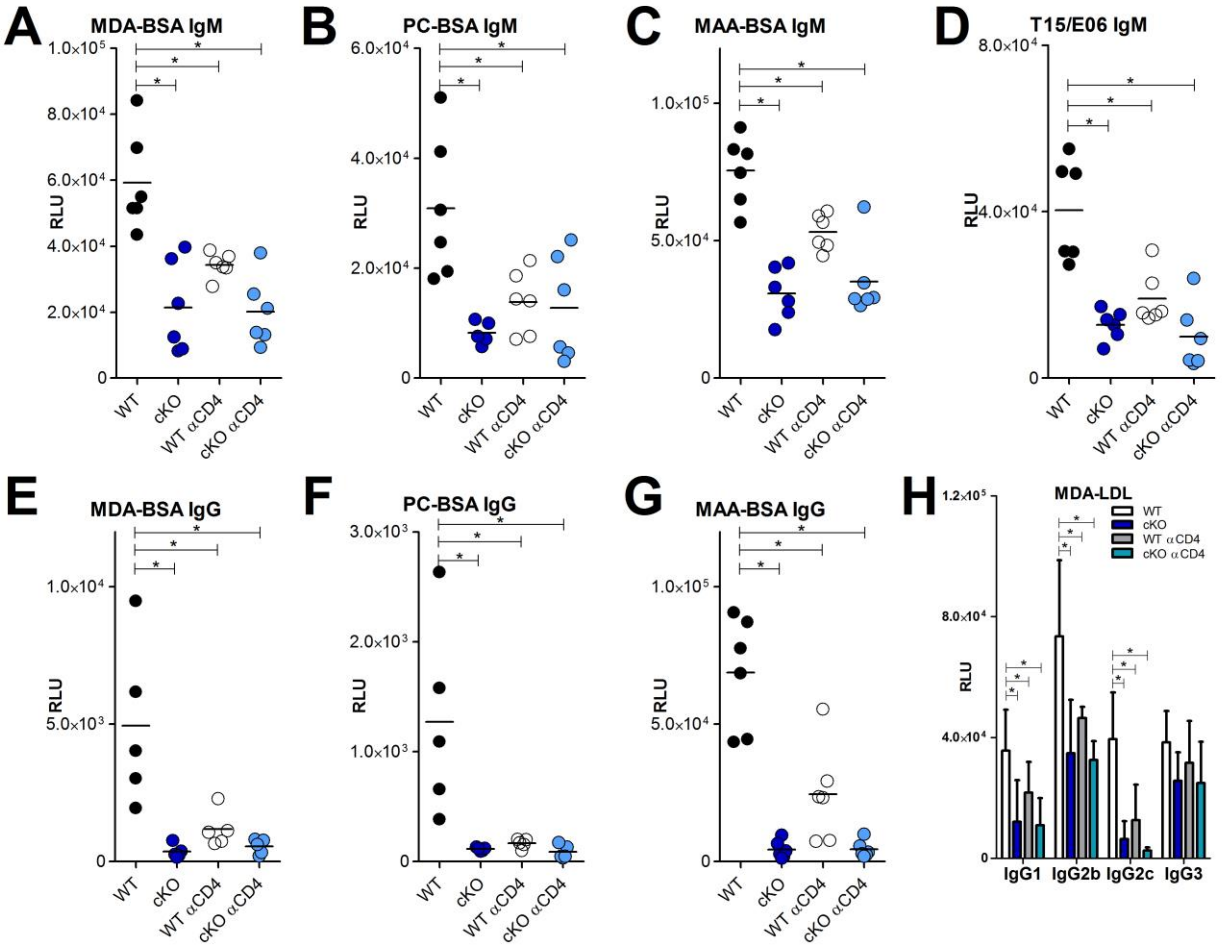

Figure 4. B cell deletion of Xbp1 in atherosclerotic mice attenuates anti-OSE antibody production.

Serum titres of anti-OSE antibodies in Ldlr/WT (WT) or Ldlr/XBP $^{B\text{-}cKO}$ mice tread with (αCD4) or without anti-CD4 antibodies. **A-C**. IgM titres against MDA, MAA and PC. **D**. T15 (AB1-2) IgM antibody titres. **E-G.** Total IgG titres against MDA, MAA and PC. **H**. IgG isotype titres against MDA-LDL. **p<0.05*.

Figure 5

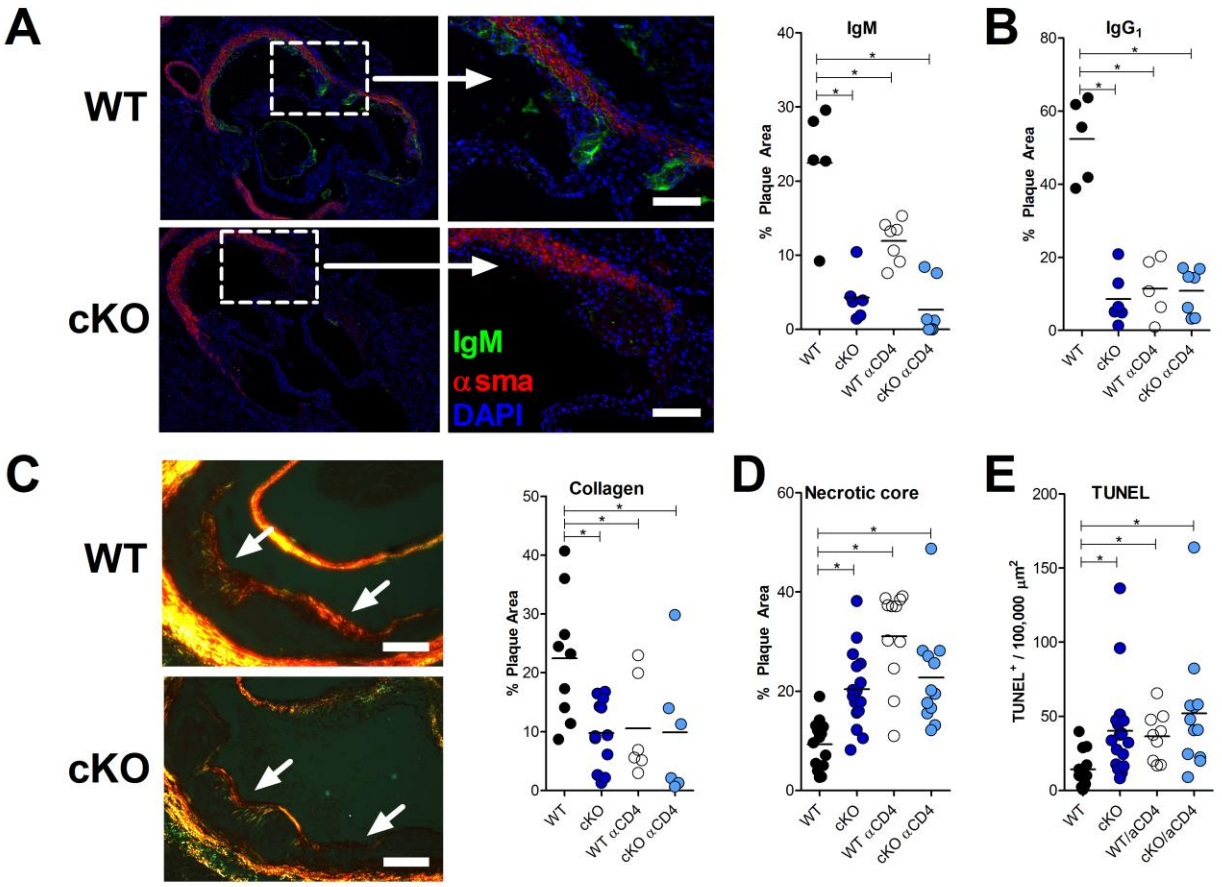

Figure 5. B Cell XBP1 deficiency increases apoptotic cell accumulation and necrotic core

Analysis of plaque composition in aortic root cryosections from Ldlr/WT (WT) or Ldlr/XBP1$^{B\text{-}cKO}$ mice tread with (αCD4) or without anti-CD4 antibodies. **A-C**. Proportions of positive staining for total IgM (***A***), IgG1 (***B***), or collagen (picrosirius red staining viewed under polarized light) (***C***). **D**. Necrotic core area. **E**. TUNEL staining, expressed per 100,000µm^2. *$p<0.05$.