B-1b Cells Secrete Atheroprotective IgM and Attenuate Atherosclerosis

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ABSTRACT

**Rationale:** B cells contribute to atherosclerosis through subset specific mechanisms. Whereas some controversy exists about the role of B-2 cells, B-1a cells are atheroprotective due to secretion of atheroprotective IgM antibodies independent of antigen. B-1b cells, a unique subset of B-1 cells that respond specifically to T cell-independent antigens, have not been studied within the context of atherosclerosis.

**Objective:** To determine whether B-1b cells produce atheroprotective IgM antibodies and function to protect against diet induced atherosclerosis.

**Methods and Results:** We demonstrate that B-1b cells are sufficient to produce IgM antibodies against oxidation specific epitopes (OSE) on LDL both in vitro and in vivo. Additionally, we demonstrate that B-1b cells provide atheroprotection after adoptive transfer into B and T cell deficient (Rag1−/− Apoe−/−) hosts. We implicate Id3 in the regulation of B-1b cells as B cell-specific Id3 knockout mice (Id3BKO Apoe−/−) have increased numbers of B-1b cells systemically, increased titers of OSE-reactive IgM antibodies, and significantly reduced diet-induced atherosclerosis compared to Id3WT Apoe−/− controls. Finally, we report that the presence of a homozygous SNP in ID3 in humans that attenuates Id3 function is associated with an increased percentage of circulating B-1 cells and anti-MDA-LDL IgM suggesting clinical relevance.

**Conclusions:** These results provide novel evidence that B-1b cells produce atheroprotective OSE-reactive IgM antibodies and protect against atherosclerosis in mice, and suggest that similar mechanisms may occur in humans.

**Keywords:** Atherosclerosis, B-1b cells, antibodies, lymphocyte, oxidation specific epitopes.

**Nonstandard Abbreviations and Acronyms:**
OSE   Oxidation-specific epitopes
BAFFR  B cell activating factor receptor
Id3   Inhibitor of differentiation 3
NAb   Natural antibodies
NHC   Natural helper cells
Id3BKO   B cell specific knockout of Id3
Id3WT   wildtype littermates to Id3BKO mice
TUNEL   Terminal deoxynucleotidyl transferase dUTP nick end labeling
LPS   Lipopolysaccharide
MDA   Malondialdehyde
CuOx   Copper oxidized
PC   Phosphorylcholine
OxPL   Oxidized phospholipid
PPS-3   Pneumococcal polysaccharide
INTRODUCTION

Murine studies provide clear evidence that B cells regulate atherosclerosis\(^1\).\(^2\) and that this effect is subset dependent. While much evidence supports a pro-atherogenic role of B-2 cells, there are experimental data supporting a protective role as well\(^1\).\(^3\)-\(^7\). Alternatively, there is near uniform agreement of an atheroprotective role for B-1a cells\(^8\)-\(^11\).

B-1 and B-2 cells are developmentally and functionally distinct subsets in mice\(^12\). B-1 cells produce the majority of total circulating IgM\(^13\), which they secrete in a T cell independent manner\(^14\). B-1 cells are further subclassified by the surface expression of CD5; B-1a cells being CD5\(^+\) and B-1b cells being CD5\(^-\). B-1a cells produce so-called germ line encoded IgM that harbor few non-templated insertions, termed natural antibodies (NAb), in response to non-antigenic activation. A substantial proportion of these antibodies bind oxidation-specific epitopes (OSE), as found on oxidized LDL (OxLDL), as well as apoptotic cells, and reduce the development of atherosclerosis\(^15\)-\(^17\). In contrast, B-1b cells are activated by both non-antigenic and antigen-dependent stimuli, producing antigen specific IgM, and to a smaller extent IgG3, and subsequently become TI memory B cells\(^18\),\(^19\). Whether B-1b cells can secrete atheroprotective OSE reactive IgM antibodies or provide protection against atherosclerosis remains undetermined.

Previous work in our lab has demonstrated that global deletion of inhibitor of differentiation 3 (Id3) resulted in early and significantly increased atherosclerosis\(^7\). Id3 is a member of the helix-loop-helix transcription factor family known to be important in lymphocyte function\(^20\). These global Id3 knockout mice developed equivalent numbers of B-2 cells, but reduced numbers of atheroprotective B-1a cells compared to wild-type\(^21\). However, this was not phenocopied by B cell-specific deletion of Id3, providing evidence that Id3 can regulate B cell subsets and atherosclerosis through effects in non-B cells. Yet the effect of B cell-specific deletion of Id3 on B-1b cells has not been reported.

A putative human equivalent to murine B-1 cells was described by Rothstein and colleagues as CD20\(^+\)CD3\(^-\) B cells that are CD27\(^+\)CD43\(^+\)\(^22\). They demonstrated that these cells functionally resemble murine B-1 cells in that they spontaneously secrete IgM, are enriched in umbilical cord blood, stimulate T cells and have tonic intracellular signaling. Our lab previously published that a non-synonymous single nucleotide polymorphism (SNP) in the coding region of the ID3 gene (rs11574 G\(\rightarrow\)A) causes an amino acid change (A\(\rightarrow\)T) and this results in decreased Id3 binding to E proteins\(^23\). Whether individuals harboring the homozygous SNP have a modified proportion of B-1 cells or OSE reactive IgM is unknown.

In the present study, we addressed the role of B-1b cells in atherosclerosis by demonstrating that B-1b cells are sufficient to produce atheroprotective antibodies reactive to OSE and that transferring B-1b cells into B and T cell deficient hosts attenuates atherosclerosis. Furthermore, we report that B cell-specific Id3 knockout (Id3\(^{BKO}\)ApoE\^-\^-\) mice develop a systemic increase in B-1b cells, increased titers of atheroprotective, OSE reactive IgM antibodies, and attenuated atherosclerosis. Finally, we report that a cohort of patients bearing the rs11574 SNP within ID3 have an increased proportion of circulating B-1 cells and OSE reactive IgM, suggesting possible clinical relevance for our murine findings in humans.
METHODS

Mice.
All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. Id3^{null} mice were a generous gift of Dr. Yuan Zhang (Duke University). CD19^{Cre/+} mice and Rag1^{-/-} mice were provided by Dr. Timothy Bender (University of Virginia). Apoe^{-/-} mice were purchased from Jackson Laboratory. All mice, purchased or generated, were backcrossed at least 10 generations to C57BL/6J mice.

Serum cholesterol determination.
Cholesterol levels were determined as previously described by the University of Virginia Medical laboratories.

Analysis of atherosclerotic lesions.
Hearts and aortas were removed and prepared as previously described. Briefly, hearts were embedded in OCT compound (Tissue-Tek) and snap frozen. Serial 5µm sections were cut by Cryostat (Leica biosystems) from the beginning of the three aortic leaflets to the aortic arch. Aortas were fixed in 4% paraformaldehyde then opened longitudinally, pinned, and stained using Sudan IV (Sigma). Plaque areas were assessed using Image-Pro Plus software (Media Cybernetics). For aortic sinus measurements, maximum plaque area was used for comparison. For aortic plaque measurements, the percentage of positive staining was used for comparison.

Immunofluorescence and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis of aortic sinus sections.
Slides of the aortic sinus, described above, were stained for macrophage content as previously described using biotinylated Mac-2 as the primary antibody (Cedarlene CL8942B) and Streptavidin Alexa Fluor 488 as the secondary antibody (Invitrogen Molecular Probes S11223) then counterstained with DAPI and mounted (Vectashield H-1500). For staining of apoptotic cell bodies the TUNEL method was used following the protocol from ApopTag Peroxidase In Situ Apoptosis Detection Kit (emdMillipore S7100). Imaging for both was done using an Olympus BX51 high magnification light microscope. Images were analyzed using ImageJ (http://imagej.nih.gov/ij/).

Adoptive transfer of B-1b and B-1a cells into Rag1^{-/-} Apoe^{-/-} hosts.
Following fluorescence-activated cell sorting (FACS) from the PerC of Apoe^{-/-} mice, 1x10^5 B-1b or B-1a cells were transferred interperitoneally (IP) into 8-week-old, male, Rag1^{-/-} Apoe^{-/-} mice. Mice were maintained on chow diet for one to four weeks following transfer then switched to Western diet for 16 weeks at the end of which time the animals were euthanized and hearts were collected for histological analysis of atherosclerotic lesions as described above.

Immunization.
Mice were immunized with DNP-KLH as described previously. Briefly, 100µg DNP-KLH in complete Freund’s adjuvant were injected IP. 21 days later mice were boosted with DNP-KLH in PBS. Blood was collected at days 0, 7, 21, and 28. Bone marrow was collected at time of euthanization.

Preparation of tissues for flow cytometry and cell sorting.
PerC cells, splenocytes, PBMCs and bone marrow (BM) cells were harvested and processed for flow cytometry as previously described.

In vitro stimulation assays.
Post electronic cell sorting, Apoe^{-/-} B-1b and B-1a cells were plated at 1x10^6 cells per well in a 96 well plate in 200 µl of B cell culture media with 50µg/mL lipopolysaccharide (LPS) (Sigma, L4391) or PBS for 72 hours. The supernatant was collected for measurement of immunoglobulins by ELISA.
**Enzyme-linked Immunosorbent Assay (ELISA).**
Specific Ab levels to given antigens in plasma from mice or humans were determined by chemiluminescent ELISA as previously described. Data were expressed either as µg/ml, based on standard curves of isotype standards (Online Figure I), or relative light units (RLU/100ms) where quantitative standards were unavailable. For these epitopes, dilution curves are shown in the supplement (Online Figure I).

**ELISPOT.**
BM cells were added to sterile MultiScreen IP-Plates (Millipore, MSIPS4510) according to manufacturer’s protocol after coating wells with unlabelled anti-mouse IgM antibody (Southern Biotech). IgM producing cells were visualized using streptavidin alkaline phosphatase (Abcam) followed with BCIP/NBT. Each spot represented an IgM secreting cell.

**Human genotyping.**
Id3 SNP (rs11574, Assay ID# C_2462609_10) genotyping was performed using the ABI Taqman SNP Genotyping assay from LifeTechnologies. All genotypes were analyzed and assigned automatically using the ABI SDS 2.3 software.

**Statistical methods.**
Data was analyzed using Prism 6.0b (GraphPad Software, Inc.). Results are displayed containing all replicated experiments and values shown are mean ± SEM.

**RESULTS**

**B-1b cells secrete MDA-LDL and CuOx-LDL IgM in response to TLR stimulation.**
B-1 cell-mediated atheroprotection has been strongly linked to the production of atheroprotective IgM. In particular, IgM that bind OSE on OxLDL, such as anti-MDA-LDL and anti-CuOx-LDL, are considered to be atheroprotective. To test whether B-1b cells have the capacity to secrete OSE targeted IgM and how that compares to B-1a cells, B-1b cells (CD19+B220+/-IgMhiCD23-CD5-) and B-1a cells (CD19+B220+/-IgMhiCD23-CD5-) were sorted at greater than 99% purity from PerC of apolipoprotein E deficient (Apoe⁻/⁻) mice and placed in cell culture with the TLR4 ligand LPS, which has been shown to stimulate antibody secretion from B-1 cells. Absolute concentration of IgM and relative concentration of anti-MDA-LDL and anti-CuOx-LDL IgM were measured using established chemiluminescent ELISA and calculated against IgM standard and pooled dilution series respectively (Online Figure IA). The data demonstrate that stimulation induced a robust IgM response from both B-1b and B-1a cells (Figure 1B). Additionally, IgM reactive to MDA-LDL and CuOx-LDL was greatly increased for both B-1b and B-1a cells compared to unstimulated controls, though cultured B-1b cells secreted lower titers compared to B-1a cells (Figure 1C&D). Titers of IgG3 were found to be unmeasurable in the culture supernatant (data not included). These data reveal that B-1b cells produce OSE-reactive IgM, though at lower titers than B-1a cells in culture.

**B-1b cells secrete measurable titers of OSE reactive IgM in vivo.**
To determine if B-1b cells secrete OSE reactive antibodies in vivo, PerC B-1b and B-1a cells were sorted from Apoe⁻/⁻ mice in the same manner as for in vitro testing. Recombination activation gene 1 deficient (Rag1⁻/⁻) mice, which are devoid of T and B cells, crossed with Apoe⁻/⁻ mice (Rag1⁻/⁻Apoe⁻/⁻) were injected with equal numbers (1x10⁵) of purified B-1b or B-1a cells, or PBS control (Figure 1E). Blood was collected four weeks after transfer and absolute titers of IgM and relative titers of IgM reactive to MDA-LDL and CuOx-LDL were calculated against IgM standard and pooled dilution series respectively.
(Online Figure IB). As expected, PBS-injected Rag1"⁻/⁻ Apoe"⁻/⁻ mice had undetectable titers of IgM while both B-1b and B-1a injected mice produced measurable titers of IgM and OSE-reactive IgM. Notably, in contrast to in vitro findings, B-1b recipient mice had higher titers of total IgM and MDA-LDL IgM than B-1a recipient mice (Figures 1F-G). Titers of CuOx-LDL IgM were equivalent compared to B-1a recipients (Figure 1H).

**B-1b cells confer atheroprotection to Rag1"⁻/⁻ Apoe"⁻/⁻ recipients.**

To determine whether B-1b cells function in an atheroprotective manner, B-1b cells were sorted as described above and transferred into Rag1"⁻/⁻ Apoe"⁻/⁻ hosts. The recipient mice were then fed a Western diet for 16 weeks. Weight and lipid analysis demonstrated that B-1b cell adoptive transfer did not significantly modify weight gain, or plasma total cholesterol, triglycerides, or HDL cholesterol levels compared to PBS controls (Online Table I). Flow cytometry was used to demonstrate that the transferred B-1b cells populated the recipient mice and that their immunophenotype did not change over the course of the 16 week diet as the transferred cells did not upregulate CD23 or CD5 on their cell surface (Figure 2B&C, Online Figure IIA&B). An average of 3.6x10⁴±0.7 CD19⁺ B cells were recovered from the PerC of the recipient mice (n=9) of which <1% were CD23⁺ and 3.7% were CD5⁺, though with lower CD5 expression than on B-1a cells from Apoe"⁻/⁻ mice. B-1b cells were also detected in the spleen, although in fewer numbers, while very few transferred cells were found in the blood (Online Figure IIB). Plasma was collected from recipient mice and absolute titers of IgM, IgG1, IgG2c, and IgG3 were measured by ELISA and calculated in comparison to immunoglobulin standards (Online Figure IIC). High titers of IgM were measured in host mice that received B-1b cells with very low levels of IgG2c and IgG3, whereas IgG1 was not detectable (Figure 2D). Additionally, relative titers of IgM reactive to native LDL (nLDL), MDA-LDL, CuOx-LDL, α-1,3-dextran, and E06/T15, (the NAb to the phosphorylcholine (PC) moiety of oxidized phospholipid (OxPL)) were measured based on pooled dilution series (Online Figure IID). The data demonstrate that the B-1b recipient mice maintained high titers of IgM antibodies reactive to MDA-LDL and CuOx-LDL as well as low titers of IgM reactive to E06/T15 (Figure 2D). Very low levels of IgM to nLDL were noted, which may reflect binding to spontaneously generated oxidative epitopes on LDL plated on the microtiter wells (Figure 2D).

Importantly, levels of IgM reactive to α-1,3-dextran, which is a classic TI bacterial surface antigen that was used as a non-OSE type antigen control, were not measurable (Figure 2D).

To compare atherosclerotic plaque size, aortic sinuses were collected for histochemical measurement of cross sectional plaque area demonstrating that B-1b recipient mice exhibited significantly reduced atherosclerosis compared to PBS recipient controls (2.05x10⁵ ± 0.26x10⁵ µm² vs 3.16x10⁵± 0.51x10⁵ µm², p<0.05, Figure 2E&F). Comparison of plaque size to total cholesterol demonstrated that there was no correlation for these values (Online Figure IIC). These data, taken together, provide evidence that B-1b cells produce atheroprotective IgM and attenuate atherosclerosis independent of T cells or other B cells.

**B cell-specific Id3 deficiency increases B-1b cell numbers in peritoneum, spleen, and blood.**

We had previously implicated Id3 in B cell-mediated atheroprotection utilizing adoptive transfer of CD43⁺ splenocytes into uMT Apoe"⁻/⁻ mice as no mouse with B cell-specific deletion of Id3 was available at that time. To directly assess the loss of Id3 specifically in B cells in an atherogenic Apoe"⁻/⁻ mouse, we generated B cell-specific Id3 knockout mice (Id3BKO) by crossing Id3fl/fl mice with CD19cre/+ mice and then bred these with Apoe"⁻/⁻ mice (Id3BKO Apoe"⁻/⁻) and confirmed the deletion with Western blot analysis as previously reported. PerC, spleen, and blood were collected from 8-week-old, male, Id3BKO Apoe"⁻/⁻ and littermate control (Id3WT Apoe"⁻/⁻) mice and B-2, B-1a, and B-1b cell subsets quantified by flow cytometry. Representative flow cytometry with our gating strategy to differentiate these subsets is depicted in Figure 3A. Id3BKO Apoe"⁻/⁻ mice contained significantly greater numbers of B-1b cells compared to Id3WT Apoe"⁻/⁻ controls. No differences in the number of B-1a or B-2 cells were detected (Figure 3B-D). Importantly, the
comparable numbers of B-1a cells suggest that deletion of Id3 did not result in down regulation of CD5 expression. Additionally, CD4 and CD8 T cell numbers were not modified (Online Figure III).

**Id3^BKO^Apoe-/^- mice have increased BM IgM producing cells and B-1b cells.**

It was recently reported that a large proportion of IgM producing B-1 cells are found in the BM of mice. To determine whether Id3^BKO^Apoe-/^- mice had increased numbers of IgM producing cells in their BM, total BM cells were extracted from leg bones of 8-week-old mice and placed into ELISPOT wells (Figure 4A). Id3^BKO^Apoe-/^- mice had significantly more IgM^+^ immunoglobulin secreting cells (ISC) in their BM than Id3^WT^Apoe-/^- mice (Figure 4B&C). A portion of the BM tissue was also analyzed by flow cytometry using the gating strategy published by Baumgarth and colleagues (Online Figure IV). BM from Id3^BKO^Apoe-/^- mice contained significantly more total B-1 cells compared to Id3^WT^Apoe-/^- mice and the difference was entirely attributable to increased B-1b cells (Figure 4D). Finally, it was determined that Id3^BKO^Apoe-/^- mice of the same age had significantly higher titers of IgM compared to Id3^WT^Apoe-/^- mice (Figure 4E). These findings demonstrate that the increased B-1b population of Id3^BKO^Apoe-/^- mice is also detected within the BM, which is responsible for a large proportion of circulating IgM.

**Id3^BKO^Apoe-/^- mice exhibit reduced Western diet induced atherosclerosis.**

To determine if B cell-specific deletion of Id3 resulted in reduced development of atherosclerosis, Id3^BKO^Apoe-/^- and Id3^WT^Apoe-/^- mice were fed a Western diet for 16 weeks, and then aortic sinuses and aortas were harvested for histochemical analysis of cross sectional lesion area and aortic lipid deposition respectively (Figure 5A). Id3^BKO^Apoe-/^- mice exhibited significantly reduced atherosclerosis within the aortic root compared to Id3^WT^Apoe-/^- mice (3.99±10^5 ±1.91±10^5 µm^2 vs. 4.96±10^5 ±1.91±10^5 µm^2, P<0.01; Figure 5B&C). Additionally, immunofluorescent macrophage staining using an anti-Mac2 antibody identified significantly decreased macrophage content within lesions of Id3^BKO^Apoe-/^- mice compared to Id3^WT^Apoe-/^- (7.18 ± 2.23% vs. 18.48 ± 3.53%, P<.05, Figure 5D&E) Finally, Tunel staining demonstrated that lesions in Id3^BKO^Apoe-/^- mice contained a significantly decreased percentage of apoptotic cell bodies than did Id3^WT^Apoe-/^- (0.37±10^3 ±0.12±10^3 % vs. 0.84±10^3 ±0.17±10^3 %, P<0.05, Figure 5F&G). *En face* aortic preparation with Sudan IV staining demonstrated that the aortas of Id3^BKO^Apoe-/^- mice had reduced lipid deposition, though not statistically significant, compared to Id3^WT^Apoe-/^- (10.62 ± 1.17% vs 12.58 ± 0.95%, P=0.10, Figure 5H&I) Lipid analysis demonstrated that there were no significant differences in body weight, weight gain, or plasma lipids at time of euthanasia (Online Table II). Additionally, B-1b cell number remained greater in Id3^BKO^Apoe-/^- mice after the 16 weeks of Western diet feeding (Online Figure V). Interestingly, although not different at baseline (Figure 3), the number of PerC B-2 cells was greater in Id3^BKO^Apoe-/^- mice compared to WT controls after Western diet feeding (Online Figure V). This effect was not seen in the blood or spleen and is of unclear consequence.

**B cell-specific deletion of Id3 increases circulating anti-OSE IgM antibodies.**

OSE IgM reduce OxLDL uptake by lesion macrophages, slowing the development of foam cells and the expansion of intimal plaques, while also binding apoptotic cell bodies, enhancing their clearance and decreasing sterile inflammation. The finding that Id3^BKO^Apoe-/^- mice had decreased intimal macrophage content and fewer apoptotic cell bodies suggests that IgM antibodies could be involved in the atheroprotection exhibited in those mice. To determine if total IgM and OSE reactive IgM were increased in Id3^BKO^Apoe-/^- mice compared to Id3^WT^Apoe-/^-, we measured total IgM and IgG, as well as IgM reactive to the OSE described in Figure 2D in the respective mice. Absolute titers of immunoglobulins were determined using standard curves (Online Figure IC). Id3^BKO^Apoe-/^- mice had significantly higher titers of total IgM compared to Id3^WT^Apoe-/^- controls with no differences detected in titers of IgG isotypes (Figure 6A). Id3^BKO^Apoe-/^- mice also contained significantly greater relative IgM titers reactive to MDA-LDL, and CuOx-LDL than Id3^WT^Apoe-/^- mice (Figure 6B). An approximately 30% increase in the amount of the E06/T15 NAb was also detected though it was not statistically significant (Figure 6B). No significant
difference was detected in IgM reactive to α-1,3-Dextran (Figure 6B, 32). Again, low levels of IgM to nLDL were noted (Figure 6B). Pooled dilution series were used for all relative titers (Online Figure IE). Importantly, real-time PCR analysis of secreted IgM (sIgM) mRNA, expressed per cell, from sorted B cell subsets from Id3BKO Apoe−/− and Id3WT Apoe−/− mice demonstrated no differences in expression suggesting that Id3 does not directly regulate IgM produced per cell (Online Figure VII). Rather, taken together, data suggest that B cell-specific Id3 deletion results in an increased number of IgM producing B-1b cells that generate OSE reactive IgM.

To determine whether loss of Id3 in Apoe−/− mice might also modify the T cell dependent (TD) immune response, Id3BKO Apoe−/− mice were immunized with DNP-KLH in complete Freund’s adjuvant and bone marrow plasma cells and plasma were analyzed (Online Figure VIA). Greater numbers of IgM+ plasma cells (PC) were measured by flow cytometry in Id3BKO Apoe−/− mice compared to Id3WT Apoe−/− mice, both those immunized with DNP-KLH and adjuvant controls (Online Figure VIB) supporting the findings of Figure 4 that Id3 is important for BM IgM production. In contrast, there were no differences in other isotype PCs providing evidence that the increase in BM IgM producing cells is not due to inhibition of isotype switching. Analysis of serum anti-DNP antibodies by ELISA demonstrated a T cell-independent increase in IgM in Id3BKO Apoe−/− mice (Online Figure VIC). Both Id3BKO Apoe−/− and Id3WT Apoe−/− mice had robust antigen specific IgG1, IgG2a, and IgG2c responses, although the IgG1 and IgG2c responses were blunted in Id3BKO Apoe−/− mice. IgG3 was significantly increased in Id3BKO Apoe−/− mice likely due to B-1b isotype switching as has been previously demonstrated 33. These findings provide evidence that the predominant effect of B cell-specific deletion of Id3 is enhanced T cell-independent IgM production possibly due to B-1b derived PCs as have been described previously 29.

Humans harboring SNP rs11574 in ID3 have increased proportion of circulating B1 cells.

We have previously shown that presence of the SNP rs11574 encodes an Id3 protein with attenuated function 23. To determine whether patients bearing the homozygous allele of rs11574 that alters Id3 function have increased B-1 cells in circulation, human peripheral blood mononuclear cells (PBMCs) were analyzed by flow cytometry and genotyped for the presence of the SNP. The flow cytometry strategy utilized was a modification of the original Rothstein strategy 22, incorporating additional stains and gates to eliminate initial concerns about possible contamination by T cells and possible overestimation of numbers. Representative flow cytometry of human B cells is presented in Figure 7A&B. Consistent with results from Rothstein and colleagues 24 27 we detected a small but clear population of circulating CD20+ cells that are live, singlet, CD3-CD27+CD43+. Quantification of the percentage of CD20+ cells from human that are CD27+CD43+ demonstrated that patients homozygous for the minor allele of the rs11574 SNP had a significantly greater percentage of B cells that are CD27+CD43+ cells in circulation (Figure 7B). These patients did not have differences in the proportion of total, naïve, or memory B cells (Figure 7A&B). Additionally, antibody titers against MDA-LDL, which has been previously shown to inversely associate with Framingham risk score, metabolic syndrome criteria 27, and CVD 35, were tested from plasma of these patients. This analysis demonstrated that the patients with the minor allele had significantly higher titers of IgM against MDA-LDL compared to patients with the common allele (Figure 7C). We did not observe any differences in titers of IgG reactive to the same epitopes (Figure 7C). These findings suggest that Id3 may also regulate the relative percentage of B-1 cells in humans, suggesting human relevance of our murine findings.
DISCUSSION

Results of the present study are the first to demonstrate that B-1b cells produce IgM to OSE and attenuate diet-induced atherosclerosis. B-1b cells are developmentally and functionally unique compared to B-1a and B-2 cells. B-1a cells produce IgM NAbs in an antigen-independent manner, whereas B-1b cells respond to T cell-independent antigens providing them the capacity to form memory. The work of Haas et al. and Alugupalli et al. established B-1b cells as the source of T cell-independent memory and support B-1b cells as targets of T cell-independent antigen directed vaccination, which has been discussed as a novel therapeutic against atherosclerosis. As such, understanding the impact of B-1b cells on atherosclerosis and elucidating factors that regulate their activity is of clear importance.

Studies presented here provide novel evidence that B-1b cells produce atheroprotective IgM antibodies. The finding that Rag1^{-/-} Apoe^{-/-} B-1b recipient mice have higher relative titers of OSE reactive IgM compared to B-1a recipients, despite transfer of equal numbers of cells, suggests that either B-1b cells produce more OSE reactive IgM or have better cell viability and persistence in vivo after transfer. IgM antibodies reactive to OSEs on OxLDL are highly conserved and their levels associate with reduced coronary artery disease and cardiovascular events. Their protective function is thought to occur by reducing the uptake of OxLDL by tissue resident macrophages and blocking the proinflammatory properties of oxidized lipid moieties. Additional protective mechanisms are attributed to IgM binding to apoptotic bodies within atherosclerotic plaques which increases their clearance reducing sterile necrosis. Taken together these data suggest that B-1b mediated production of OSE reactive IgM antibodies could be important for their atheroprotective function. Our data underscore the importance of future studies to determine if IgM production is essential for B-1b-mediated atheroprotection and to elucidate other potential atheroprotective pathways.

Of note, the magnitude to which a single injection of B-1b cells reduced atherosclerosis in Rag1^{-/-} Apoe^{-/-} mice was modest. B-1a cells transferred into splenectomized hosts (3 injections of 1x10^5 cells over 8 weeks) resulted in a greater reduction in diet-induced atherosclerosis compared with our one injection of 1x10^5 B-1b cells prior to 16 weeks of Western diet feeding. Studies to directly compare the relative atheroprotective contributions of equal numbers of injected B-1b cells to B-1a cells are underway in our lab. Nevertheless, B-1b cells are not the only cell type mediating atheroprotection they clearly contribute significantly to protection from diet-induced atherosclerosis.

Id3 has been implicated in atherosclerosis in mice and humans. Yet, the specific mechanisms whereby Id3 regulates plaque development are just beginning to be elucidated. Id3 is a broadly expressed transcription factor known to be important throughout development. Previous studies by our lab have reported that global loss of Id3 in Apoe^{-/-} mice leads to enhanced atherogenesis. Follow-on studies demonstrated that Id3^{-/-} Apoe^{-/-} mice had reduced IL-33-stimulated IL-5 production by natural helper cells. This defect, and not loss of Id3 in B cells, led to reduced B-1a cell numbers in Id3^{-/-} Apoe^{-/-} mice. Id3 has also been implicated in the regulation of VCAM-1 expression by vessel wall cells with associated increased accumulation of macrophages in lesions. In addition, Id3 regulates aortic homing of CD43^{-/-} splenocytes (primarily B-2 cells) through regulation of the expression of chemokine receptors such as CCR6. These findings from Id3^{-/-} Apoe^{-/-} mice underscore the importance of defining cell type-specific effects of Id3 and support the use of B cell-specific Id3 knockout mice to identify B cell-specific mechanisms that may impact on atherosclerosis.

An early study by Pan et al suggested that Id3 may be important for the B-2 mediated T cell dependent immune response. In the present study, chow fed Id3^{BKO} Apoe^{-/-} mice responded to T cell-dependent immunization though in a blunted manner (Online Figure V) raising the possibility that loss of Id3 in B-2 cells could also contribute to an atheroprotective phenotype. However, IgG isotype titers were not different between Id3^{BKO} Apoe^{-/-} and Id3^{WT} Apoe^{-/-} mice after Western diet feeding suggesting that the TD antigen response was likely not important for the atheroprotection we saw in our Id3^{BKO} Apoe^{-/-} mice.
is possible that Id3 may regulate activation state or other B-2 functions given that the Id3\textsuperscript{BKO}Apoe\textsuperscript{-/-} is knocked out for all B cell subsets. Thus, it will be important in future studies to establish whether Id3-mediated regulation of B-2 cells is important for their function in atherosclerosis.

Results of the present study confirm prior findings that B cell-specific deletion of Id3 did not alter the B-2 or B-1a cell population\textsuperscript{21} and provide novel evidence that the numbers of B-1b cells in Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} mice are significantly greater than in WT control. Interestingly, in contrast to global knockout Id3\textsuperscript{-/-}Apoe\textsuperscript{-/-} mice, Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} mice exhibited significantly reduced atherosclerosis in the aortic sinus though not in the aorta, possibly due to a physiological difference in the development of atherosclerosis between the sinus and aorta\textsuperscript{45}. Taken together, results provide evidence that Id3 regulates atherosclerosis through unique cell-type dependent mechanisms. Notably, patients both heterozygous and homozygous for the ID3 SNP at rs11574 have increased clinical measures of cardiovascular disease\textsuperscript{23}, yet this same SNP is associated with an apparently specific increase in the percentage of B-1 cells.

The role of B-1 cells in human immunity is poorly understood owing to the lack of a clearly defined human B-1 cell subset. Recently, Rothstein and colleagues have identified a population of CD20\textsuperscript{+} B cells that are CD27\textsuperscript{-}CD43\textsuperscript{+} and possess key features of murine B-1 cells including spontaneous production of IgM \textsuperscript{22, 34}. More recently this subset has been linked to secretion of atheroprotective IgM after T cell-independent immunization \textsuperscript{46}. It is unknown whether the subclassification of B-1 cells into B-1a and B-1b is appropriate in humans as it is in mice. Interestingly, we have shown that a human cohort homozygous for the SNP rs11574, which expresses a modified Id3 protein with reduced function, have increased B-1 cells in circulation as a percentage of total B cells without a difference in total naive, or memory B cells (Figure 7). Additionally, the same patients had increased titers of IgM against MDA-LDL raising the possibility of a functional association although additional studies of larger cohorts will be needed to confirm these findings. Taken together, these findings suggest attenuated Id3 function associates with increased B-1 cells and could serve as a target for enhancing B-1 directed therapies in the future.

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DISCLOSURES
Drs. Tsimikas and Witztum are co-inventors and receive royalties from patents owned by UCSD on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins. Dr. Tsimikas currently has a dual appointment with UCSD and as an employee of Isis Pharmaceuticals. Dr. Witztum is a consultant to Isis Pharmaceuticals, CymaBay and Intercept.
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**FIGURE LEGENDS**

**Figure 1:** B-1b cells produce IgM reactive to MDA-LDL and CuOx-LDL in vitro and in vivo. A) B-1b (CD19^+220^-IgM^+CD23^+CD5^-) and B-1a (CD19^+220^-IgM^+CD23^+CD5^-) cells were sorted from PerC of *Apoe^−/−* mice and placed in culture then stimulated with LPS to induce antibody secretion. Culture media was collected after 72 hours and total IgM, anti-MDA-LDL IgM and anti-CuOx-LDL IgM concentration was determined by ELISA. B-D) Quantification of total IgM (µg/ml) (B), anti-MDA-LDL IgM (RLU/100ms) (C) and anti-CuOx-LDL IgM (RLU/100ms) (D). E) B-1b and B-1a cells were sorted from PerC of *Apoe^−/−* mice and transferred IP into *Rag1^−/−* *Apoe^−/−* mice. Plasma was collected 4 weeks later and measured for total IgM and OSE reactive IgMs by ELISA. F-H) Quantification of total IgM (F), anti-MDA-LDL IgM (G), and CuOx-LDL IgM (H). Data are mean ± SEM.ND = not detectable.

**Figure 2:** B-1b cells produce atheroprotective IgM antibodies and confer atheroprotection to *Rag1^−/−* *Apoe^−/−* recipients. A) PerC B-1b cells were sorted, as described in Figure 1A, from *Apoe^−/−* mice and 1x10⁵ cells (n=9) or PBS (n=6) were injected IP into 8-week-old chow fed, male, *Rag1^−/−* *Apoe^−/−* recipients which were then fed a Western diet for 16 weeks. PerC B cells were recovered for flow cytometry, plasma was collected for antibody measurements and aortic sinuses collected for lesion analysis. B-C) Representative flow cytometry plots and quantification of recovered B-1b cells from PerC of *Rag1^−/−* *Apoe^−/−* hosts after 16 weeks of Western diet feeding. D) Absolute titers of IgM, IgG1, IgG2c and IgG3, quantified in µg/ml, and relative titers of IgM antibodies reactive against native LDL (nLDL), AB1-2 (T15/E06 anti-idiotype), MDA-LDL, CuOx-LDL, and α-1,3-Dextran reported as RLU/100msE) Representative images of maximum lesion area within the aortic sinus as determined by Oil Red O staining from 10x magnification images. F) Quantification of maximum lesion area from aortic sinus sections. Data are mean±SEM, ND = Not detectable, unpaired, two-tailed, Students T-test was used to compare B-1b cell numbers, two-tailed Mann-Whitney test was used to compare lesion areas due to unequal distribution of variance. *p<0.05 **p<0.01.

**Figure 3:** B cell-specific deletion of Id3 results in increased B-1b cell numbers in PerC, spleen, and blood A) Representative flow cytometry plots subsetting B-2 (CD19^+220^high), B-1a (CD19^+220^high/low IgM^+CD23^low/hi CD5^-), and B-1b cells (CD19^+220^mid/low IgM^+CD5^-) from PerC of Id3^WT*Apoe^−/−* and Id3^BKO*Apoe^−/−* mice.. B-D) Quantification of B cell subsets from chow fed, 8-week-old male Id3^WT*Apoe^−/−* (n=12) and Id3^BKO*Apoe^−/−* mice (n=18) from PerC (B), spleen (C), and blood (D). Data are presented as the mean±SEM. Unpaired two-tailed Students T-test was used to compare B cell subsets from PerC, two-tailed Mann-whitney test was used to compare spleen and blood samples after D’Agostino & Pearson normality test revealed non-parametric distribution. *p<0.01. ***p<0.001. ****p<0.0001.

**Figure 4:** B cell-specific loss of Id3 increases bone marrow B-1b cell numbers and total bone marrow IgM producing cells A) BM was collected from Id3^KO*Apoe^−/−* (n=8) and Id3*KO*Apoe^−/−* (n=9) mice and total BM cells were analyzed for IgM production by ELISPOT while a small portion was immunophenotyped by flow cytometry. B) Representative images of ELISPOT wells showing IgM positive spots from Id3^WT*Apoe^−/−* and Id3^BKO*Apoe^−/−* BM samples compared to negative control. C) Quantification of IgM+ BM cells from ELISPOT calculated from total BM cells. D) Quantification of total B-1, B-1a, and B-1b cells from BM. Data are mean ± SEM. E) Comparison of total IgM in 8-week-old, litter matched Id3^KO*Apoe^−/−* and Id3^KO*Apoe^−/−* mice as determined by ELISA. Data are mean ± SEM.Unpaired, two-tailed Students T-test was used to compare differences, *p<0.05. **p<0.01.

**Figure 5:** B cell specific deletion of Id3 reduces diet induced atherosclerosis. A) 8-week old, male, chow fed Id3^WT*Apoe^−/−* (n=10) and Id3^KO*Apoe^−/−* (n=7) were fed a Western diet for 16 weeks then plasma, aorta, and aortic sinus were collected for analysis. B-C) Representative images and quantification of maximum lesion areafrom 4x magnification images. D-E) Representative images and quantification of Mac2^+ lesion area normalized to total plaque area from 10x magnification images. A = Adventitia, P = plaque, L = lumen. F-G) Representative images and quantification of ‘Tunel’ area normalized to plaque area from 10x images. Enhanced image is zoomed in from boxed area to show positive staining. H-I) Representative images and...
quantification of Sudan IV staining of aortas prepared \textit{en face}. Two-tailed Mann-Whitney test was used to compare measured values. *p≤0.05, **p<0.01

**Figure 6:** B cell-specific loss of Id3 results in increased titers of atheroprotective IgM antibodies after Western diet feeding. Plasma was collected from Id3\textsuperscript{WT} Apoe\textsuperscript{-/-} and Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} mice fed a Western diet for 16 weeks and absolute titers of immunoglobulin isotypes and relative titers to indicated antigens were determined by chemiluminescent ELISA. \textbf{A)} Comparison of absolute titers of IgM and IgG isotypes reported as µg/ml. \textbf{B)} Comparison of relative IgM titers to indicated antigens as reported in legend of Figure 2 reported as RLU/100ms. Values are mean ± SEM, unpaired, two-tailed Students T-test was used to compare differences. *p<0.05, **p<0.01, ***p<0.001

**Figure 7:** Patients homozygous for the minor allele at rs11574 in the ID3 gene (encoding an Id3 protein with attenuated function\textsuperscript{23}) have increased percentage of B-1 cells and IgM reactive to MDA-LDL. DNA was isolated from PBMCs for genotyping of the rs11574 SNP (ancestral allele GG N=50, minor allele AA N=8). Representative flow cytometry and quantitation of B cells by ID3 genotype at rs11574 for (\textbf{A}) total B cells and (\textbf{B}) memory B cells (CD27\textsuperscript{+}CD43\textsuperscript{-}), naïve B cells (CD27\textsuperscript{-}CD43\textsuperscript{-}) and, B-1 cells (CD27\textsuperscript{+}CD43\textsuperscript{+}). \textbf{C)} Comparison of IgM and IgG antibody titers reactive to MDA-LDL by ID3 genotype at rs11574 measured in RLU/100ms by ELISA. Data from experiments comparing genotypes are presented in Box-and-Whiskers format with 95% CI and outliers as dots. Unpaired two-tailed Students T-test was used to compare differences. *p<0.05.
Novelty and Significance

What Is Known?
- Murine B cells regulate atherosclerosis based on subset specific functions; innate B-1a cells are atheroprotective and adaptive B-2 cells have multiple functions. B-1b cells have not been studied in the context of atherosclerosis.
- Inhibitor of Differentiation 3 (Id3) regulates B cell development and atherosclerosis through multiple regulatory pathways.
- A putative human B-1 equivalent was described as CD20+CD3−CD27+CD43+CD70− and functionally resembles murine B-1 cells.

What New Information Does This Article Contribute?
- B-1b cells secrete atheroprotective IgM antibodies.
- B cell-specific loss of Id3 causes a significant increase in B-1b cell numbers suggesting it is an important regulator of B-1b cells.
- Patients with a single nucleotide polymorphism (SNP) in the ID3 gene at rs11574, which attenuates Id3 function, have increased B-1 cells as a percentage of total B cells and increased titers of IgM to MDA-LDL in the circulation.

B cells have been demonstrated to have both atheroprotective and atherogenic functions based on subset and context. To date the contributions to atherosclerosis of B-1b cells, an important subset for T cell-independent immunity, have not been studied. Here we show that B-1b cells secrete IgM antibodies reactive to oxidative epitopes on LDL and that these cells are atheroprotective when introduced into B and T cell deficient Rag1−/−Apoe−/− mice. We implicate the helix-loop-helix transcription factor Id3 in the regulation of B-1b cells as B cell-specific Id3 knockout mice (Id3BKO Apoe−/−) developed significantly increased numbers of B-1b cells systemically without modifying the number of other B cell subsets. Additionally, Id3BKO Apoe−/− mice had significantly increased titers of atheroprotective IgM and developed attenuated atherosclerosis. Finally, patients homozygous for the Id3 SNP at rs11574, previously shown to attenuate Id3 function, have an associated increase in putative B-1 cells as a percentage of total B cells in the circulation and increased titers of IgM to MDA-LDL compared to patients with the common allele. Taken together, our findings suggest targeting Id3 in B cells may attenuate atherosclerosis by augmenting atheroprotective B cell numbers and that this could be relevant in humans.
Figure 4

A

Id3<sup>WT</sup>Apo<sup>e</sup>-/- (n = 8)
Id3<sup>BKO</sup>Apo<sup>e</sup>-/- (n = 9)

Total bone marrow cells → ELISPOT → Flow Cytometry

B

WT  BKO  Control

BM

D

B-1 cells x 10<sup>3</sup>

Total B-1  B-1a  B-1b

C

IgM<sup>+</sup> ISC/whole tissue

E

IgM Bound (μg/ml)

- Id3<sup>WT</sup>Apo<sup>e</sup>-/-
- Id3<sup>BKO</sup>Apo<sup>e</sup>-/-
Figure 6

A

Immunoglobulin Bound (μg/ml)

IgM IgG1 IgG2c IgG3

B

IgG Bound (RLU/100μL)

nLDL T15/E06 MDA-LDL CuOx-LDL 1,3 α-Dextran

Id3WT Id3 KO

*** ** *
Figure 7

A

CD20

CD3

B cells (% Live CD3+/CD20+)

GG

AA

RS11574

B

Memory B cells (% CD3+CD20+)

GG

AA

RS11574

B cells (% CD3+CD27+)

CD27

CD43

Naive B cells (% CD3+CD27+)

GG

AA

RS11574

C

IgM

anti-MDA-LDL (RLU)

GG

AA

RS11574

IgG

anti-MDA-LDL (RLU)

GG

AA

RS11574
B-1b Cells Secrete Atheroprotective IgM and Attenuate Atherosclerosis
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Detailed Methods

Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. Id3<sup>fl/fl</sup> mice were a generous gift of Dr. Yuan Zhang (Duke University). CD19<sup>Cre/+</sup> mice and Rag1<sup>-/-</sup> mice were provided by Timothy Bender (University of Virginia). Apoe<sup>−/−</sup> mice were purchased from Jackson Laboratory. Id3<sup>fl/fl</sup> mice were bred to the Apoe<sup>−/−</sup> line and then with CD19<sup>Cre/+</sup> mice to develop B cell specific Id3 knockouts as previously described<sup>1</sup>. Rag1<sup>-/-</sup> mice were bred with Apoe<sup>-/-</sup> to generate Rag1<sup>-/-</sup>Apoe<sup>-/-</sup> mice. All mice, purchased or generated, were backcrossed at least 10 generations to C57BL/6J mice. Mice were fed either a standard chow diet or Western Diet (Tekland, 7012 or TD.88137). Mice were euthanized in all experiments by CO<sub>2</sub> asphyxiation. Only male mice were used for all experiments.

Serum Cholesterol Determination

Cholesterol levels were determined as previously described by the University of Virginia Medical laboratories<sup>2</sup>.

Analysis of atherosclerotic lesions

Hearts were removed and prepared as previously described<sup>3</sup>. Briefly, mice were perfused by left ventricular puncture with heparinated PBS to avoid clotting. Hearts were separated from the aorta distal to the aortic sinus. The lower third of the heart was removed by scalpel and the remaining heart was vertically embedded with cut edge down in OCT compound (Tissue-Tek) then wrapped in aluminum foil and snap frozen by floating the mold on top of liquid nitrogen for 60 seconds. Blocks were then left on dry ice to completely freeze and stored at -80°C. Serial 5µm sections were cut by Cryostat (Leica biosystems) from the beginning of the three aortic leaflets to the aortic arch. Slides were stored at -80°C until stained. For lesion analysis, 1/4-1/5 of the total slides, equally spaced, were stained with Oil Red O lipid stain and counterstained with hemotoxylin (both from Sigma). Sections were imaged using an Olympus BX51 high magnification light microscope. Aortas were prepared as previously described<sup>2</sup>. Briefly, aortas were fixed in 4% paraformaldehyde then opened longitudinally, pinned, and stained using Sudan IV (Sigma). Aortas were imaged with a Nikon D70 DSLR camera. Plaque areas were assessed using Image-Pro Plus software (Media Cybernetics). For aortic sinus measurements, maximum plaque area measured from each mouse was used for comparison. For aortic plaque measurements, total percentage of the aorta that was covered by plaque was used for comparison.

Immunofluorescence and Tunel analysis of aortic sinus sections

Slides of the aortic sinus, described above, were stained for macrophage content as previously described<sup>2</sup> using biotinylated Mac-2 as the primary antibody (Cedarlene CL8942B) and Streptavidin Alexa Fluor 488 as the secondary antibody (Invitrogen Molecular Probes S11223) then counterstained with DAPI and mounted (Vectashield H-1500). For staining of apoptotic cell bodies the TUNEL method was used following the protocol from ApopTag Peroxidase In Situ Apoptosis Detection Kit (emdmMillipore S7100). Imaging for both was done using an Olympus BX51 high magnification light microscope. Images were analyzed using ImageJ (http://imagej.nih.gov/ij/).

Adoptive transfer of B-1b cells into Rag<sup>1<sup>-</sup></sup>Apoe<sup>-/-</sup> hosts

Adoptive transfer of B-1b cells into Rag<sup>1<sup>-</sup></sup>Apoe<sup>-/-</sup> hosts
Following electronic cell sorting, as described below, 1x10^{5} B-1b cells were transferred intraperitoneally (IP) into 8-week-old Rag1^{-/}Apoe^{-/} mice. Mice were maintained on chow diet for one week following transfer then switched to Western Diet for 16 weeks at the end of which time the animals were euthanized and hearts were collected for histological analysis of atherosclerotic lesions within the aortic roots as described above.

**Immunizations**

8-10-week-old male Id3^{BKO}Apoe^{-/} and Id3^{WT}Apoe^{-/} mice were immunized with the T cell dependent antigen DNP-KLH as described previously. Briefly, mice were immunized IP with 100 µg DNP-KLH in complete Freund’s adjuvant or adjuvant alone as control then boosted with 100 µg DNP-KLH on day 21 in PBS or PBS alone as control. Blood was collected on days 0 (prior to immunization), 7, and 21 (prior to boost). On day 28 mice were euthanized and blood was collected by cardiac puncture. Bone marrow was harvested and treated, as described below, for flow cytometric analysis of plasma cells (PC).

**Preparation of tissues for Flow Cytometry and Cell Sorting**

PerC cells, splenocytes, PBMCs and bone marrow (BM) cells were harvested and single cell suspension were prepared as previously described. Cells were blocked for Fc receptors using anti-FCRIII/II (CD16/32, FCR-4G8, Life Tech) then stained for cell surface markers using fluorescently-conjugated antibodies for 20 minutes at 4°C. Cells were washed in PBS and stained with a fixable live/dead stain diluted in PBS for 20 minutes at 20°C then fixed in 2% PFA in PBS for 10 minutes prior to resuspending in FACS buffer (PBS with 0.05% NaN3 and 1% BSA). For FAC sorting, cells were resuspended in modified FACS buffer (PBS with 1% BSA) and 4′,6-Diamidino-2-Phenylindole (DAPI) live/dead stain then immediately taken to sorting facility. B-1b cells were sorted to better than 99% purity from their parent gate. Flow cytometry antibodies: CD5 (PE, 53-7.3), CD19 (PECy7 or APCef780, 1D3), CD21 (APC, 8D9, 7G6), CD23 (PECy7, B3B4), CD43 (FITC, S7), B220/CD45R (APC, RA3-6B2), IgD (FITC, 11-26.2a), and IgM (e450 or FITC, II/41, R6-60.2), Ter119 (Biotin, Ter119), NK1.1 (Biotin, PK136), Gr-1 (Biotin, RB6-8C5), CD11b (Biotin, M1/70), CD138 (PE, 281-2). All antibodies were purchased from eBioscience, BD Bioscience, and Biolegend. Immunoglobulin isotype antibodies used for plasma cell intracellular staining were conjugated to FITC and purchased from Southern Biotech (IgG1 clone SB77e, IgG2a clone SB84a, IgA clone 11-44-2), BD (IgG2c clone R19-15) and Abcam (IgG3 clone ab97259). Intracellular staining for immunoglobulin isotypes was done using reagents and protocols from the BD Cytofix/Cytoperm kit (BD, 554714). Live/Dead discrimination was determined by LIVE/DEAD fixable yellow staining (Invitrogen) or DAPI. For flow cytometry of human samples, PBMCs were isolated from blood using SepMate tubes per the manufacturer’s instructions (Stemcell Technologies, 15425). Briefly, blood was diluted with an equal volume of wash buffer: PBS with 2% FBS (Gibco). The diluted sample was carefully added down the side of the SepMate tube containing Ficoll-Paque Plus (GE Healthcare, 17-1440-02). Tubes were centrifuged at 1200g for 10 minutes then the buffy coat layer was removed, washed and resuspended for staining. Antibodies were from BD – CD3 (PE-CF594, SK7), CD27 (BV421, M-T271), and eBioscience – CD20 (APC-H7, 2H7), CD43 (FITC, 84-3C1). Electronic cell sorting was carried out at the University of Virginia Flow Cytometry Core on an Influx cell sorter (BD Bioscience) using BD FACS Software Sorter Software. Immunophenotyping was performed on a CyAn ADP (Beckman Coulter) and analyzed with FlowJo software (Tree Star Inc). All gates were determined using fluorescence minus one (FMO) controls.

**In vitro stimulation assays**
Post electronic cell sorting, \textit{Apoe}^{-}\textit{B}-1b cells were plated at 1-4x10^4 cells per well in a 96 well plate in 200 µl of B cell culture media: complete RPMI 1640 containing 10% heat inactivated FBS (Hyclone), 10mM HEPES, 1x nonessential amino acids, 1mM sodium pyruvate, 100 U/mL penicillin and 100 mg/mL streptomycin, 0.55 mM 2-Mercaptoethanol with 50µg/mL LPS (Sigma, L4391), 100mM CPG (ODN 1668, Invivogen), or PBS for 72 hours. All culture reagents are from Gibco unless otherwise specified. Media and cells were taken up and centrifuged. The supernatant was collected for measurement of immunoglobulins by ELISA.

\textit{Enzyme-linked Immunosorbent Assay (ELISA)}

Specific Ab levels to given antigens in plasma from mice were determined by chemiluminescent ELISA as previously described. In brief, Microfluor® 2 White “U” Bottom Microtiter® plates (Thermo Labsystems, Franklin, MA, USA) were coated with various antigens at 5 µg/mL PBS overnight at 4°C. The plates were blocked with 1% BSA in TBS, serially diluted plasma was added, and the plates incubated for 1.5h at room temperature. Bound plasma Ig isotype levels were detected with various anti-mouse Ig isotype-specific alkaline phosphatase (AP) conjugates using LumiPhos 530 solution, and a Dynex Luminometer (Dynex Technologies, Chantilly, VA, USA). The following goat AP-conjugated secondary Ig isotype-specific Abs were used; anti-mouse IgM (µ-chain specific) (Sigma-Aldrich) and anti-mouse IgG1, IgG2c, and IgG3 (all Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Data were expressed as relative light units counted per 100 milliseconds (RLU/100 ms) and converted to absolute Ig values from simultaneously run standard curves for each given isotype. For each set of mice, plasma pools were made and used for formal dilution curves to determine optimal dilution for each antigen to use in binding assays. A specific non-saturating dilution was chosen for each antigen, and then plasma samples from each mouse were assayed to determine mean and SD for that determination. Antigens used were prepared as previously described. IgM E06 titers were determined by plating AB1-2, a T15-antiidiotype, and then determining the amount of IgM bound to AB1-2 using the anti-mouse IgM as above.

Ab levels against MDA-LDL and apoB-immune complexes (ICs) in human plasma were measured by chemiluminescent ELISA as previously described. Briefly, MDA-LDL (5 µg/ml) was coated on microtiter well plates, plasma was added, and IgG or IgM antibodies binding to MDA-LDL was detected with alkaline phosphatase labeled goat anti-human IgG or (Sigma). ApoB-100 ICs were detected by plating murine monoclonal antibody MB47 to bind a saturating amount of human apoB. Plasma was added and IgG or IgM antibodies binding to the captured apoB were detected with alkaline phosphatase labeled goat anti-human IgG or IgM.

Antibody titers against DNP were measured as previously described. Briefly, 96 well plates were coated with DNP-BSA (10µg/ml) overnight. Samples were incubated on plates for 2 hours then washed and alkaline phosphatase conjugated, isotype specific, secondary antibodies were added for 2 hours then DnPP reagent was added. The mean optical density at 450 nm was measured and compared to a standard curve developed from pooled serum in order to determine RLU/100ms

\textit{ELISPOT}

Single cell suspension of BM was prepared as described above. Sterile MultiScreen IP-Plates (Millipore, MSIPS4510) were used for the assay according to manufacturer’s protocol. Wells were coated with unlabelled anti-mouse IgM antibody (Southern Biotech) and incubated overnight at 4°C. The following day the antibody solution was removed and the membrane was washed and then blocked with RPMI 1640+10% FCS for 2 hours at 37°C. A suspension of 1x10^5 cells/ml was prepared then 2.5x10^5 cells were added to the first well then serially diluted
for each subsequent well incubated overnight at 37°C in a cell culture incubator (5% CO₂). Cells were decanted then biotin-labeled anti-mouse IgM antibody (1:5000 dilution) was added to each well and incubated 2 hours. Following washing streptavidin alkaline phosphatase (Abcam) was added and incubated 30 min at room temp. Again following washing BCIP/NBT was added and incubated until spots became visible. Each spot on the membrane indicated an antibody secreting cell. Wells were imaged under a dissecting microscope (Zeiss) then spots were counted manually. The ideal concentration of cell was determined based on visible spots for counting.

**Human Genotyping**

Id3 SNP (rs11574, Assay ID# C_2462609_10) genotyping was performed using the ABI Taqman SNP Genotyping assay from LifeTechnologies. Briefly, DNA was isolated from whole blood using the Gentra Puregene kit (Qiagen) according to the manufacturer’s instructions. PCR was performed on the ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA) in 5 μL reaction volume. For each PCR, 1 μL genomic DNA (~10 ng) was mixed with 2.5 μL 2× TaqMan Genotyping Master Mix (P/N 4371355), 0.25 μL 20× TaqMan SNP genotyping assay mix and 1.25 μL of nuclease free water. Assays were loaded onto 384 well plates (Life Technologies, 4309849) and PCR conditions were one cycle at 95 °C for 10 min, 40 cycles at 92 °C for 15 s, 60 °C for 1 min. All genotypes were analyzed and assigned automatically using the ABI SDS 2.3 software.

**Real Time PCR**

Total RNA was isolated from FACS purified peritoneal B cells using the RNeasy Plus Micro kit with gDNA elimination columns (Qiagen). RNA (1 μg) was reversed transcribed with SuperScript III First-Strand Synthesis System by Oligo d(T) tailing (Invitrogen). Secreted IgM was normalized by the ΔΔCq method to 18S with SYBR® Green real-time PCR (SsoFast™ EvaGreen® Supermix, Bio-Rad). PCR reactions were always performed with at least duplicate wells using the C1000 Thermal Cycler and CFX96 Real Time system (Bio-Rad). Primers were used as follows: sIgM, forward primer (5’-GGA GAG ACC TAT ACC TGT GTT GTA GG-3’) and reverse primer (5’-TGA GCG CTA GCA TGG TCA ATA GCA G-3’); 18S forward primer (5’-CGG CTA CCA CAT CCA AGG AA-3’), reverse primer (5’-AGC TGG AAT TAC CGC GGC GGC-3’).

**Statistical Methods**

To test if data sets fit a Gaussian distribution, a D’Agostino-Pearson omnibus normality test was used. If data was normal, a two-tailed student’s t-test was performed. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. To compare differences in more than two data sets, a one-way analysis of variance (ANOVA) and Holm-Sidak or Tukey’s multiple comparisons test were used. Data was analyzed using Prism 6.0b (GraphPad Software, Inc.). Results are displayed containing all replicated experiments and values shown are mean ± SEM.
Online Figure I: Standard curves and dilution series for isotype and antigen specific ELISAs. 

A) IgM standard curve and dilution series for total IgM, MDA-LDL IgM, and CuOx-LDL IgM used for Figures 1B-D. 

B) IgM standard curve and dilution series for total IgM, MDA-LDL IgM, and CuOx-LDL IgM used for Figures 1F-H. 

C) Standard curves for IgM, IgG1, IgG2c, and IgG3 used to calculate absolute titers for Figure 2D and Figure 6A. 

D) Dilution series for IgM, IgG1, IgG2c, IgG3, MDA-LDL IgM, CuOx-LDL IgM, native LDL, T15/E06, and 1,3,α-Dextran used for Figures 2D-E. 

E) Standard curves for IgM, IgG1, IgG2c, and IgG3 used to calculate
absolute titers for Figure 2D and Figure 6A. D) Dilution series for IgM, IgG1, IgG2c, IgG3, MDA-LDL IgM, CuOx-LDL IgM, native LDL, T15/E06, and 1,3,α-Dextran used for Figures 2D-E. E) Dilution series for IgM, IgG1, IgG2c, IgG3, MDA-LDL IgM, CuOx-LDL IgM, native LDL, T15/E06, and 1,3,α-Dextran used for Figures 6A-B.

Online Figure II: B-1b cells are found in the spleen of Rag1<sup>−/−</sup>Apoe<sup>−/−</sup> host mice but not in blood and plasma cholesterol does not correlate with lesion area from aortic sinus. B-1b cell quantification after collection from Rag1<sup>−/−</sup>Apoe<sup>−/−</sup> host fed Western diet for 16 weeks from the (A) spleen and (B) blood. C) Analysis of correlation between total plasma cholesterol (mg/dl) and max lesion area (µm²). Data are mean ± SEM for B-1b cell numbers and individual XY values with linear regression (solid line) and 95% confidence interval (dotted lines) for correlation analysis. Two-tailed students T-test was used to compare B-1 b cell numbers, linear regression was used for correlation analysis. ***p<0.001, NS = non-significant.
Online Figure III: Comparison of CD4⁺ and CD8⁺ T cells from 8-week-old, chow fed, male Il3⁻/⁻ ApoE⁻/⁻ and Il3⁺/⁺ ApoE⁻/⁻ mice. A) Representative flow cytometry gating of splenic CD45⁺ cells then gated on CD3⁺ population and then gated on CD4⁺ or CD8⁺ populations. B-D) Quantification of T cell subsets from PerC (B), spleen (C), and blood (D). Data are mean ± SEM.

Online Figure IV: Representative gating of bone marrow B-1a and B-1b cells. Adapted from the gating described in Choi et al, Eur J Immunol, 2012.
Online Figure V: Quantification of B cell subsets from PerC, spleen, and blood recovered from 16-week Western Diet fed \textit{Id3}^{WT}\textit{Apoe}\textsuperscript{−/−} and \textit{Id3}\textsuperscript{BKO}\textit{Apoe}\textsuperscript{−/−} mice. Data are mean ± SEM. Two-tailed Students T-test was used to compare differences between genotypes. **p<0.01, ***p<0.001.
Online Figure VI: Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} mice respond to T cell dependent immunization with increased IgM and IgG3 and slightly blunted IgG1 and IgG2c compared to Id3\textsuperscript{WT} Apoe\textsuperscript{-/-} mice. A) Study design. 8-10 week-old Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} and Id3\textsuperscript{WT} Apoe\textsuperscript{-/-} were immunized with 100µg DNP-KLH in complete Freund’s adjuvant (Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} n = 5, Id3\textsuperscript{WT} Apoe\textsuperscript{-/-} n = 5) or complete Freund’s adjuvant alone (Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} n = 5, Id3\textsuperscript{WT} Apoe\textsuperscript{-/-} n = 3) then boosted at day 21 with DNP-KLH in PBS or PBS alone. Blood was drawn on days 0, 7, 21, and 28 (days 0 and 21 prior to immunization). Bone marrow was collected from mice on day 28 and isotype specific PCs were measured by flow cytometry. B) Absolute counts of isotype specific PCs from DNP-KLH, or adjuvant control, immunized mice. Data presented are mean ± SEM. C) Relative anti-DNP antibody measurements determined by ELISA using isotype specific secondary antibodies for IgM, IgG1, IgG2a, IgG2c, IgG3, and IgA. Data are presented as the mean of repeat measures over time ± SEM. One-way ANOVA and Tukey’s multiple comparisons test were used to compare differences in PC counts. Two-way ANOVA and Tukey’s multiple comparisons test were used to compare differences across groups on individual days. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Online Figure VII

Online Figure VII: Loss of Id3 in B cells does not modify the amount of IgM expressed per cell. Expression analysis of IgM mRNA transcripts was done in Id3\textsuperscript{BKO} Apoe\textsuperscript{-/-} and Id3\textsuperscript{WT} Apoe\textsuperscript{-/-} PerC B cell subsets and normalized to the housekeeping gene 18S rRNA. Data are mean ± SEM.
Online Table I: Weight and lipids from Rag-/-Apoe-/- mice injected with B-1b cells (or PBS control) and fed a Western diet for 16 weeks

<table>
<thead>
<tr>
<th>Injection</th>
<th>PBS (n=6)</th>
<th>B-1b cells (n= 9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>36.09± 1.87</td>
<td>32.89± 1.90</td>
<td>NS</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>11.55± 0.66</td>
<td>8.05± 2.34</td>
<td>NS</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>1247± 162</td>
<td>964± 89</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>262.3± 29.43</td>
<td>223.3± 29.34</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>46.50± 2.63</td>
<td>36.67± 4.63</td>
<td>NS</td>
</tr>
</tbody>
</table>

Online Table II: Weight and lipids from Id3 WT and Id3 BKO mice fed a Western diet for 16 weeks

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Id3 WT (n= 11)</th>
<th>Id3 BKO (n= 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>38.62± 0.89</td>
<td>40.08± 0.86</td>
<td>NS</td>
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<tr>
<td>Weight gain (g)</td>
<td>12.52± 1.35</td>
<td>14.56± 0.93</td>
<td>NS</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>1476± 65.0</td>
<td>1514± 100.3</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>358.6± 23.3</td>
<td>387.9± 29.2</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>43.64± 3.19</td>
<td>42.60± 4.13</td>
<td>NS</td>
</tr>
</tbody>
</table>
Works Cited


