B-1B CELLS ARE A NOVEL ATHEROPROTECTIVE B CELL SUBSET AND ARE NEGATIVELY REGULATED BY ID3

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Abstract

Vascular disease remains the leading cause of death globally through its sequelae myocardial infarction and stroke. The primary contributor to vascular disease, atherosclerosis, is defined by intimal plaque formation in response to lipid deposition. Atherosclerosis is a chronic, progressive disease of the medium to large size arteries and has been well described as a product of low grade inflammation.

B cells are highly involved in the development and progression of atherosclerosis, responding to atherogenic antigen and aggregating in the vascular adventitia. The contributions of B cells to atherosclerosis are subset dependent. Adaptive B-2 cells are considered atherogenic though they can also have atheroprotective function based on the context and model used to study them. Innate B-1a cells are atheroprotective through the secretion of IgM natural antibodies reactive to oxidized LDL. B-1b cells, a unique subset of B-1 cells that are able to provide T cell-independent immune memory, are also atheroprotective and secrete IgM reactive to oxidation-specific epitopes on oxidized LDL.

Inhibitor of differentiation 3 (Id3) is a helix-loop-helix transcription factor known to regulate B cell development and homeostasis as well as atherosclerosis. Studies in a B cell specific Id3 knockout model ($Id3^{BKO}$) revealed that these mice have a

significantly expanded B-1b cell population in all compartments without a difference in B-1a or B-2 numbers. Additional studies demonstrated that $Id3^{BKO}$ mice developed attenuated atherosclerosis and increased titers of atheroprotective IgM supporting B-1b cells as atheroprotective and suggesting Id3 selectively negatively regulates their numbers. Attempts to define a mechanism by which Id3 regulates B-1b cells have been inconclusive.

Given their contributions to T cell independent memory, B-1b cells are a novel target for immunization strategies against atherosclerosis and Id3 could be an important target for B-1 directed therapies.

Dedication and Acknowledgements

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List of Abbreviations

APC	antigen presenting cell
Apoe	apolipoprotein E
ATLO	artery tertiary lymphoid organ
BAFF	B cell activating factor
BAFFR	BAFF receptor
BCR	B cell receptor
bHLH	basic HLH
BM	bone marrow
Breg	regulatory B cell
BrdU	bromodeoxyuridine
BTK	Bruton’s tyrosine kinase
CCR	C-C chemokine receptor
CXCR	C-X-C chemokine receptor
CD	cluster of differentiation
CLP	common lymphoid progenitor
Cre	causes recombination enzyme
CuOx-LDL	copper oxidized LDL
CHD	coronary heart disease
CVD	cardiovascular disease
DAMP	danger associated molecular pattern

DNP-KLH	2,4-dinitrophenyl conjugated to Keyhole Limpet Hemocyanin
E06	Apoe autoantibody 6
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FO	follicular
GC	germinal center
GM-CSF	granulocyte-macrophage colony stimulating factor
HDL	high-density lipoprotein
HLH	helix-loop-helix
4-HNE	4-hydroxynonenal
HSC	hematopoietic stem cells
Id3	inhibitor of DNA binding 3 or inhibitor of differentiation 3
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IP	interperitoneal
IRA	innate response activating B cells
LDL	low-density lipoprotein
Lin	lineage
LLPC	long lived plasma cell
LPS	lipopolysaccharide
mAb	monoclonal antibody

Mac	macrophage
Mac2	macrophage marker 2
MBL	mannose binding lectin
MDA	malondialdehyde
MHCII	major histocompatibility complex 2
μMT	mu heavy chain mutant
MZ	marginal zone
NAb	natural antibody
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHC	natural helper cell
OSE	oxidation specific epitope
oxCL	oxidized cardiolipin
oxLDL	oxidized LDL
PBMC	peripheral blood mononuclear cells
PC	phosphorylcholine
PCR	polymerase chain reaction
PerC	peritoneal cavity
PPS-3	pneumococcal polysaccharide serotype 3
PRR	pattern recognition receptor
PVAT	perivascular adipose tissue
RAG	recombination activating gene
RTX	Rituximab (moncoclonal αCD20 Ab)

RLU	relative light unit
sIgM	secreted IgM
SLPC	short lived plasma cell
SNP	single nucleotide polymorphism
SR	scavenger receptor
T15	T15 idiotype
TD	T cell dependent
TDT	terminal deoxynucleotide transferase
Th	helper T cell
TI	T cell independent
TLR	toll-like receptor
VSMC	vascular smooth muscle cell

Chapter 1

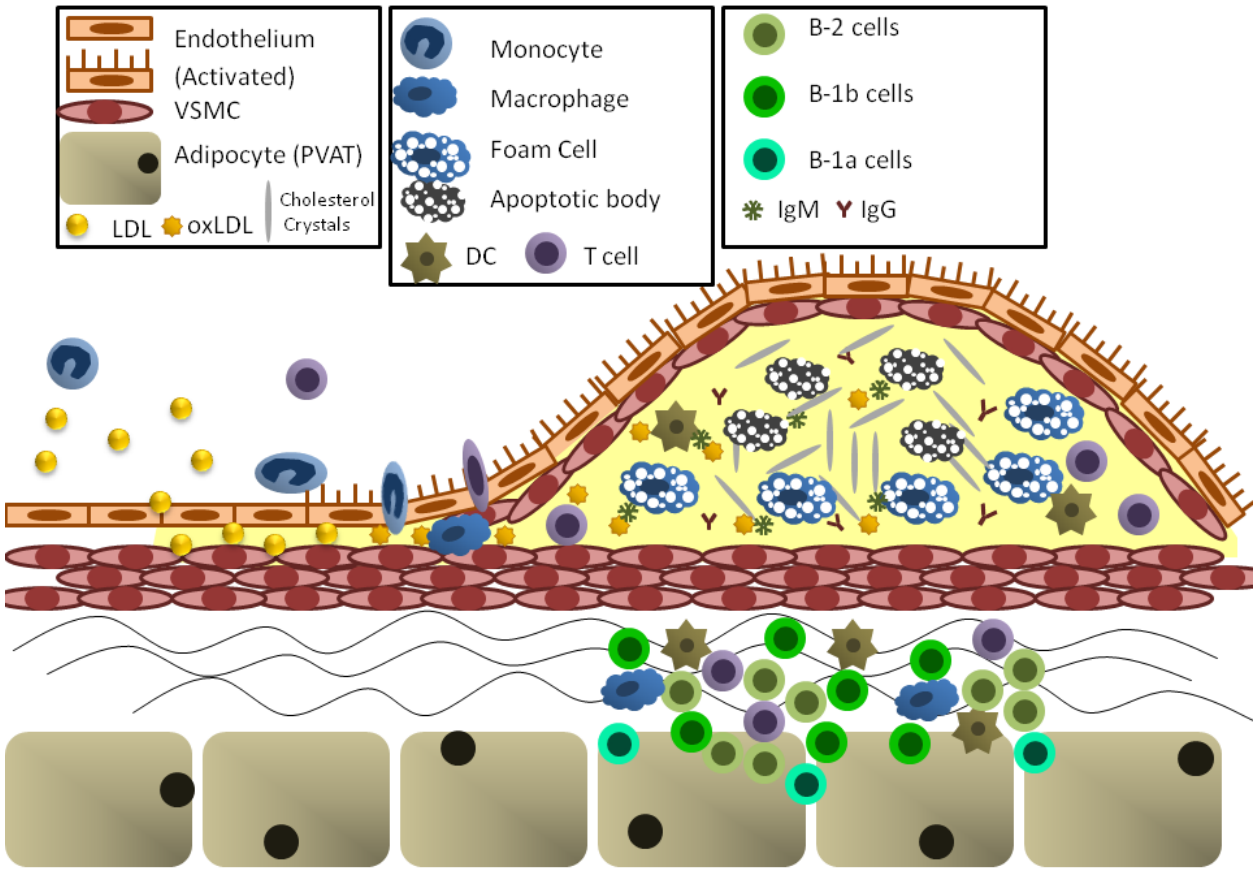
Introduction

Impact and Pathogenesis of Atherosclerosis

Cardiovascular disease (CVD) remains the leading global cause of morbidity, accounting for at least 17.3 million deaths annually [1]. The primary contributor to CVD is atherosclerosis, a chronic inflammatory disease of large and medium sized arteries defined by subintimal accumulation of lipids and progressive expansion of vascular lesions [2, 3]. Traditional risk factors for atherosclerosis include obesity, hypertension, dyslipidemia, diabetes, and smoking status. While these risk factors frequently present in patients that develop clinical manifestations of atherosclerosis, they fail to predict the majority of acute events which occur in asymptomatic individuals [4-6]. The Framingham Heart Study demonstrated that half of patients present with unheralded myocardial infarction or sudden cardiac death as their first manifestation of coronary artery disease [7]. Approximately two-thirds of acute coronary syndromes occur following rupture of atherosclerotic plaque initially less than 50% stenosed which may explain the development of acute events in asymptomatic individuals [8-10]. This suggests that the current means of testing for and treatment of CVD is missing a large portion of the population at risk for atherothrombotic events and requires us to look beyond the contributions of traditional risk factors for answers.

Early lesions, called ‘fatty streaks’, form as a result of low-density lipoprotein (LDL) aggregation in the subendothelial space of the vascular wall (**Figure 1**). Subendothelial retention of LDL is the driving mechanism for the development of advanced atherosclerosis [11]. The retained lipids are enzymatically and non

Figure 1: Pathogenesis of atherosclerosis. Deposition of luminal LDL into the subendothelial space and its subsequent oxidation cause endothelial dysfunction (activation) leading to monocyte, T cell, and dendritic cell (DC) extravasation. Monocytes convert to macrophages and engulf oxLDL becoming lipid laden foam cells. Lesion macrophages and T cells secrete inflammatory cytokines and chemokines that recruit other leukocytes. Uncleared apoptotic foam cells release cholesterol and cellular debris forming a lipid filled, acellular, necrotic core. B cell subsets, T cells, macrophages, and DCs home to the adventitia and peri-vascular adipose tissue (PVAT). OSE reactive IgM and IgG can also be found in the atherosclerotic lesion bound to oxLDL and apoptotic cell bodies.


Endothelium
(Activated)
VSMC
Adipocyte (PVAT)
LDL
oxLDL
Cholesterol
Crystals
Monocyte
Macrophage
Foam Cell
Apoptotic body
DC
T cell
B-2 cells
B-1b cells
B-1a cells
IgM
IgG

enzymatically oxidized producing a mix of modified lipid species that causeendothelial dysfunction. A hallmark of this is the expression of adhesion molecules in the vascular lumen and secretion of chemokines which attract monocytes, T cells and dendritic cells (DCs), which extravasate into the subintimal space [3]. Activated immune cells in the subintimal space secrete inflammatory cytokines and chemokines which contribute to a positive feedback loop for the recruitment of more leukocytes and establish an inflammatory milieu leading to plaque formation [12, 13]. Macrophages take up oxLDL by scavenger receptors (SR) in an attempt to clear the microenvironment, becoming the prototypic lipid laden foam cells [2, 14]. As foam cells die they release debris and cholesterol which, when not cleared, leads to the formation of a lipid filled necrotic core with an overlying collagen rich fibrous cap [15, 16]. Expansion of the lesion over time causes stenosis of the artery and ischemia of the surrounding tissue. Erosion or rupture of the fibrous cap exposes thrombotic material from the necrotic core to the circulation inducing thrombus formation and tissue infarction[17].

The vascular adventitia regulates intimal lesion formation

The vascular adventitia is formed primarily of connective tissue and adipocytes from the surrounding perivascular adipose tissue (PVAT) and serves as a host to progressive leukocyte accumulation during atherosclerotic lesion development [18, 19]. Adventitia has been demonstrated to play an important role in neo-intimal formation in response to manipulation [20-22]. For example placement of a silastic

collar around the carotid artery of Western diet fed rabbits induced rapid plaque development possibly due to disruption of the adventitial vasa vasorum [21], suggesting adventitial disruption can directly contribute to plaque development. Furthermore, balloon overstretch of porcine coronary arteries induced rapid proliferation within the adventitia prior to intimal expansion suggesting that adventitial cells can communicate and possibly home into intima during lesion formation [22]. Finally, advanced plaques, in mouse models of atherosclerosis, have been demonstrated to be in communication with the adventitia both through vasa vasorum and lymphatic-like conduits suggesting antigenic material from the lesion can be phagocytosed by cells in the adventitia and presented for lymphocytic activation [18].

B Cells in Atherosclerosis

A functional role for B cells in atherosclerosis was hypothesized in response to the findings that immunization of mice with oxLDL reduced atherosclerosis in hypercholesterolemic rabbits and mice, suggesting a protective humeral immune response [23, 24]. Subsequently, two landmark studies from 2002 suggested that B cells function in an atheroprotective manner. Hansson's group demonstrated that splenectomy, which depletes B and T cells, resulted in dramatically increased atherosclerosis compared to sham controls and adoptive transfer of B cells into the splenectomized mice rescued the normal phenotype and provided added protection compared to sham [25]. Major and colleagues demonstrated that lethally irradiated *Ldlr*$^{-/-}$ mice receiving bone marrow (BM) from B cell-deficient mice

(μMT) developed significantly greater atherosclerosis compared with mice receiving wild type BM [26]. The atheroprotective function of B cells was hypothesized to be, in part, due to their production of protective IgM antibodies against oxidized phospholipids [25, 27, 28]. This notion was supported in a subsequent study demonstrating that mice unable to secrete IgM (sIgM) developed significantly augmented atherosclerosis compared to control mice [29].

More recently, subset specific functions have been described. Selective depletion of B-2 cells by either pharmacological [30, 31] or genetic means [32, 33] resulted in significant reduction of atherosclerosis suggesting an atherogenic role for B-2 cells. However, other studies have suggested potential atheroprotective functions for B-2 cells [34]. Alternatively, adoptive transfer of B-1a cells into splenectomized *Apoe*$^{-/-}$ mice was demonstrated to attenuate atherosclerosis suggesting that these cells are atheroprotective [35].

B cells accumulate in the vascular adventitia

Lymphocytes have long been known to locate at sites of plaque formation. For example, Gerlis described “foci of inflammatory cells” within the adventitia of coronary arteries in patients that suffered acute MI in 1956 [36]. Development of targeting antibodies allowed for characterization of cells via immunohistochemistry which proved that these foci contained B cells [37-39]. Importantly, a recent study by Hamze and colleagues, using laser capture microdissection to analyze individual lymphocytes in diseased coronary arteries,

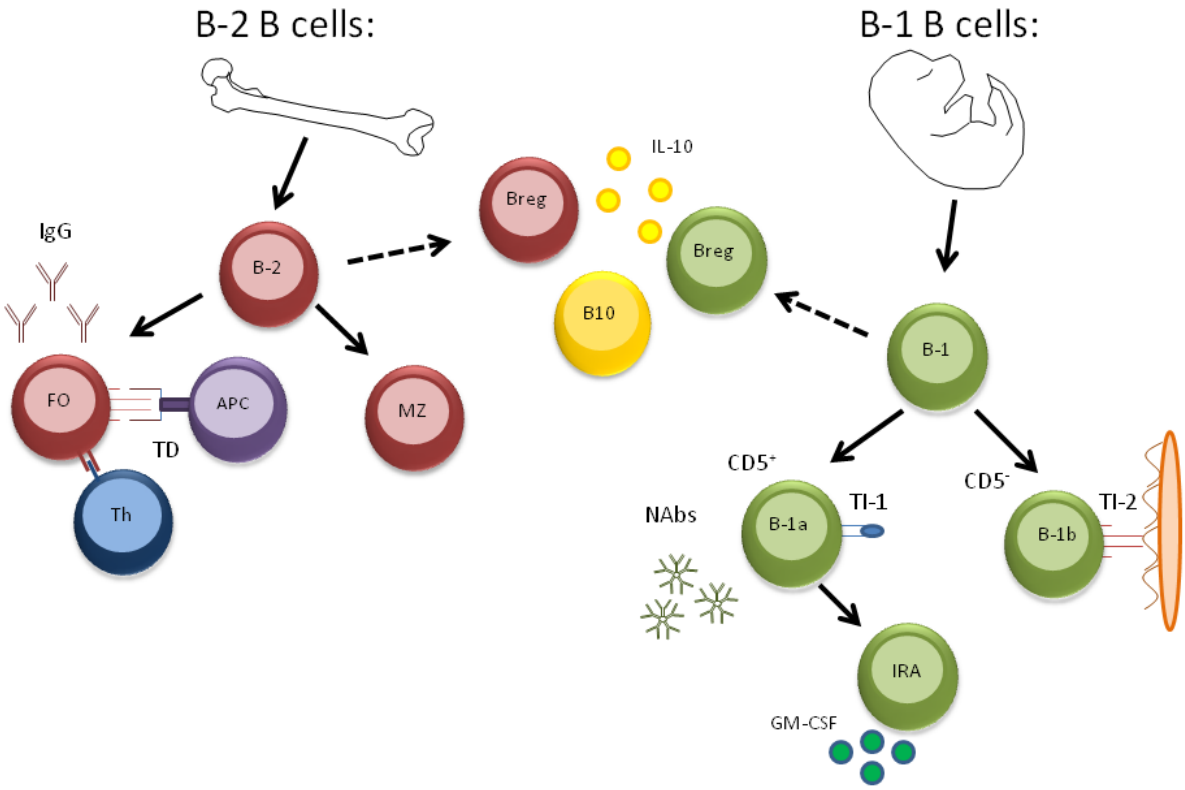
demonstrated that the majority of vascular B cells were present in the adventitia of arteries and that they primarily expressed an activated plasmablast phenotype suggesting the cells were active at sites of disease [40]. This finding was supported by the work of Habenicht and colleagues, which revealed that aged apolipoprotein E deficient mice (*Apoe*$^{-/-}$) developed aortic tertiary lymphoid organs (ATLO) containing mature B cell follicles within the adventitia at sites of lesion development [19, 41]. Though ATLOs have not been discovered in younger mice it has been previously demonstrated that B cells are enriched in atherogenic aortas, home to the adventitia overlying intimal lesions, and that B cell subsets can become differentiated within the adventitia [19, 34, 42-44]. The functional importance of B cells within the adventitia is complex and poorly understood giving to the varied functions of murine B cell subsets described below.

Murine B Cell Subsets

In mice there have been described two developmentally and functionally distinct lineages of B cells termed B-1 and B-2 B cells (**Figure 2**).

B-2 cells, also called conventional B cells, develop in the bone marrow (BM) from common lymphoid progenitors (CLPs) and contribute to adaptive immunity by the production of high affinity, class switched, antibodies reactive to extrinsic pathogens. B-2 development is a highly regulated, multi-step, process that

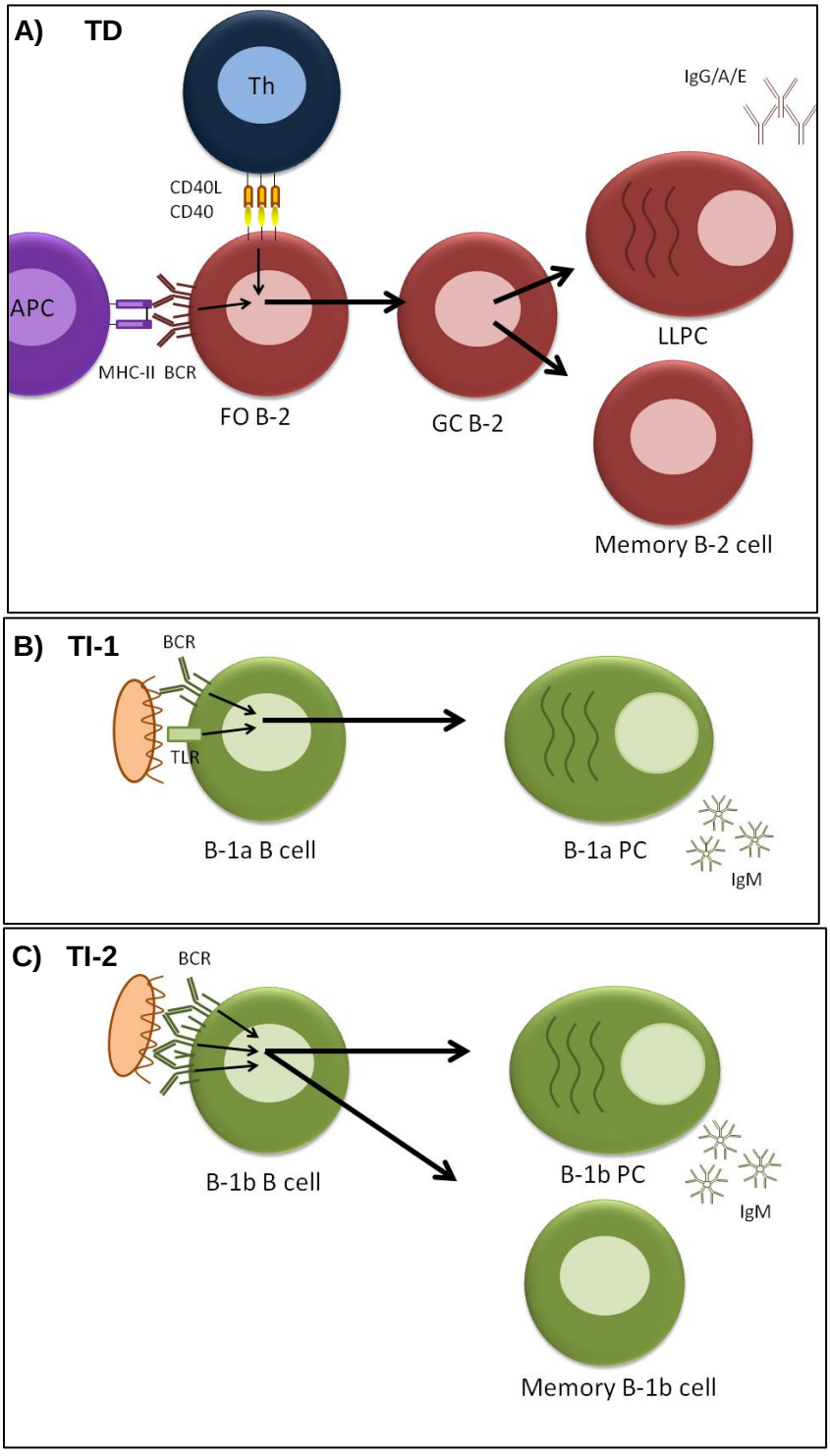
Figure 2: Murine B cell subsets. Conventional B-2 B cells develop in the bone marrow and reside in follicles or marginal zone. B-2 cells produce high affinity, class switched immunoglobulins in a T cell dependent manner. B-1 B cells develop in the fetal liver and are subdivided by surface CD5 expression into $CD5^+$ B-1a cells and $CD5^-$ B-1b cells. B-1a cells are activated by T cell independent type 1 (TI-1) antigens through toll like receptor (TLR) signaling, secreting IgM natural antibodies (NAb). B-1b cells are additionally activated by T cell independent type 2 (TI-2) antigens through the B cell receptor. Activated B-1a cells under inflammatory conditions can become innate response activating (IRA) B cells that secrete GM-CSF. Regulatory B cells (Bregs) can be derived from B-2, B-1 or possibly a unique B10 lineage. They are defined by IL-10 secretion and immunosuppression.


B-2 B cells:
B-1 B cells:
IgG
B-2
FO
APC
TD
Th
MZ
IL-10
Breg
Breg
B10
B-1
CD5+
B-1a
TI-1
NAbs
CD5-
B-1b
TI-2
IRA
GM-CSF

develops a broad repertoire of antigen specificities to non-self antigens while removing auto-reactive B cell clones [45]. Upon maturation, B-2 cells escape the BM and migrate to secondary lymphoid organs such as the spleen and lymph nodes where they locate in B cell follicles (FO B cells) or in the splenic marginal zone (MZ B cells). B-2 cells are short-lived and continuously circulate in search of their cognate peptide antigen. They are activated only when they bind antigen presented on the major histocompatibility complex II (MHCII) by antigen presenting cells (APCs) in the presence of CD4 T cells, which provide secondary signaling for example through CD40/CD40L interaction[46] (**Figure 3**). The products of B-2 cell activation are highly specific, class switched, antibodies made by short- and long-lived plasma cells (SLPC/LLPC) or memory B cells primed for repeat antigen exposure [47, 48].

B-1 cells develop early in ontogeny and contribute primarily to the innate immune system by responding to non-peptide T cell-independent (TI) antigen such as carbohydrate or lipid moieties on neo- or non-self structures [49]. In mice, B-1 cells develop from a unique precursor in the fetal liver and make up the majority of B cells in the neonate [50-52]. Upon maturation, B-1 cells reside in serosal cavities such as the peritoneum and pleura, and they self-renew, in contrast to B-2 cells [53, 54]. Two distinct subsets of B-1 cells have been described in mice based on the expression of the T cell marker CD5, B-1a cells ($CD5^+$) and B-1b cells ($CD5^-$) [55, 56]. B-1a cells produce IgM antibodies in the absence of antigen exposure, called natural antibodies (NAbs), which are polyreactive and low affinity with few

Figure 3: T cell dependent and T cell independent B cell activation: **A)** T cell dependent (TD) antigen is made of short peptide bound to the MHC-II complex on APCs. When B-2 cells are met with cognate TD antigen, a weak activating signal occurs through the B cell receptor complex (BCR). This signal is not sufficient to activate the cell. However in the presence of a $CD4^+$ helper T cell (Th) a secondary signal, such as the one delivered by CD40L through CD40 activates the B cell to undergo the germinal center response (GC). From the GC come class switched short- and long-lived plasma cells (LLPCs) and memory B-2 cells that are prone for reactivation upon repeat antigen presentation. **B)** T cell independent (TI) antigen are lipid or carbohydrate moieties that act as danger associated molecular patterns (DAMPs) that activate patter recognition receptors (PRRs) such as toll like receptors (TLRs). TI type 1 activation (TI-1) occurs through the combined signaling of the BCR and TLR which activates B-1a (and B-1b) cells to become short lived plasma cells but not memory B-1 cells. **C)** TI type 2 activation (TI-2) occurs in response to highly repetitive TI antigen which causes aggregation of BCR on the surface of B-1b cells. The combined BCR signaling activates B-1b cells to become LLPCs and memory B-1b cells.


A) TD
Th
IgG/A/E
CD40L
CD40
APC
MHC-II BCR
FO B-2
GC B-2
LLPC
Memory B-2 cell
B) TI-1
BCR
TLR
B-1a B cell
B-1a PC
IgM
C) TI-2
BCR
B-1b B cell
B-1b PC
IgM
Memory B-1b cell

nucleotide inclusions and reactive to primarily auto-antigens and pathogen associated molecular patterns (PAMPs) [49, 53, 57, 58]. B-1a cells are activated by the combined presence of antigen and mitogenic stimulation through toll-like receptors (TLRs) which has been termed the T cell-independent type 1 response (TI-1) [59]. B-1b B cells have generally been considered a sister population to B-1a cells. Their initial discovery was as a minor subset of B cells with similar distribution and function to B-1a cells but without surface expression of CD5 [56]. It was observed in this initial publication, and subsequent studies, that the bone marrow of adult mice is far better at resupplying B-1b cells compared to B-1a suggesting they are derived from separate progenitors or the common progenitor becomes more prone to developing B-1b cells in maturity [56]. A recent publication by Ghosn and colleagues demonstrated that hematopoietic stem cells (HSCs) transferred into irradiated hosts were able to reconstitute all B cell subsets except for B-1a cells [60] supporting the concept that B-1a and B-1b cells develop from different progenitors. B-1b cells can respond to either TI-1 stimuli or in an antigen dependent manner toward highly repetitive carbohydrate or lipid moieties that cause extensive cross-linking of the B cell receptor (BCR) called the TI-2 response [59]. BCR cross-linking delivers an extended activating signal to the B cell in a Bruton's tyrosine kinase (BTK) dependent manner, overcoming the need for secondary signaling by T cells [61]. An important result of TI-2 signaling is the development of TI memory [62] discussed below.

A final classification of B cells are regulatory B cells (Bregs), which are functionally defined by their ability to suppress the immune response primarily through the production of the anti-inflammatory cytokine IL-10 or through direct interaction with other leukocytes [63]. Several types of murine Bregs exist, including B1a-derived and B2-derived Bregs as well as possibly a distinct subset called B10 cells [64, 65]. Similar to mice there is phenotypic heterogeneity in human IL-10 expressing Bregs. Epidemiological studies have demonstrated that functional or numerical deficits in Bregs associate with autoimmune diseases such as multiple sclerosis (MS) and systemic lupus erythematosis (SLE) [63, 66]. Moreover, induction of IL-10 expression from B cells appears to protect from MS suggesting that Bregs may be important in protecting against immune mediated diseases [67].

B-2 B cells in atherosclerosis

Work published by the groups of Toh and Mallat both demonstrated that selective depletion of B-2 cells was accomplished by treatment with a monoclonal antibody (mAb) against the pan B cell marker CD20 [30, 31] and this attenuated atherosclerosis. Supporting their findings, both groups published that genetic deletion of the B cell activating factor receptor (BAFFR) selectively reduced B-2 cell numbers, while generally retaining B-1 cells, and similarly attenuated atherosclerosis [32, 33]. Additionally, supporting their depletion studies, Kyaw et al demonstrated that adoptive transfer of 5 million splenic B-2 cells, taken from C57BL/6 mice, into atherogenic lymphocyte deficient (*Rag2*$^{-/-}$*γc*$^{-/-}$*Apoe*$^{-/-}$) and

atherogenic B cell deficient (*μMTApoe*$^{-/-}$) mice resulted in significantly increased atherosclerosis compared to PBS or peritoneal B-1 cell transfer [30]. Anti-CD20 treatment, as published by Ait-Oufella et al, caused a profound loss of B-2 cells and associated with reduced titers of IgG against epitopes on oxLDL and increased ratios of IgM/IgG to the same epitopes [31]. Additionally, it was observed that mice treated with the mAb had reduced T cell activation and accumulation in atherosclerotic lesions and that T cells were shifted from IFNγ secretion (Th1) to IL-17 secretion (Th17) suggesting B-2 cells, possibly via auto-reactive IgG secretion, augment the inflammatory milieu within lesions.

Alternatively, it has been demonstrated that B-2 B cells have atheroprotective properties under certain conditions. Findings from our lab revealed that adoptive transfer of 30 or 60 million splenic CD43^{-} B-2 cells from *Apoe*$^{-/-}$ mice significantly reduced Western diet-induced atherosclerosis in *μMT*/*Apoe*$^{-/-}$ mice suggesting these cells were atheroprotective [34]. This apparent contradiction to the findings of Kyaw and colleagues may suggest that prior B cell exposure to lipid antigen, as would certainly occur in hypercholesterolemic *Apoe*$^{-/-}$ mice, could impact their functional contribution to atherosclerosis. Indeed, we have also demonstrated that transfer of 60 million B-2 cells derived from C57BL/6 mice into *μMTApoe*$^{-/-}$ mice did not reduce atherosclerosis [68] suggesting that primed B-2 cells but not naïve B-2 cells could respond to atherosclerotic conditions in an atheroprotective manner. Alternatively, more advanced subsetting of B-2 cells, based on

activation status, tissue residence, or antigen specificity, could better explain the contrasting results from these studies.

B-1 B cells and atherosclerosis

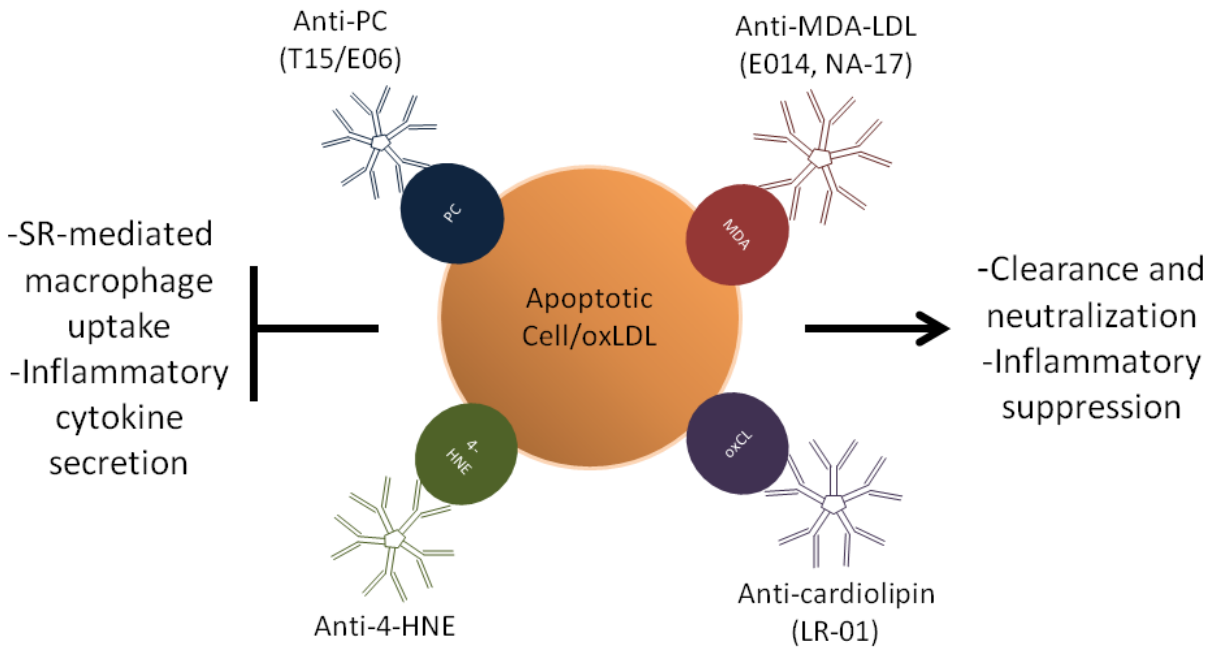
B-1a cells

Toh and colleagues demonstrated that splenectomizing *Apoe*$^{-/-}$ mice resulted in selective depletion of peritoneal B-1a cells in association with augmented atherosclerosis [35]. By supplementing the splenectomized mice with IP injections of peritoneal B-1a cells they were able to restore the B-1a population by approximately 70% and these mice developed significantly attenuated atherosclerosis compared to both splenectomized and sham control mice. Additional findings from this work suggested B-1a mediated atheroprotection was IgM dependent as adoptive transfer of B-1a cells unable to secrete IgM (sIgM$^{-/-}$) were able to fully restore the peritoneal B-1a population but did not develop reduced atherosclerosis compared to splenectomized controls.

Atheroprotective IgM NAbs bind modified self epitopes, such as oxidation specific epitopes (OSEs), found on oxLDL and apoptotic cells [69] (**Figure 4**). Oxidative products such as malondialdehyde (MDA) and phosphorylcholine (PC), generated on LDL within atherosclerotic plaques, are highly immunogenic and anti-MDA-LDL and anti-PC antibodies are measured in high titers within plasma and lesions of mice and humans [23, 27, 70, 71]. Indeed, OSE reactive IgMs are highly conserved making up as much as 30% of the total NAb repertoire in mice [72].

OSE on LDL and apoptotic cells act as danger associated molecular patterns (DAMPs) within the vascular lesion and are recognized by SR and other pattern recognition receptors (PRRs) [73]. The atheroprotective mechanisms of OSE reactive IgM are thought to be two fold. First, they act in a "housekeeping" capacity to aid in the clearance of apoptotic bodies through the recruitment of complement factors C1q and Mannose Binding Lectin (MBL) which direct their uptake by macrophages [74]. Secondly, they are anti-inflammatory by competing with SR mediated uptake of oxLDL which reduces inflammatory cytokine secretion by macrophages [75, 76]. The prototypic OSE reactive NAb is T15/E06, which binds to phosphorylcholine (PC) on the surface of oxLDL, though not native LDL, and apoptotic cells [77]. Binder and colleagues demonstrated that immunization of atherogenic *Ldlr*$^{-/-}$ mice with *Streptococcus pneumoniae*, which expresses PC on its cell wall, greatly increased production of circulating T15/E06 IgM and decreased the extent of atherosclerosis [78]. The same group showed that *Ldlr*$^{-/-}$ mice that were lethally irradiated and received IL-5$^{-/-}$ bone marrow had significantly reduced titers of T15/E06 and developed greatly increased atherosclerosis compared to IL-5$^{+/+}$ bone marrow donor controls [79]. IL-5 is a strong mitogen for B-1a proliferation supporting the atheroprotective role of B-1a cells due to the production of NAbs. A number of alternative OSE reactive IgM clones have been described with reactivities to MDA (E014 and NA-17) and oxidized cardiolipin (oxCL, LR01) [80]. All of these epitopes are formed upon lipid peroxidation of LDL and on apoptotic cells underscoring the critical importance of OSE reactive IgM NAbs in atherosclerosis. A possible additional mechanism for

Figure 4: Mechanisms of atheroprotective IgM: OSE reactive IgM recognize neo-epitopes on oxLDL and apoptotic cells such as phosphorylcholine (PC), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and oxidized cardiolipin (oxCL). Bound antibodies compete with scavenger receptor (SR) mediated uptake of oxLDL by macrophages reducing their production of inflammatory cytokines/chemokines. Additionally, the OSE reactive IgM can induce clearance of apoptotic cells avoiding the development of sterile necrosis. Finally, N-terminal sialylation of IgM can induce tolerance and suppress inflammatory response by macrophages and T cells. Figure adapted from **Figure 1**, Tsiantoulas, Gruber, and Binder, B-1 Cell Immunoglobulin Directed Against Oxidation-Specific Epitopes. 2013. *Frontiers in Immunology* [80].


Anti-PC
(T15/E06)
Anti-MDA-LDL
(E014, NA-17)
PC
MDA
-SR-mediated
macrophage
uptake
-Inflammatory
cytokine
secretion
Apoptotic
Cell/oxLDL
-Clearance and
neutralization
-Inflammatory
suppression
4-
HNE
oxCL
Anti-4-HNE
Anti-cardiolipin
(LR-01)

NAb mediated atheroprotection is induction of immune tolerance towards TI antigens due to the post-translational N-glycosylation of TI immunoglobulins. Research by Hess et al demonstrated that TI immunization induced secretion of suppressive sialylated IgG in contrast to TD immunization which associated with inflammatory agalactosylated and asialylated IgG [81]. More recently, studies by Colucci et al. demonstrated that terminally sialylated IgM from human plasma were internalized by T cells resulting in inhibition of T cell proliferation and activation [82]. These findings suggest that sialylated IgM NAb could suppress the hallmark inflammation that characterizes atherosclerosis, though it remains to be determined whether these antibodies are present in atherosclerotic plaques.

Similar to the findings that B-2 cells are both atherogenic and atheroprotective, recent identification of a novel subset of B-1a cells, called innate response activating (IRA) B cells by Rauch and colleagues [83] raised the possibility that B-1a cells also have contrasting functions. They demonstrated that IRA B cells, derived from peritoneal B-1a cells, within the spleen produce granulocyte-macrophage colony-stimulating factor (GM-CSF), an atherogenic growth factor which promotes the differentiation of inflammatory Ly-6C^{high} monocytes [83, 84]. The same group subsequently demonstrated that mice deficient in GM-CSF producing B cells were protected from atherosclerosis suggesting IRA B cells are indeed atherogenic though surprisingly through regulation of a Th1 mediated mechanism of atherogenesis [85]. This example of a potentially atherogenic B cell derived from “atheroprotective” B-1a cells underscores the notion that a

dichotomy that B-1 B cells attenuate and B-2 B cells aggravate atherosclerosis is likely too simplistic.

B-1b cells

B-1b cells have not been studied in the context of atherosclerosis. However their unique ability to generate TI immune memory allows for speculation that they could have atheroprotective function. B cell-mediated immunological memory is defined by the continued presence of antibodies, long lived plasma cells (LLPCs) and memory B cells after the clearance of antigen [86]. TD and TI antigens can both induce memory formation, though with differing features. TD antigens induce germinal center (GC) formation involving follicular B cells, follicular helper T cells (T_{FH}), and follicular dendritic cells (FDCs) which produce high affinity, class-switched, LLPCs and memory B cells [87]. In contrast, TI antigens do not induce GC formation, resulting in predominantly IgM secreting LLPCs and low affinity memory B cells [62]. Importantly, B-1b cells have been demonstrated to be the primary source of TI memory formation. The work of Alugupalli and colleagues demonstrated that B-1b cells, but not B-2 or B-1a cells, from convalescent mice were able to confer long lasting immunity against the relapsing/remitting fever of *Borrelia hermsii* infection when transferred to T and B cell deficient (*Rag1*$^{-/-}$) hosts [88]. In addition they demonstrated that *Borrelia hermsii* infection induced B-1b cell expansion and that mouse models deficient for T cells, B-2 cells or B-1a cells were all able to eliminate and become resistant to repeat infection suggesting that B-1b cells were key to the memory response.

Their findings were supported by the work of Haas et al which demonstrated that immunization with pneumococcal polysaccharide (PPS-3) induced antigen specific IgM titers in *Rag1*$^{-/-}$ mice that had received B-1b cells and that this was sufficient to protect the host mice from *Streptococcus pneumoniae* infection [89]. Indeed it has now been demonstrated that TI immunization can induce memory B cell and PC development [90, 91] suggesting the TI memory response is comparable to that developed by TD immunization. Better understanding of TI memory will be important for future treatment strategies. Indeed, in humans, TI vaccination strategies have been in use for decades. The anti-pneumococcal vaccine, first introduced in 1983, is made of pneumococcal polysaccharide from the 23 most common serotypes of *S. pneumoniae* and confers protection for upwards of a decade after immunization [62]. Given that IgM reactive to OSE on LDL are immunodominant in mice [72] and that these IgM antibodies are atheroprotective [79], determining a strategy to boost their production by immunization is a novel means of treatment that would be both long lasting and cost efficient. Research is underway to find effective small molecule antigens to use for atheroprotective vaccines [92]. What must be established is whether B-1b cells produce OSE reactive antibodies in the context of atherosclerosis and to what antigens they respond.

Regulatory B cells in atherosclerosis:

While it is unknown how Bregs contribute to atherosclerosis, given their production of IL-10 and ability to suppress other immune mediated conditions

such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis, and colitis, it is natural to speculate that they could be atheroprotective [93-95]. Bregs were demonstrated to accumulate in the aorta in an L-selectin dependent manner [43]. The same work demonstrated that a decrease in aortic Breg cells was associated with decreased IL-10 within the aortic tissue and increased overall aortic leukocyte content, suggesting Bregs are important contributors of IL-10 in atherosclerotic conditions. It is well established that depletion of IL-10 in mice aggravates atherosclerosis, associating with increased infiltration of inflammatory cells and increased production of inflammatory cytokines [96, 97]. Additional functions attributed to Breg cells include activation of Tregs through IL-10 and secondary signal presentation, suppression of inflammatory Th1 and Th17 cells due to IL-10 production and expression of FasL, and suppression of cytotoxic $CD8^+$ T cells due to IL-10 and TNF-α secretion [63]. Whether these immunoregulatory mechanisms occur under atherosclerotic conditions is unknown.

B Cells in human vascular disease

A recent study of cases taken from the Framingham Heart study used genome wide association (GWAS) to develop gene co-expression networks and identify independent genes that associate with coronary heart disease (CHD) [98]. This study found that genes associated with B cell activation were the most strongly enriched in controls but not in coronary artery disease cases, suggesting B cells may play an important protective role in human atherosclerosis. However, few

studies have compared B cells and B cell subsets to clinical measures of CVD. One study demonstrated that circulating $CD80^+$ late phase activated B cell numbers correlate with carotid intimal medial thickness (cIMT), an established risk factor for adverse cardiovascular events, suggesting that this subset contributes to atherosclerosis [99]. More recently, a prospective study of frozen peripheral blood mononuclear cells (PBMCs) from 700 patients addressed the correlation of the costimulatory molecules CD40 and CD86 on B cells and cardiovascular events [100]. This study demonstrated that patients in the highest tertile of percentage of $CD40^+$ B cells had a significantly reduced risk of stroke. In contrast patients in the highest tertile of numbers of $CD86^+$ B cells were at a significantly increased risk of stroke event. These findings suggest B cell costimulatory surface markers are important regulators of the suppressive or activating function of B cells and could be used to predict susceptibility to cardiovascular events.

Adding to the difficulty in this field is our poor understanding of human B cell subsets. The B-1a cell subset in mice is recognized by its surface expression of the T cell marker CD5 and its spontaneous production of NAbs. This is not recapitulated in humans as multiple B cell subsets are known to express CD5 [101]. In 2011, Griffin and colleagues used a clever approach whereby they identified a subset of circulating human B cells that were CD19, CD20, CD27, and CD43 positive and CD70 negative that shared functional characteristic of murine B-1 cells. These cells spontaneously produce IgM, stimulate T cells to proliferate, and

demonstrate tonic intracellular signaling [102]. Furthermore, the subset was enriched in human umbilical cord blood much like murine B-1 cells. The same group has recently described a Breg-like human B cell population they termed "Orchestrator" B cells (B1orc) which are $CD11b^+$ B-1 cells that spontaneously secrete IL-10 and suppress T cell activation [103]. There have been challenges to this group's findings arguing that the CD19, CD20, CD27, and CD43 positive B cells in humans may be pre-plasmablasts [54, 104-108], highlighting the difficulties in identifying the human equivalents of murine subsets. A recent review by Rothstein and Quach details discovery and subsequent technical challenges that have come from the description of the putative human B-1 cell subset [109].Recently, two groups independently demonstrated that increased numbers of putative human B-1 cells associated with the TI *Streptococcus pneumoniae* immunization, suggesting, like murine B-1 cells, the putative human B-1 subset is important for the TI antibody response [110, 111]. Importantly, the work by Leggat et al. used fluorescently tagged PPS to label PPS-specific B cells, demonstrating that the majority of positive cells were $CD27^+CD43^+$ B-1 cells [110]. Interestingly where this group described that the majority of the B-1 cells analyzed after immunization were $CD5^+$, Verbinnen et al described the opposite, that the majority were $CD5^-$, in their cohort. It will be important to determine whether CD5 is an important marker for possible subdifferentiation of the human B-1 subset. Additionally, it has not been studied if these cells produce NAb to OSE or whether their numbers are associated with circulating levels of NAb to OSE and atherosclerosis.

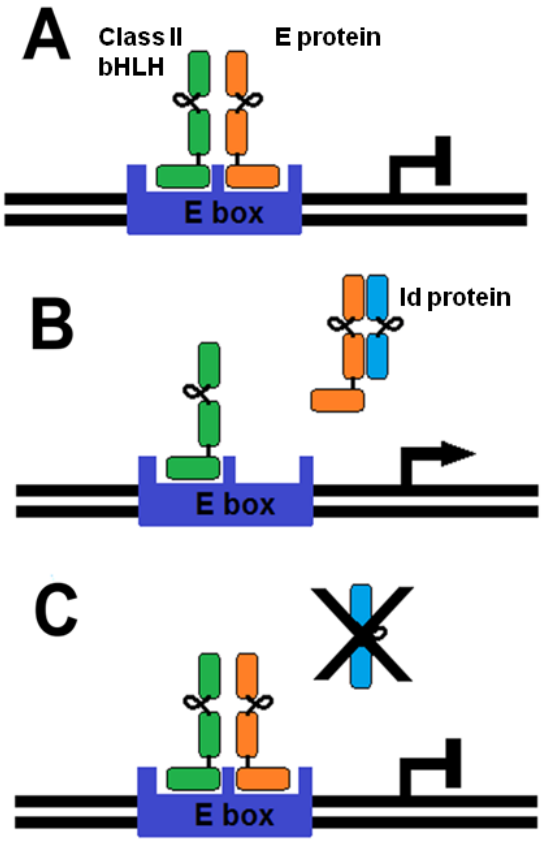
It has been well established that leukocytosis is independently associated with CHD [112] and poor outcomes after cardiovascular events [113-115] suggesting that circulating leukocyte counts can act as biomarkers for disease progression and outcome. Though less established, it has been demonstrated that circulating lymphocyte counts have an inverse relationship to recurrent ischemic events and cardiovascular mortality [112]. Indeed, the ratio of neutrophils to lymphocytes is associated with of acute vascular events and CHD severity and 3 year outcome [116]. Importantly, increased neutrophil to lymphocyte ratio has been shown to have independent prognostic value in cardiovascular events [117]. A better understanding of the association between B cell subsets and atherosclerosis could provide a clearer picture of the functional relevance of lymphocytes to cardiovascular disease in humans and possibly provide better prognostic measures than neutrophil to lymphocyte ratio. Importantly, characterization of human B cell subsets may help identify individuals at higher risk of acute atherothrombotic events.

Id3 regulates B cell homeostasis and atherosclerosis

The basic helix-loop-helix (bHLH) transcription factors are key regulators of cell growth and differentiation [118]. These factors are defined by the presence of an N-terminal basic DNA binding domain which binds to the conserved E box consensus sequence (G/ACAXXTGG/A) and a C-terminal helix-loop-helix domain used for dimerization to other HLH proteins [119]. There are three classes of bHLH proteins: Class I, called E proteins, are expressed in most tissues,

though they are not ubiquitous, and can homodimerize with other E proteins or heterodimerize with tissue specific class II HLH proteins to activate or repress transcription. Four E proteins exist in mammals: E12 and E47 which are both splice variants of the E2A gene, E2-2, and HEB. E2A in particular has been demonstrated to play essential roles in the development, survival, and proliferation of lymphocytes [119]. Class III HLH factors are the inhibitor of differentiation (Id) proteins which are unique in that they lack a basic DNA binding domain while maintaining the HLH domain [119]. Id proteins function by heterodimerizing to E proteins through the HLH domain, blocking E protein homo- or heterodimerization and inhibiting DNA binding thus acting as dominant negative inhibitors (or activators) of transcription [120] (**Figure 5**). Four Id proteins (Id1-4) exist in mammals and are highly expressed during development and in proliferating cells. Their expression diminishes in terminally differentiated and quiescent cells except in hematopoietic cells where Ids are expressed in mature lymphocytes [121]. Id3 is a regulator of B cell development, function, and antibody production [122, 123], and thymocyte development [124, 125]. Initial studies in Id3 deficient mice ($Id3^{-/-}$) demonstrated equivalent numbers of total B cells compared to WT but decreased proliferation in response to BCR crosslinking and attenuated antibody response to TD and TI immunization [123]. These findings could have been influenced by the mixed genetic background of the model however, as our lab has demonstrated that $Id3^{-/-}$ mice on a pure C57BL/6 background actually develop increased titers of total IgM [34].

Figure 5: Inhibitor of differentiation mechanism of transcriptional regulation. A) Class 1 helix-loop-helix (HLH) transcription factors, E proteins, can homo- or hetero- dimerize to E proteins or class 2, tissue specific, HLH proteins, respectively through their HLH domain allowing their basic domains to bind conserved sequences on DNA called ‘E-boxes’. **B)** Inhibitors of differentiation (Id) proteins lack a basic domain and compete to dimerize with E proteins thus inhibiting their DNA binding capacity acting as dominant negative regulators of transcription. **C)** Deletion of Id proteins disinhibits E protein dimerization.


A
Class II
bHLH
E protein
E box
B
Id protein
E box
C
E box

Other work in our lab has demonstrated that global deletion of Id3 significantly aggravates atherosclerosis after Western diet feeding suggesting it is atheroprotective [34]. Additionally it was demonstrated that Id3 was important for B cell mediated atheroprotection, as adoptive transfer of $CD43^-$ splenic B cells from *Id3*$^{-/-}$*ApoE*$^{-/-}$ mice into *μMTApoE*$^{-/-}$ mice did not modify atherosclerosis after Western diet feeding compared to vehicle controls, whereas *ApoE*$^{-/-}$ B cells substantially attenuated atherosclerosis [34]. Subsequent studies demonstrated that *Id3*$^{-/-}$*ApoE*$^{-/-}$ mice had significantly reduced numbers of B-1a cells compared to *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice and reduced T15/E06 IgM titers [42]. Follow-on studies revealed that B-1a numbers were not regulated by Id3 in B cells but rather due to upstream regulation of IL-33 stimulated IL-5 secretion by Id3 in innate type 2 natural helper cells (NHCs) [42]. These findings, and other work in the lab [126, 127], demonstrate that Id3 has broad regulatory functions that contribute to atherosclerosis, underscoring the importance of cell specific deletion models for future studies.

Id3 appears to have an important role in human atherosclerosis [128]. Our group assessed single nucleotide polymorphisms (SNP) in the human *ID3* gene from individuals in the Diabetic Heart Study. One of six of the tagged SNPs tested, rs11574, was found to be significantly associated with increased cIMT, a measurement of subclinical atherosclerosis. This SNP is found within the C-terminal coding exon of *ID3*, which is associated with its dominant negative function. The polymorphism is the replacement of guanine with adenine which

results in the substitution of the ancestral alanine with threonine at amino acid 105. To determine whether the modified Id3 had reduced interaction with E proteins, the ancestral Id3 (105A) and the polymorphic Id3 (105T) were transfected into NIH-3T3 cells that were co-transfected with FLAG-tagged E12. Co-immunoprecipitation demonstrated that 105T had significant reduction in binding affinity to E12 suggesting Id3105T has attenuated function compared to Id3105A [128]. Whether the presence of Id3105A associates with modified B cell populations or immunoglobulin titers has not been addressed.

Project Rationale

At the outset of this project, the dichotomy of B cell subset functions had been established and Id3 had been demonstrated in our lab to be an important regulator of atherosclerosis and B-1a cell numbers [42, 129]. The publication by Perry et al. described the generation of a B cell-specific knock out of Id3 ($Id3^{BKO}$) and from these mice it was demonstrated that Id3 did not regulate B-1a cell numbers in a B cell dependent-manner [42]. It had been previously observed that global Id3 knockout mice ($Id3^{-/-}$) had substantially greater numbers of B-1b cells compared to $Id3^{+/+}$ mice. From the generation of the $Id3^{BKO}$ mice it was again observed that these mice had significantly greater numbers of B-1b cells compared to $Id3^{WT}$. However, neither of these observations had been published. We hypothesized that Id3 was an important regulator of B-1b cells and that B-1b cells were atheroprotective and sufficient to generate OSE reactive IgM antibodies known to be atheroprotective.

In chapter 3 we present evidence that B-1b cells are an important atheroprotective B cell subset and secrete atheroprotective antibodies. Additionally, we demonstrate that mice with B cell-specific loss of Id3 had significantly greater numbers of the B-1b cell subset in all compartments and attenuated atherosclerosis. Sorted B-1b cells from *Apoe*$^{-/-}$ mice were sufficient to produce IgM antibodies reactive to OSE on oxLDL both *in vitro* and *in vivo*. Additionally, adoptive transfer of B-1b cells into B and T cell deficient *Rag1*$^{-/-}$ *Apoe*$^{-/-}$ mice significantly attenuated atherosclerosis. Finally, patients homozygous for the rs11574 SNP, which causes attenuated Id3 function, were revealed to have an increase of putative B-1 cells as a percentage of total B cells and increased titers of anti-MDA-LDL IgM compared to patients homozygous for the common allele. These studies are the first to test the contribution of B-1b cells to atherosclerosis and demonstrate that Id3 is important for homeostatic regulation of B-1b cells.

In Chapter 4, we explore possible mechanisms by which Id3 regulates B-1b cells. Multiple stimulatory conditions did not reveal a difference in proliferation or survival of B-1b cells from Id3^{BKO}*Apoe*$^{-/-}$ mice compared to Id3^{WT}*Apoe*$^{-/-}$ mice. Quantification of B cell subsets in 4-week-old mice demonstrated that Id3^{BKO}*Apoe*$^{-/-}$ mice had significantly increased numbers of B-1b cells prior to maturity however this did not correspond with increased numbers of B-1 progenitors. Finally, Id3^{BKO}*Apoe*$^{-/-}$ B-1 progenitors when cultured did not demonstrate a difference in expansion or maturation compared to Id3^{WT}*Apoe*$^{-/-}$

progenitors. Additional studies are necessary to determine the specific regulatory mechanism.

Chapter 2

Materials and Methods

Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. *Id3*$^{fl/fl}$ mice were a generous gift of Dr. Yuan Zhang (Duke University). *CD19*$^{Cre/+}$ mice and *Rag1*$^{-/-}$ mice were provided by Timothy Bender (University of Virginia). *Apoe*$^{-/-}$ mice were purchased from Jackson Laboratory. *Id3*$^{fl/fl}$ mice were bred to the *Apoe*$^{-/-}$ line and then with *CD19*$^{Cre/+}$ mice to develop B cell specific Id3 knockouts as previously described [42]. *Rag1*$^{-/-}$ mice were bred with *Apoe*$^{-/-}$ to generate *Rag1*$^{-/-}$*Apoe*$^{-/-}$ 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_2 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 [34].

Analysis of atherosclerotic lesions

Hearts were removed and prepared as previously described [35]. 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 [34]. 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 [34] 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 cells into Rag1$^{-/-}$Apoe$^{-/-}$ hosts

Following electronic cell sorting, as described below, 1x10^{5} B-1b cells were transferred interperitoneally (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[123]. 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).

LPS injections and proliferation analysis

8-12-week-old male Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice were injected IP with the TLR 4 ligand LPS (Sigma, L4391) to induce proliferation as described previously[130]. Briefly, mice were injected IP with 40 µg LPS in 200 µl sterile PBS or 200 µl sterile PBS as control, 48 hours prior to euthanization. Mice were then injected IP with 1.5 mg BrdU (BD-Fisher, 550891), in 150 µl of PBS, 24 hours and 12 hours prior to euthanization. PerC and splenic B cells were collected and prepared for flow cytometric analysis as described below.

Western diet feeding and proliferation/survival analysis

8-12-week-old male Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice were placed on Western diet, or maintained on chow as controls, for 4 weeks. The mice were injected IP with 1.5 mg BrdU (BD-Fisher, 550891) 24 hours prior to euthanization. PerC, splenice, and BM B cells were collected and prepared for flow cytometric analysis as described below. During staining, samples were split evenly with one half used for intracellular anti-BrdU staining following manufacturer's protocols from BD-Fisher (BD FITC BrdU Flow kit, 559619) and the other used for staining active caspase 3/7 following manufacturers protocols from Life Technologies (CellEvent Caspase-3/7 Green flow cytometry assay kit, C10427).

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 [34, 131]. Briefly, peritoneal lavage was done with DMEM media supplemented with 10% heat

inactivated FBS, 2 mM L-glutamine, 1X Penicillin/Streptomycin, and 2 mM EDTA (all purchased from GIBCO). 4 ml media were injected through the peritoneal wall, the mouse was agitated to detach cells then a small hole was opened in the peritoneal membrane to recollect lavage fluid. Spleen were removed from the peritoneal cavity and placed in 70 µm cell strainers (Falcon, 352350) in 30 mm dishes filled with 10 ml of supplemented DMEM. Spleens were homogenized using the plunger of a 1 ml syringe and media was transferred into 15 ml falcon tubes. Blood was collected by cardiac puncture and 100 µl were transferred into tubes containing heparin. 1 ml erythrocyte lysis buffer (ammonium chloride, potassium carbonate, and EDTA in water) was added and the tube was left rotating for 5 mins then diluted with PBS. BM was harvested from the femur and tibia of each leg. Muscle and fascia were removed from leg bones and the heads of each bone were cut to expose the BM compartment. BM was flushed using cold PBS injected through a 27G needle into a 15 ml falcon tube. BM cells were separated from stromal tissue after incubating for 15 minutes by transferring supernatant using a transfer pipette.

Cell suspensions were blocked for Fc receptors using anti-FCγRIII/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% NAN_3 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.

Intracellular staining for immunoglobulin isotypes was done using reagents and protocols from the BD Cytofix/Cytoperm kit (BD, 554714). Intracellular staining for BrdU incorporation…(look at kit details). Staining for active caspase 3/7 was done using… (find kit details)

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.

Electronic cell sorting was carried out at the University of Virginia Flow Cytometry Core on an Influx cell sorter (BD Bioscience) using BD FACS Sortware 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.

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). Live/Dead discrimination was determined by LIVE/DEAD fixable yellow staining (Invitrogen) or DAPI. All human antibodies were from BD – CD3 (PE-CF594, SK7), CD27 (BV421, M-T271), and eBioscience – CD20 (APC-H7, 2H7), CD43 (FITC, 84-3C1).

In vitro stimulation assays

Post electronic cell sorting, *Apoe*$^{-/-}$ 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) 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.

In vitro B-1 progenitor culture

B-1 progenitors were sorted as described above and placed into culture at 1-3x10^3 cells/well of a 6-well dish as previously described. B-1 progenitors were cultured as described previously [51]. Briefly, stromal OP9 cells were cultured to a monolayer on transwell columns in stromal culture media (αMEM supplemented with 5% FCS, 1 mM L-glutamine, 100 U/ml penicillin/streptomycin all purchased from Gibco). Post-electronic cell sorting, B-1 progenitors were washed then resuspended in B-1 progenitor culture media (RPMI medium supplemented with 10% FCS, 50 μM β-mercaptoethanol, 1 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 50 μg/ml gentimycin all purchased from Gibco) containing 20ng/ml IL-3, 20ng/ml IL-6, 20ng/ml stem cell factor, (all from Life Tech), 10ng/ml FLT3 ligand (Peprotech), and 10ng/ml recombinant murine TSLP (R&D Systems) in 6-well dishes and OP9 monolayers were placed onto culture wells. Cells were observed for (48-72 hours) then fed with new media every 48 hours once they reached 20% confluency. For flow cytometric cell staining, transwell columns were carefully removed and kept sterile and B-1 culture media was agitated by pipette to separate cells from the bottom of the well. One half of the total culture media was removed for analysis and new media was added then OP9 cells were replaced.

Enzyme-linked Immunosorbent Assay (ELISA)

Specific Ab levels to given antigens in plasma from mice were determined by chemiluminescent ELISA as previously described [78, 92]. 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 [132]. 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[123]. 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

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 $1x10^6$ 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% CO_2). 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-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.

Chapter 3

B-1b cells secrete atheroprotective IgM and attenuate atherosclerosis

As published in Rosenfeld, S.M. et al. B-1b cells secrete atheroprotective IgM and attenuate atherosclerosis. *Circ Res* (2015)

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 (Id3^{BKO}*Apoe*$^{-/-}$) have increased numbers of B-1b cells systemically, increased titers of OSE-reactive IgM antibodies, and significantly reduced diet-induced atherosclerosis compared to Id3^{WT}*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.

Introduction

Murine studies provide clear evidence that B cells regulate atherosclerosis[25, 26] 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[26, 30, 32-34, 133]. Alternatively, there is near uniform agreement of an atheroprotective role for B-1a cells [35, 71, 78, 129].

B-1 and B-2 cells are developmentally and functionally distinct subsets in mice[54]. B-1 cells produce the majority of total circulating IgM[134], which they secrete in a T cell-independent manner[135]. 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 germline 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[72, 136, 137]. 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[90, 138]. 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[34]. Id3 is a member of the helix-loop-helix transcription factor family known to be important in lymphocyte function[122]. These global Id3 knockout mice developed equivalent numbers of B-2 cells, but reduced numbers of atheroprotective B-1a cells compared to wild-type[42]. 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 $CD27^{+}CD43^{+}$ [102]. 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->A) causes an amino acid change (A->T) and this results in decreased Id3 binding to E proteins [128]. 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.

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[35]. In particular, IgM that bind OSE on oxLDL, such as anti-MDA-LDL and anti-CuOx-LDL, are considered to be atheroprotective [139]. 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 ($CD19^{+}B220^{+/-}IgM^{hi}CD23^{-}CD5^{-}$) and B-1a cells ($CD19^{+}B220^{+/-}IgM^{hi}CD23^{-}CD5^{+}$) 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[91] (**Figure 6A**). Absolute concentration of IgM and relative concentration of anti-MDA-LDL and anti-CuOx-LDL IgM were measured using established chemiluminescent ELISA[78, 92] and calculated against IgM

Figure 6: B-1b cells produce IgM reactive to MDA-LDL and CuOx-LDL *in vitro* and *in vivo*. A) B-1b ($CD19^{+}B220^{+/-}IgM^{hi}CD23^{-}CD5^{-}$) and B-1a ($CD19^{+}B220^{+/-}IgM^{hi}CD23^{-}CD5^{+}$) 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

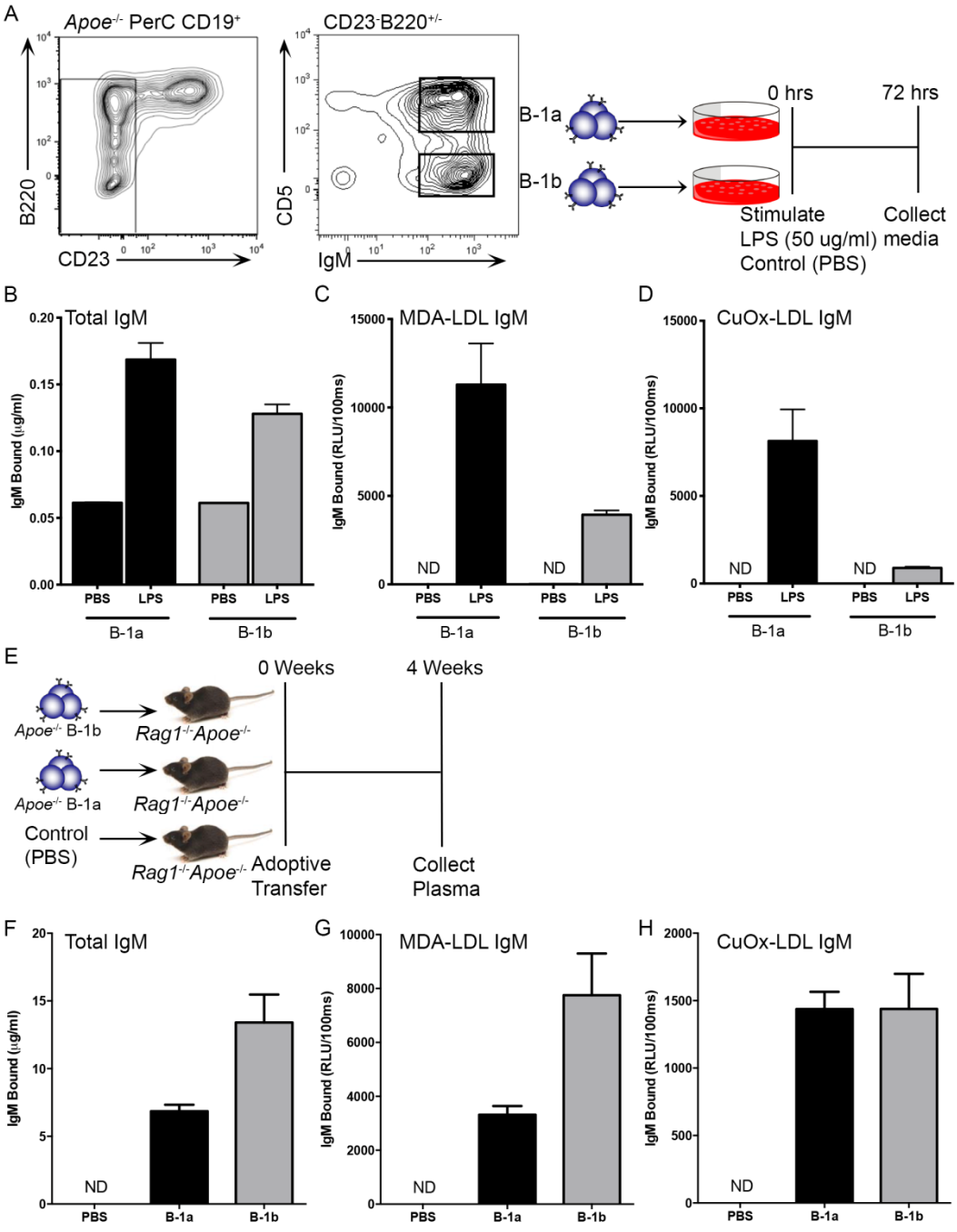
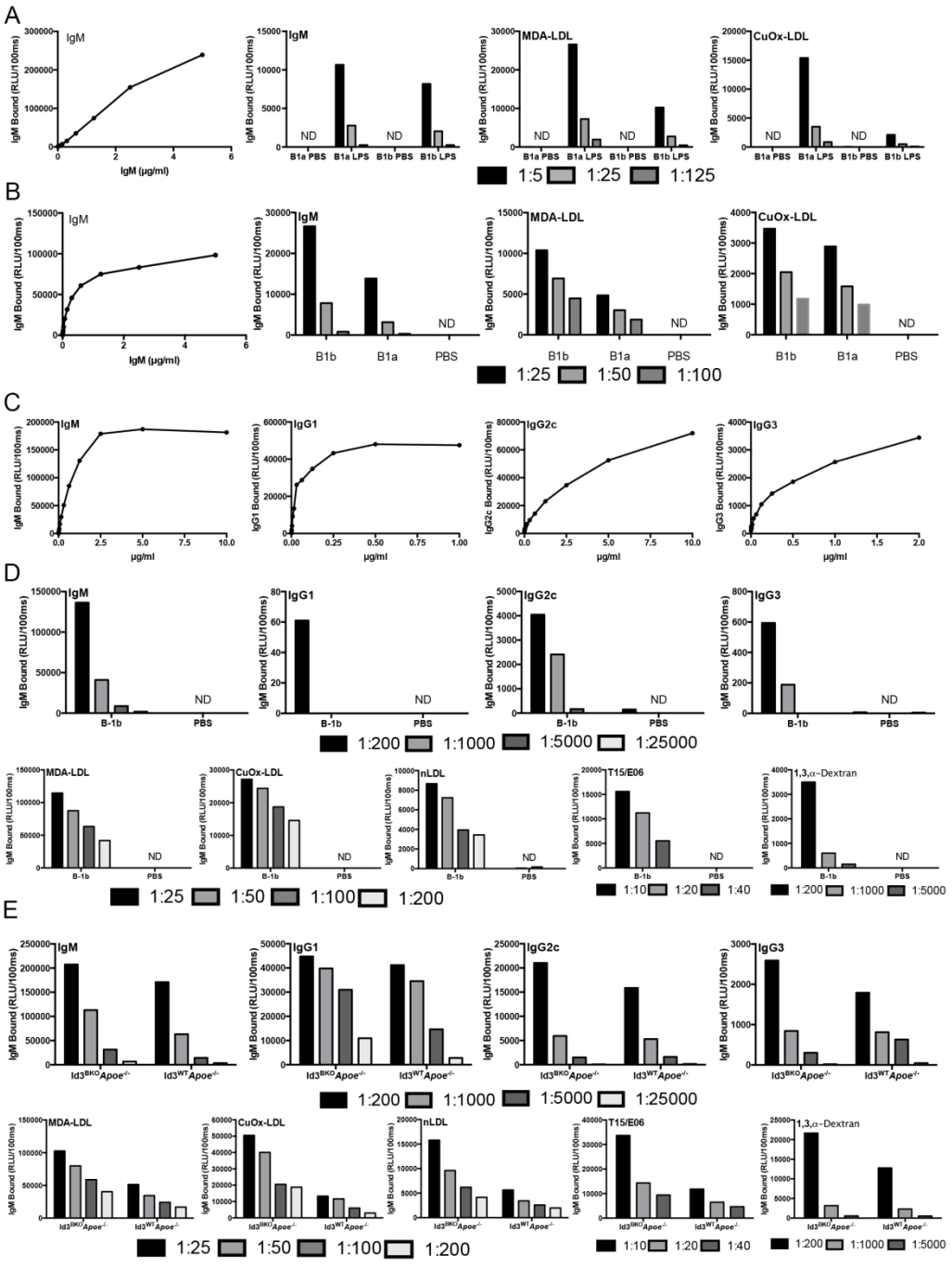

A
Apoe-/- PerC CD19+
B220
CD23
CD23-B220+/-
CD5
IgM
B-1a
B-1b
0 hrs
72 hrs
Stimulate
LPS (50 ug/ml)
Control (PBS)
Collect
media
B
Total IgM
IgM Bound (µg/ml)
PBS
LPS
B-1a
B-1b
C
MDA-LDL IgM
IgM Bound (RLU/100ms)
ND
D
CuOx-LDL IgM
E
0 Weeks
4 Weeks
Apoe-/- B-1b
Rag1-/-Apoe-/-
Apoe-/- B-1a
Control
(PBS)
Adoptive
Transfer
Collect
Plasma
F
Total IgM
G
MDA-LDL IgM
H
CuOx-LDL IgM

Figure 7: 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 6B-D**. **B)** IgM standard curve and dilution series for total IgM, MDA-LDL IgM, and CuOx-LDL IgM used for **Figures 6F-H**. **C)** Standard curves for IgM, IgG1, IgG2c, and IgG3 used to calculate absolute titers for **Figure 8D** and **Figure 8A**. **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 8D-E**. **E)** Standard curves for IgM, IgG1, IgG2c, and IgG3 used to calculate absolute titers for **Figure 8D** 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 8D-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**


A
IgM
IgM Bound (RLU/100ms)
IgM (µg/ml)
MDA-LDL
CuOx-LDL
ND
B1a PBS
B1a LPS
B1b PBS
B1b LPS
1:5
1:25
1:125
B
1:25
1:50
1:100
B1b
B1a
PBS
C
IgG1
IgG2c
IgG3
µg/ml
D
B-1b
1:200
1:1000
1:5000
1:25000
nLDL
T15/E06
1,3,α-Dextran
1:10
1:20
1:40
E
Id3BKOApoe-/-
Id3WTApoe-/-

standard and pooled dilution series respectively (**Figure 7A**). The data demonstrate that stimulation induced a robust IgM response from both B-1b and B-1a cells (**Figure 6B**). 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 6C&D**). Titers of IgG3 were 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 (*Rag*1$^{-/-}$) mice, which are devoid of T and B cells, crossed with *Apoe*$^{-/-}$ mice (*Rag1*$^{-/-}$*Apoe*$^{-/-}$) were injected with equal numbers (1x10^5) of purified B-1b or B-1a cells, or PBS control (**Figure 6E**). 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 (**Figure 7B**). 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, trigylcerides, or HDL cholesterol levels compared to PBS controls (**Table 1**). 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 8B&C, Figure 9A&B**). An average of $3.6x10^4$±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 (**Figure 9**). 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 (**Figure 7C**). 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 8D**). Additionally, relative titers of IgM reactive to native

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

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

Figure 8: 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 1x10^5 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/100ms**E)** 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

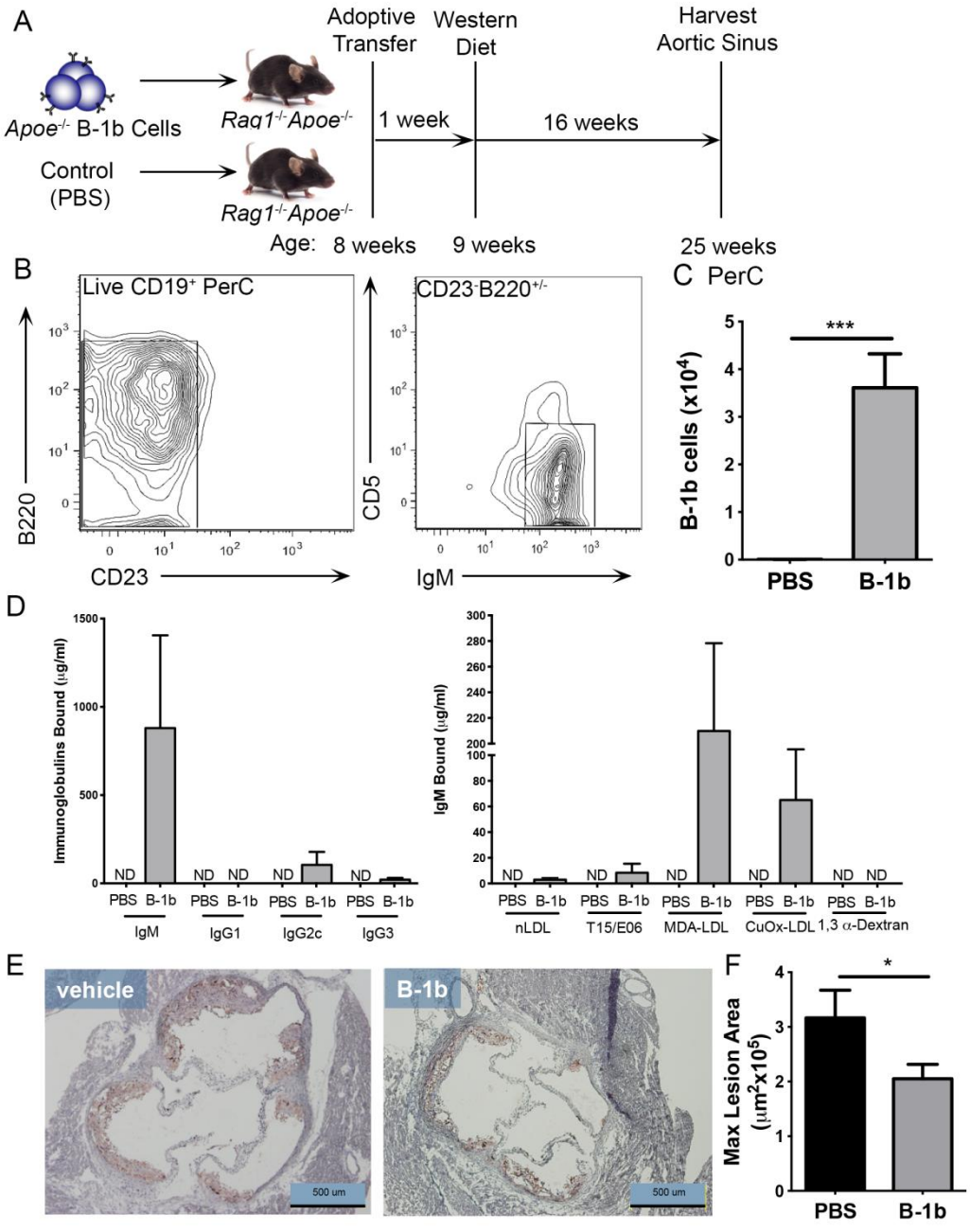
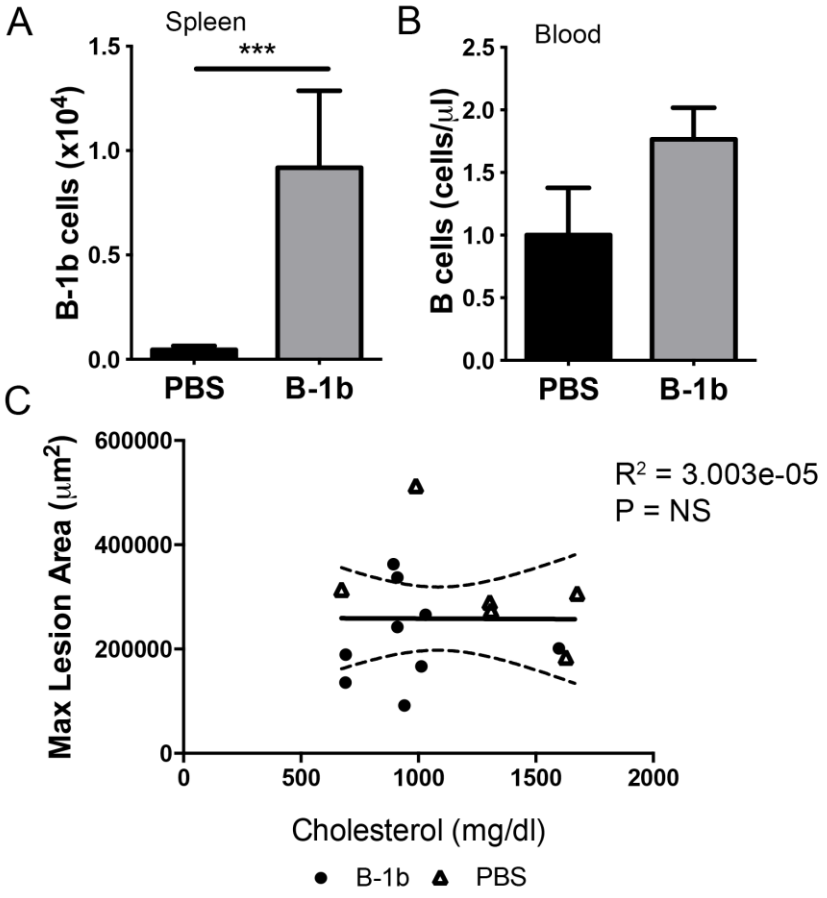

A
Adoptive Transfer
Western Diet
Harvest Aortic Sinus
Apoe-/- B-1b Cells
Rag1-/-Apoe-/-
1 week
16 weeks
Control (PBS)
Rag1-/-Apoe-/-
Age: 8 weeks
9 weeks
25 weeks
B
Live CD19+ PerC
B220
CD23
CD23-B220+/-
CD5
IgM
C PerC
***
B-1b cells (x10^4)
PBS
B-1b
D
Immunoglobulins Bound (μg/ml)
IgM
IgG1
IgG2c
IgG3
IgM Bound (μg/ml)
nLDL
T15/E06
MDA-LDL
CuOx-LDL
1,3 α-Dextran
E
vehicle
B-1b
500 um
F
*
Max Lesion Area (μm2x10^5)
PBS
B-1b

Figure 9: **B-1b cells are found in the spleen of *Rag1*$^{-/-}$*Apoe*$^{-/-}$ 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*$^{-/-}$*Apoe*$^{-/-}$ 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 (μm^2). 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


A
Spleen
***
B-1b cells (x10^4)
1.5
1.0
0.5
0.0
PBS
B-1b
B
Blood
B cells (cells/μl)
2.5
2.0
1.5
1.0
0.5
0.0
PBS
B-1b
C
Max Lesion Area (μm^2)
600000
400000
200000
0
0
500
1000
1500
2000
Cholesterol (mg/dl)
R^2 = 3.003e-05
P = NS
B-1b
PBS

LDL (nLDL), MDA-LDL, CuOx-LDL, α-1,3-dextran, and E06/T15, (the NAb to the phosphorylcholine (PC) moiety of oxidized phospholipid (OxPL)[136]) were measured based on pooled dilution series (**Figure 7D**). 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 8D**). 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 8D**) 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 8D**). 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 ($2.05x10^5 \pm 0.26x10^5$ μm^2 vs $3.16x10^5 \pm 0.51x10^5$ μm^2, $p<0.05$, **Figure 8E&F**). Comparison of plaque size to total cholesterol demonstrated that there was no correlation for these values (**Figure 9C**). 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[34] 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 (Id3^{BKO}) by crossing *Id3*$^{fl/fl}$ mice with *CD19*$^{cre/+}$ mice and then bred these with *Apoe*$^{-/-}$ mice (Id3^{BKO}*Apoe*$^{-/-}$) and confirmed the deletion with Western blot analysis as previously reported[42]. PerC, spleen, and blood were collected from 8-week-old, male, Id3^{BKO}*Apoe*$^{-/-}$and littermate control (Id3^{WT}*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 10A**. Id3^{BKO}*Apoe*$^{-/-}$ mice contained significantly greater numbers of B-1b cells compared to Id3^{WT}*Apoe*$^{-/-}$ controls. No differences in the number of B-1a or B-2 cells were detected (**Figure 10B-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 (**Figure 11**).

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 [131]. 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 12A**). Id3^{BKO}*Apoe*$^{-/-}$ mice had significantly more IgM^{+} immunoglobulin secreting cells (ISC) in their BM than Id3^{WT}*Apoe*$^{-/-}$ mice (**Figure 12B&C**). A portion of the BM tissue was also analyzed by flow cytometry using the gating

Figure 10: 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 ($CD19^{+}B220^{high}$), B-1a ($CD19^{high}B220^{mid/low}IgM^{high}CD5^{+}$), and B-1b cells ($CD19^{high}B220^{mid/low}IgM^{high}CD5^{-}$) 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

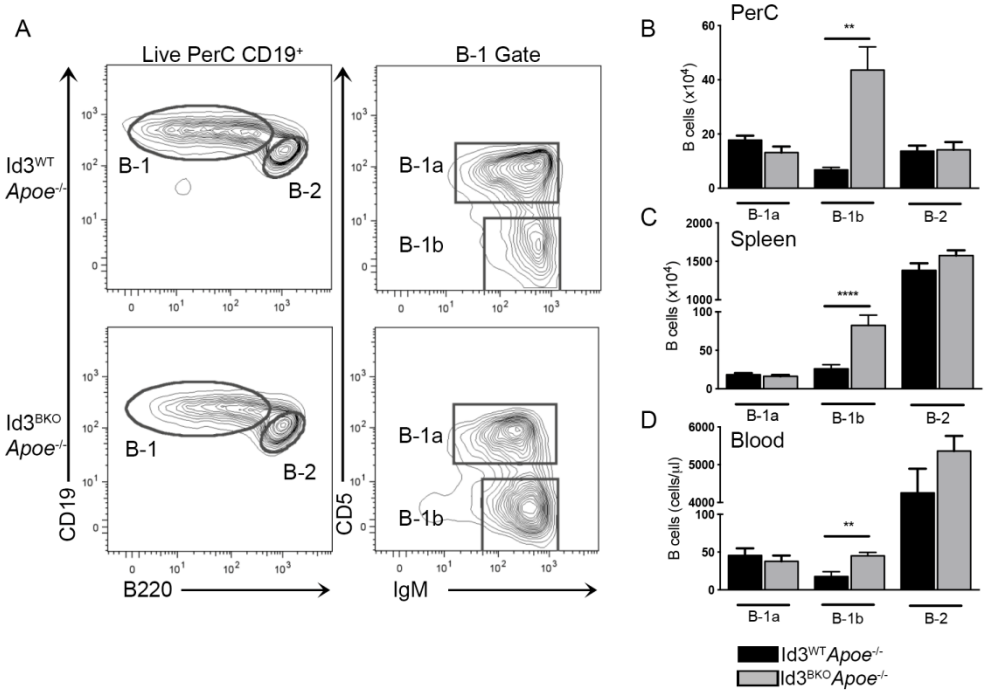
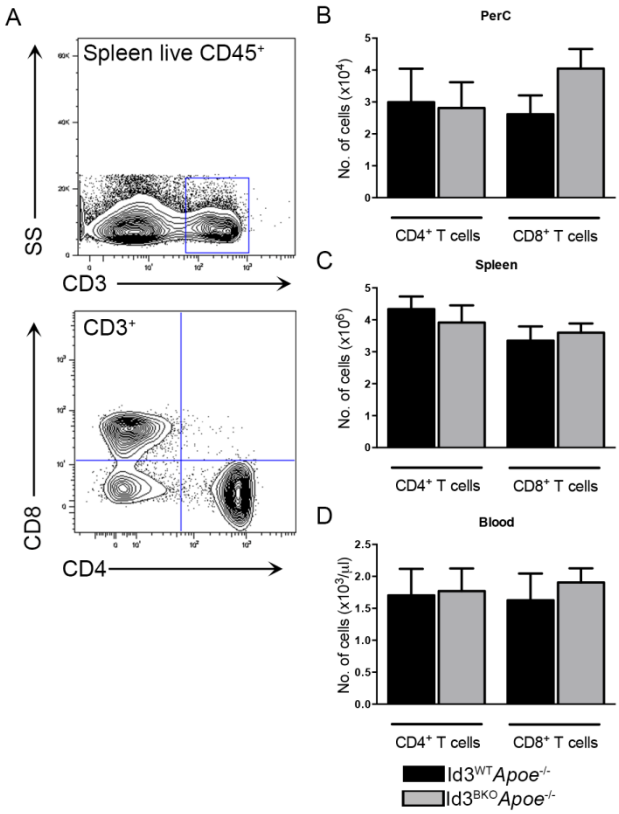

A
Live PerC CD19+
B-1 Gate
Id3WT Apoe-/-
Id3BKO Apoe-/-
B-1
B-2
B-1a
B-1b
CD19
B220
CD5
IgM
B
PerC
B cells (x10⁴)
**
B-1a
B-1b
B-2
C
Spleen
****
D
Blood
B cells (cells/μl)
Id3WT Apoe-/-
Id3BKO Apoe-/-

Figure 11: **Comparison of $CD4^+$ and $CD8^+$ T cells from 8-week-old, chow fed, male $Id3^{BKO}$*Apoe*$^{-/-}$ and $Id3^{WT}$*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.


A
Spleen live CD45+
SS
CD3
CD3+
CD8
CD4
B
PerC
No. of cells (x10^4)
CD4+ T cells
CD8+ T cells
C
Spleen
No. of cells (x10^6)
CD4+ T cells
CD8+ T cells
D
Blood
No. of cells (x10^3/µl)
CD4+ T cells
CD8+ T cells
Id3WT Apoe-/-
Id3BKO Apoe-/-

strategy published by Baumgarth and colleagues (**Figure 13**, [131]). 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 12D**). 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 12E**). 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 [131].

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 14A**). Id3^{BKO}*Apoe*$^{-/-}$ mice exhibited significantly reduced atherosclerosis within the aortic root compared to Id3^{WT}*Apoe*$^{-/-}$ mice (3.99x10^5 ±.19x10^5 μm^2 vs. 4.96x10^5 ±.19x10^5 μm^2, P<0.01, **Figure 14B&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 14D&E**) Finally, Tunel staining demonstrated that lesions in Id3^{BKO}*Apoe*$^{-/-}$ mice contained a significantly decreased percentage of apoptotic cell bodies than did

Figure 12: 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^{WT}*Apoe*$^{-/-}$ (n=8) and Id3^{BKO}*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^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice as determined by ELISA. Data are mean ± SEM.Unpaired, two-tailed Students T-test was used to compare differences, *$p<0.05$, **$p<0.01$

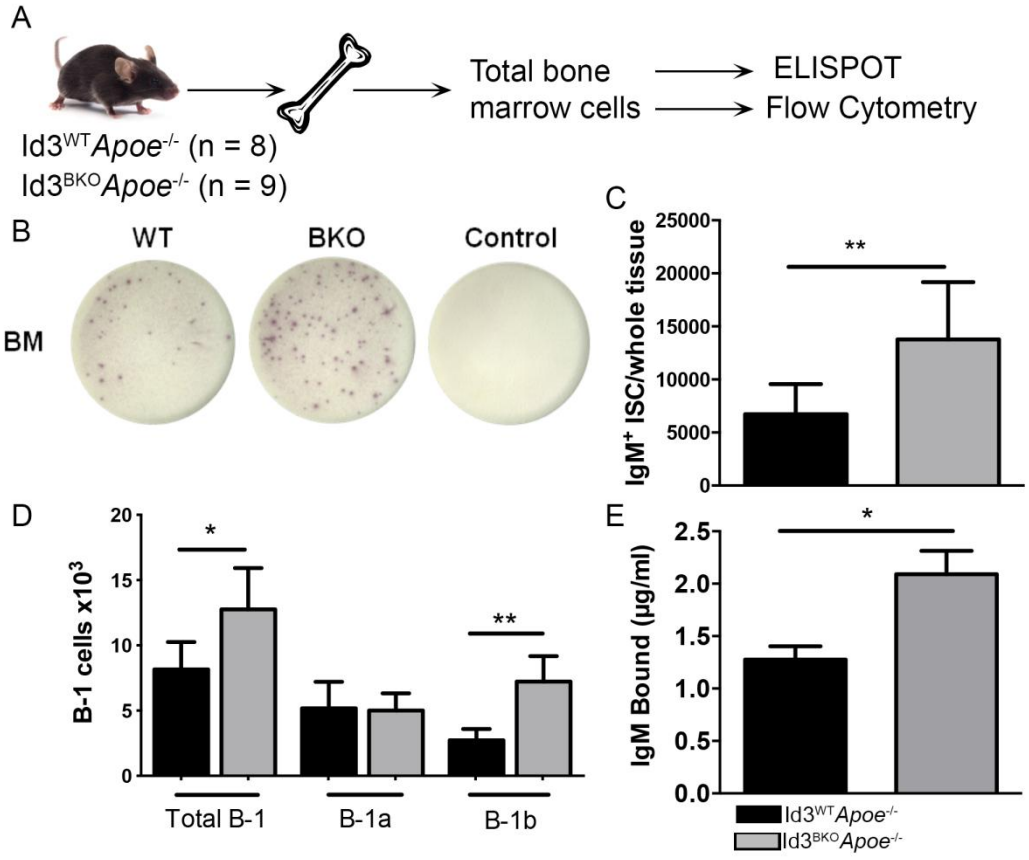
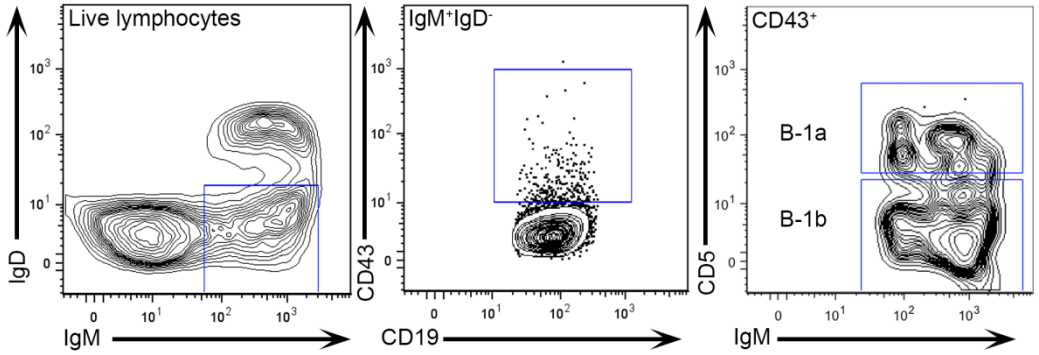

A
Total bone marrow cells
ELISPOT
Flow Cytometry
Id3WTApoe-/- (n = 8)
Id3BKOApoe-/- (n = 9)
B
WT
BKO
Control
BM
C
IgM+ ISC/whole tissue
25000
20000
15000
10000
5000
0
**
D
B-1 cells x10^3
20
15
10
5
0
*
**
Total B-1
B-1a
B-1b
E
IgM Bound (µg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
*
Id3WTApoe-/-
Id3BKOApoe-/-

Figure 13: **Representative gating of bone marrow B-1a and B-1b cells.**

Adapted from the gating described in Choi et al, *Eur J Immunol*, 2012.


Live lymphocytes
IgD
IgM
IgM+IgD-
CD43
CD19
CD43+
B-1a
B-1b
CD5
IgM

Id3^{WT}*Apoe*$^{-/-}$ ($0.37*10^{-3}$ ± $0.12*10^{-3}$ % vs. $0.84*10^{-3}$ ± $0.17*10^{-3}$ %, $P<0.05$, **Figure 14F&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 14H&I**) Lipid analysis demonstrated that there were no significant differences in body weight, weight gain, or plasma lipids at time of euthanasia (**Table 2**). Additionally, B-1b cell number remained greater in Id3^{BKO}*Apoe*$^{-/-}$ mice after the 16 weeks of Western diet feeding (**Figure 15**). Interestingly, although not different at baseline (**Figure 10**), the number of PerC B-2 cells was greater in Id3^{BKO}*Apoe*$^{-/-}$ mice compared to WT controls after Western diet feeding (**Figure 15**). 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 [78, 140] while also binding apoptotic cell bodies, enhancing their clearance and decreasing sterile inflammation [141]. 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 8D** in the respective mice. Absolute

Figure 14: B cell specific deletion of Id3 reduces diet induced atherosclerosis. A) 8-week old, male, chow fed $Id3^{WT}$*Apoe*$^{-/-}$ (n=10) and $Id3^{BKO}$*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 *en face*. Two-tailed Mann-Whitney test was used to compare measured values. *p≤0.05, **p<0.01

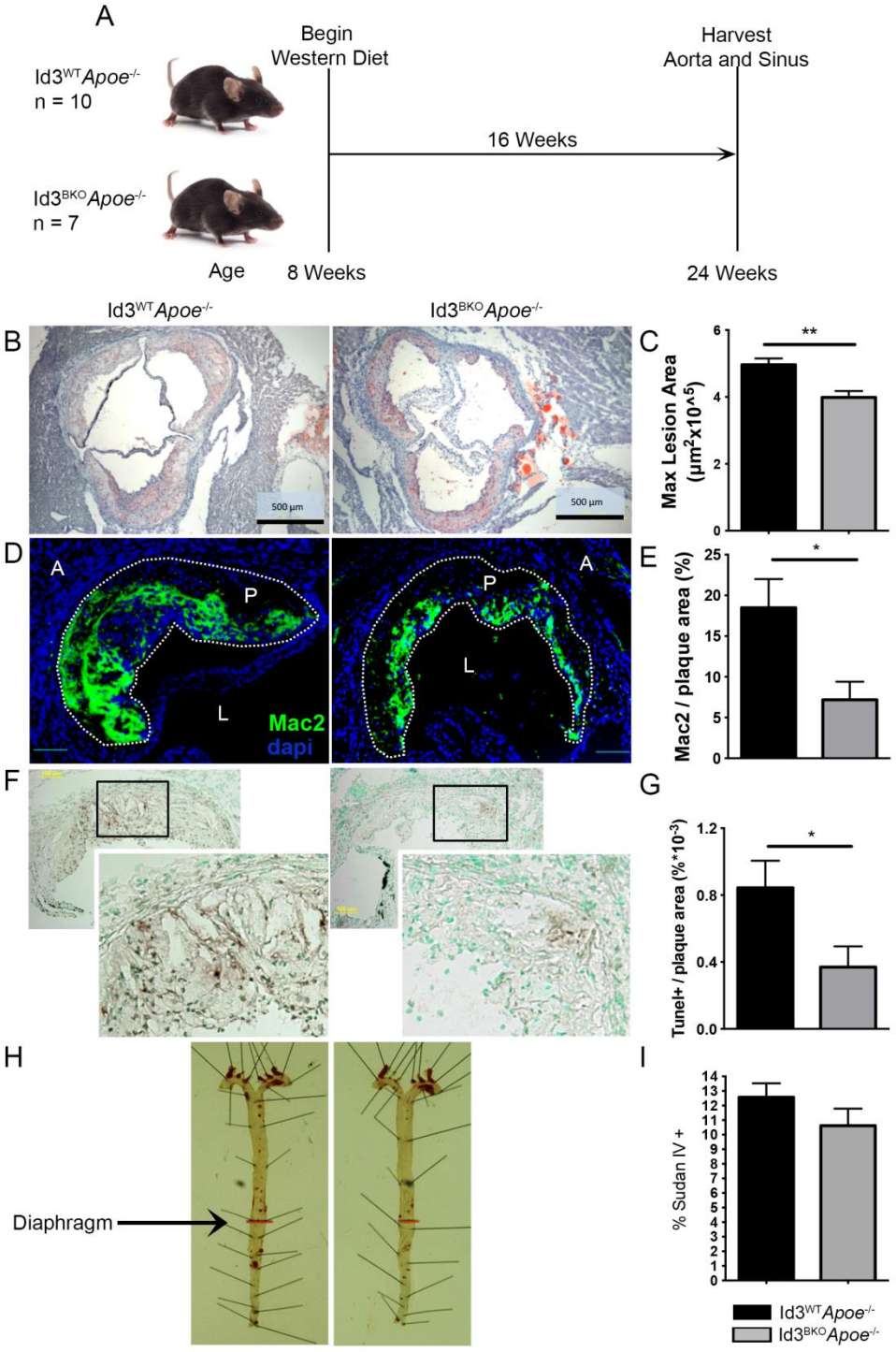
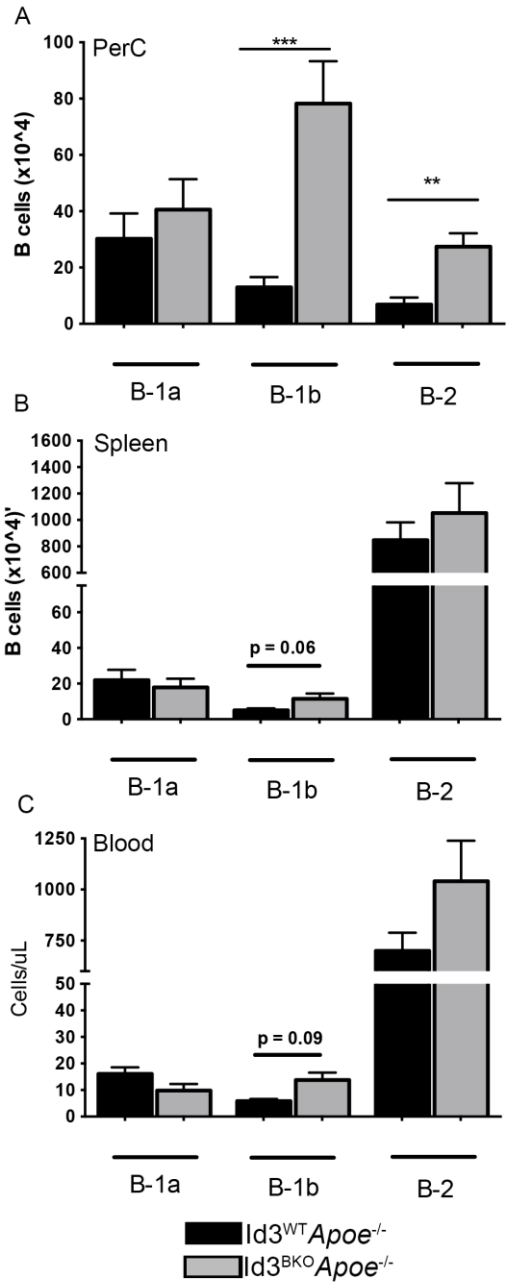

A
Id3WT Apoe-/-
n = 10
Id3BKO Apoe-/-
n = 7
Begin
Western Diet
16 Weeks
Harvest
Aorta and Sinus
Age
8 Weeks
24 Weeks
Id3WT Apoe-/-
Id3BKO Apoe-/-
B
500 µm
C
Max Lesion Area
(µm2x10^5)
**
D
A
P
L
Mac2
dapi
E
Mac2 / plaque area (%)
*
F
G
TunelH+ / plaque area (%*10-3)
*
H
Diaphragm
I
% Sudan IV +
Id3WT Apoe-/-
Id3BKO Apoe-/-

Table 2: Weight and lipids from $Id3^{WT}$ and $Id3^{BKO}$ mice fed a Western diet for 16 weeks

Genotype	$Id3^{WT}$ (n= 11)	$Id3^{BKO}$ (n= 7)	P
Weight (g)	38.62± 0.89	40.08± 0.86	NS
Weight gain (g)	12.52± 1.35	14.56± 0.93	NS
Total Cholesterol (mg/dL)	1476± 65.0	1514± 100.3	NS
Triglycerides (mg/dL)	358.6± 23.3	387.9± 29.2	NS
HDL (mg/dL)	43.64± 3.19	42.60± 4.13	NS

Figure 15: Quantification of B cell subsets from PerC, spleen, and blood recovered from 16-week Western Diet fed Id3^{WT}*Apoe*$^{-/-}$ and Id3^{BKO}*Apoe*$^{-/-}$ mice. Data are mean ± SEM. Two-tailed Students T-test was used to compare differences between genotypes. **p<0.01, ***p<0.001


A
PerC
B cells (x10^4)
100
80
60
40
20
0
***
**
B-1a
B-1b
B-2
B
Spleen
B cells (x10^4)'
1600
1400
1200
1000
800
600
60
40
20
0
p = 0.06
B-1a
B-1b
B-2
C
Blood
Cells/uL
1250
1000
750
50
40
30
20
10
0
p = 0.09
B-1a
B-1b
B-2
Id3WT Apoe-/-
Id3BKO Apoe-/-

titers of immunoglobulins were determined using standard curves (**Figure 7C**). 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 16A**). Id3^{BKO}*Apoe*$^{-/-}$ mice also contained significantly greater relative IgM titers reactive to MDA-LDL, and CuOx-LDL than Id3^{WT}*Apoe*$^{-/-}$ mice (**Figure 16B**). An approximately 30% increase in the amount of the E06/T15 NAb was also detected though it was not statistically significant (**Figure 16B**). No significant difference was detected in IgM reactive to α-1,3-Dextran (**Figure 16B, [142]**). Again, low levels of IgM to nLDL were noted (**Figure 16B**). Pooled dilution series were used for all relative titers (**Figure 7E**). Importantly, real-time PCR analysis of secreted IgM (sIgM) mRNA, expressed per cell, from sorted B cell subsets from Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice demonstrated no differences in expression suggesting that Id3 does not directly regulate IgM produced per cell (**Figure 17**). 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, Id3^{BKO}*Apoe*$^{-/-}$ mice were immunized with DNP-KLH in complete Freund's adjuvant and bone marrow plasma cells and plasma were analyzed (**Figure 18A**). Greater numbers of IgM^{+} plasma cells (PC) were measured by flow cytometry in Id3^{BKO}*Apoe*$^{-/-}$ mice compared to Id3^{WT}*Apoe*$^{-/-}$ mice, both those immunized with DNP-KLH and adjuvant controls (**Figure 18B**)

Figure 16: B cell-specific loss of Id3 results in increased titers of atheroprotective IgM antibodies after Western diet feeding. Plasma was collected from Id3^{WT}*Apoe*$^{-/-}$ and Id3^{BKO}*Apoe*$^{-/-}$ 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. **A)** Comparison of absolute titers of IgM and IgG isotypes reported as µg/ml. **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

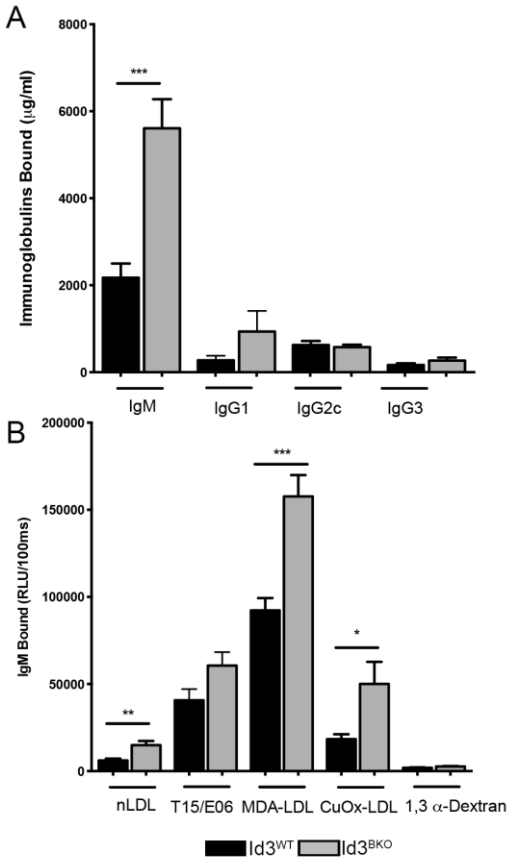
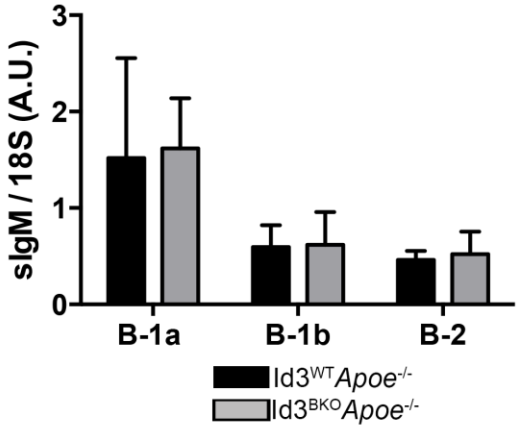

A
Immunoglobulins Bound (μg/ml)
8000
6000
4000
2000
0
***
IgM
IgG1
IgG2c
IgG3
B
IgM Bound (RLU/100ms)
200000
150000
100000
50000
0
***
**
*
nLDL
T15/E06
MDA-LDL
CuOx-LDL
1,3 α-Dextran
Id3WT
Id3BKO

Figure 17: 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 $Id3^{BKO}Apoe^{-/-}$ and $Id3^{WT}Apoe^{-/-}$ PerC B cell subsets and normalized to the housekeeping gene 18S rRNA. Data are mean ± SEM.

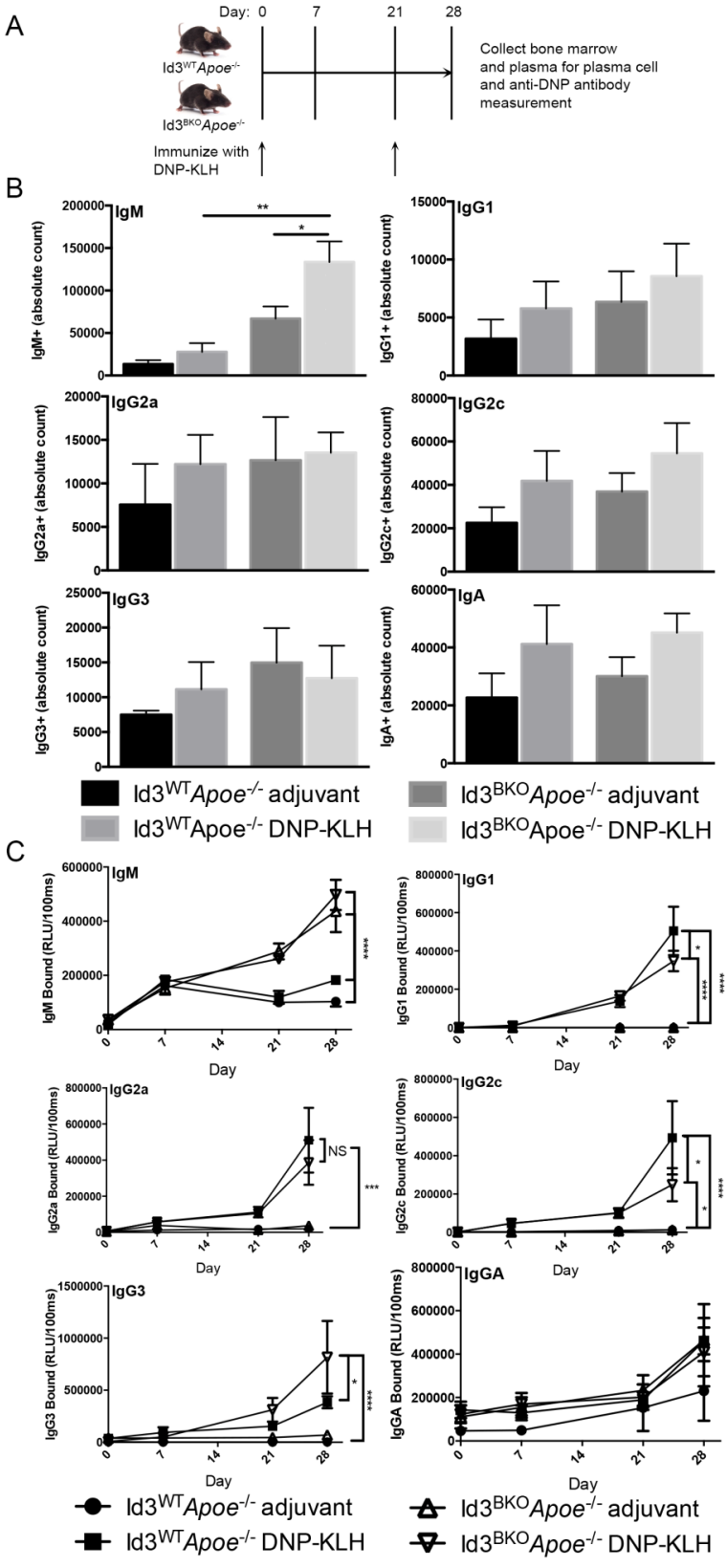

sIgM / 18S (A.U.)
3
2
1
0
B-1a
B-1b
B-2
Id3WT Apoe-/-
Id3BKO Apoe-/-

supporting the findings of **Figure 12** 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 $Id3^{BKO}$*Apoe*$^{-/-}$ mice (**Figure 18C**). Both $Id3^{BKO}$*Apoe*$^{-/-}$ and $Id3^{WT}$*Apoe*$^{-/-}$ mice had robust antigen specific IgG1, IgG2a, and IgG2c responses, although the IgG1 and IgG2c responses were blunted in $Id3^{BKO}$*Apoe*$^{-/-}$ mice. IgG3 was significantly increased in $Id3^{BKO}$*Apoe*$^{-/-}$ mice likely due to B-1b isotype switching as has been previously demonstrated[89]. These findings provide evidence that the predominant effect of B cell-specific deletion of Id3 is enhanced T cell-independent IgM production as a result of increased B-1b cell numbers and possibly due to increased B-1b derived PCs as have been described previously[91].

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 [128]. 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[102], incorporating additional stains and gates to eliminate initial concerns

Figure 18: Id3^{BKO}*Apoe*$^{-/-}$ mice respond to T cell dependent immunization with increased IgM and IgG3 and slightly blunted IgG1 and IgG2c compared to Id3^{WT}*Apoe*$^{-/-}$ mice. A) Study design. 8-10 week-old Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ were immunized with 100µg DNP-KLH in complete Freund's adjuvant (Id3^{BKO}*Apoe*$^{-/-}$ n = 5, Id3^{WT}*Apoe*$^{-/-}$ n = 5) or complete Freund's adjuvant alone (Id3^{BKO}*Apoe*$^{-/-}$ n = 5, Id3^{WT}*Apoe*$^{-/-}$ 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

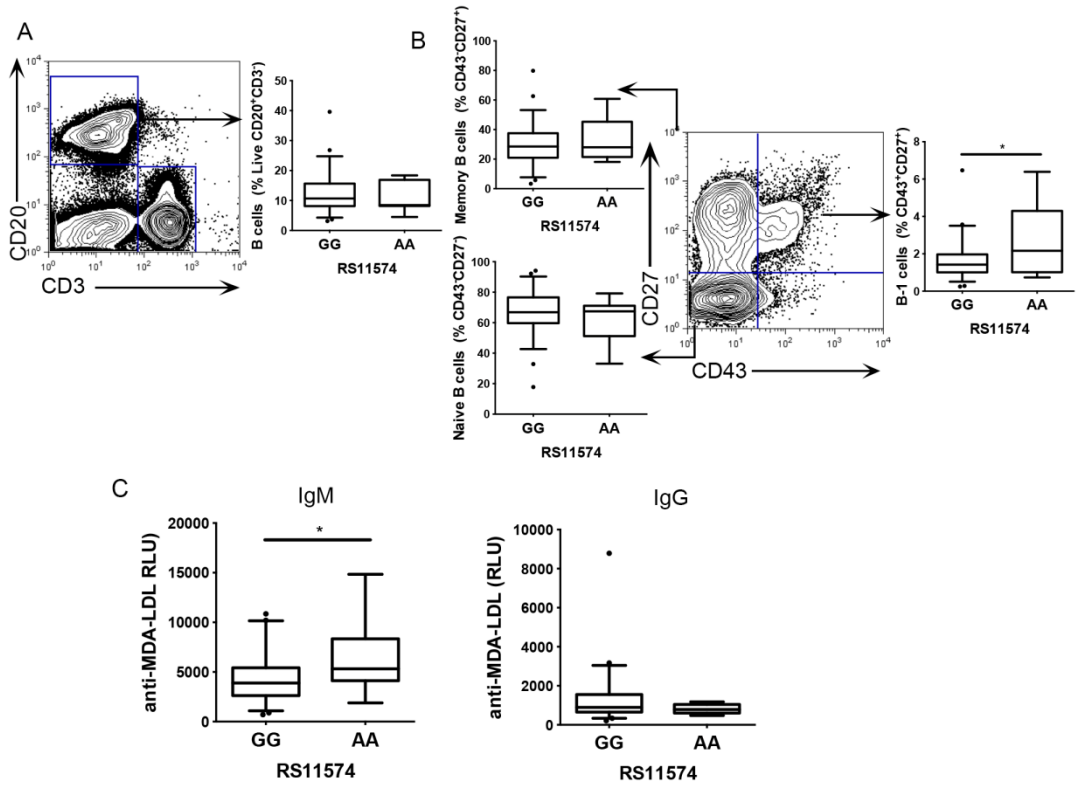

A
Day: 0 7 21 28
Id3WTApoe-/-
Id3BKOApoe-/-
Collect bone marrow and plasma for plasma cell and anti-DNP antibody measurement
Immunize with DNP-KLH
B
IgM
IgG1
IgG2a
IgG2c
IgG3
IgA
IgM+ (absolute count)
IgG1+ (absolute count)
IgG2a+ (absolute count)
IgG2c+ (absolute count)
IgG3+ (absolute count)
IgA+ (absolute count)
Id3WTApoe-/- adjuvant
Id3WTApoe-/- DNP-KLH
Id3BKOApoe-/- adjuvant
Id3BKOApoe-/- DNP-KLH
C
IgM Bound (RLU/100ms)
IgG1 Bound (RLU/100ms)
IgG2a Bound (RLU/100ms)
IgG2c Bound (RLU/100ms)
IgG3 Bound (RLU/100ms)
IgGA Bound (RLU/100ms)
Day
NS

about possible contamination by T cells and possible overestimation of numbers. Representative flow cytometry of human B cells is presented in **Figure 19A&B**. Consistent with results from Rothstein and colleagues[109] [102] 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 19B**). These patients did not have differences in the proportion of total, naïve, or memory B cells (**Figure 19A&B**). Additionally, antibody titers against MDA-LDL, which has been previously shown to inversely associate with Framingham risk score, metabolic syndrome criteria[132], and CVD[70], 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 19C**). We did not observe any differences in titers of IgG reactive to the same epitopes (**Figure 19C**). 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 [60, 143].

Figure 19: Patients homozygous for the minor allele at rs11574 in the ID3 gene (encoding an Id3 protein with attenuated function[128]) 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 (**A**) total B cells and (**B**) memory B cells ($CD27^{+}CD43^{-}$), naïve B cells ($CD27^{-}CD43^{-}$) and, B-1 cells ($CD27^{+}CD43^{+}$). **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. *$p<0.05$.


A
CD20
CD3
B cells (% Live CD20+CD3-)
GG
AA
RS11574
B
Memory B cells (% CD43-CD27+)
Naive B cells (% CD43-CD27-)
CD27
CD43
B-1 cells (% CD43+CD27+)
C
IgM
anti-MDA-LDL RLU)
IgG
anti-MDA-LDL (RLU)

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 [54, 62]. The work of Haas et al [89] and Alugupalli et al [88] 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 [90, 138, 140, 144]. 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 [72, 145, 146]. Their protective function is thought to occur by reducing the uptake of oxLDL by tissue resident macrophages[78] and blocking the proinflammatory properties of oxidized lipid moieties [141]. Additional protective mechanisms are attributed to IgM binding to apoptotic bodies within atherosclerotic plaques which increases their clearance reducing sterile necrosis [141, 147]. 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[35]. 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, while 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[34, 128]. 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.[122] Previous studies by our lab have reported that global loss of Id3 in *Apoe*$^{-/-}$ mice leads to enhanced atherogenesis[34]. Follow-on studies demonstrated that *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice had reduced IL-33-stimulated IL-5 production by natural helper cells[42]. 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[127]. In addition, Id3 regulates aortic homing of $CD43^-$ splenocytes (primarily B-2 cells) through regulation of the expression of chemokine receptors such as CCR6[34]. 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[123]. In the present study, chow fed $Id3^{BKO}$*Apoe*$^{-/-}$ mice responded to T cell-dependent immunization though in a blunted manner (**Figure 18**) 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. It is possible that Id3 may regulate activation state or other B-2 functions given that the $Id3^{BKO}$*Apoe*$^{-/-}$ 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[42] and provide novel evidence that the numbers of B-1b cells in $Id3^{BKO}$*Apoe*$^{-/-}$ mice are significantly greater than in

WT control. Interestingly, in contrast to global knockout *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice, Id3^{BKO}*Apoe*$^{-/-}$ 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[148]. 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[128], 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^{+} B cells that are CD27^{+}CD43^{+} and possess key features of murine B-1 cells including spontaneous production of IgM [102, 109]. More recently this subset has been linked to secretion of atheroprotective IgM after T cell-independent immunization [149]. 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, naïve, or memory B cells (**Figure 19**). 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.

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 and are atheroprotective
- B cell-specific loss of Id3 causes 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 circulation.

Summary

B cells have been demonstrated to have both atheroprotective and atherogenic functions based on subset and context. To date the contributions of B-1b cells, an important subset for T cell-independent immunity, to atherosclerosis have not been studied. Here we provide the first evidence that B-1b cells secrete atheroprotective IgM antibodies reactive to oxidative epitopes on LDL and 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 (Id3^{BKO}*Apoe*$^{-/-}$) developed significantly increased numbers of B-1b cells systemically without modifying the number of other B cell subsets. Additionally, Id3^{BKO}*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 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.

Chapter 4

Id3 regulates B-1b cell numbers early in post-natal life by an unknown mechanism

Abstract

Rationale: B cell-specific Id3 knockout mice ($Id3^{BKO}$) have systemically increased numbers of B-1b cells compared to $Id3^{WT}$ without modifications in number of the B-1a or B-2 subsets. Id3 has been shown to regulate cellular development, proliferation, and survival however the mechanism whereby Id3 regulates B-1b cells has not been determined.

Objective: To determine the mechanism regulated by Id3 that selectively induces B-1b cell expansion

Methods and Results: We demonstrate that $Id3^{BKO}$ B-1b cells do not have uniformly increased bromodeoxyuridine (BrdU) incorporation under stimulated or homeostatic conditions compared to $Id3^{WT}$ suggesting loss of Id3 modifies cell turnover differentially based on tissue. Additionally, we demonstrate that caspase 3/7 activity and Annexin V staining are not modified in $Id3^{BKO}$ B-1b cells suggesting Id3 does not regulate B-1b cell apoptosis. Immunophenotyping of 4-week-old $Id3^{BKO}$ mice demonstrated that a significant difference in B-1b cells is already present early in post-natal life. Finally, B-1 progenitor culture demonstrated that $Id3^{BKO}$ progenitors expanded less though had more IgM^{+} pro-B cells as a percentage of $CD19^{+}$ cells after 12 days in culture compared to $Id3^{WT}$ progenitors suggesting Id3 possibly regulates B-1 progenitor commitment.

Conclusions: No obvious regulatory mechanisms were determined to explain the observed difference in B-1b cell number due to loss of Id3 though findings were suggestive that Id3 could regulate progenitor development.

Introduction

Two lineages of B cells, B-1 and B-2, have been described in mice, which are developmentally and functionally unique [53, 54]. B-1 cells develop early in ontogeny from the fetal liver and inhabit the serosal cavities responding to T cell independent (TI) danger associated molecular patterns (DAMPs). B-2 cells are bone marrow (BM) derived and continuously circulate through lymphatic organs in search of cognate peptide antigen presented by antigen presenting cells (APCs) in the presence of CD4 T cells [150]. B-1 cells are further sub classified as B-1a and B-1b based on the surface expression of CD5, B-1a are $CD5^+$ and B-1b are $CD5^-$. There is evidence that these subsets are in fact separate lineages as B-1b cells can be derived from adult hematopoietic stem cells (HSCs) whereas B-1a cells cannot [60]. Additionally, it has been observed that the mature bone marrow is not capable of fully repopulating the B-1 compartment and that the B-1 cells that come from the mature bone marrow are predominantly B-1b cells giving rise to a layered model of B-1 cell development with early waves of fetal derived cells regulating later influx from mature BM progenitors [51, 54, 151, 152]. Little is known about unique factors that regulate individual B cell subsets.

The helix-loop-helix (HLH) transcription factors are essential regulators of cell development and differentiation [118, 121]. They are broadly, though not ubiquitously, expressed early in development, in proliferating cells, and in hematopoietic cells [118]. Class 3 HLH proteins are called inhibitors of differentiation (Id) and lack the basic DNA binding domain used by other HLH

proteins to directly regulate transcription, acting rather as dominant negative regulators of HLHs by heterodimerizing and restricting their DNA binding capacity. The 4 known Id proteins (Id1-4) are highly conserved. Inhibitor of differentiation 3 (Id3) regulates B cell development, function, and homeostasis [120, 122, 153]. Mice globally deleted for Id3 (*Id3*$^{-/-}$) develop functional B cell compartments however it was described in *Id3*$^{-/-}$ mice from a mixed genetic background that there were reduced IgG antibody titers in response to TD and TI antigens [123]. Additionally, studies with *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice on a pure C57BL/6 background demonstrated these mice had significantly fewer B-1a cells due to reduced IL-33 dependent IL-5 production by NHCs [42]. The cell specific mechanisms whereby Id3 regulates B cells are unknown.

Our lab has previously demonstrated that B cell-specific Id3 deletion results in significantly increased numbers of B-1b cells in the peritoneal cavity (PerC), spleen, blood, and BM without reciprocal reduction in B-1a or modification of B-2 cell numbers. How B cell-specific loss of Id3 causes such a specific expansion of B-1b cells and how this was maintained as far out as 6 months of age is unknown. Id3 has been demonstrated to regulate cell cycle genes such as P21^{Cip1} [154, 155] as well as cell survival genes in a caspase 2 dependent pathway [153] demonstrating that Id3 is a regulator of proliferation and survival under mature conditions. Additionally, ectopic expression of Id3 in B cell progenitors was sufficient to induce apoptosis demonstrating that Id3 could regulate early B cell development [122]. Preliminary studies from our lab demonstrated that CFSE

diluted more rapidly in B-1b cells from Id3^{BKO}*Apoe*$^{-/-}$ mice under normal conditions suggesting Id3 may regulate the self-renewal or proliferation of B-1b cells [156]. From this we hypothesized that the increased B-1b population in Id3^{BKO} mice was due to an increased rate of proliferation or self-renewal of the mature cells.

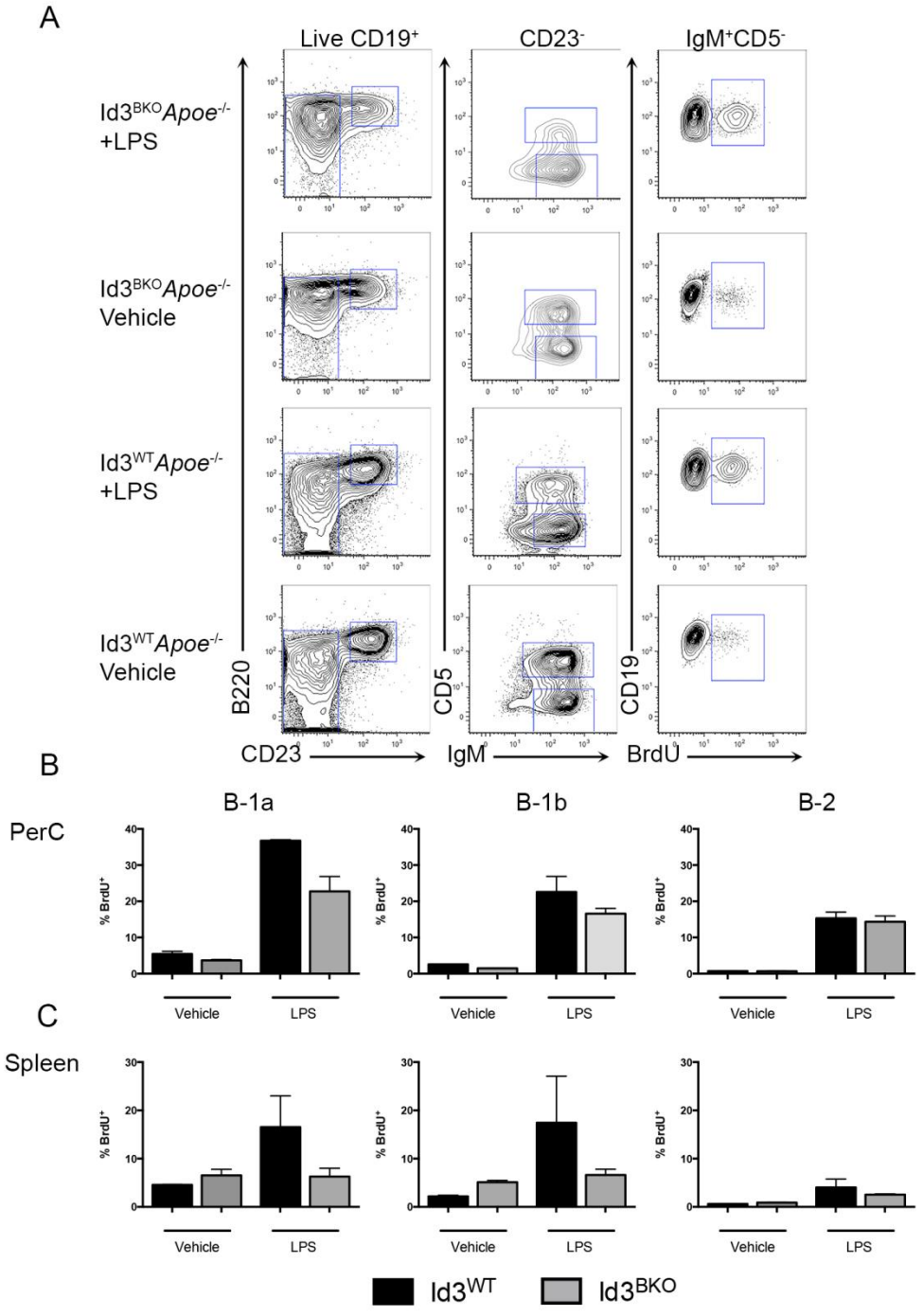
Here we demonstrate that BrdU incorporation is not uniformly increased in B-1b cells from Id3^{BKO}*Apoe*$^{-/-}$ mice under homeostatic conditions (self-renewal) or in response to stimulation (induced). Neither self-renewal or induced proliferation were enhanced in PerC by loss of Id3 though self-renewal was increased in spleen. Moreover, these studies demonstrate that B-1 proliferation due to LPS stimulation is attenuated due to loss of Id3. Additionally, assessment of caspase 3 and 7 activity and annexin V staining revealed no difference in survival of B-1b cells from Id3^{BKO}*Apoe*$^{-/-}$ mice. Finally, B-1b cell numbers were quantified in 4 week old Id3^{BKO}*Apoe*$^{-/-}$ mice demonstrating a significant increase was present prior to maturity suggesting the increased numbers of B-1b cells were possibly due to increased B-1 progenitor commitment. However, *in vitro* culture of sorted Id3^{BKO}*Apoe*$^{-/-}$ progenitors from BM did not result in increased mature B-1b cells compared to Id3^{WT}*Apoe*$^{-/-}$ though progenitor commitment was possibly increased.

Results:

Id3 deficient B-1b cells do not demonstrate increased self-renewal or LPS induced proliferation

Previous studies of Id3^{BKO}*Apoe*$^{-/-}$ mice demonstrated that CFSE injected IP had increased dilution in B-1b cells compared to Id3^{WT}*Apoe*$^{-/-}$ B-1b cells raising the possibility that B cell specific Id3 deletion increases B-1b cell proliferation or self-renewal[156]. To test this hypothesis, bromodeoxyuridine (BrdU), which intercalates into dividing DNA, was injected IP into Id3^{WT}*Apoe*$^{-/-}$ mice after injection of the TLR4 ligand lipopolysaccharide (LPS) or vehicle control (PBS). Cell division under stimulated conditions was considered proliferative whereas division under control conditions was considered self-renewal in B-1 cells (B-2 cells do not self renew). PerC lavage fluid and spleen were stained for B cell surface markers and intracellular measurement of BrdU (**Figure 20A**). The findings demonstrated indeed LPS stimulation induced significant proliferation of all three B cell subsets (**Figure 20B**). However, quantification of BrdU^{+} B cells as a percentage of their subsets demonstrated that this was not different between Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice under either stimulated or control conditions within the PerC (**Figure 20B**) suggesting loss of Id3 does not modify proliferation or self-renewal of PerC B-1b cells. Comparison of splenic B-1b cells from control mice revealed a non-significant increase in BrdU^{+} cells of Id3^{BKO} mice compared to Id3^{WT} suggesting loss of Id3 could have differing outcomes based on tissue (**Figure 20B**). Interestingly, LPS stimulation did not cause proliferation of B-1

Figure 20: Id3^{BKO}*Apoe*$^{-/-}$ B-1b cells do not exhibit consistently increased self-renewal or proliferation due to LPS stimulation. Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice (8 weeks old, chow fed, male, litter matched, n=3 for all groups) were injected IP with LPS or vehicle (PBS) 48 hours prior to euthanization and BrdU 24 and 12 hours prior to euthanization to measure BrdU incorporation into proliferating (LPS) or self-renewing (PBS) B cells. **A)** Representative flow cytometry plots from PerC of all four groups demonstrating the gating strategy for B cell subsets. B-2 cells are CD19^{+}B220^{high}CD23^{+}, B-1a cells are CD19^{+}B220$^{+/-}$CD23^{-}IgM^{+}CD5^{+}, B-1b cells are CD19^{+}B220$^{+/-}$CD23^{-} IgM^{+}CD5^{-}. All gates were set using FMO controls. **B)** Quantification of the percentage of BrdU^{+} B cells from PerC and Spleen.

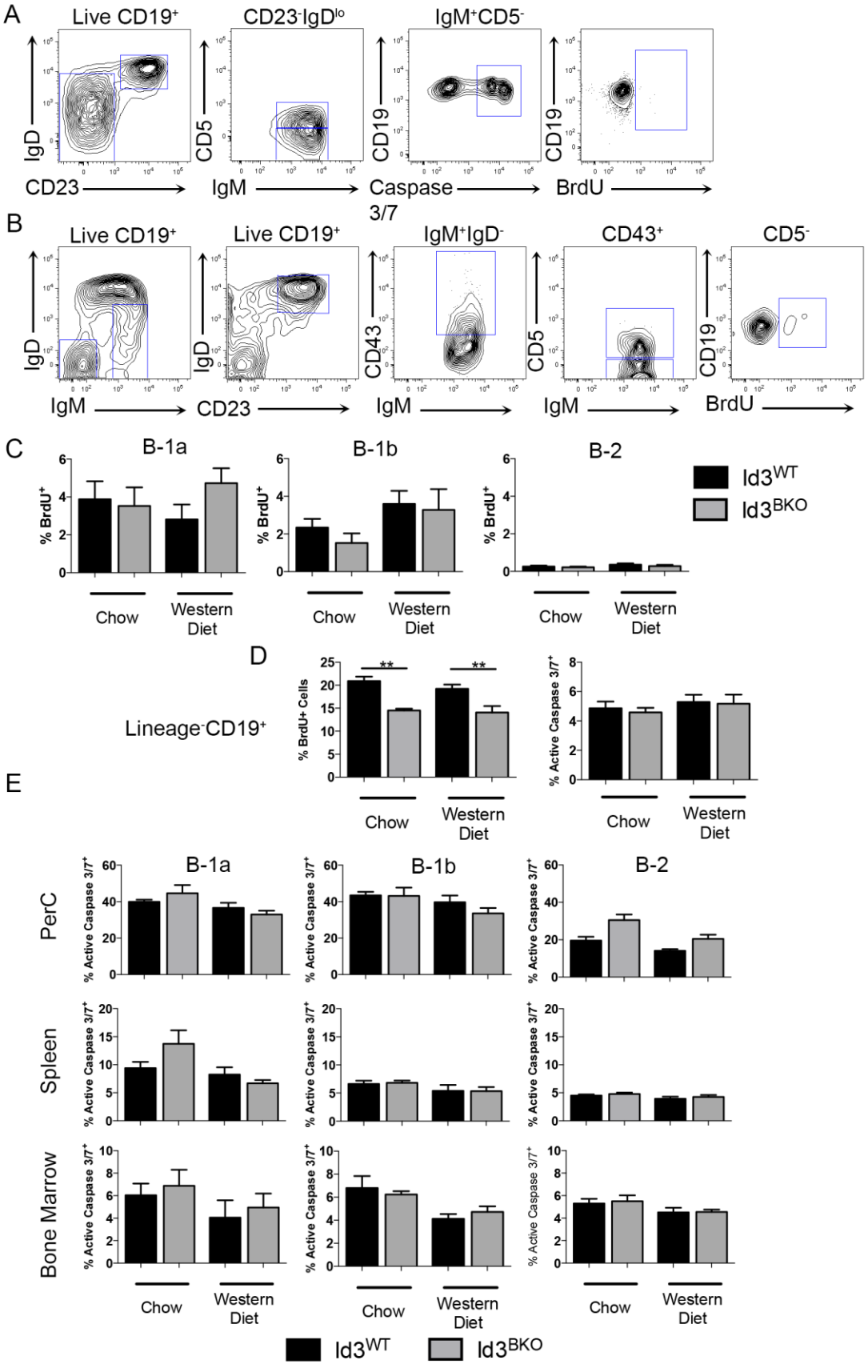

A
Live CD19+
CD23-
IgM+CD5-
Id3BKOApoe-/- +LPS
Id3BKOApoe-/- Vehicle
Id3WTApoe-/- +LPS
Id3WTApoe-/- Vehicle
B220
CD23
CD5
IgM
CD19
BrdU
B
B-1a
B-1b
B-2
PerC
% BrdU+
Vehicle
LPS
C
Spleen
Id3WT
Id3BKO

subsets in the spleens of Id3^{BKO}*Apoe*$^{-/-}$ mice compared to vehicle control where it was increased for Id3^{WT}*Apoe*$^{-/-}$ B-1 cells suggesting loss of Id3 potentially mediates proliferation in secondary lymph organs or the systemic distribution of B-1 cells. Further studies are required to explore these possibilities.

Bone marrow Id3^{BKO}*Apoe*$^{-/-}$ B-1b cells do not exhibit increased proliferation due to Western diet feeding

Though previous findings did not support the hypothesis that proliferation is increased in B-1b cells due to loss of Id3 it was hypothesized that the acute inflammatory conditions created by LPS could have obscured a potential difference. We had previously observed that Western diet feeding caused a substantial increase in B cell populations in the PerC of *Apoe*$^{-/-}$ mice globally deficient in Id3 (Perry, personal communication) suggesting that Western diet may provide an Id3-dependent proliferative stimulus. Surprisingly, 4 weeks of Western diet feeding did not significantly expand the number of B cells in either Id3^{BKO}*Apoe*$^{-/-}$ or Id3^{WT}*Apoe*$^{-/-}$ mice (data not shown). Additionally, IP injection of BrdU 24 hours prior to euthanization revealed very little BrdU uptake in B cells from PerC or spleen making analysis unfeasible (**Figure 21A)**. However, analysis of bone marrow (BM) B cells, based on the gating described by Nicole Baumgarth's group [131], revealed measurable BrdU uptake allowing for comparison of B cell proliferation (**Figure 21B**). The data demonstrated a substantially greater percentage of both B-1 subsets that were BrdU positive compared to B-2 cells. Additionally there was a non-significant increase in BrdU^{+}

Figure 21: B cell-specific loss of Id3 does not increase proliferation or self-renewal or decrease apoptosis in mice fed Western diet for 4 weeks. 8-12 week old $Id3^{BKO}$*Apoe*$^{-/-}$ and $Id3^{WT}$*Apoe*$^{-/-}$ mice were fed either Western diet (n= 6 and 7, respectively) or chow (n=5 and 7, respectively) for 4 weeks and then injected IP with BrdU 24 hours prior to euthanization. **A)** Representative flow cytometry plots of PerC B cell subsetting with BrdU and active caspase 3/7 gating of B-1b cells. **B)** Representative flow cytometry plots of BM B cell subsetting as described by Choi et al, *Eur J Immunol*, 2012[131] with additional BrdU gating of B-1b cells. **C)** Quantification of $BrdU^+$ BM B cells as a percentage of their subset. **D)** Quantification of $BrdU^+$ and active caspase $3/7^+$ $CD19^+$$lineage^-$ B cells (Lineages include B220, IgM, IgD, CD23, CD43, and CD5). **E)** Quantification of active caspase $3/7^+$ B cells from PerC, spleen, and BM as a percentage of their subset.


A
Live CD19+
CD23-IgDlo
IgM+CD5-
IgD
CD23
CD5
IgM
CD19
Caspase 3/7
BrdU
B
Live CD19+
Live CD19+
IgM+IgD-
CD43+
CD5-
IgD
IgM
IgD
CD23
CD43
IgM
CD5
IgM
CD19
BrdU
C
B-1a
B-1b
B-2
% BrdU+
Chow
Western Diet
Id3WT
Id3BKO
D
Lineage-CD19+
% BrdU+ Cells
**
**
% Active Caspase 3/7+
E
PerC
Spleen
Bone Marrow
B-1a
B-1b
B-2
% Active Caspase 3/7+
Chow
Western Diet
Id3WT
Id3BKO

B-1b cells from mice fed WD compared to those fed chow diet and that difference was greater in Id3^{BKO}*Apoe*$^{-/-}$ mice than for Id3^{WT}*Apoe*$^{-/-}$ mice (**Figure 21C**). However, there was no difference in the percentage of BrdU^{+} B-1b cells when comparing similar feeding conditions between Id3^{BKO}*Apoe*$^{-/-}$ mice and Id3^{WT}*Apoe*$^{-/-}$ mice (**Figure 21C**) suggesting Id3 does not regulate proliferation or self-renewal of BM B-1b cells. Surprisingly, the only significant difference observed between B cell subsets from Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice was when BrdU incorporation was measured in CD19^{+} cells that were otherwise lineage negative (IgM, IgD, B220, CD43, CD23, CD5) demonstrating a significantly reduced percentage of BrdU^{+} lin^{-}CD19^{+} B cells from Id3^{BKO}*Apoe*$^{-/-}$ mice regardless of feeding status. These lin^{-}CD19^{+} B cells were either B-1 progenitors which are defined as CD19^{+}B220^{-}Lin^{-}AA4.1^{+} or class switched plasmablasts that retained CD19 expression, suggesting loss of Id3 reduces proliferation in either of these subsets.

Apoptosis is not modified due to loss of Id3 in B-1b cells.

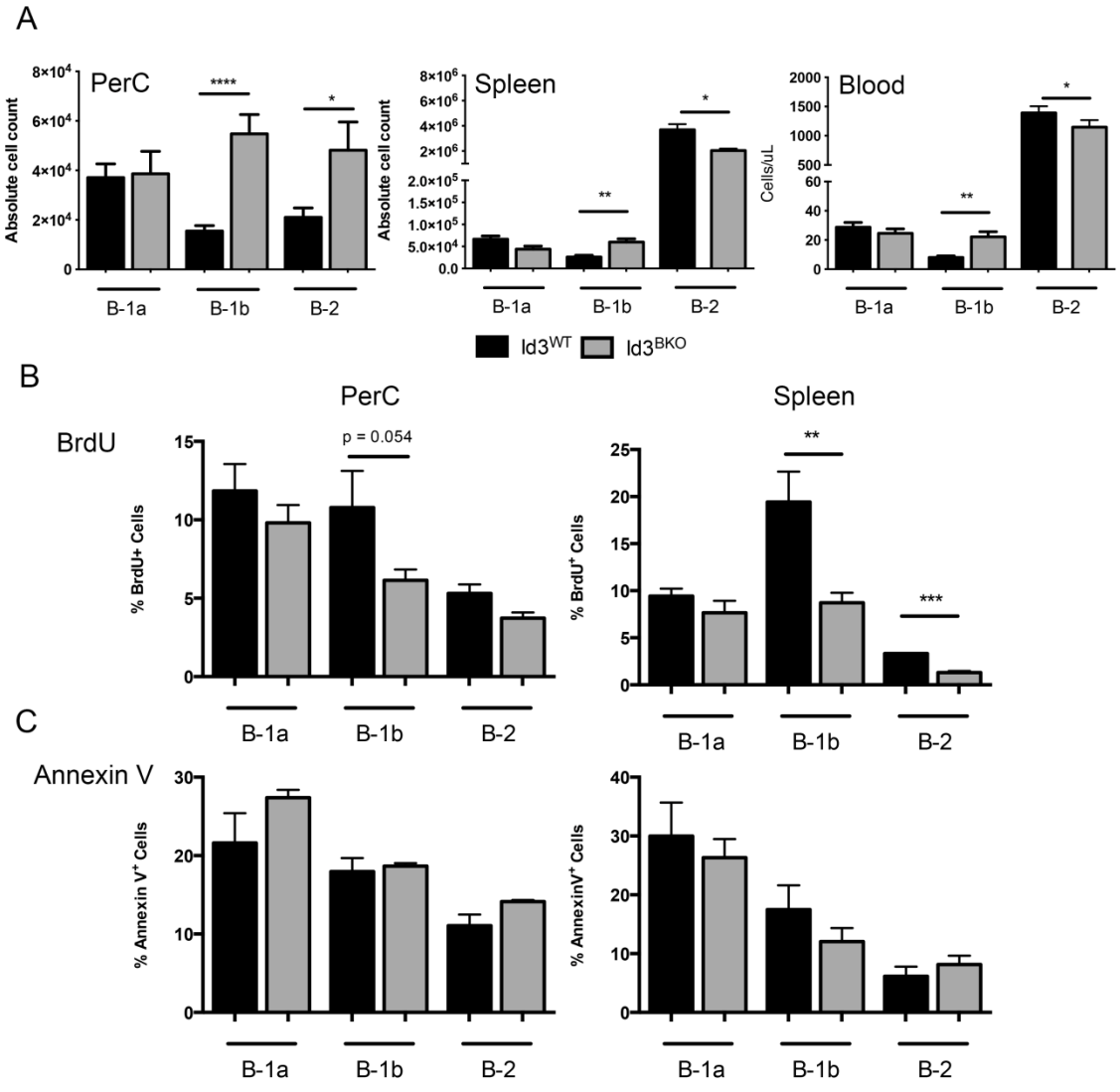
Id3 has been demonstrated to regulate cell survival and has the capacity to induce apoptosis. As we did not observe a difference in the proliferation or self-renewal of B-1b cells due to B cell-specific deletion of Id3 we hypothesized that the increase in B-1b cell numbers could be due to increased resistance to apoptosis. To test this we utilized the activation of capases 3 and 7 as indicators of commitment to apoptosis in mice fed either chow or Western diet for 4 weeks **(Figure 21A**). Measurement of active caspases 3/7 in PerC, spleen, and BM B

cells demonstrated no differences between Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ B-1b cells (**Figure 21E**) suggesting loss of Id3 does not decrease apoptosis. Interestingly, a non-significant reduction in active capase 3/7 was observed in some B cell subsets (PerC B-2, splenic B-1a, BM B-1a) in Western diet fed mice compared to chow fed, suggesting Western diet feeding of *Apoe*$^{-/-}$ mice may reduce apoptosis among B cells.

4 week old Id3^{BKO}*Apoe*$^{-/-}$ mice have significantly increased B-1b cell numbers but reduced self-renewal

It was previously demonstrated that 8-12 week old Id3^{BKO}*Apoe*$^{-/-}$ mice had up to 6 fold greater numbers of B-1b cells than Id3^{WT}*Apoe*$^{-/-}$ controls and that this fold difference was maintained up to 24 weeks of age after 16 weeks of Western diet feeding [157]. To determine whether this difference is established early after birth (0-4 weeks post-natal) or develops at maturity (6-8 weeks), B cell subsets were quantified from PerC, spleen, and blood of 4-week-old Id3^{BKO}*Apoe*$^{-/-}$ and Id3^{WT}*Apoe*$^{-/-}$ mice (n=5 & 7). These data demonstrated that indeed Id3^{BKO}*Apoe*$^{-/-}$ mice had significantly greater numbers of B-1b cells compared to Id3^{WT}*Apoe*$^{-/-}$ mice (**Figure 22A**). Additionally, it was observed that B-2 cells were significantly increased in the PerC but reduced in the spleen and blood suggesting loss of Id3 modifies B-2 cell distribution (**Figure 22A**). To determine whether at this early age B-1b cells were dividing more frequently due to loss of Id3, self-renewal was measured over 48 hours under unstimulated conditions. Quantifying the percentage of BrdU positive B cells demonstrated that Id3^{BKO}*Apoe*$^{-/-}$ B-1b cells

Figure 22: 4 week old Id3^{BKO}*Apoe*$^{-/-}$ mice develop significantly greater B-1b numbers but reduced BrdU incorporation under normal conditions. 4-week-old chow fed Id3^{BKO} and Id3^{WT} mice were injected with BrdU at 24 and 12 hours prior to euthanization then tissues were collected for B cell subset immunophenotyping. **A)** Absolute numbers of B cell subsets (subsetting based on work in Rosenfeld et al, *Circ Res*, 2015[157]) from PerC (x10^4), Spleen (x10^5&6), and blood (cells/µl). **B)** Intracellular BrdU incorporation and Annexin V staining of B cell subsets from PerC and spleen (%) (gating based on the strategy presented in **Figure 21**, Annexin V^{+} gate was based on an FMO). Data are mean±SEM. 2-tailed Student's *t* test was used to compare differences between genotypes. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001


A
PerC
Spleen
Blood
Absolute cell count
Cells/uL
B-1a
B-1b
B-2
Id3WT
Id3BKO
B
BrdU
PerC
Spleen
p = 0.054
% BrdU+ Cells
% BrdU+ Cells
C
Annexin V
% Annexin V+ Cells
% AnnexinV+ Cells

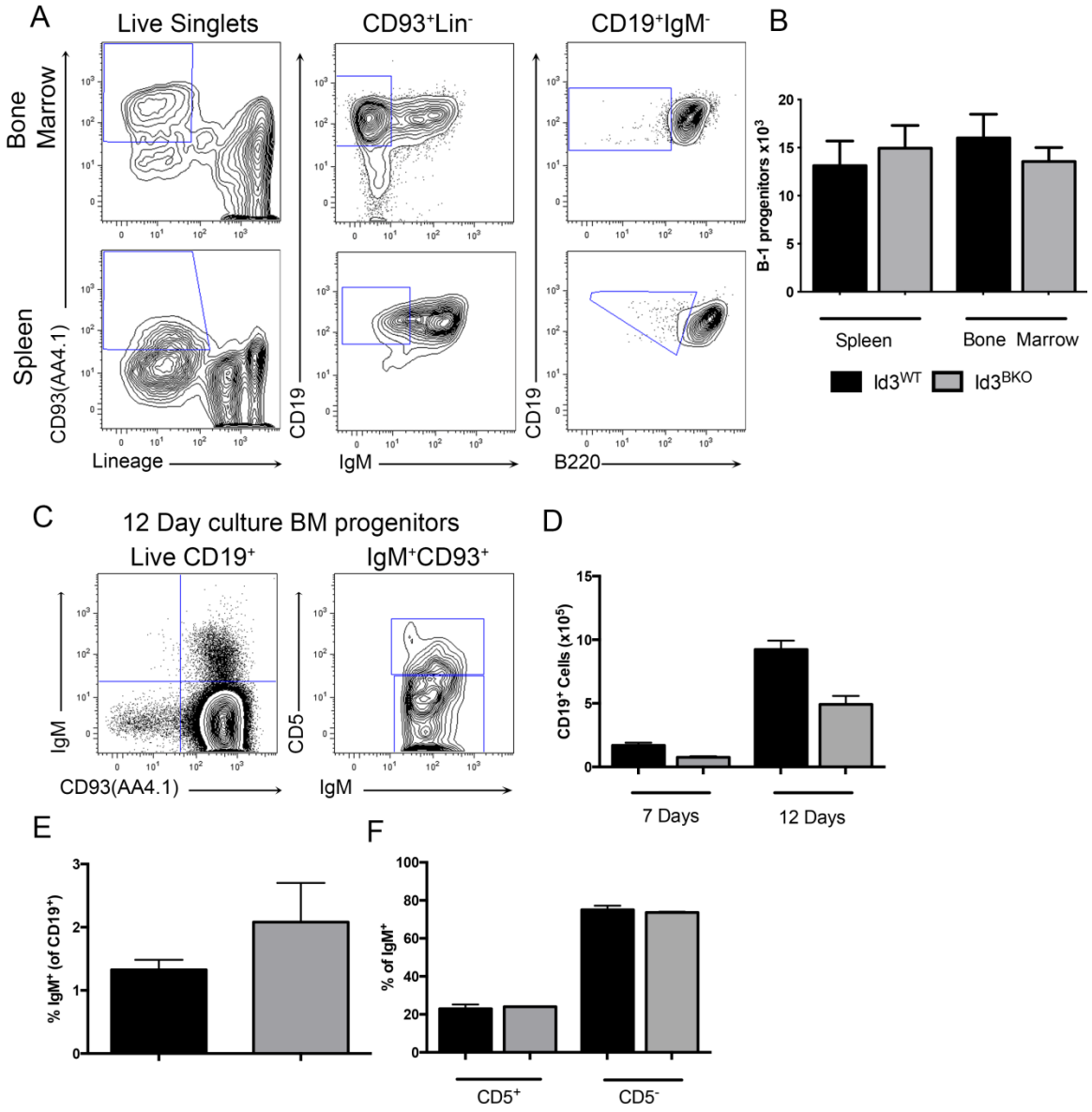
had significantly less BrdU incorporation in the spleen and a trending decrease in the PerC (p=0.054) compared to Id3^{WT}*Apoe*$^{-/-}$ (**Figure 22B**). These data suggest that loss of Id3 causes the B-1b cells to divide less frequently at 4 weeks of age. A similar difference was observed for Id3^{BKO}*Apoe*$^{-/-}$ B-2 cells though not B-1a cells (**Figure 22B**). To test whether apoptosis was decreased due to loss of Id3, Annexin V was used to stain phosphoserine which indicates commitment to apoptosis when present on the cell surface. Quantification of Annexin V staining demonstrated no differences between B cell subsets in the PerC or spleen (**Figure 22C**) confirming the findings from **Figure 21** that loss of Id3 does not modify survival of B-1b cells.

Cultured BM B-1 progenitors from Id3^{BKO}*Apoe*$^{-/-}$ mice do not develop into greater numbers of B-1b cells

B-1 cells develop from a unique progenitor early during ontogeny in the fetal liver and at maturity from the spleen, and BM [51]. B-1 progenitors were discovered when a small population of CD43^{+}AA4.1^{+} pre-pro-B cells were identified that expressed CD19 and not B220. Classic development of B cell progenitors progress into pre-pro-B cell stage by first expressing B220 then becoming B220/CD19 double positive pro-B cells. These cells when transferred were able to populate the B-1 compartment but did not produce B-2 or MZ B cells and were predominantly B-1b. To determine whether loss of Id3 increased the number of B-1 progenitors, BM and spleen cells were isolated and analyzed by flow cytometry (**Figure 23A**) which demonstrated that Id3^{BKO}*Apoe*$^{-/-}$ mice did not have

increased numbers of B-1 progenitors in their BM or spleen (**Figure 23B**). To determine whether loss of Id3 from B-1 progenitors caused them to expand or mature to B-1 cells more rapidly they were sorted and placed into culture following the strategy described by Montecino-Rodriguez et al. (**Figure 23A**). B-1 progenitors from both genotypes survived and expanded in culture. Analysis of the surface phenotype of the cultured cells after 12 days demonstrated that the vast majority maintained their pre-pro B cell phenotype ($AA4.1^{+}IgM^{-}$) where a small percentage upregulated IgM on their cell surface becoming pro-B cells ($AA4.1^{+}IgM^{+}$) (**Figure 23C**) Quantification of the total number of $CD19^{+}$ cells after 7 and 12 days in culture demonstrated a substantial decrease in total $Id3^{BKO}$*Apoe*$^{-/-}$ cells compared to $Id3^{WT}$*Apoe*$^{-/-}$ (**Figure 23D**) suggesting that loss of Id3 reduces the expansion of cultured B-1 progenitors. This finding could be in agreement with the observation from **Figure 21C** that $Lin^{-}CD19^{+}$ BM cells demonstrated decreased homeostatic turnover. Interestingly, though the number of $Id3^{BKO}$*Apoe*$^{-/-}$ $CD19^{+}$ cells was reduced, the percentage of $CD19^{+}$ cells that were IgM^{+} pro-B cells was increased (**Figure 23E**) suggesting loss of Id3 potentially induces more progenitors towards maturity. Further analysis of the IgM^{+} cells demonstrated that they were strongly skewed toward $CD5^{-}$ and this ratio was not changed due to loss of Id3 (**Figure 23F**).

Figure 23: Numbers of B-1 progenitors are not modified in BM or spleen of $Id3^{BKO}$ mice and $Id3^{BKO}$ BM B-1 progenitors produce fewer cells in culture but develop an increased percentage of IgM^+ pro-B cells. B-1 progenitors were sorted from BM of $Id3^{BKO}$ and $Id3^{WT}$ (n=2 in duplicate) and placed into culture based on the strategy developed by Montecino-Rodriguez et al, *Nat Immunol,* 2006 [51]. Cells were collected and analyzed by flow cytometry after 7 and 12 days. **A)** Representative flow cytometry of B-1 progenitor gating from spleen and BM, B-1 progenitors are Lin^-CD93^+(AA4.1)$CD19^+B220^-IgM^-$. Lineages markers include CD11b, Gr-1 (Ly-6G), Ly-6C, Ter119, NK1.1, CD8, TCRα/β and TCRγ/δ. **B)** Quantification of B-1 progenitor numbers from spleen and BM. **C)** Representative flow cytometry of resulting cells from B-1 progenitors cultured for 12 days. **D)** Quantification of total $CD19^+$ cells from B-1 progenitor culture after 7 and 12 days (x10^5). **E)** Quantification of IgM^+ cells (% of $CD19^+$) after 12 days in culture. **F)** Quantification of $CD5^{+/-}$ IgM^+ cells (% of IgM^+) from B-1 progenitor culture. Data are mean±SEM.


A
Live Singlets
CD93+Lin-
CD19+IgM-
Bone Marrow
Spleen
CD93(AA4.1)
Lineage
CD19
IgM
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B220
B
B-1 progenitors x10^3
Spleen
Bone Marrow
Id3WT
Id3BKO
C
12 Day culture BM progenitors
Live CD19+
IgM+CD93+
IgM
CD93(AA4.1)
CD5
IgM
D
CD19+ Cells (x10^5)
7 Days
12 Days
E
% IgM+ (of CD19+)
F
% of IgM+
CD5+
CD5-

Discussion

In this chapter several lines of evidence were presented that attempted to decipher the mechanism whereby Id3 selectively regulates the expansion of B-1b cells as was previously observed [157]. Measuring self-renewal and proliferation under homeostatic and proliferative conditions respectively, revealed that neither of these were uniformly increased due to loss of Id3 in B-1b cells. In fact, self-renewal of B-1b cells from 4 week old mice was reduced in $Id3^{BKO}$ B-1b cells. Additionally, cell turnover due to apoptosis was measured, which demonstrated that loss of Id3 did not decrease the number of apoptotic B-1b cells. Finally, B-1 progenitors from the BM and spleen were quantified demonstrating that there was no difference in their numbers due to loss of Id3 and that Id3 deficient B-1 progenitors when cultured expanded less than $Id3^{WT}$ but had an increased percentage of IgM^{+} cells. Importantly, each of these studies represents a single experiment and any significant findings bear repeating.

Whereas we hypothesized, based on our prior observations, that B cell-specific loss of Id3 would result in increased proliferation or self-renewal of B-1b cells, it was observed in multiple conditions (LPS stimulation, homeostatic turnover) and tissues (spleen and BM) that this was actually reduced compared to controls. Given that Id3 is known to positively associate with proliferation through its regulation of $p21^{cip1}$ [154] or IL-5 [42] this is not surprising though it runs contrary to the observed expanded B-1b population in $Id3^{BKO}$ mice. This could be due to negative feedback from increased IgM titers given that mice unable to secrete

IgM (sIgM) develop significantly increased numbers of B-1 cells [158] and this is reversed by administration of polyclonal IgM [159]. It has been previously demonstrated that Id3^{BKO}*Apoe*$^{-/-}$ mice have increased circulating titers of total IgM [157] therefore this could contribute to the reduced cell turnover observed in these mice. Supporting this was the reduced rate of Id3^{BKO} B-1a cell BrdU incorporation measured in **Figure 20b**, which demonstrated that both B-1 subsets were equally affected in that study. An interesting question is whether BM resident B-1 cells regulate B-1 progenitor development rate as it was previously demonstrated that Id3^{BKO} mice have increased BM B-1b cells and IgM secreting cells (ISCs)[157]. Regardless, this potential feedback regulation fails to explain how loss of Id3 induces, or sustains, the increased B-1b cell numbers at the outset. Studies measuring the expansion of B-1b cells after adoptive transfer into B cell deficient hosts could avoid this homeostatic feedback and reveal physiological differences regulated by Id3 in B cells.

Complicating these findings are the various differences in self-renewal and proliferation observed based on the tissues analyzed. Loss of Id3 in PerC B-1b cells does not appear to modify self-renewal or proliferation whereas splenic B-1b cells have increased self-renewal compared to Id3^{WT}. Additionally, loss of Id3 appears to attenuate splenic B-1 cell proliferation. B-1 cells, especially B-1a cells, have been demonstrated to migrate to the spleen after stimulation where they secrete high levels of IgM [160-162]. It is possible that the differences in turnover observed between spleen and PerC are a result of Id3 mediated activation or

homing from LPS stimulation. In particular, it was demonstrated that LPS induced B-1 homing to the spleen is associated with CXCR4 upregulation [162] We are currently exploring the regulation of CXCR4 by Id3 in B cells.

A central assumption of this work was that B-1b cells are a homogenous population regulated by Id3. This assumption however is potentially false as the heavy chain repertoires of B-1 cells have been demonstrated to change whether they are supplied from the fetal liver or bone marrow [163]. During early fetal development B-1 cells do not express terminal deoxynucleotide transferase (TDT), an enzyme that randomly inserts non-templated nucleotides into the N regions of the heavy chain, where the V-D-J regions are joined, to increase antibody diversity and affinity [164]. Thus, early B-1 cells have essentially germline encoded heavy chains. This changes over time as B-1 cells are supplied from the bone marrow where they express TDT and have increased non-templated N-region insertions suggesting that, as a whole, B-1 cells are largely heterogeneous [163, 165]. Additionally, B-1 progenitors from the adult BM (an amalgam of non-HSC derived B-1a and HSC derived B-1b progenitors [60] which are currently inseparable) have an attenuated capacity to repopulate the B-1 compartment, and those B-1 cells that do populate the PerC have increased insertions and more B-2 like antibody repertoires suggesting that fetal and mature progenitors themselves differ [152, 163]. An essential study that could clarify findings from this project would be to measure the amount of non-templated N-region insertions within the expanded B-1b population found in $Id3^{BKO}$ mice.

Demonstrating that Id3 deficient B-1b cells had decreased insertions compared to WT would suggest that the increased B-1b numbers were supplied by fetal progenitors and thus Id3 regulated early B-1b cell development or expansion. Alternatively, if $Id3^{BKO}$ B-1b cells had increased insertion rates it would suggest that they were BM derived and that Id3 negatively regulated either B-1b development in maturity or the turnover of BM derived B-1b cells.

Much has been described for the homeostatic requirements of B-2 cells though less is known for B-1a and B-1b cells. Differences between B-2 cell, B-1a and B-1b cell homeostatic maintenance include their mitogenic response to different cytokines such as BAFF [166], IL-7 [53], IL-5 [167], and IL-9 [168], and self-renewal as regulated by Cyclin genes [169]. Under the assumption that negative feedback, as described above, obscured the difference in Id3 mediated proliferation, Id3 could potentially regulate a B-1b cell specific cytokine receptor. For instance, it was demonstrated that IL-9 deficient mice had profoundly reduced B-1 cells and that X-linked immunodeficient mice (*xid*) which lack B-1 cells had restored B-1b numbers when crossed with IL-9 transgenic mice [168, 170]. It could be speculated that Id3 might negatively regulate IL-9 receptor mediated B-1b proliferation and the loss of Id3 would increase B-1b cells in an IL9 dependent manner. The regulatory mechanism controlled by Id3 in B cells also must account for the observed increase in B-2 cell numbers in PerC of 4-week old mice and reduction in numbers within spleen and blood (**Figure 3A**). This observation is supported by previous findings that PerC B-2 cells were significantly increased in 24-week

$Id3^{BKO}$ mice after 16 weeks of Western diet feeding though there were no differences in the spleen or blood [157]. This could suggest that PerC B-2 cells are under different regulatory requirements than those circulating or in the lymphatics or alternatively, as has been previously demonstrated [34], Id3 could be regulating homing factors that regulate the distribution of B-2 cells. Future studies should address the PerC B-2 cell population and whether it is important in atheroprotection.

B-1 progenitors were described as $Lin^{-}CD19^{+}B220^{-}AA4.1^{+}$ cells able to generate B-1a and B-1b cells when transferred into immunedeficient host mice [51]. However, whether they have unique developmental requirements compared to B-2 progenitors is poorly understood. It was demonstrated that B-1 progenitors, like B-2, develop from BM hematopoietic stem cells (HSCs) and common lymphoid progenitors (CLPs) suggesting B-1 and B-2 cells have a shared ancestry [171]. The same research demonstrated that B-1 progenitors are dependent on IL-7Rα expression similar to B-2 cell progenitors, demonstrating common developmental requirements. However, the B-1 progenitors are not affected by loss of Ig gene recombination as exhibited by their sustained numbers in RAG mice in contrast to B-2 progenitors suggesting divergent developmental pathways [171]. Additionally, it was demonstrated that highly sorted BM HSCs were unable to reconstitute the B-1a compartment suggesting a hetergenous population of B-1 progenitors resides in the adult BM, derived from HSCs and another undetermined stem cell population [60]. Moreover, different cytokine requirements

were demonstrated for B-2 and B-1 progenitors as B-1 progenitors can develop when supplemented with thymic-stromal lymphopoietin (TSLP) whereas B-2 progenitors require IL-7 [51]. Here we cultured B-1 progenitors to determine whether loss of Id3 caused the cells to be more proliferative or to commit more toward the B-1b cell lineage. By demonstrating that $Id3^{BKO}$ B-1 progenitors had decreased numbers compared to $Id3^{WT}$ after 12 days in culture, it suggests that Id3 is an important positive regulator of progenitor turnover. The additional finding that $Id3^{BKO}$ B-1 progenitors developed a greater percentage of IgM^{+} cells compared to $Id3^{WT}$ begins to suggest that loss of Id3 could accelerate the commitment to maturity from the progenitor cells. To confirm this finding B-1 progenitors will need to be sorted and transferred into immunedeficient or irradiated mice and the resultant populations of cells quantified. We would hypothesize that $Id3^{BKO}$ B-1 progenitors would give rise to increased numbers of mature B-1b cells compared to $Id3^{WT}$.

Chapter 5

Discussion

As a common, chronic, progressive disease involving environmental, genetic, and immunological factors, elucidating the contributions of one cell type, or subset of a cell type, to atherosclerosis is particularly difficult. Yet, the continuing cost of the disease to human health both in morbidity and mortality [172], despite improved screening and treatment modalities, underscores the importance of our sustained effort to determine the key regulatory mechanisms controlling atherosclerosis. Rapid advances in methods of immune modulation, such as the use of monoclonal antibodies (mAbs) to antagonize or induce immune responses, demands further research into the broad inflammatory response that is a hallmark of atherosclerosis. As these strategies become more common in treating autoimmunity or oncology, the potential effects this could have on cardiovascular disease should be assessed and murine data should be translated into the human immune system despite the difficulties involved.

We have presented evidence using murine models of atherosclerosis that B-1b cells are atheroprotective and secrete OSE-reactive IgM antibodies. A number of questions remain from this work including whether B-1b cell mediated atheroprotection is IgM dependent, whether B-1b cells have varying local and systemic functions in atherosclerosis, and whether B-1b cells can produce OSE-reactive antibodies in response to immunization? Additionally, it was demonstrated that Id3 is an important negative regulator of B-1b cells in mice. However, attempts to determine the mechanism controlling this observation were inconclusive leaving numerous questions to be answered. Finally, data was

presented demonstrating an association between an increased proportion of circulating human B-1 cells and the presence of an uncommon homozygous SNP at rs11574 in the *Id3* gene which has been shown to express an Id3 protein with attenuated function [128]. What this association implies for future targeting and immunization strategies towards human B-1 cells has not been explored. In this chapter these remaining questions will be discussed and future experiments designed to address these questions will be highlighted. Additionally, the importance of these murine findings will be assessed in the context of human atherosclerosis as possible new biotheranostic targets.

B-1b cells are atheroprotective and secrete atheroprotective IgM antibodies

B-1b cells are protective against atherosclerosis when adoptively transferred into *Rag1*$^{-/-}$*Apoe*$^{-/-}$ host mice and Id3^{BKO}*Apoe*$^{-/-}$ mice demonstrate a selective enhancement of B-1b cell numbers and attenuated atherosclerosis. A potential mechanism whereby B-1b cells mediate atheroprotection is the secretion of IgM reactive to oxidation-specific epitopes (OSE) on LDL. B-1b cells secrete these anti-MDA-LDL and anti-CuOx-LDL IgM in response to TLR stimulation in culture and *in vivo* after Western diet feeding. Whether B-1b cell mediated atheroprotection is IgM dependent is undetermined. It was demonstrated that adoptive transfer of B-1a cells unable to secrete IgM (sIgM) into spelenctomized *Apoe*$^{-/-}$ mice failed to attenuate atherosclerosis confirming that B-1a mediated atheroprotection is through IgM secretion [35]. Adoptive transfer experiments were attempted using sIgM B-1b cells into *Rag1*$^{-/-}$*Apoe*$^{-/-}$ hosts however it was

determined that cells did not populate the host mice after 16 weeks of Western diet feeding. This is in agreement with previous studies that demonstrated B-1 cells depend on IgM for survival and homeostasis [173]. Further optimization, or an alternative host model, is needed to answer this vital question. In the event that B-1b cell mediated atheroprotection is demonstrated to be IgM dependent this will support the established paradigm that OSE reactive IgM are atheroprotective [29, 35, 78, 79, 146, 174]. Additional studies comparing the relative production of these antibodies between B-1b and B-1a cells are currently underway. Alternatively, demonstration that atheroprotection is not, or not entirely, IgM dependent would suggest studies to determine an alternative protective mechanism should be undertaken. B-1 derived Bregs secrete IL-10 and directly mediate immune cell suppression [63-65] supporting the pursuit of studies measuring B-1b derived IL-10 expression and secretion by intracellular flow cytometry and ELISA from sorted and cultured B-1b cells, respectively. Additionally, direct *in vitro* suppression assays either by co-culture of B-1b cells with polarized macrophages or T cells or treatment of polarized cells with conditioned media from cultured B-1b cells would provide evidence for regulatory function. Additionally, it has been demonstrated that ‘macrophage-like’ B-1 cells, based on function and surface phenotype, develop in the PerC [175], which warrants study in the context of athersclerosis. Exploring whether B-1a or B-1b cells are more prone to develop macrophage phenotypic characteristics and whether this could be important for atherosclerosis could reveal a novel mechanism for B-1b mediated atheroprotection.

Alteration of normal immune function through the use of targeting therapies toward specific immune cells or molecules carries the potential to have a profound impact on patients. One aspect to pursuing this line of treatment may be unraveling the mechanism by which B cells modulate local development of atherosclerosis from the adventitia as compared to their systemic function [18]. B cells aggregate within the aortic adventitia in relation to atherosclerotic lesions [34, 44] and work in our lab has demonstrated that B-1b cells are enriched in ATLOs of aged atherogenic mice (unpublished observation). However, the functional significance of this local accumulation is not understood. Combined efforts to subset the local B cell populations and measure antibody production by ELISPOT are currently being optimized in order to determine whether local B-1 cells actively produce anti-MDA-LDL IgM. Following clarification of B cell function within the adventitia, molecular targeting to vascular B-1b cells for the delivery of atheroprotective antigens could be used to enhance atheroprotective B-1b function. Furthermore, local treatment with B-1 mitogens such as IL-5, which in mice induces B-1 proliferation [79] and in humans is positively associated with levels of NAbs against oxLDL [176], or others such as CD40 or IL-9 could serve a protective role by increasing B-1 cell numbers and protective antibody titers.

Active immunization against atherosclerosis is a promising form of immune modulation as it could be used to cheaply induce a lasting immune response against common atherogenic antigens. Early evidence supporting this concept

was provided by Palinski and colleagues when they showed that immunization of hyperlipidemic rabbits with malondialdehyde-modified LDL (MDA-LDL) induced high titers of antibodies reactive to oxLDL and protected against atherosclerosis [23]. Binder and colleagues corroborated these findings when they demonstrated that immunization of *Apoe*$^{-/-}$ mice with Streptococcus pneumonia, which expresses the same phosphorylcholine epitope on its cell surface as that bound by T15 and EO6 on oxLDL, induced high titers of oxLDL reactive IgM and protected against atherosclerosis [78]. These findings suggest that immunization against oxidation specific epitopes (OSEs) could be an effective treatment option [140].

Regardless of whether B-1b mediated atheroprotection is IgM dependent, there is strong evidence that OSE reactive IgM are able to decrease the development of atherosclerosis [23, 78-80, 177]. The contribution of B-1b cells to T cell-independent (TI) immunity has been explored in the context of infectious disease, demonstrating a unique capacity to provide long-term antibody coverage against TI-2 antigens [88, 89]. Importantly, whether B-1b cells can secrete OSE reactive IgM in response to antigen and provide anti-OSE memory has not been studied. We are currently testing this by transferring B-1b or B-1a cells into *Rag1*$^{-/-}$*Apoe*$^{-/-}$ mice, as previously described, then immunizing the mice with MDA-LDL and pneumococcal polysaccharide (PPS-3), which is known to induce secretion of T15/E06 [78]. From these mice we will compare the relative titers of OSE reactive IgM to mice immunized with vehicle control (PBS) and measure atherosclerosis

with the hypothesis that B-1b recipients immunized with MDA-LDL/PPS-3 will develop higher titers of OSE IgM and reduced atherosclerosis compared to B-1a recipients and vehicle controls. Early measurements of antibody titers from these mice 7 days after immunization did not demonstrate a substantial antigen specific response though this is possibly due to the early time point. In the event that we do not demonstrate a response to the immunization this will require further optimization of the immunization 'cocktail' or introduction of an adjuvant. A recent publication by Cehn et al. demonstrated that the addition of dexamethasone to an immunization with a heat shock protein 60 antigen induced antigen specific responses from B-1 cells and reduced atherosclerosis [178]. We could optimize our immunization strategy to include dexamethasone. Additional studies to measure the longevity of sustained antibody production and to analyze the phenotype of the immunized cells should also be pursued. Importantly, we should assess whether immunization with MDA-LDL induces memory formation through repeat immunizations.An alternative immunization strategy would be to immunize to induce immunological tolerance thus causing the immune system to ignore atherogenic antigens. Such strategies against antigenic peptides such as apoB-100 on LDL, which induces an inflammatory adaptive immune response, have been discussed [179]. Though immune tolerance is mediated by Tregs, Bregs are likely involved and can be derived from B-1 cells [65]. Bregs can suppress Th1 responses and activate Tregs in mice [180] suggesting that immune tolerance could be achieved through B-1 targeting. Though work in animal models support immunization strategies as novel treatment options, more work is needed to fully

describe the relevant epitopes involved in the human atherosclerotic immune response.

The N-terminal glycosylation state of IgM is important for its suppressive function [82]. Whether OSE-reactive IgM secreted by B-1b cells are sialylated has not been measured. Comparing the extent of sialylated IgM from TLR stimulated B-1b and B-1a cells will reveal if there are differences in the N-terminal glycosylation state of antibodies produced by the different subsets. Additionally, measurement of sialylation of OSE-reactive IgM, captured on plates coated with MDA-LDL, would reveal a potential novel mechanism whereby OSE IgM are atheroprotective. Furthermore, this can be carried over to the antibodies measured after immunization. Perhaps different antigens induce increased sialylation which would be hypothesized to better suppress inflammation.

Id3 is an important negative regulator of B-1b cells

Id3 is important for B cell development and homeostasis [34, 42, 123] however these findings primarily came from globally deleted or *in vitro* models. Observations that B cell-specific Id3 knockout ($Id3^{BKO}$) mice had systemically increased numbers of B-1b cells in comparison to $Id3^{WT}$ mice without significant changes in B-1a cell numbers, and inconsistent changes in B-2 cell numbers and distribution, suggested that Id3 selectively negatively regulates B-1b cells. Studies to determine the mechanism utilized by Id3 demonstrated that it does not appear to regulate proliferation or self-renewal of mature B-1b cells. However this

could have been obscured by feedback regulation mediated by increased IgM titers [54, 173]. Additional studies suggested that loss of Id3 potentially mediated BM B-1 progenitor commitment to IgM^{+} pro-B cells however this was measured entirely in culture and would require adoptive transfer studies to allow quantification of mature B-1b cells from the progenitors.

An important first step for this project going forward is to determine from where the expanded B-1b cell population was derived. This would be achieved by measurement of non-templated N-region insertions. Single cell sorting and sequencing first described by Kantor et al. demonstrated that approximately 20% of B-1b cells from the PerC of 22-week-old Balb/c mice had no N-region insertions, which strongly suggests they did not express TdT and were derived from fetal progenitors [165]. A more recent study by Holodick et al demonstrated that B-1a cells from PerC of Balb/c mice were 55% non inserted at 6-8 weeks of age but this decreased to 30% at 24 weeks of age suggesting the influx of B-1 cells from the bone marrow introduces cells with increased insertions [163]. Based on these studies, single cell sorting and heavy chain sequencing could be done to measure individual VDJ sequences and calculate the number of non-templated insertions at each N-region. The percentage of non-inserted B-1b cells from $Id3^{BKO}$ mice could then be compared to $Id3^{WT}$. If it is demonstrated that the percentage of non-inserted B-1b cells is higher in $Id3^{BKO}$ mice the findings would suggest Id3 negatively regulates fetal progenitors whereas if the percentage is

the same or lower this would suggest Id3 regulates the maintenance or development of B-1b cells from mature progenitors.

In the event that Id3 regulates fetal development of B-1b cells, studies to determine the mechanism regulated by Id3 in the fetal liver would be indicated. B-1b cells can be derived from progenitors in the fetal liver as early as embryonic day 11 (E11) [181]. Prior to that, non-hematopoeitic progenitors from the para-aortic splanchnopleura region (PAS) and yolk-sac are restricted to B-1a and MZ lineages [51, 52]. $Id3^{BKO}$ mice have a 3 fold increase in B-1b cells within 4 weeks of age. Studies that measure the difference in B-1b cells present in the fetal liver and spleen early in post-natal life could determine at what time-point this difference first develops. Since the B cell specific deletion of Id3 is driven by the CD19 promoter and B-1 progenitors are defined as $CD19^{+}B220^{-}$ pre-pro B cells [51], it can be assumed that Id3 expression is deleted at the earliest stages after B cell commitment. The results from these studies would determine at what time-point future studies should be conducted. Adoptive transfer of fetal B-1 progenitors from $Id3^{BKO}$ and $Id3^{WT}$ mice into irradiated or immune deficient hosts would be able to then determine whether the loss of Id3 in B-1 progenitors generates a greater number of mature B-1b cells.

Alternatively, if N-region insertion studies suggest that loss of Id3 increases the B-1b cells supplied by the adult bone marrow or the homeostatic turnover of mature B-1b cells, these alternatives could be studied with additional BM progenitor culture and adoptive transfer experiments similar to those described

above. The latter alternative would support the IgM mediated regulatory mechanism described above and would require adoptive transfer experiments with mature B-1 cells, measuring proliferation/self-renewal and survival as were done in studies from Chapter 4. These experiments would utilize host mice lacking IgM, such as the sIgM or Rag1$^{-/-}$ mouse models, to avoid the feedback regulation. Id3 has been demonstrated to regulate proliferation and self-renewal through p21^{Cip1} [154, 155] and survival through a caspase 2-dependent pathway [153]. Additionally, B-1a and B-2 cells have differential signaling through the CyclinD3-cdk4 complex to control their proliferative response to PMA stimulation; however this has not been examined in B-1b cells [182]. These candidates could be measured by a combination of expression and functional analysis in the event that a difference in proliferation, self-renewal, or survival is demonstrated in subsequent experiments.

Possible Id3 regulatory targets in B-1b cells

BCR signaling through the classical NF-κB pathway is critical for B-1 but not B-2 cell development [181, 183]. For instance, deletion of NF-κB1 (p50), the DNA binding subunit of the classical pathway, reduces all B cell subsets but predominantly B-1 cells [184, 185]. Additionally, mutation of Bruton tyrosine kinase (BTK), as occurs in X-linked immunodeficient mice (*xid*), or PLCγ2, both immediately downstream of BCR signaling, depletes mature B-1 cells without eliminating B-2 cells [186, 187]. In contrast, signaling through the alternative NF-κB pathway, as induced by B cell-activating factor (BAFF)/BAFFR signaling, is critical for B-2 cell development but

not essential to B-1 cells [183]. This was exhibited by studies of mice deleted for NF-κB2 (p52/p100), the alternative pathway equivalent to NF-κB1, which demonstrated complete loss of B-2 cells but retention of B-1 cells though with reduced numbers [188, 189]. Based on the evidence that loss of Id3 selectively increases B-1b and not B-1a cells this supports a hypothesis that Id3 does not mediate the classical NF-κB signaling pathway, particularly given that genetic deletion models of proteins involved with this pathway predominantly lose B-1a cells compared to B-1b cells [183]. Additionally, though *xid* mice have decreased B-1b cell numbers they retain adequate function and can respond to TI-2 immunization suggesting BCR signaling is a stronger regulator of B-1a than B-1b cell development [190]. More likely, if Id3 regulates NF-κB signaling, it is through the alternative pathway. To date, there have been no studies connecting Id3 to either NF-κB signaling pathway. BAFFR is expressed on B-1b cells and its deletion is associated with decreased B-1b cells in a dose dependent manner, suggesting BAFFR signaling regulates B-1b maintenance [191]. Flow cytometric analysis of BAFFR on $Id3^{BKO}$ B-1b cells could demonstrate whether loss of Id3 increases the amount of BAFFR surface expression. Observing that B-2 but not B-1a cell numbers are increased in 4 week old BKO mice and after Western diet feeding supports this hypothesis, as B-2 cells and not B-1a cells are sensitive to BAFF signaling. *In vitro* stimulation with BAFF could also demonstrate whether $Id3^{BKO}$ B-1b cells have increased responsiveness to BAFF signaling.

An important mitogen for B-1b cell proliferation is the Th2 derived IL-9. IL-9 transgenic mice develop a greatly increased population of PerC B-1 cells that are primarily B-1b cells [170]. Additionally, *xid* mice crossed with IL-9 transgenic mice were restored for B-1b cell numbers, though not function, with no recovery of B-1a cell numbers. These findings suggest IL-9 signaling has divergent importance between B-1 subsets. IL-9R expression analysis and responsiveness of B-1b cells from $Id3^{BKO}$ mice would determine whether loss of Id3 increases IL-9R expression or IL-9 mediated B-1b cell proliferation. Finding that Id3 regulates IL-9 dependent B-1b responsiveness would be similar to the finding that Id3 regulates natural helper cell (NHC) but not Th2 secretion of IL-5 in response to IL-33 stimulation [42].

Biotheranostic implications of B cells and atherosclerosis

B cells and their products as novel Biomarkers

Clinicians and researchers have invested significant effort and resources attempting to identify biomarkers which can predict the risk of adverse cardiovascular events in individuals with subclinical atherosclerosis, as there are currently no reliable means to identify unstable atherosclerotic plaques that are prone to rupture. Biomarkers such as CRP, Lp-PLA2, or MPO and non-invasive testing such as coronary calcium scoring, coronary computed tomographic angiography, carotid ultrasound to assess intima-media thickness, and assessment of endothelial function with flow-mediated vasodilation, along with

traditional risk factors, have been proposed to aid clinicians in risk-stratifying patients. Given our rapidly growing knowledge regarding the role of B cells in atherosclerosis, perhaps novel biomarkers of disease can be developed from circulating B cells or their products.

Immunoglobulins: Studies involving circulating antibodies have shown associations between immunoglobulin isotype and atherosclerosis. In patients with prior assessment of coronary artery disease burden with coronary angiography, total titers of IgG were positively associated with the burden of CAD whereas titers of IgM had an inverse relationship with CAD burden [146]. This could suggest that IgG and IgM are atherogenic and atheroprotective respectively. More specifically, the titers of anti-MDA-LDL IgM and IgG were found to again have opposing association (IgG positive and IgM negative) to inflammatory biomarkers of CAD[132]. Additionally, it was reported that circulating IgA associated with disease burden through a multivariate analysis from patients assessed by carotid and femoral angiography. In this cohort IgA titers independently associated with atherosclerosis suggesting IgA was atherogenic [192]. Alternatively, these findings could be purely associative wherein circulating antibodies could serve as novel biomarkers of disease. Two recent studies looked at the response of oxidative biomarkers, including oxidized phospholipid epitopes on circulating apolipoprotein B-100 (apoB) and IgG and IgM apoB immune complexes, to lipid-lowering statin therapies in patients with stable CAD or ACS. Both cohorts demonstrated that apoB levels increase in response to statins whereas immune

complexes decrease[174, 193] suggesting that circulating antibodies could also potentially serve as indicators for efficacy of therapy.

B cell homing markers: The idea of modifying leukocyte homing as a means of treating atherosclerosis has become popular based on findings that monocyte subsets differentially express the chemokine receptors CCR2 and CX3CR1. Classical monocytes ($CD14^{+}CD16^{-}$), which positively associate with cardiovascular events, express high levels of CCR2 whereas non-classical ($CD14^{+}CD16^{+}$) express CX3CR1 and negatively associate with carotid atherosclerosis [194] suggesting homing factors could be used as targets or diagnostic markers for cardiovascular disease. Our group recently demonstrated that splenic B cells isolated by CD43 negative selection traffic to the aorta and provide atheroprotection in *Apoe*$^{-/-}$ mice [34]. Moreover, $CCR6^{-/-}$ B cells were impaired for trafficking to the aorta after adoptive transfer relative to WT B cells and did not provide atheroprotection. These data suggests that the trafficking of B cells to the aorta plays an important role in B cell-mediated atheroprotection in mice. Other factors implicated in B cell homing to sites of atherosclerosis include L-selectin [44] and CCR7 [41], though these findings remain to be established in humans. This raises the question of whether homing factors such as chemokine receptors, selectins, and integrins act as important factors in the function of human B cells in atherosclerosis. Furthermore, as with CD14 and CD16 expression on monocytes, we could speculate that surface homing receptors on B cells could be used to assess outcomes in cardiovascular disease. Further

research is required to confirm these murine findings and to establish whether differential expression of homing factors on B cell subsets correlate with atherosclerosis in humans.

B cell depletion – Treatment with biologics:

With the common use of immunosuppressive mAbs, commonly called biologics, for the treatment of autoimmunity and oncology and the expanding evidence that immune cells have atheroprotective functions, it is important that we determine the potential impact of immunosuppressive biologics on the outcomes of cardiovascular disease. It is well established that there is a strong association between autoimmune rheumatic disease and atherosclerosis [195]. Indeed, acute coronary disease is the primary cause of premature mortality in patients with autoimmune diseases and patients with autoimmunity have been shown to have greater inflammation and plaque instability in their coronary arteries at autopsy [196, 197]. Rheumatoid arthritis is associated with the presence of auto-antibodies such as rheumatoid factor (RF) that form immune complexes found in affected joints [198]. Furthermore, B cells contribute to rheumatoid arthritis through antigen presentation to T cells and cytokine production [199]. The use of the mAb Rituximab (RTX) which causes broad B cell depletion has become a common clinical practice in the treatment of rheumatoid arthritis, vasculitis, systemic lupus erythematosus (SLE), and other rheumatologic diseases refractory to anti-TNFα treatment or immunosuppresive therapy [200]. Long-term B cell depletion using RTX is thought to improve outcomes through the reduction in autoreactive

antibodies in comparison to long lasting protective antibodies though it has not been assessed what this does to human B-1 cells or titers of circulating OSE-reactive IgM [201]. It was hypothesized that this is due to preferential depletion of short lived auto-reactive B cells compared to long-lived plasma cells. While RTX delivery attenuates atherosclerosis development in mice [30, 31], it is poorly understood whether RTX treatment in humans is associated with reduced subclinical atherosclerosis or CV events. Early findings from several cohort studies suggest RTX treatment could be beneficial for cardiovascular outcomes but they are limited in size and scope. Thirty-eight patients receiving RTX treatment showed improved flow-mediated dilation after 24 months and slightly improved carotid intima-media thickness [202]. These findings were supported by a study that reported five patients treated with RTX were measured to have improved flow-mediated dilation of the brachial artery, and a transient decrease in carotid intima-media thickness, as well as improved lipid profiles [203]. Another study reported improved lipid profiles after six months in patients whose RA positively responded to RTX treatment compared with those that responded poorly [204]. In contrast, a study of rheumatoid arthritis patients measuring arterial stiffness after 12 months of RTX therapy measured no improvement in arterial stiffness or inflammatory markers but did measure increased LDL with HDL levels unchanged [205]. These studies are small in size and use surrogate markers rather than acute cardiovascular events making it difficult to draw larger conclusions. Additionally, none of these studies assessed the effect RTX treatment had on B cells. However they raise the interesting possibility that RTX

treatment could be beneficial for CV outcomes. A meta-analysis of randomized controlled trials comparing RTX therapy in patients with rhematologic disease demonstrated no association with short-term risk of adverse cardiovascular events [144]. As atherosclerosis is a chronic, progressive disease, longer time points are required in order to determine if RTX induced B cell depletion alters cardiovascular outcomes in patients with rheumatologic disease.

Other means of B cell depletion in the context of autoimmunity that have been proposed include neutralizing antibodies against BAFF, or its ligand APRIL, or neutralizing antibodies against the pan-B cell surface markers CD19 and CD22 [206]. Clinical trials using a mAb reactive to BAFF have made it through Phase II for the treatment of RA and SLE [207]. A second means of blocking BAFF and APRIL is being tested using a TACI-Fc fusion protein which is in phase II and III testing for various autoimmune disorders [208]. The possible effects these treatments will have on cardiovascular disease and circulating B-1 cells have not been addressed.

Limitations and future considerations

An important caveat in the field is that the vast majority of findings implicating immune cells to atherosclerosis come from murine models which do not recapitulate thrombotic complications of human atherosclerosis [209]. Furthermore, distinct differences exist between the immune systems of humans and mice, as well as between various inbred mouse strains [210]. Of particular import, it is known

that humans and mice express different subtypes of immunoglobulins. Specifically, humans express IgG 1-4 and two subtypes of IgA, IgA1 and IgA2, whereas mice have an alternative IgG2b, no IgG4 and only IgA1 [210]. Furthermore, humans and mice express different IgG receptors with varying affinities and expression patterns [211]. This could have significant impact on potential therapeutics seeking to induce or target antibody production against atherosclerosis. Considering these differences between humans and mice, a stronger emphasis should be placed on assessing the impact of B cells on atherosclerosis in humans, both through large cohort studies tracking CV events and basic research using human tissue and blood samples in conjunction with plaque characterization using advanced imaging modalities (e.g. IVUS).

An additional limitation in the field is our dependence on immune-depleted mouse models such as SCID, Rag, or μMT which are deficient for various leukocytes. The advantage of these models is the ability to conduct adoptive transfer experiments without immune rejection. However, eliminating leukocytes from the model also eliminates interacting partners and severely modifies the complex network of regulation that makes up the immune system, limiting what we are able to conclude from any of our findings and leading to disparate findings based on model, cell of origin, or prior cell stimulus. This is not to say these models are not useful or question the findings derived from them but it will be important for the field to embrace cell specific targeting strategies, such as

inducible cell type specific knockout models, and to commit ourselves to finding more immune-sufficient animal models.

The contributions of murine B cells to atherosclerosis differ by subset. B-1a mediated atheroprotection is due to the secretion of IgM reactive to OSE on oxLDL. Here we have demonstrated for the first time that B-1b cells are similarly atheroprotective and additionally secrete OSE reactive IgM under atherosclerotic conditions. B-1b mediated immune memory toward polysaccharide and lipid TI-2 antigens strongly promote B-1b cells as targets for future research into immunization strategies. Considering scale and cost, vaccination against atherosclerosis would provide immense benefit due to the longevity and affordability of the treatment. We additionally demonstrated that Id3 is a selective negative regulator of B-1b cells in mice and possibly in humans. Though the mechanism is still undetermined, this underscores the importance of cell specific models and suggests Id3 could serve as an important target for B cell modulation in the future. With advances in molecular targeting and improved understanding of B cell specific (or better B-1 cell specific) surface markers, small molecule inhibitors of Id3 could be selectively delivered in liposomes or microbubbles to B cells in an attempt to induce B-1 cell expansion while avoiding off target effects. This would also be an effective way to deliver atheroprotective antigen for B-1 cell stimulation.

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