THE IMPACT OF ID3 ON NATURAL IMMUNITY IN ATHEROSCLEROSIS

Heather Perry
Dallas, PA

B.S., University of Pittsburgh, 2008

A dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Pathology

University of Virginia
December, 2013

Abstract

Coronary artery disease remains the leading cause of death worldwide. The underlying cause of coronary artery disease, atherosclerosis, is the formation of plaques in the arterial wall that can rupture, resulting in heart attacks and stroke. The pathogenesis of atherosclerosis can initiate early in childhood with the deposition of lipoproteins in the wall, leading to the activation of vessel wall cells and innate and adaptive immune responses. Current therapy directed toward lowering pathogenic lipoproteins in the circulation have reduced morbidity and mortality. However, disease burden remains high, suggesting that additional approaches are needed. Therapies targeting inflammation have emerged as the next frontier and anti-inflammatory agents are in clinical trials to reduce cardiovascular events. As such, understanding the complex cellular and molecular mechanisms in the immune pathogenesis of atherosclerosis may help identify those who will benefit from immune-modulatory therapeutic approaches.

Inhibitor of DNA binding-3 (Id3) is a transcription factor protein that has been reported to be important in murine and human atherosclerosis. Id3-deficient atherogenic (*Id3*$^{-/-}$*Apoe*$^{-/-}$) mice display early onset atherosclerosis. Compared to control mice, *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice contain fewer proliferating B-1a B cells, an immune cell that is a subset of B cells and can protect from atherosclerosis through the secretion of natural IgM antibodies. Additionally, *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice contained lower amounts of a natural IgM antibody, E06, in the circulation

compared to control mice. The generation of a B cell specific, Id3-deficient mouse revealed that these effects were not due to the loss of Id3 in B cells. Instead, *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice contained lower amounts of interleukin-5 (IL-5), a cytokine important for B-1a B cell proliferation and natural IgM antibody production. Furthermore, reduced amounts of IL-5 was detected in natural helper cells isolated from *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to control mice. Lastly, proliferating B-1a B cells and IL-5 producing natural helper cells were identified in the aorta and surrounding tissue. Data provides evidence for a novel role for Id3 in promoting IL-5 production by natural helper cells and subsequent proliferation of B-1a B cells.

Prior studies suggest that another mechanism for protection from atherosclerosis in Id3-deficient mice might be due to the loss of Id3 in B cells. To determine if the loss of Id3 in B cells exacerbates atherosclerosis, B cell specific, Id3-deficient and littermate control mice were placed on a Western diet to induce atherosclerosis. Surprisingly, B cell specific, Id3-deficient mice displayed attenuated atherosclerosis compared to controls. B cell specific, Id3-deficient mice also contained a greater number of B-1b B cells and larger amounts of a natural IgM antibody, T15-Id IgM, in the circulation compared to control mice after Western diet. Atherosclerotic plaques of B cell specific, Id3-deficient mice had fewer apoptotic cells and less macrophage content. These associations suggest that B-1b B cells may protect from atherosclerosis; a hypothesis that has yet to be tested.

Dedication and Acknowledgements

The University of Virginia and surrounding community has been a superbly nurturing environment for my graduate studies in biomedical research. Foremost, I would like to thank my advisor, Coleen McNamara, for her excellence in mentoring scientific and personal matters with patience, kindness, and never-ending enthusiasm. From her I have learned many aspects of becoming a successful scientific leader including keeping an open-mind about a scientific field, maintaining relationships with a diverse set of collaborators, laboratory management, and, hopefully, writing skills. I will forever cherish my time spent in her laboratory and will always remember the indispensable skills and qualities she has instilled in me during my graduate training.

I am very grateful for others who have been instrumental in the progression of my graduate training at the University of Virginia. Tim Bender was always willing to dedicate his time and effort to my research and would challenge me to reach and defend conclusions without over-interpreting data. Janet Cross has been an outstanding chair for my thesis committee and would constantly lend an ear to my queries and grievances. I would also like to thank the rest of my committee members, Loren Erickson, Adam Goldfarb and Ken Tung for their continued support and guidance. I am also grateful for Joanne Lannigan, Mike Solga, Sebastien Coquery, and Lesa Campbell in the flow cytometry core facility for providing technical support and Michael Kidd for his excellent administrative support. Shawn Fahl and the rest of the Bender laboratory as well as the

Erickson laboratory and Stacey Gorski have been incredibly helpful in providing technical advice and assistance. Lastly, I am very thankful to have such supportive laboratory members, especially Dan Harmon. He has always been insightful and supportive as a coworker and friend, of which I am truly appreciative. I would like to thank others past and present in the McNamara laboratory, Stephanie Oldham, Jim Garmey, Sam Morris-Rosenfeld, Jen Kaplan, and Melissa Marshall for their assistance. There have also been rotation students, Kirsti Campbell and Dee Das, who have been very helpful with this project.

I attribute much of where I am today to the love and support of my family and friends. To my friends, life is what you make of it and I think we can successfully say we have made the most of it here in Charlottesville. There are so many great past memories and I look forward to the many that will come. My Aunt Eileen and her family and my Uncle Jim and his family have provided support in countless different ways. They originally inspired me to pursue scientific research and have always been great mentors in my life. My sister Sarah, brothers Tom, Josh and I have prevailed in the face of adversity by relying on the tight foundation we have established as siblings. They are my best friends and I know we will always be able to depend on each other. The values my parents have imparted in me such as dedication, stamina, problem solving, leadership, accountability, self-motivation and strategic thinking have become important qualities applicable in graduate school. They as well as my

grandparents have always displayed unconditional love and encouragement. Lastly, I would like to dedicate this dissertation to my late grandmother, Wilma Lansberry. To this day, I still consider her my role model and superhero.

Table of Contents

List of Figures

Chapter 1:

Chapter 2:

None.

Chapter 3:

List of Tables

Chapter 1:

Chapter 2:

None.

Chapter 3:

Chapter 4:

List of Abbreviations

AID	activation-induced deaminase
Apoe	apolipoprotein E
ATLO	artery tertiary lymphoid organ
BAFF	B cell activating factor
BAFFR	BAFF receptor
BCR	B cell receptor
bHLH	basic HLH
BMMC	bone marrow derived mast cell
Breg	regulatory B cell
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
CD	cluster of differentiation
Cre	causes recombination enzyme
CypB	cyclophilin b
E06	Apoe autoantibody 6
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FALC	fat associated lymphoid cluster

FO	follicular
Foxp3	forkhead box P3
GATA3	GATA binding protein 3
γ_c	gamma chain
HDL-C	high-density lipoprotein cholesterol
HLH	helix-loop-helix
Id3	inhibitor of DNA binding 3 or inhibitor of differentiation 3
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
LDL	low-density lipoprotein
Ldlr	LDL receptor
Lin	lineage
Mac	macrophage
Mac2	macrophage marker 2
MAPK	mitogen activated kinase-like protein
MDA	malondialdehyde
μMT	mu heavy chain mutant
MZ	marginal zone
NAb	natural antibody
NH cell	natural helper cell

oxLDL	oxidized LDL
PC	phosphocholine
PCR	polymerase chain reaction
PD-L2	programmed cell death 1 ligand 2
PerC	peritoneal cavity
PRR	pattern recognition receptor
PVAT	perivascular adipose tissue
Rag	recombination activating gene
RLU	relative light unit
RORα	related orphan receptor A
sIgM	secreted IgM
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
T15-Id	T15 idiotype
Th	helper T cell
TI	T cell independent
TLR	toll like receptor
VCAM1	vascular cell adhesion molecule 1

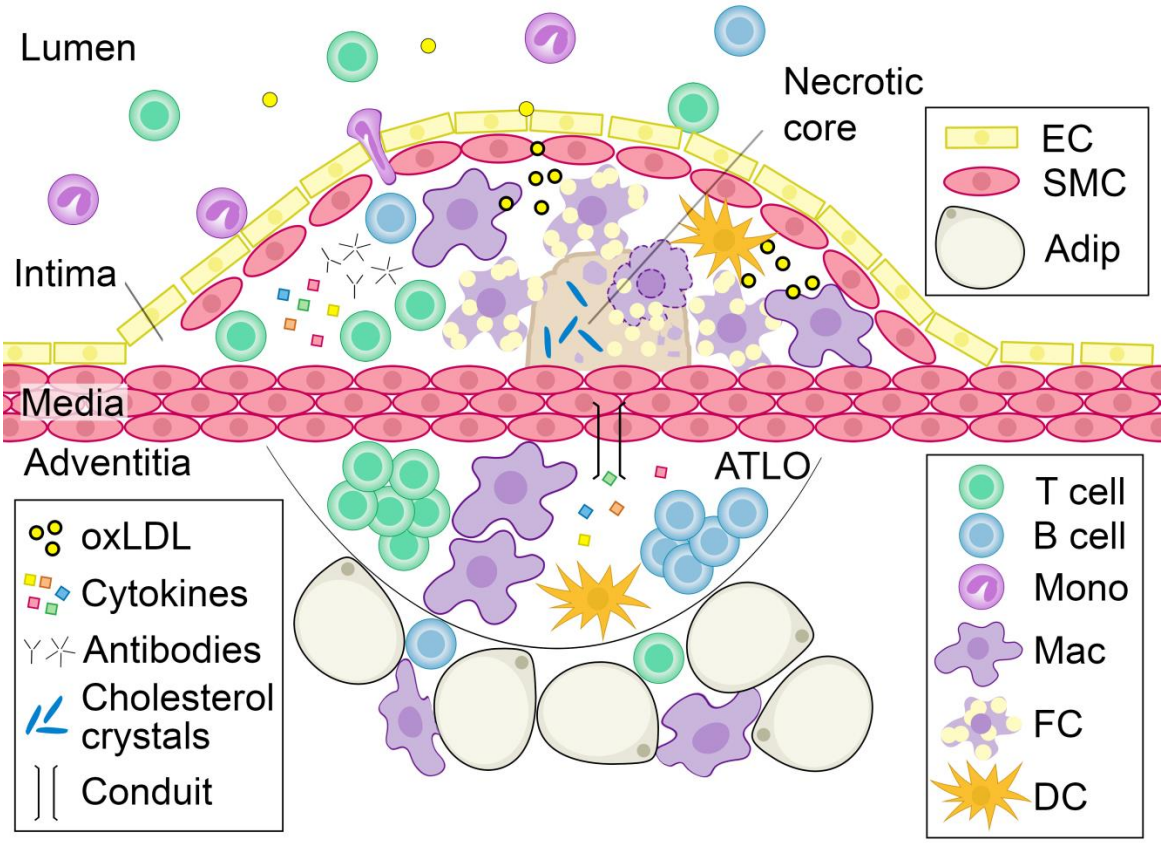
Chapter 1

Introduction

Pathogenesis of atherosclerosis

Atherosclerosis and its attendant sequelae of heart attacks and strokes remains a leading cause of death and disability in Westernized countries[1]. Substantial work over the last several decades has clearly established atherosclerosis as a chronic inflammatory disease of the blood vessel wall[2]. Immune cells including macrophages, dendritic cells, mast cells, neutrophils, T cells and B cells regulate the atherogenic process[3-6]. As such, immune-modulatory therapy holds promise as the next frontier for improving prevention of atherosclerotic cardiovascular disease[7-9]. Deposition of lipids such as low-density lipoprotein (LDL) in the subendothelial space of the intima and other injurious stimuli have been implicated as initial inflammatory triggers[10]. Vessel wall enzymes can act on deposited lipids to generate modifications in the lipids, such as oxidation, that serve both directly and indirectly as inflammatory signals[11-14]. Inflammatory cells are then recruited to the arterial wall, promoting progression of plaques through a host of interconnected mechanisms[5,15-18]. For example, recruited monocytes differentiate into macrophages within the plaque. There they engulf oxidized lipids, becoming foam cells, which secrete chemokines and other cytokines that further promote immune cell infiltration and activation including additional monocytes. Furthermore, many of these lipid-laden macrophages subsequently undergo apoptosis and necrosis, releasing their contents into the extracellular space and contributing to the necrotic core[2] (Figure 1). Factors such as hypoxia, oxidative stress, and pro-inflammatory cytokines

Figure 1. Characteristics of advanced atherosclerotic disease. As luminal LDL is deposited into the subendothelial space of the blood vessel wall, it becomes oxidized. Oxidized lipids trigger the recruitment of leukocytes to the subendothelial space. Monocytes differentiate into dendritic cells or tissue macrophages that engulf oxidized lipids and become foam cells. Without effective clearance of these apoptotic-prone cells, they accumulate, secrete pro-inflammatory cytokines and undergo apoptosis and necrosis, releasing their lipid contents into the extracellular space to create a necrotic core. Vessel wall and immune cell production of cytokines and immunoglobulins can further exacerbate atherosclerosis. ATLOs are found in the adventitia of diseased vessels and are composed of T cells, B cells, macrophages, and other leukocytes. Soluble factors can cross into the media through conduits and may contribute to plaque development. Abbr.: oxidized LDL (oxLDL), aortic tertiary lymphoid organ (ATLO), endothelial cell (EC), smooth muscle cell (SMC), adipocyte (Adip), monocyte (Mono), macrophage (Mac), foam cell (FC), dendritic cell (DC).

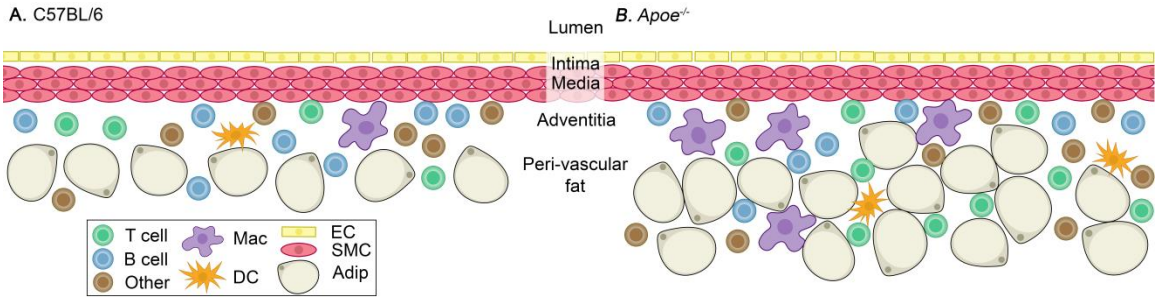

Lumen
Necrotic core
EC
SMC
Adip
Intima
Media
Adventitia
ATLO
oxLDL
Cytokines
Antibodies
Cholesterol crystals
Conduit
T cell
B cell
Mono
Mac
FC
DC

also induce apoptosis in vascular wall cells and other leukocytes in the atherosclerotic plaque[19]. Apoptotic cell burden can contribute to the necrotic core and/or may initiate plaque erosion and if not contained can lead to the unstable syndromes that result in heart attack and stroke.

The adventitia in atherosclerosis

In addition to immune cell entry into the plaque via the blood vessel lumen, immune cells are also found in the adventitia, or outer layer of the vessel wall. In fact, aortic tertiary lymphoid organs (ATLOs) have been identified in the aortic adventitia of aged mice at sites of advanced intimal plaque (Figure 1). Conduit networks, similar to those that filter soluble substances within lymph nodes[20] and facilitate lymphocyte organization in the white pulp of the spleen[21] connect the adventitia with the vessel wall[22,23]. At homeostasis, dendritic cells, macrophages, T cells and B cells are present in the adventitia and peri-aortic adipose tissue in wildtype and hyperlipidemic, apolipoprotein E knockout (*Apoe*$^{-/-}$) mice without atherosclerosis[24,25] (Figure 2), indicating that the aortic adventitia and surrounding perivascular fat (PVAT) is a homeostatic niche for specific leukocytes including B cells.

Figure 2. Immune cells at homeostasis in the adventitia of C57BL/6 and *Apoe*$^{-/-}$ mice. A healthy blood vessel is composed of an endothelial layer, an intima in between the endothelial layer and smooth muscle cell layer, and a smooth muscle cell layer (media) surrounded by the adventitia and peri-vascular fat. **(A)** The adventitia of a C57BL/6 mouse contains T cells, B cells, macrophages, dendritic cells, and others (neutrophils, natural killer cells, and natural killer T cells) [24,26]. **(B)** *Apoe*$^{-/-}$ mice have an increase in the number of T cells and macrophages, with a lesser increase in dendritic cells and other cells (neutrophils, natural killer cells, and natural killer T cells) compared to C57BL/6 [24]. Abundant adventitial B cells persist in the *Apoe*$^{-/-}$ mouse[24,25]. They also have an expanded peri-vascular fat pad compared to **(A)**. Abbr.: macrophage (Mac), dendritic cell (DC), endothelial cell (EC), smooth muscle cell (SMC), adipocyte (Adip).


A. C57BL/6
Lumen
B. Apoe-/-
Intima
Media
Adventitia
Peri-vascular fat
T cell
B cell
Other
Mac
DC
EC
SMC
Adip

Historical perspective on lymphocytes in atherosclerosis

Lymphocytes have long been identified in the adventitia and plaque of diseased arteries. In his 1915 book, *Diseases of the Arteries, Including Angina Pectoris,* Sir Thomas Clifford Allbutt noted that, "Round cell growth in the adventitia in arteriosclerosis is correlated with absorption of depraved matter from the diseased intima"[27], suggesting that lymphocytes might serve to protect against the accumulation of lipids and necrotic material in blood vessel walls. Subsequent histological studies confirmed the presence of lymphocytes in diseased blood vessels, underscoring the hypothesis that lymphocytes may be important regulators of atheroma development[28,29]. Yet, it was not until the advent of immunohistological reagents in the 1980s which allowed the visualization of specific types of lymphocytes that the presence of B cells in atherosclerotic plaques and the adventitia of diseased blood vessels were clearly confirmed[30-32]. A wealth of active and passive immunization studies in mice and the identification of atheroprotective IgM antibodies implicated B cells in attenuating atherosclerosis[33-40].

B cell subsets

B cells can be divided into two developmentally distinct lineages, B-1 and B-2. These lineages arise in overlapping waves within a layered immune system where B-1 B cell development predominates in the fetus and B-2 B cell

development in the adult. B-2 B cells include follicular B cells and marginal zone B cells; and B-1 B cells include B-1a B and B-1b B cells[41-45]. Common surface markers used to identify these B cell subsets are outlined in Table 1. Conventional follicular B-2 B cells undergo isotype switching and affinity maturation in the spleen and lymph nodes in response to T-dependent antigens to either become plasma cells that secrete large amounts of antibody, or memory B cells with the ability to produce specific antibodies upon re-exposure to the same antigen[46-49]. Unlike conventional follicular B-2 B cells of the adaptive immune system, marginal zone (MZ) B cells are considered part of the innate immune system. Marginal zone B cells reside in the spleen and are positioned to immediately respond to antigens in the blood that are filtering through the spleen[50,51].

Mature, adult B-1 B cells develop from fetal tissues including the liver and bone marrow, and less so from progenitors in the adult spleen and bone marrow[41,52-56]. Mature B-1 B cells are primarily found in serosal cavities and the spleen, and have the capacity to self-renew. B-1 B cells are largely T cell-independent and produce the majority of IgM antibodies that recognize self and foreign antigens[44,57,58]. B-1a B cells spontaneously produce natural IgM antibodies, which constitutes most of the serum IgM at homeostasis[59-63]. Additionally, their antibody repertoire is biased toward self-reactivity[64-68]. B-1b B cells have a distinct role from B-1a B cells in that they can be induced to secrete antibodies and proliferate by cross-linking the BCR on antigen specific B cells in

Table 1. B cell Subset Surface Markers

Subset	**Surface Markers***	**Citation**
Follicular	$CD19^{+}$ $B220^{+}$ IgM^{dull} IgD^{hi} $CD21^{mid}$ $CD23^{+}$	69
Marginal zone	$CD19^{+}$ $B220^{+}$ IgM^{hi} IgD^{dull} $CD1d^{hi}$ $CD21^{hi}$ $CD23^{-}$	69
B-1a	$CD19^{+}$ $B220^{low/mid}$ IgM^{hi} IgD^{dull} $CD43^{+}$ $CD11b^{+}$ $CD5^{+}$	70
B-1b	$CD19^{+}$ $B220^{low/mid}$ IgM^{hi} IgD^{dull} $CD43^{+}$ $CD11b^{+}$ $CD5^{-}$	70

*Surface markers define follicular and marginal zone B cells in the spleen, and B-1 B cells in the peritoneal cavity

a clonal manner. This response includes producing IgM or isotype switching to IgG3 or IgA with limited affinity maturation, and may lead to a memory-like phenotype[71-75].

Regulatory B cells (Bregs) are defined by their ability to inhibit autoimmune pathogenesis and restore tissue homeostasis mainly through production of IL-10[76-80]. Varying proportions of IL-10-producing B cells are found in several B cell subsets such as transitional 2-MZ precursor cells, MZ cells, and B-1a B cells, making it difficult to define a particular set of surface markers for Bregs. At present, the lack of clarity with respect to surface immunophenotype makes understanding Breg development challenging[76].

B cells in atherosclerosis

Beginning in 2002, studies emerged directly testing the hypothesis that B cells modulate atherosclerosis. Early studies reported that splenectomy of *Apoe*$^{-/-}$ mice exacerbated atherosclerosis compared to the sham control group[81]. Adoptive transfer of splenic B cells from atherosclerotic *Apoe*$^{-/-}$ mice not only rescued these mice from the atherogenic effects of splenectomy, but also reduced atherosclerosis to significantly less than that observed in the non-splenectomized controls. In addition, adoptive transfer of B cells, but not T cells, from atherosclerotic *Apoe*$^{-/-}$ mice to non-splenectomized, sham-operated mice significantly attenuated atherosclerosis[81]. Consistent with these findings, another

study showed increased atherosclerosis in atherogenic low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice transplanted with bone marrow from B cell deficient (*μMT*) mice compared to *Ldlr*$^{-/-}$ mice transplanted with bone marrow from control C57BL/6 mice[82]. Additional studies have suggested a protective role for B cells in atherosclerosis. *Ldlr*$^{-/-}$ mice unable to secrete IgM (*sIgM*) had accelerated atherosclerosis compared to control *Ldlr*$^{-/-}$ mice when fed a Western diet[83]. Also, there was attenuated Western diet-induced atherosclerosis in B cell deficient *μMT Apoe*$^{-/-}$ mice with the adoptive transfer of splenic B cells from *Apoe*$^{-/-}$ mice[25]. Taken together, these studies indicate that B cells protect from Western diet-induced atherosclerosis.

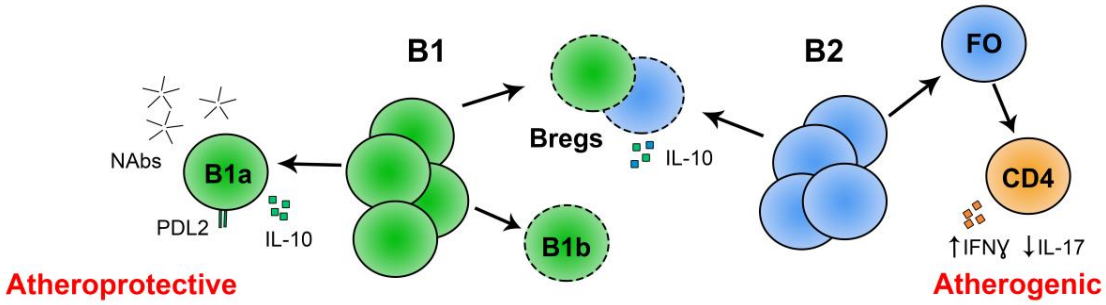
However, in 2010 two groups utilized an anti-CD20 monoclonal antibody to predominantly deplete B-2 cells, and less so B-1a B cells, in *Apoe*$^{-/-}$ mice and found attenuation of Western diet-induced atherosclerosis[84,85]. Confirmation of an atherogenic role for B-2 cells was provided by these same two groups in studies using atherosclerosis-prone mice null for B cell activation factor receptor (*Baffr*$^{-/-}$) or anti-BAFFR treated mice[86 87,88]. *Baffr*$^{-/-}$ mice lack B-2 B cells that require BAFF for survival, such as follicular or marginal zone B cells[89,90]. *Baffr*$^{-/-}$*Apoe*$^{-/-}$ mice or *Apoe*$^{-/-}$ mice treated with anti-BAFFR developed less severe atherosclerosis compared to control *Apoe*$^{-/-}$ mice when fed an atherogenic diet[86,88]. Additionally, *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from *Baffr*$^{-/-}$ mice had less Western diet-induced atherosclerosis compared to *Ldlr*$^{-/-}$ mice reconstituted with bone marrow from C57BL/6 mice[87]. These studies suggest that B-2 B cells

can aggravate atherosclerosis development. The apparent discrepancy in findings between studies early on suggesting an atheroprotective role for B cells and those more recent suggesting an atherogenic role for B cells may be explained by unique roles for specific B cell subsets in regulating atherosclerosis (Figure 3).

B-2 B cells in atherosclerosis

B cell depletion studies have suggested that B-2 B cells are an atherogenic B cell subset[84-88], but the mechanisms by which B-2 B cells can aggravate atherosclerosis are incompletely understood. Anti-CD20 treatment was associated with an increase in the percentage of IL-17+ T cells (Th17 cells), and IL-17A neutralization abrogated anti-CD20 attenuation of atherosclerosis[85]. These results imply that IL-17 may mediate B-2 B cell aggravation of atherosclerosis (Figure 3). However, the role for IL-17 in atherosclerosis remains controversial[91-97]. In addition to increasing Th17 cells, anti-CD20 treatment was also associated with a decrease in CD4 T cell secretion of the Th1 cytokine IFNγ, and reduced proliferation and activation of splenic CD4 T cells[85,87]. Several pro-atherogenic roles for Th1 cells have been identified and reviewed[5,6,9,22,98-100]. Depletion of B-2 B cells was also associated with decreased T cells in the atherosclerotic plaque [85,87,88], suggesting that B-2 B cells may aggravate atherosclerosis by regulating T cells in the aorta as well as the spleen.

Figure 3. Known and putative roles for B cell subsets in atherosclerosis. Conventional, follicular (FO) B-2 B cells may promote atherosclerosis by skewing CD4 T cell differentiation to IFNγ producing Th1 cells and away from IL-17 producing Th17 T cells. The role of Bregs in atherosclerosis is not yet determined, but they may attenuate atherosclerosis by secretion of IL-10. Peritoneal B-1a B cells attenuate atherosclerosis through production of IgM, and potentially IL-10. PD-L2 is expressed on anti-phosphocholine (PC) B-1a B cells, potentially marking atheroprotective cells within this subset. The role of B-1b B cells in atherosclerosis is unknown. *(- - -) Role in atherosclerosis not yet reported.


B1
B2
FO
NAbs
B1a
PDL2
IL-10
Bregs
IL-10
B1b
CD4
↑IFNγ ↓IL-17
Atheroprotective
Atherogenic

B-2 B cells may also aggravate atherosclerosis by producing pathogenic antibodies. B-2 B cell depletion was associated with a reduction in total serum IgG including IgG1, IgG2a, IgG2c, as well as IgG1 and IgG2a in the atherosclerotic plaque. Furthermore, B-2 cell depletion resulted in a reduction in serum IgG against modified lipids, oxLDL and malondialdehyde LDL (MDA-LDL). Consistent with the predominant depletion of B-2 cells and not B-1a B cells, there were only modest decreases in total IgM and IgM against MDA-LDL and oxLDL[84,85,87,88]. Interestingly, univariate analysis revealed that serum levels of IgG and IgM to oxLDL have divergent associations with coronary artery disease in humans. IgM to OxLDL was inversely associated with coronary artery disease while IgG was positively associated[101]. Mechanisms whereby adaptive immunoglobulins might regulate plaque development are poorly understood. Downstream of antibody production, B cells may indirectly regulate atherosclerosis in an antigen-independent manner when IgG immune complexes bind to Fc gamma receptors (FcγR). Activating FcγRs have been implicated as being pro-atherogenic [102] and inhibitory FcγRIIb anti-atherogenic[103,104]. Additionally, IgE and its Fc receptor present on mast cells, FcεR1α, are pro-atherogenic[105].

B-2 B cells may also promote atherosclerosis by altering other inflammatory mediators in the aorta. The loss of BAFFR in *Apoe*$^{-/-}$ mice resulted in decreased immunostaining of VCAM1, CD11c, CD83 and proliferating cell nuclear antigen, and reduced gene expression of the inflammatory markers

tumor necrosis factor α, IL-1β, and CCL2 in the atherosclerotic lesion[88]. Taken together, recent studies suggest that B-2 B cells are atherogenic, yet the specific role for certain types of B-2 B cells in atherosclerosis such as FO, MZ, and Bregs is unclear.

B-1 B cells in atherosclerosis

The adoptive transfer of B-1a B cells attenuated splenectomy-aggravated atherosclerosis, providing direct evidence for an atheroprotective role for B-1a B cells. B-1a B cells produce IgM antibodies that have long been implicated in atheroprotection[106]. Indeed, adoptive transfer of B-1a B cells from *sIgM* mice did not protect from splenectomy-aggravated atherosclerosis[107].

The mechanisms whereby B-1a B cell-derived IgM can protect from atherosclerosis have been best characterized using the prototypic monoclonal IgM antibody, E06[37,108-112]. E06 is structurally and functionally similar to the classic antibody T15 and is produced by B-1a B cells at homeostasis in germ-free mice[110,111] suggesting that E06 IgM is a natural antibody. E06 was cloned from spleens of Western diet fed *Apoe*$^{-/-}$ mice and is able to bind oxidized LDL (oxLDL) in serum and in atherosclerotic lesions[113]. E06 inhibits uptake of oxLDL by macrophages[109,114] preventing foam cell formation, inhibiting release of pro-inflammatory cytokines in the atherosclerotic lesion. Additionally, E06 and other T15-idiotype (T15-Id) containing NAbs bind to oxidation-specific epitopes,

including phosphocholine (PC), on membrane phospholipids of proinflammatory apoptotic cells and mediate apoptotic cell clearance[111,115]. Effective clearance of apoptotic cells and oxLDL neutralization reduces proinflammatory effects on other vessel wall cells[16,106,108,112,116]. Natural IgM antibodies, typified by E06, provide one mechanism whereby B-1a B cells can attenuate atherosclerotic plaque progression[117].

Characterization of the impact of splenectomy and B-1a B rescue on atherosclerotic plaques provided *in vivo* support for atheroprotective mechanisms originally described *in vitro* [14,106,107,117]. Splenectomy led to reduced atherosclerotic lesion IgM content and increased the size of the necrotic core within the lesion[107]. Large lesional necrotic cores typify advanced unstable plaques and are linked to failed apoptotic cell clearance[16,118]. Consistent with previous work demonstrating increased IgM in atherosclerotic lesions of *Rag*$^{-/-}$ mice after adoptive transfer of B-1 cells[111], adoptive transfer of B-1a B cells to splenectomized mice increased lesional IgM. The increased IgM was associated with a reduced necrotic core, an effect that was lost when the transferred B-1a B cells came from *sIgM* null mice[107]. These data suggest that the increased necrotic core could be due to failed IgM-mediated clearance of apoptotic foam cells. Indeed, adoptive transfer of B-1a B cells reduced splenectomy-induced lesional apoptosis. However, it is not clear that this effect was dependent on IgM, as there was a trend toward an increase in apoptotic cells in lesions from mice receiving B-1a B cells null for *sIgM* compared to wildtype, but it was not

statistically significant[107]. Consistent with this observation, *sIgM*.*Ldlr*$^{-/-}$ mice had only a non-significant trend toward an increase in lesion apoptotic cell content compared to control *Ldlr*$^{-/-}$ mice[83]. Additional studies will be needed to fully address the role of B-1a B cell-derived IgM in apoptotic clearance *in vivo*. Moreover, IgM-dependent effects of B-1a B cells may need to be tested in a model without splenectomy as B-1a B cells may need the spleen for full IgM production. B-1a B cells in the peritoneal cavity spontaneously secrete low amounts of IgM, but in the spleen, they are 'super-secretors'[119], suggesting that the spleen may be important for B-1a B cells to produce large amounts of IgM antibodies against modified lipids that are protective against atherogenesis. In addition, these results raise the possibility that B-1a B cells may also regulate apoptotic cell clearance by mechanisms independent of IgM. B-1a B cells are known to produce cytokines involved in atheroprotection[120] and future studies are needed to determine if production of cytokines might be another mechanism whereby B-1a B cells protect from atherosclerosis.

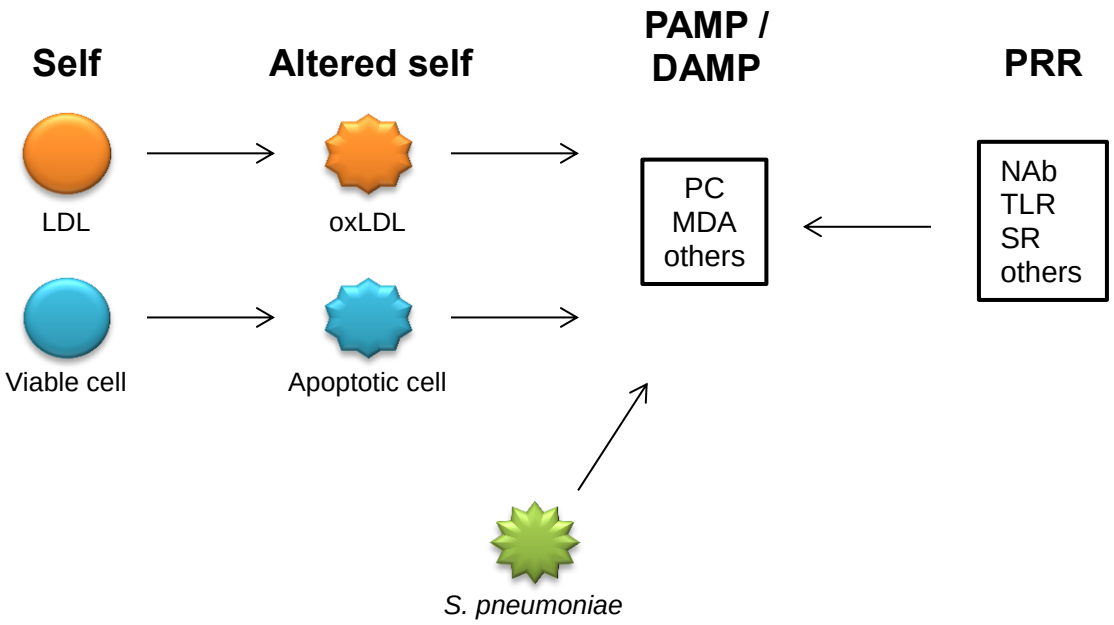
The sister population to B-1a B cells, B-1b B cells, also produce IgM and can undergo clonal expansion in response to T-independent (TI) antigens[44,57,121]. Yet, the role of B-1b B cells in atherosclerosis is unknown (Figure 3). Insight into a potential role for B-1b B cells in atherosclerosis may come from studies on the immune response to TI pathogens. In infection models of *B. hermsii*[74] or *Salmonella*[75] and immunization models of *S. pneumoniae*[73], B-1b cells have a distinct role from B-1a B cells in that they can proliferate in a clonal manner in

response to antigen and provide a protective antibody response by isotype switching to IgG3 or IgA, or increasing natural IgM titers upon pathogen re-challenge, suggesting that they may be able to provide long-lasting protection from pathogens[37 75]. Mounting a TI-2 response to pneumococcal vaccination can protect from atherosclerosis through inducing an anti-PC IgM response[37], inferring that B-1b cells could provide long-lasting protection from atherosclerosis in an antigen-specific manner potentially through production of IgM.

Innate immune responses in atherosclerosis

Innate immunity plays a major role in initial defense against disease by using natural receptors that discriminate 'self' versus 'neo-self' epitopes. In the context of atherosclerosis, self-epitopes such as native LDL can become modified after oxidation to become oxLDL and express neo-self epitopes, including MDA and PC (Figure 4). Another classic example is lipid-laden macrophages that undergo apoptosis and expose the PC group on oxidized phospholipids in their plasma membrane[14]. Oxidation-specific epitopes are recognized by pattern recognition receptors (PRR) in innate immunity, including natural antibodies, toll like receptors, scavenger receptors, and soluble receptors[14,106]. Oxidation-specific epitopes are a class of danger signals termed danger associated molecular pattern or 'DAMPS' in the case of oxidized lipids and are considered pathogen

Figure 4. Innate immune responses in atherosclerosis**.** Oxidizing conditions in atherosclerosis induce LDL to become oxLDL and cellular apoptosis which can then express oxidation-specific epitopes PC, MDA. Microbial pathogens such as *S. pneumoniae* also display molecular mimics of oxidation-specific epitopes. Oxidation-specific epitopes are considered DAMPs when displayed on oxLDL and PAMPs on apoptotic cells and pathogens. PRRs in the innate immune system, such as natural antibodies, toll-like receptors, scavenger receptors and soluble receptors, recognize DAMPs and PAMPs and initiate responses in vascular disease and infection. Abbr.: Danger associated molecular pattern (DAMP), pathogen associated molecular pattern (PAMP), pattern recognition receptor (PRR), natural antibody (NAb), toll-like receptor (TLR), scavenger receptor (SR). Adapted from Witztum and Lichtman, *Annu. Rev. Pathol. Mech. Dis.* 2014.


Self
Altered self
PAMP / DAMP
PRR
LDL
oxLDL
PC
MDA
others
NAb
TLR
SR
others
Viable cell
Apoptotic cell
S. pneumoniae

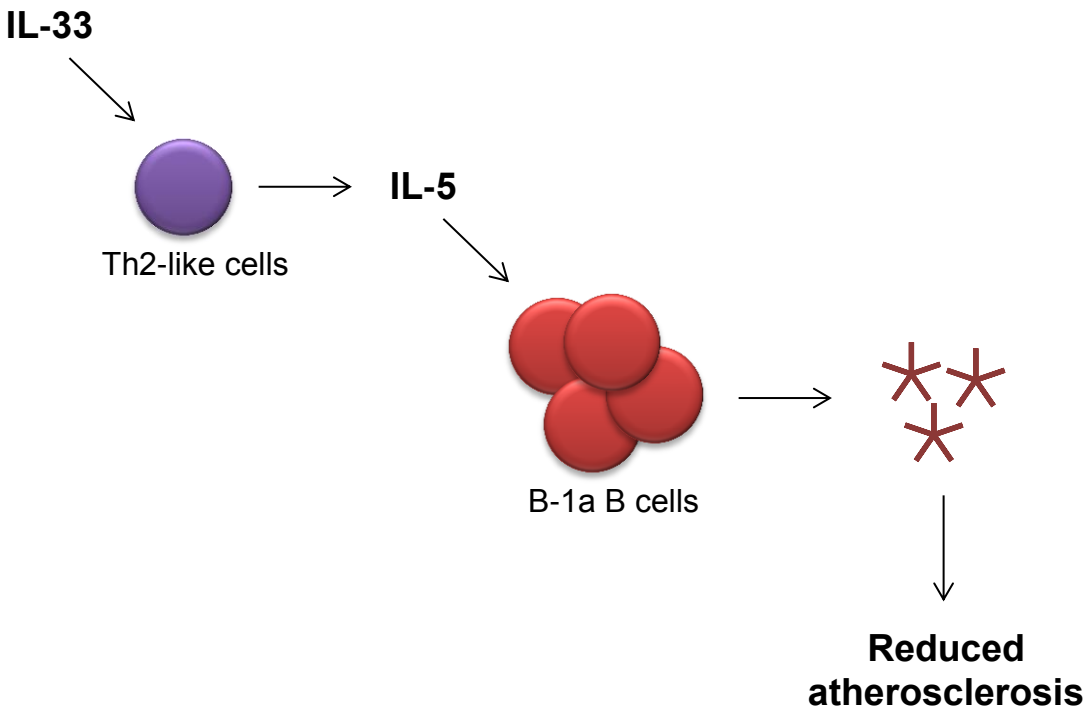
associated molecular patterns or ‘PAMPS’ when expressed on apoptotic cells or as molecular mimics on microbes[122].

One group of natural antibodies that recognize the PC group on oxidized phospholipids and apoptotic cells are the T15-Id antibodies which are present in atherosclerotic plaques[110,111]. A turning point in the progression of atherosclerosis is the failure to resolve inflammation, which usually involves the suppression of cell infiltration, effective clearance of apoptotic cells, and promotion of cell efflux from the arterial wall. However, when there are defects in these mechanisms, or when these mechanisms are overwhelmed, atherosclerotic plaques progress to dangerous plaques capable of rupture[16].

IL-33/IL-5 axis in atherosclerosis

The IL-33/IL-5 axis is atheroprotective most likely by promoting B-1a B cell proliferation and E06 production[14,40,123] (Figure 5). Treatment with IL-33, an IL-1β cytokine family member, in *Apoe*$^{-/-}$ mice results in decreased atherosclerosis and is associated with an increase in serum levels of IL-5, a Th2-associated cytokine that is important for survival and proliferation of B-1a B cells[124,125] and is atheroprotective[123,126]. Immunization with MDA-LDL induced IL-5 production by splenocytes, increased plasma IL-5 and E06 levels, and reduced atherosclerosis. In addition, *Il5*$^{-/-}$ mice have reduced levels of E06 in the plasma compared to

Figure 5. The IL-33/IL-5 axis in protection from atherosclerosis. The IL-1β cytokine, IL-33, induces IL-5 production by Th2-like cells such as Th2 T cells, mast cells, and NH cells. IL-5 is important for B-1a B cell proliferation and production of IgM such as E06 that has been shown to prevent oxLDL uptake by macrophages and facilitate clearance of apoptotic cells.


IL-33
Th2-like cells
IL-5
B-1a B cells
Reduced atherosclerosis

controls, while treatment with IL-5 increases E06 levels in wildtype mice[40]. Lastly, *Ldlr*$^{-/-}$ mice reconstituted with *Il5*$^{-/-}$ bone marrow have decreased plasma E06 levels and increased atherosclerosis compared to mice reconstituted with control bone marrow[40]. Many cell types including Th2 T cells and the recently discovered natural helper (NH) cell can be stimulated by IL-33 to produce IL-5.

Natural helper cells

NH cells belong to an emerging arm of the innate lymphoid cell (ILC) family, the group 2 ILC helper subset[127-131]. Group 2 ILCs are important in clearing extracellular parasites, flu pathology, and allergy[128,131-141]. More specifically, NH cells have key roles in helminth expulsion and allergic lung inflammation[137,138,140]. NH cell development is most likely from the bone marrow and is dependent on Id2, RORα, GATA3, γ_c and IL-7 [129,139,140,142-144]. NH cells are organized into fat-associated lymphoid clusters (FALCs) in the mesenteric adipose depot at homeostasis and can also be found in the lung under pathologic conditions. No specific markers have been identified that define NH cells and they are therefore defined by the lack of lineage markers (lin$^-$) and positive for Sca-1, CD117 (c-kit), CD44, CD90 and the IL-33R (T1/ST2)[139,140]. NH cells produce mainly Th2-associated cytokines IL-2, IL-4, IL-5, IL-6, and IL-13, in response to a variety of stimuli including IL-2, IL-25, and IL-33. Co-culture experiments demonstrate that NH cells directly support B-1 cell proliferation

similar to IL-5 alone and *in vivo* studies show that they can support B-1 proliferation in *Rag2*$^{-/-}$ mice[140]. However, it is unknown if NH cells participate in atherosclerosis.

HLH transcription factors

The basic helix-loop-helix (bHLH) family of transcription factors is highly conserved and plays a role in the differentiation and growth of a variety of cell types[145-147]. These factors are characterized by the presence of two domains. The basic domain binds DNA by recognizing a hexanucleotide “E-box” sequence and the HLH domain at the carboxy-terminus facilitates dimerization of these factors. Within the bHLH family, there are several subgroups including E-proteins, tissue-specific bHLH factors and inhibitors of DNA binding (Ids).

There are six E-proteins: E12 and E47 which are splice variants from the E2A gene, ITF-1, ITF-2a and ITF-2b which are from the E2-2 gene and HEB. These widely expressed factors function as homodimers or as heterodimers and are highly conserved among mammals. HLH factors are part of a complex gene-regulatory network of lineage specific transcription factors that determine lymphocyte development and activation[148-157].

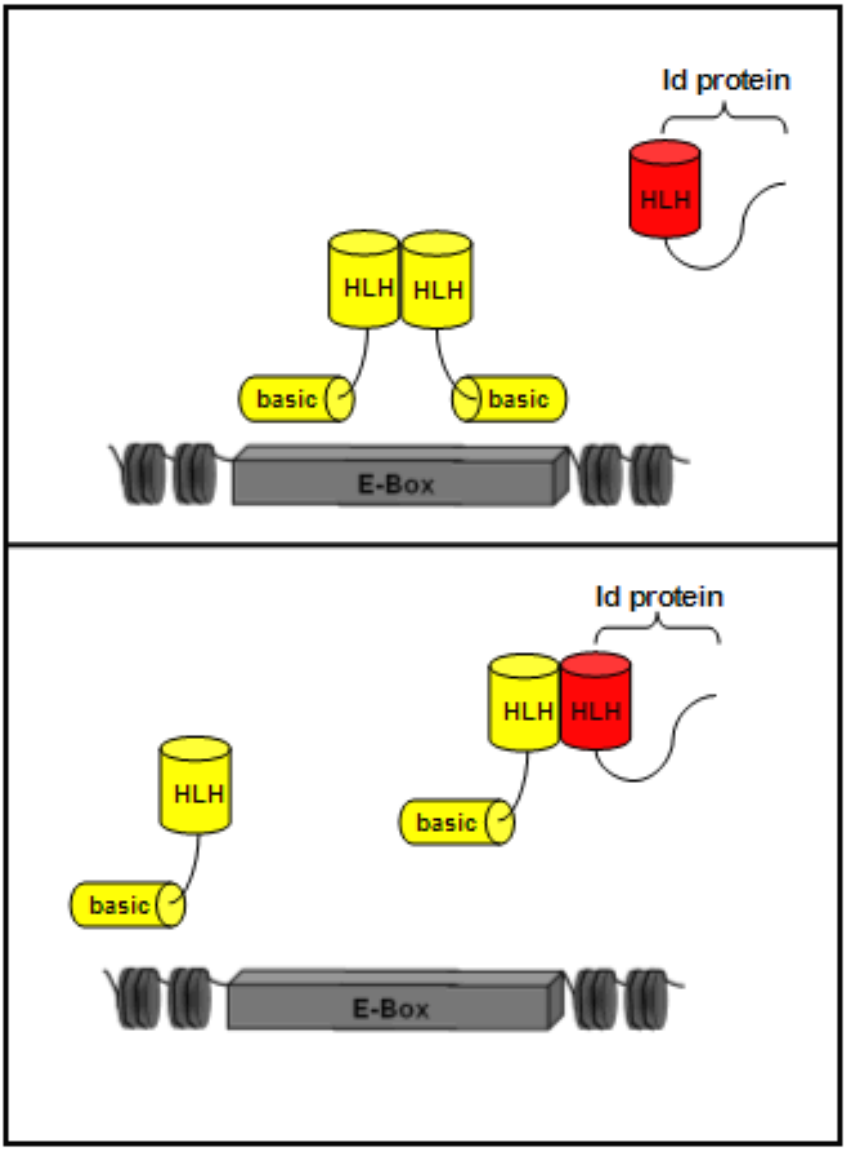
The Ids are a unique subset of HLH factors. While the Ids possess a HLH domain, they lack the basic domain required for DNA binding. Through the HLH domain, they heterodimerize with the E-proteins, or other bHLH family members,

and disrupt E-protein dimers, thereby inhibiting the ability of those factors to activate or repress transcription[158,159] (Figure 6). Because of this mechanism of action, the Ids are considered dominant negative inhibitors of DNA binding and transcription. The four known mammalian Id genes (Id 1-4) have overlapping, non-redundant function[145,147] and are broadly but not uniformly expressed. Levels tend to be high during development and in proliferating cells, while they are low in healthy adult tissues, terminally differentiated or quiescent cells[147,159]. One exception is the hematopoietic system, where Id proteins are expressed even in mature lymphocytes[160]. Ids are also re-expressed in disease states, including cancer, autoimmune and vascular disease[147,161-163].

Id3 and B cell function

Most effort in studying the role of Id and E-proteins in B cells has been focused on lymphocyte development[157,164]. In 1999, Yuan Zhaung's group characterized the Id3 deficient mouse and found defects in B cell proliferation and clonal expansion[154]. Id3 knockout mice contain normal numbers of B and T lymphocytes, but have reduced levels of IgG1 and IgG2a prior to immunization and an impaired antibody response to type 2 TI antigens. Full B cell activation has two steps, first, activation signals from the B-cell receptor (BCR) as indicated by surface marker expression and calcium signaling and second, proliferation

Figure 6. Paradigm of HLH function. bHLH family members can hetero- or homodimerize through their HLH domain enabling their basic domain to bind to conserved regions on DNA termed ‘E-boxes.’ Id proteins lack the basic domain, thereby sequestering bHLH proteins from DNA and regulating transcription.


Id protein
HLH
HLH
HLH
basic
basic
E-Box
Id protein
HLH
HLH
HLH
basic
basic
E-Box

and/or differentiation. B cells from *Id3*$^{-/-}$ mice have a normal first step of the activation response downstream of B-cell receptor (BCR) signaling, but they cannot proliferate in response to surface BCR cross-linking. In B cells from wildtype mice, BCR crosslinking leads to rapid upregulation of Id3, suggesting that Id3 is downstream from BCR crosslinking and is important for clonal expansion. Further evidence suggests that Id3 may be important in mature B cell somatic hypermutation and class switch recombination[165]. Id3, in concert with E-proteins, can directly regulate transcription of the *Aicda* gene[165] which produces the enzyme AID that may directly or indirectly induce DNA mutations[166]. These data suggest that Id3 may regulate adaptive B cell humoral responses.

Project rationale and summary

At the inception of this project, the role of B cells in atherosclerosis was emerging to be subset dependent, with B-2 B cells atherogenic, and B-1a B cells atheroprotective[2]. Prior studies in the lab demonstrated that adoptive transfer of splenic B cells to a B cell-deficient mouse attenuated Western diet-induced atherosclerosis, an effect that was dependent on Id3[25,167]. Id3 was also shown to modulate B cell homing and vessel wall adhesion molecule expression[25,167] yet, Id3 may also regulate factors involved in innate immunity as loss of Id3 in *Apoe*$^{-/-}$ mice resulted in very early onset of diet-induced atherosclerosis[25]. We hypothesized that Id3 may be an important regulator of B-1a B cells and natural immunity.

In Chapter 3, we provide evidence that Id3 may be an important regulator of natural immunity in atherosclerosis. Mice lacking Id3 had significantly fewer B-1a B cells in the spleen and peritoneal cavity and reduced levels of E06 in the serum. B cell-specific deletion of Id3 revealed that this effect was not due to the loss of Id3 in B cells. IL-33 induced abundant, Id3-dependent IL-5 production in NH cells, but not Th2 or mast cells and delivery of IL-5 to Id3-deficient mice restored B-1a B cell proliferation. Proliferating B-1a B cells and IL-5 producing NH cells in response to exogenous IL-33 stimulation were identified in the aorta and surrounding adventitia/PVAT. These studies are the first to identify NH and B-1a B cells in the aorta and provide evidence that Id3 is a key regulator of NH cell IL-5 production and B-1a B cell homeostatic maintenance.

In Chapter 4, we suggest that the B cell specific deletion of Id3 may have an intrinsic role in B-1b B cells and attenuate atherosclerosis. The B cell specific loss of Id3 on an atherogenic background resulted in a greater number of B-1b B cells and elevated levels of IgM in the serum. After Western diet feeding, there was a systemic increase in the number of B-1b B cells and levels of IgM and T15-Id IgM. B cell specific Id3 knockout mice had a decrease in atherosclerotic plaque area that contained less macrophage content and fewer apoptotic cells. Data in the B cell specific Id3 knockout mouse model suggests that Id3 may regulate B-1b B cells in atherosclerosis.

Chapter 2

Materials & Methods

Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. *Apoe*$^{-/-}$ male mice were purchased from Jackson Laboratory (Stock # 002052). *Id3*$^{-/-}$ and *Id3*$^{fl/fl\ 162}$ mice were a generous gift of Dr. Yuan Zhang (Duke University). *Id3*$^{-/-}$ mice were bred to the *Apoe*$^{-/-}$ background to obtain *Id3*$^{-/-}$*Apoe*$^{-/-}$ as previously described[25]. *CD19*$^{cre/+}$ mice were gifted by Timothy Bender (University of Virginia). *Id3*$^{fl/fl}$ *mice were bred to the Apoe*$^{-/-}$ mouse, and then to *CD19*$^{cre/+}$ mice to generate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$. These mice were then bred to *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice to generate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{cre/+}$ and littermate control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice. Id3-GFP mice were a generous gift from the Murre Lab (University of California San Diego). All mice were purchased from Jackson on a pure C57BL/6J background or mice were backcrossed 10 generations to pure C57BL/6J except Id3-GFP mice. Studies were with male mice fed a constant chow (Teklad 7012) or standard Western diet, 42% kcal from fat (Teklad TD.88137). All mice were euthanized by CO_2 inhalation as approved by the ACUC.

Determination of serum lipid levels

Whole blood was harvested from non-fasting mice at the time of sacrifice by right ventricular puncture. Blood was centrifuged at 2,000 xg for five minutes, after which the serum layer was removed to a clean tube. Cholesterol and

triglyceride determinations were performed by the University of Virginia Medical Laboratories using an Architect 8000 series analyzer.

Flow cytometry

Aortas were harvested as previously described[25] except with the peri-aortic adipose tissue left intact and all lymph nodes carefully removed. Aortas were chopped in PBS + 20 mM HEPES with 200 U mL^{-1} Coll XI (Sigma), 60 U mL^{-1} DNaseI (Sigma), 125 U mL^{-1} Hyaluronidase I (Sigma) and 50 U mL^{-1} Coll I (Sigma) and placed in a 37°C shaking incubator for 45 minutes. After centrifugation, adipocytes were aspirated. The cell suspension was washed and strained through a 40 µm filter. Mesenteric cells were prepared as described elsewhere[140]. Briefly, mice were transcardially perfused with PBS +20 U mL^{-1} heparin after CO_2 euthanasia. Mesenteric adipose tissue was carefully removed of mesenteric lymph nodes and separated from intestines, and finely chopped in 5 mL of DMEM with 2 mg mL^{-1} Collagenase I (Worthington Biochemical Co.) and 4% BSA (Sigma), then digested at 37°C in a shaking incubator for 45 minutes. The supernatant containing adipocytes after centrifugation was aspirated. After washing the cells in HBSS with 10% HI-FBS, cells were strained through a 40 µm filter. Peritoneal cells were harvested by lavage with 4 mL DMEM+ (DMEM, 1X L-glut, 1X P/S, 5% HI-FBS). Spleens were filtered through a 70 µm filter in DMEM+ and red blood cells lysed with AKC lysis buffer. Pairs of peri-aortic lymph nodes were combined and filtered through a 70 µm filter in DMEM+. Peritoneal cavity

cells, splenocytes, lysed blood, lymph nodes, mesenteric cells, digested aortas or cultured cells were first blocked for Fc receptors (FCR-4G8, invitrogen), then stained for cell surface markers, followed by a live/dead stain, and/or intracellular markers. For intracellular staining, the FIX & PERM® kit (Invitrogen GAS004) was used as per manufacturer's instructions. Flow cytometry antibodies CD3ε (500A2), CD4 (GK1.4, RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD43 (S7) CD44 (IM7), CD45 (30F11), CD45R/B220 (RA3-6B2), CD49b (DX5), CD90 (53-2.1), CD117 (ACK2), CD196 (29-2L17), FcεR1α (MAR-1), IgM (II/41), IFNγ (XMG1.2), IL-4 (BVD6-24G2), IL-5 (TRFK5), Ly6G (RB6-8C5), NK1.1 (PK136), Sca-1 (D7), SiglecF (E50-2440), TCRβ (H57-597), TCRγδ (GL3) and Ter119 (TER119) were purchased from eBioscience or BD Bioscience and T1/ST2 (DJ8) from MDbioscience. Live/dead discrimination was determined by LIVE/DEAD® fixable yellow cell staining (Invitrogen) or dapi. Cells were run on a CyAN ADP (Beckman Coulter) or sorted on a Reflection Cell Sorter (iCyt) and analyzed with FlowJo software (Tree Star Inc) using fluorescence minus one (FMO) controls for gate determination. Counting beads were used for quantification as per manufacturer's instructions (CountBright™ Absolute Counting Beads, Molecular Probes).

ELISA

Whole blood was harvested from mice by right ventricular puncture and peritoneal fluid was harvested by lavage with 1 mL PBS at the time of sacrifice. For murine E06, ELISA plates (Costar, 3590) were coated with anti-PC T15 (AB1-2, ATCC). For IgM, IgG1, IgG2b, IgG2c, or IgG3, ELISA plates were coated with their respective capture antibody from Southern Biotech, anti-mouse IgM (1020-01), IgG1 (1070-01), IgG2b (1090-01), IgG2c (1079-06), IgG3 (1100-01) overnight. Plates were then washed with a plate washer and blocked with BSA-PBS for two hours at room temperature. Then plates were washed and serum was added at a dilution of 1:250 (E06), 1:1000 (IgM), or 1:10000 (IgG1, IgG2b, IgG2c, IgG3) for two hours at room temperature. After washing, the secondary antibody, anti-IgM AP (Southern Biotech 1020-04) for E06 and IgM or IgG1-AP (1070-04), IgG2b-AP (1090-04), IgG2c-AP (1079-04), IgG3-AP (1100-04) was added for one hour. The plate was developed with PNPP (Sigma N1891) then stopped with a strong base. For IL-5 levels, mice were i.p injected with 125 ng of mIL-33 (CF, R&D) reconstituted in PBS every three days for 7 days. Serum and peritoneal fluid were then harvested for concentration of IL-5 in undiluted samples (BD OptEIA™, BDbiosciences as per manufacturer's instructions). IL-33 was measured undiluted by the mouse/rat Quantikine ELISA kit (R&D). Plates were read at 405 nm with 570 nm subtraction. Data was considered not detectable if below the sensitivity of the assay.

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). E06 gene expression was normalized by the ΔΔCq method to 18S as determined by TaqMan real-time PCR (SsoFast™ Probes Supermix, Bio-Rad). Secreted IgM or Id3 was also normalized by the ΔΔCq method to 18S or cyclophilin 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: E06 IgHV, forward primer (5'-CTG TGC AAG AGA TTA CTA CGG TAG-3') flanked the E06 IgH V and D junction, the reverse primer (5'-AGG ACT GAC TCT CTG AGG AGA CG-3') flanked the JH and mu chain junction for amplifying E06 IgM, but not T15 IgA. The fluorescent probe (6FAM CGC CCC AGA CAT CGA AGT ACC AG TAMRA, Applied Biosystems) matched to the E06 IgH D and J junction; 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') and probe (6FAM TGC TGG CAC CAG ACT TGC CCT C TAMRA, Applied Biosystems); Id3 forward primer (5'-TGC TAC GAG GCG GTG TGC TG-3') and reverse primer (5'-TGT CGT CCA AGA GGC TAA GAG GCT-3'); CypB

forward primer (5'-TGC CGG AGT CGA CAA TGA T-3') and reverse primer (5'-TGG AGA GCA CCA AGA CAG ACA-3'). For Id3 expression in NH cells, mesenteric adipose tissue was processed and cell suspensions were incubated with CD90.2 microbeads. CD90+ cells were positively selected on an LS column as per manufacturer's instructions (Miltenyi Biotech). Cells were washed in PBS + 5% BSA and sorted for live, singlet, CD45+Lin-Sca1+CD117+ fraction by FACS. Id3 (Invitrogen, Cat. # 4331182) and 18S (Invitrogen Cat. # 4331182) transcripts were determined in purified cells using the TaqMan® Gene Expression Cells-to-CT™ Kit (Invitrogen) as per manufacturer's instructions.

In vivo proliferation

1.5 mg of BrdU was administered i.p. and peritoneal cells or spleens were harvested one or two days later and BrdU was detected by flow cytometry as per manufacturer's kit instructions (FITC BrdU Flow Kit, BD Pharmingen). In some experiments, 1 µg of IL-5 was administered every day for 5 days, including 1.5 mg of BrdU on day 5. For CFSE dilution experiments, mice were intraperitoneally (i.p.) injected with 1 mL of 10 µm CFSE (CFDA, SE-mixed isomers, Invitrogen). After 7 days, peritoneal cells were harvested and stained for flow cytometry as described above.

Apoptosis assay

Peritoneal lavage cells were washed in cold PBS and suspended in binding buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCL, 2.5 mM $CaCl_2$). PerC cells were then incubated with Annexin V (BD Pharmigen, 556420) and B cell surface marker antibodies for 15 min at room temperature. After washing with binding buffer, cells were incubated with dapi and analyzed by flow cytometry.

Western blot analysis

Splenic B cells were isolated by CD19 microbeads (Miltenyi Biotech) with >99% pure CD19+ B cells. 10 x 10^6 cells were resuspended in 200 µL lysis buffer (1% DOC, 1% NP40, 50mM Tris pH 7.6, 1 mM EDTA, 150mM NaCl, 0.1% SDS with protease inhibitor cocktail), incubated on ice for 30 minutes, and centrifuged for 10 minutes at 11,600 xg, 4°C. The infranatants were separated from the pellet. Samples were assayed for protein concentration with the Bio-Rad DC protein assay (catalog #500-0111), adjusted to equal concentration with lysis buffer, and supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% Tris-glycine gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (BioRad). Equal protein loading was confirmed by staining the membrane with immunodetection of β-tubulin. Western blotting was carried out using an antibody to Id3 (0.1 µg ml^{-1},

CalBioreagents, catalog #M097) or β-tubulin (16 µg ml^{-1}, Cell Signaling Technology, Inc. catalog #2146) followed by horseradish peroxidase-linked secondary antibody (Jackson). Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with ECL reagent (Amersham Pharmacia Biotech). Densitometry was analyzed by ImageJ.

Cell Culture

Mesenteric cells were isolated as above and plated at 2 x 10^5 cells per well in a 96 well plate with 200 µL of complete RPMI 1640 (cRPMI, RPMI-1640 medium containing 10% HI-FBS, 50 mM 2-mercaptoethanol, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin, 1X nonessential amino acids, 10 mM HEPES, 1 mM sodium pyruvate), and 10 ng mL^{-1} mrIL-33 (CF, R&D) for 4 days without golgi transport inhibitor.

For Th2 cells, 7 x 10^5 splenic CD4+ cells isolated by macs column purification with CD4 microbeads (Miltenyi Biotech) were stimulated with soluble anti-CD3/anti-CD28 Dynabeads® (GIBCO) and a combination of 10 ng mL^{-1} mIL-4 (peprotech), 5 ng mL^{-1} mIL-2 (R&D), 10 ng mL^{-1} mrIL-33 for four days. Cells were then stimulated for six hours with 50 ng mL^{-1} PMA and 1 µg mL^{-1} ionomycin. During the last three hours, golgiplug (BDbioscience) was added to the culture. Cells were then harvested for flow cytometry and polarization was confirmed with intracellular staining of IL-4.

Mast cells and basophils were derived by culturing bone marrow harvested from femurs with 20% WEHI-3b conditioned media. After 7 days, cells in suspension were collected and plated with cRPMI and 10 ng mL^{-1} mrIL-33 for 5 days. On day 6, golgiplug was added to the culture for the last 4 hours, and cells were harvested flow cytometry and stained for mast cells ($FcεR1α^{+}$, $CD49b^{-}$, $CD117^{+}$) and basophils ($FcεR1α^{+}$, $CD49b^{+}$, $CD117^{-}$) and intracellular IL-5 and IFNγ.

Eosinophils were derived from bone marrow as described by others[168,169]. Briefly, bone marrow was harvested from femurs and seeded for 5 days in cRPMI with 10 ng mL^{-1} mrIL-33 (CF, R&D). On day 6, cells were harvested for eosinophils ($CD45^{+}SiglecF^{+}$) and intracellular IL-5 and IFNγ detection by flow cytometry as above.

IL-5 Promoter-Reporter Assay

BJAB cells, a lymphoid cell line derived from a human lymphoma of B lymphocyte type, co-electroporated with 2 µg of human IL-5 promoter-luciferase construct, pLightSwitch-IL5 (pLS-IL5, Switchgear Genomics) and 10 µg of empty pEF4 or pEF4 human Id3 expression vector previously described[170]. GFP was used as an electroporation efficiency control. After 24 hours, cells were harvested for luciferase chemiluminescence and normalized to total protein.

Atherosclerosis measurement

At eight weeks of age, mice were placed on a Western diet and 16 weeks later, mice were euthanized and perfused with PBS + 20 U mL^{-1} heparin. The heart including the aortic sinus was removed and placed in OCT (Tissue-Tek, Andwin Scientific) then snap frozen in $N_{2(l)}$ and stored at -80°C. Cryostat sections of 6 µm were cut from the beginning of the three aortic leaflets to the aortic arch. Five slides containing four sections taken every 30 µm per mouse were stained for oil-red-O and counterstained with hemotoxylin and eosin. Plaque volume was quantified using ImagePro 5.0 software.

Immunohistochemistry

Fresh frozen sections in OCT were selected at 30 µm intervals from the start of the aortic valves. OCT was removed by DPBS with Ca^{2+} and Mg^{2+} then fixed in cold acetone for 10 minutes at 4°C. Slides were air dried for 10 minutes then washed twice with dH_2O and blocked with DPBS containing fish scale gelatin for one hour at room temperature. Mac-2 (Biotinylated rat anti-mouse, Cedarlene CL8942B) or isotype control (Rat IgG2a Biotinylated, Cedarlene CLCR2A15) at 1:100 was incubated in a humidified chamber overnight in blocking buffer. Slides were then washed five times with DPBS. For Mac-2, secondary (Streptavidin Alexa Fluor 488, Invitrogen Molecular Probes S11223) was added at 1:100 and incubated in a humidified chamber for 30 minutes at

room temperature. Slides were then washed three times in DPBS with the last wash in dH_2O. Lastly, slides were mounted with anti-fade + DAPI reagent (Vectashield H-1500). Area per intima area was measured independently by two investigators as the area within the intima divided by the total area of the intima on one section per slide, 5 slides per mouse, using ImagePro Plus 7.0 software.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed to assess cellular apoptosis using the EMD Millipore (Billerica, MA) ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100) according to the manufacturer's instructions. Briefly, slides with fresh frozen sections of the aortic sinus in OCT were fixed with 1% PFA in PBS, pH 7.4 for 10 minutes at room temperature and washed twice in PBS then placed in 2:1 ethanol:acetic acid for 5 minutes at -20°C. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in PBS. Tissues were incubated in TdT for 1 hour at 37°C, then incubated in anti-digoxigenin conjugate for 30 minutes at room temperature, treated with peroxidase substrate (10mL PBS + 1 tablet DAB, Acros 7411-49-6 + 7.5 µL 30% H_2O_2) for 3 minutes, and counterstained with 0.5% methyl green (DAKO S-1962). Slides were allowed to air dry and mounted with Vectamount (H-5000) permanent mounting medium. One section was chosen per slide every 30 µm and 5 slides were averaged per mouse. Nuclei were considered apoptotic if completely brown and had morphometric features of apoptosis including cell shrinkage, nuclear condensation, or fragmentation and expressed as TUNEL+

per intima area by two independent investigators using ImagePro Plus 7.0 software.

Statistics

To test if data sets fit a Gaussian distribution, a D'Agostino-Pearson omnibus normality test was used. If data was normal and had equal variance, a student's t-test was performed. If data sets had unequal variance, a t-test with Welch's correction was used. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. Data was analyzed using Prism 6.0b (GraphPad Software, Inc.). Results are displayed containing all replicated experiments and values shown are mean only or mean ± SEM.

Chapter 3

The Helix-Loop-Helix Factor Id3 Regulates IL-5 Expression and B-1a B cell Proliferation

As published in: Perry, H. M. et al. Helix-Loop-Helix Factor Inhibitor of Differentiation 3 Regulates Interleukin-5 Expression and B-1a B-Cell Proliferation. Arterioscler Thromb Vasc Biol, doi:10.1161/ATVBAHA.113.302571 (2013).

Abstract

Objective: Natural immunity is emerging as an important mediator of protection from atherogenesis. Natural IgM antibodies that recognize oxidation-specific epitopes on LDL or phospholipids and the B-1a B cells that produce them, attenuate atherosclerosis. We previously demonstrated that *Apoe*$^{-/-}$ mice globally deficient in the helix-loop-helix protein Id3 develop early diet-induced atherosclerosis. Furthermore, B cell-mediated attenuation of atherosclerosis in B cell-deficient mice was dependent on Id3. Here we sought to determine if Id3 regulates B-1a B cells and the natural antibodies that they produce, and identify mechanisms mediating these effects.

Approach & Results: Id3 deficient mice contained significantly fewer B-1a B cells in the spleen and peritoneal cavity and reduced serum levels of the natural antibody E06. B cell-specific deletion of Id3 revealed that this effect was not due to the loss of Id3 in B cells. IL-33 induced abundant, Id3-dependent IL-5 production in the recently identified innate lymphoid cell, the natural helper (NH) cell, but not Th2 or mast cells. Additionally, delivery of IL-5 to Id3-deficient mice restored B-1a B cell proliferation. B-1a B cells were present in aortic samples also contained NH cells. Aortic NH cells produced IL-5, a B-1a B cell mitogen in response to IL-33 stimulation.

Conclusion: These studies are the first to identify NH and B-1a B cells in the aorta and provide evidence that Id3 is a key regulator of NH cell IL-5 production and B-1a B cell homeostasis.

Introduction

Atherosclerosis is a chronic inflammatory disease of the blood vessel wall that can lead to heart attacks and stroke. Despite current therapy targeting traditional risk factors, atherosclerosis remains the leading cause of death in Westernized countries[1]. Substantial work over the last several decades clearly established a key role for the immune system in atherosclerosis development and progression[4,5]. As such, immune modulation holds promise as an effective addition to current prevention approaches. Thus, a deeper understanding of the contributions of various immune cells to atherogenesis is of key importance. While abundant evidence implicates macrophages and some T cell subsets in promoting inflammation in the vessel wall[5,6,16,22], B cells have emerged as another important immune cell that can modulate atherogenesis. Characterization of atheroprotective antibodies, passive and active immunization studies[33-40], and adoptive transfer studies of splenic B cells from *Apoe*$^{-/-}$ mice[81,82] support an atheroprotective role for B cells in mice. Yet, recent studies suggest that the role of B cells is subset dependent, where B-2 B cells may be atherogenic and B-1a B cells protective[2].

A subset of B cells from the B-1 lineage, the B-1a B cell[41,44], has been reported to rescue the enhanced atherosclerosis caused by splenectomy. Atheroprotection was demonstrated to be dependent on the ability of the B-1a B cells to secrete IgM natural antibodies (NAbs)[107]. B-1a B cells are considered part of the innate immune system, develop from fetal tissues, have a high

capacity to undergo homeostatic proliferation[41,44,52,54-56], and spontaneously secrete IgM NAbs[40,107,111]. IgM NAbs are present prior to pathogen exposure, recognize self-antigens for housekeeping functions[14,63] and are thought to be the product of natural selection. A representative IgM NAb, E06, has been reported to be atheroprotective through recognition of oxidation-specific epitopes on oxidized LDL and apoptotic cells, blocking the uptake of oxidized LDL by macrophage scavenger receptors and mediating apoptotic cell clearance[14,108,109,111,114,171]. While B-1a B cells are important because of the IgM NAbs they produce, the factors that regulate B-1a B cells in atherosclerosis are poorly understood. IL-5 is an important atheroprotective cytokine known to promote B-1a B cell proliferation and E06 production[40,124,125,172]. Many cell types including Th2 T cells and the recently discovered natural helper (NH) cells produce IL-5. Moreover, NH cells produce large amounts of IL-5 relative to other IL-5 producing cells[140].

NH cells belong to an emerging arm of the innate lymphoid cell family: the group two innate lymphoid cell (ILC) helper subset[127-131]. NH cells are organized into fat-associated lymphoid clusters (FALCs) in the mesenteric adipose depot. No specific markers have been identified that define NH cells and they are therefore defined by lineage negative (Lin^-: CD3ε, CD4, CD5, CD8α, CD11b, CD11c, CD19, CD45R/B220, FcεR1α, Ly6G, NK1.1, TCRβ, TCRγδ and Ter119). They are positive for Sca-1, CD117 (c-kit), CD44, CD90 and the IL-33R (T1/ST2)[139,140]. NH cells produce mainly Th2-associated cytokines in response to

a variety of stimuli, including IL-33. Direct evidence with co-culture experiments demonstrates that NH cells support B-1 B cell proliferation, similar to IL-5 alone[140].

Inhibitor of differentiation 3 (Id3), a helix-loop-helix (HLH) protein, is a widely expressed dominant negative regulator of gene transcription that acts through interaction with DNA binding bHLH proteins, such as E-proteins[173]. These HLH proteins are part of a complex gene-regulatory network of lineage specific transcription factors that orchestrate lymphocyte development and activation[148-157]. Studies suggest that Id3 is also important in human and murine atherosclerosis. In humans, the *ID3* gene contains a single nucleotide polymorphism (SNP) at rs11574. This non-synonymous SNP results in an amino acid substitution in the carboxy-terminus of the ID3 protein which attenuates ID3 antagonism of the E-protein, E12[170]. Notably, this SNP is associated with increased carotid intimal media thickness in humans, suggesting that loss of Id3 function may promote vascular disease. In murine models, global deletion of Id3 results in increased atherosclerosis in both *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice[25,167,170]. Moreover, Id3 was necessary for B cell-mediated attenuation of atherosclerosis in B cell-deficient/*Apoe*$^{-/-}$ mice[25,167]. Id3 has also been reported to modulate B cell homing and vessel wall adhesion molecule expression[25,167], yet Id3 may also regulate factors involved in innate immunity as loss of Id3 in *Apoe*$^{-/-}$ mice resulted in very early onset of diet-induced atherosclerosis[25]. These results raise the

interesting hypothesis that Id3 may be an important regulator of B-1a B cells and natural immunity.

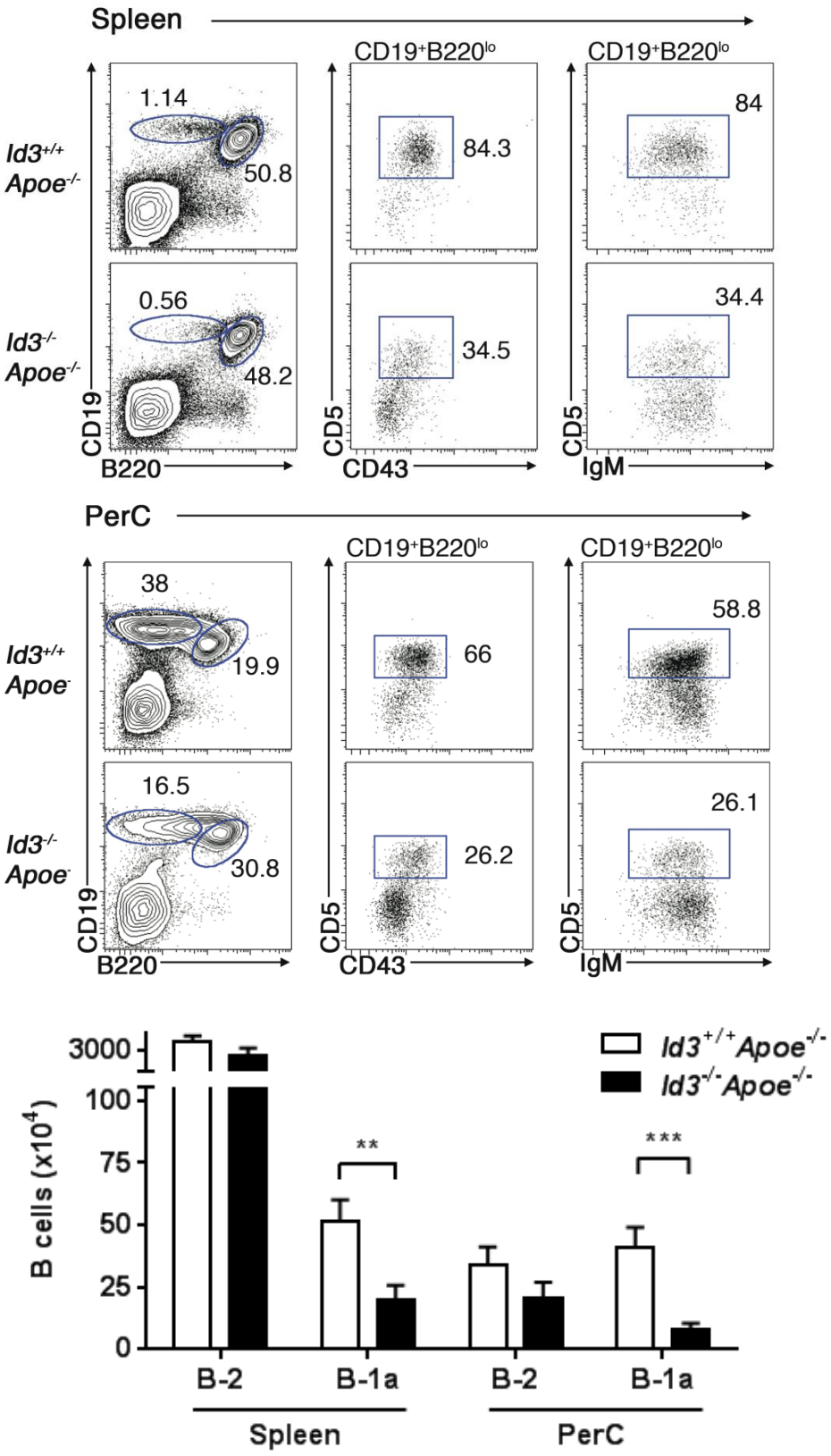
The present study demonstrates that Id3 is important for maintenance of splenic and peritoneal cavity B-1a B cells and serum levels of E06. However, Id3 regulation of the B-1a B cell pool is due to the loss of Id3 in a non-B cell population as the B-1a B cell number was unchanged in mice with B cell-specific loss of Id3. Loss of Id3 significantly reduced NH cell production of the B-1a B cell mitogen, IL-5. In addition to the mesentery, NH cells are present and produce IL-5 in response to IL-33 stimulation in the aortic adventitia/surrounding perivascular adipose tissue (PVAT). B-1a B cells were also found in the aortic adventitia/surrounding PVAT. These results provide the first evidence that NH cells and B-1a cells are present in the aorta and implicate Id3 as a key regulator of NH cell IL-5 production and B-1a B cell homeostasis.

Results

Id3 regulates B-1a B cell number in* Apoe$^{-/-}$ *mice. Previous studies demonstrated that B-1a B cells are atheroprotective by producing IgM NAbs[37,40,107,110-112] . To determine if Id3 regulates B-1a B cell number in *Apoe*$^{-/-}$ mice, B-1a B cells in *Id3*$^{+/+}$*Apoe*$^{-/-}$ and *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice were quantified by flow cytometry. The gating strategy has been previously described[90,174] and Figure 7 presents representative flow cytometry plots that identify B-1a B cells (CD19^{+}B220^{lo}CD5^{+}CD43^{+}IgM^{hi}) and B-2 B cells (CD19^{+}B220^{hi}) in both the spleen and peritoneal cavity (PerC). Results reveal significantly fewer B-1a B cells in both the spleen and PerC in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice (Figure 7). In contrast, and consistent with previous reports[25,154], the absolute number of B-2 B cells in the spleen and PerC of *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice was equivalent compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice (Figure 7).

Id3 has been reported to be necessary for protection from atherosclerosis in *Apoe*$^{-/-}$ mice[25,170]. To investigate if the global loss of Id3 resulted in alterations of other immune cell populations important in atherosclerosis, flow cytometry was performed for splenic CD4+ T cells, CD4+Foxp3+ regulatory T cells (Tregs), CD8+ T cells, and peritoneal CD4+ T cells, CD8+ T cells, and F4/80+ macrophages in *Id3*$^{-/-}$*Apoe*$^{-/-}$ and *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice. No differences were observed in the proportions of these populations (data not shown). Cholesterol levels also effect atherogenesis[4] and there was no difference in the serum lipid

Figure 7. *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice have fewer B-1a B cells in the spleen and PerC compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ *mice*. Representative flow cytometry plots (**top**) and quantification (**bottom**) of B-2 B cells (CD19^{+}B220^{hi}) and B-1a (CD19^{+}B220^{lo}CD5^{+}CD43^{+}IgM^{hi}) in the spleen and peritoneal cavity lavage (PerC) of *Id3*$^{+/+}$*Apoe*$^{-/-}$ (n=13) or *Id3*$^{-/-}$*Apoe*$^{-/-}$ (n = 12) mice at 8 weeks of age as measured by flow cytometry. ** $p < 0.01$, *** $p < 0.001$.


Spleen
CD19+B220lo
CD19+B220lo
Id3+/+ Apoe-/-
Id3-/- Apoe-/-
1.14
50.8
0.56
48.2
84.3
34.5
84
34.4
CD19
B220
CD5
CD43
CD5
IgM
PerC
CD19+B220lo
CD19+B220lo
Id3+/+ Apoe-
Id3-/- Apoe-
38
19.9
16.5
30.8
66
26.2
58.8
26.1
CD19
B220
CD5
CD43
CD5
IgM
Id3+/+Apoe-/-
Id3-/-Apoe-/-
B cells (x10^4)
3000
100
75
50
25
0
**
***
B-2
B-1a
B-2
B-1a
Spleen
PerC

profiles of *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to control mice (Table 2) consistent with previous observations[25].

Consistent with the decreased number of B-1a B cells in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice, we detected less E06 antibody in the serum of 8-week-old *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to control mice (Figure 8A). To determine if the lower amount of serum E06 antibody is due to reduced production of E06 on a per-cell basis, B-1a B cells were isolated from the PerC by FACS and analyzed for the amount of mRNA encoding the secreted form of E06 IgM. The gating strategy for FACS purification of B-1a and B-2 B cells is depicted in Figure 8B. There was no difference in E06 mRNA in B-1a B cells from *Id3*$^{-/-}$*Apoe*$^{-/-}$ compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice (Figure 8C), providing evidence that the lower amount of serum E06 antibody is not due to reduced production of E06, but more likely due to fewer B-1a B cells in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice. To determine if the loss of Id3 altered proliferation of B-1a B cells, BrdU or CFSE was administered i.p. to *Id3*$^{+/+}$*Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice. PerC cells were harvested for BrdU incorporation twenty-four hours later or for CFSE dilution seven days later in B-1a and B-2 B cells by flow cytometry (Figure 9A). A decrease in the proportion of B-1a B cells that incorporated BrdU as well as CFSE dilution was detected in *Id3*$^{-/-}$*Apoe*$^{-/-}$ compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice. Consistent with no difference in B-2 B cell number associated with the loss of Id3, there was no difference in the proportion of B-2 B cells that incorporated BrdU or diluted CFSE. In addition, there was no

Table 2. Average serum lipid profiles of *Id3*$^{+/+}$*Apoe*$^{-/-}$ and *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice at eight to ten weeks of age**.**

	Id3$^{+/+}$*Apoe*$^{-/-}$	*Id3*$^{-/-}$*Apoe*$^{-/-}$
n	8	8
Cholesterol (mg/dL)	314.2 ± 23.9	376.0 ± 34.5
HDL-C (mg/dL)	35.63 ± 3.6	36.75 ± 1.4
Non HDL-C (mg/dL)	252.6 ± 13.9	303.4 ± 33.6
Triglycerides (mg/dL)	184.3 ± 31.7	215.1 ± 20.7

Values are mean ± SEM. No comparisons are statistically significant.

Figure 8. Id3 is necessary for normal serum levels of E06. **(A)** E06 levels in the serum of *Id3*$^{+/+}$*Apoe*$^{-/-}$ or *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice as measured by ELISA. **(B)** Gating strategy to sort B cells (dapi^{-}, singlets, lymphs, CD3ε^{-}, B-2: CD19^{+}B220^{hi}, B-1a: CD19^{+}B220^{lo}, CD5^{+}) and post-sort purity of B-1a cells. **(C)** E06 sIgM normalized to total sIgM transcript levels in peritoneal B cell subsets, B-2 (CD19^{+}B220^{hi}) and B-1a (CD19^{+}B220^{lo} CD5^{+}) B cells as measured by real-time PCR from three independent cell sorts. ** $p < 0.01$. Abbr.: lymphocytes (lymphs).

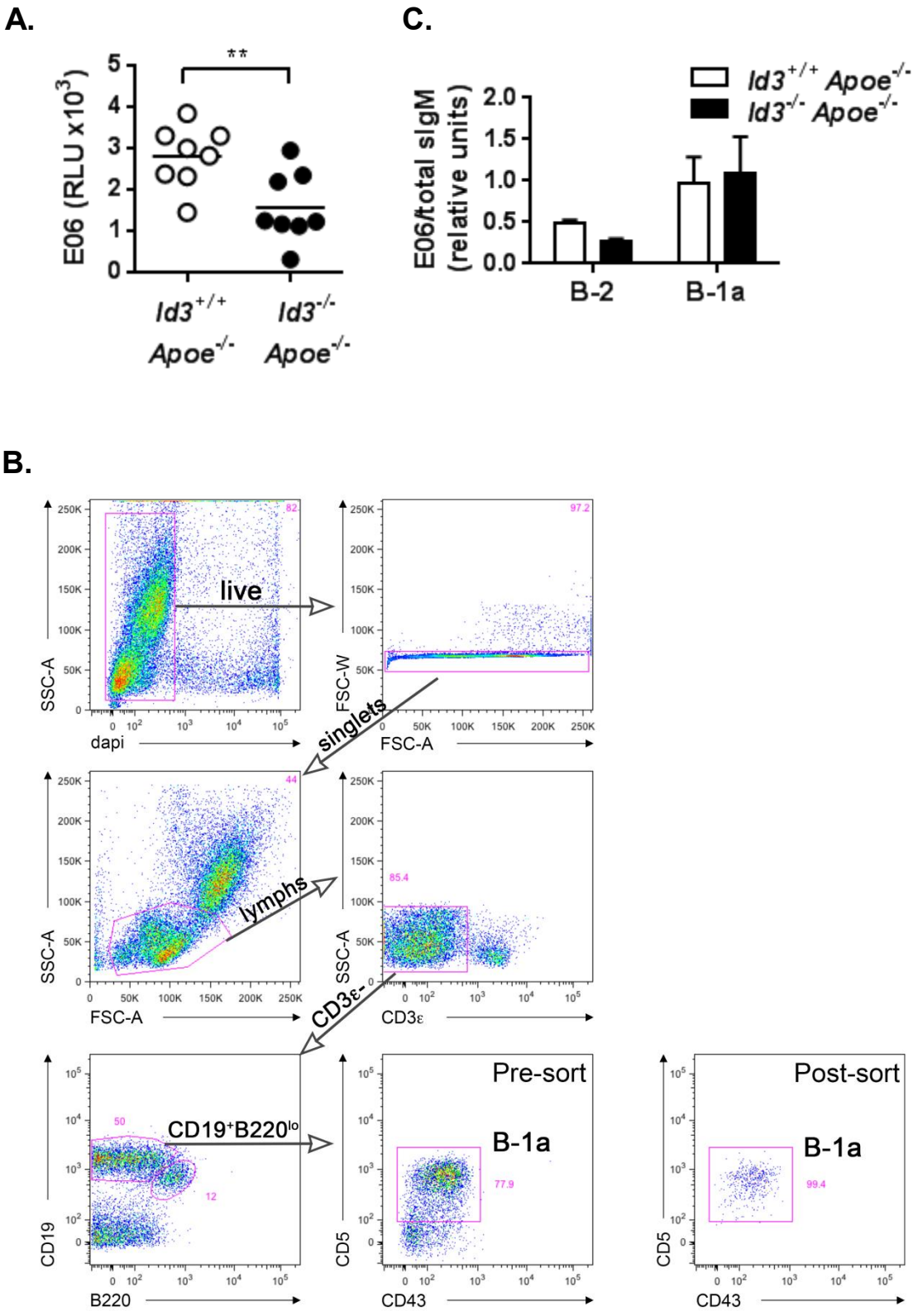
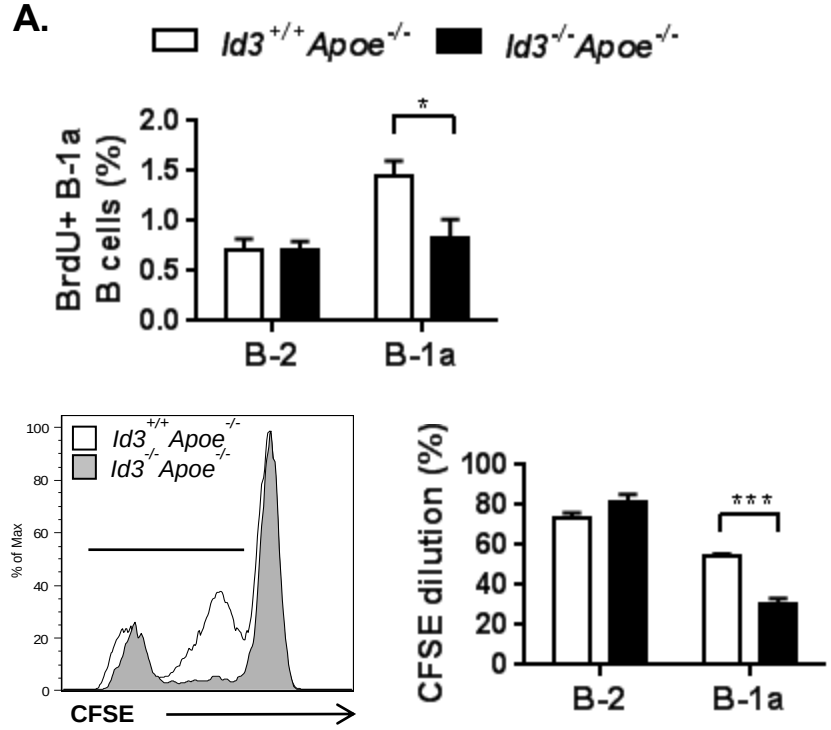
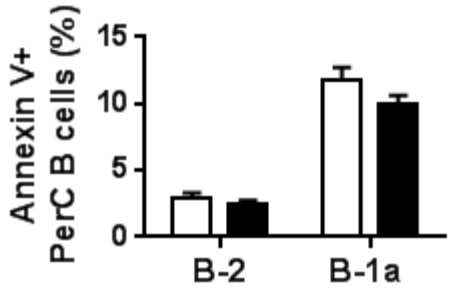

A.
E06 (RLU x10^3)
**
Id3+/+ Apoe-/-
Id3-/- Apoe-/-
C.
E06/total sIgM (relative units)
Id3+/+ Apoe-/-
Id3-/- Apoe-/-
B-2
B-1a
B.
live
singlets
lymphs
CD3ε-
CD19+B220lo
Pre-sort
B-1a
Post-sort
B-1a
SSC-A
dapi
FSC-W
FSC-A
CD3ε
CD19
B220
CD5
CD43

Figure 9. Reduced proliferation of B-1a B cells in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice. **(A)** BrdU incorporation (top) and CFSE dilution (bottom) or **(B)** Annexin V^{+} in PerC B cell subsets of *Id3*$^{+/+}$*Apoe*$^{-/-}$ (n = 6) or *Id3*$^{-/-}$*Apoe*$^{-/-}$ (n = 7) mice *in vivo*. * $p < 0.05$, *** $p < 0.001$.

A.


Id3+/+Apoe-/-
Id3-/-Apoe-/-
BrdU+ B-1a B cells (%)
2.0
1.5
1.0
0.5
0.0
*
B-2
B-1a
Id3+/+ Apoe-/-
Id3-/- Apoe-/-
% of Max
100
80
60
40
20
0
CFSE
CFSE dilution (%)
100
80
60
40
20
0
***
B-2
B-1a

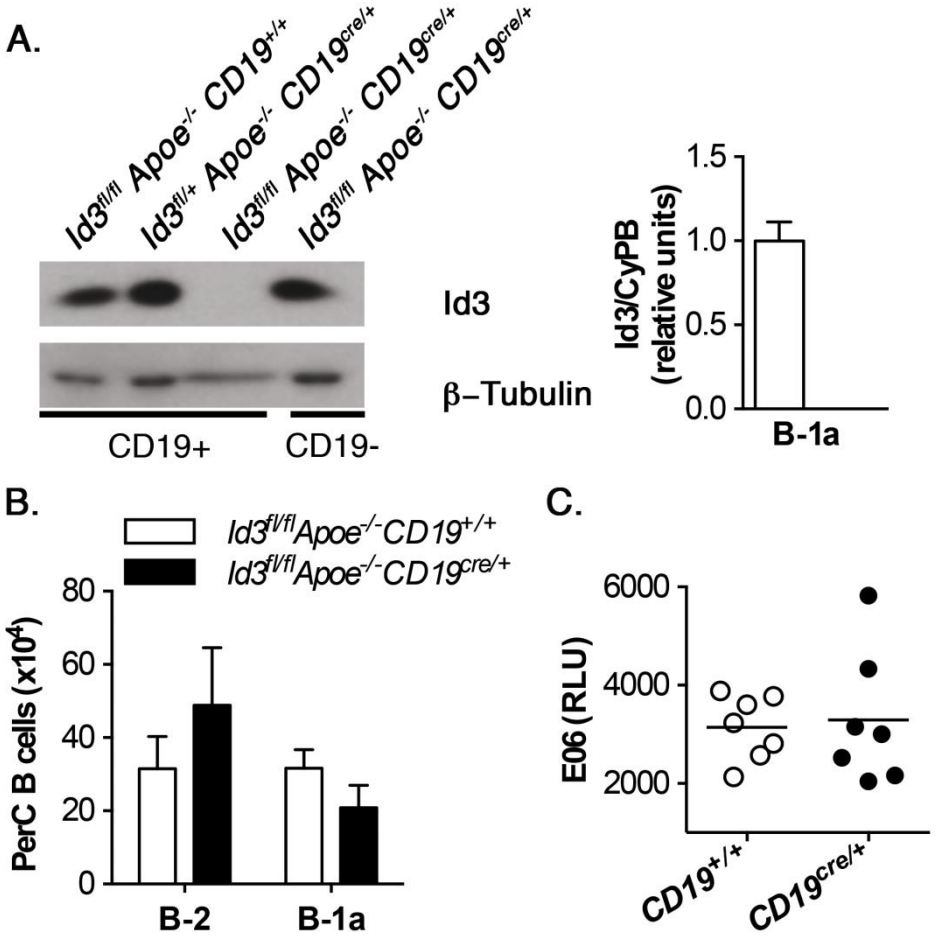
B.


Annexin V+ PerC B cells (%)
15
10
5
0
B-2
B-1a

difference in the proportion of peritoneal B-1a or B-2 B cells that were positive for Annexin V, a marker of apoptosis (Figure 9B). These results suggest that the reduced number of B-1a B cells in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice is due to reduced B-1a B cell proliferation.

To determine if the reduced number of B-1a B cells in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice was due to the loss of Id3 in B cells, *Id3* was specifically deleted in B cells on the *Apoe*$^{-/-}$ background. These mice were generated by first crossing a floxed *Id3* allele onto the *Apoe*$^{-/-}$ background to produce *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ mice. These mice were then crossed with *Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice to produce *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ and *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ control mice. Western blotting of lysates from splenic B cells demonstrated the loss of Id3 protein in B cells from *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to controls (Figure 10A). Furthermore, *Id3* mRNA was not detectable by real-time PCR in sorted B-1a B cells from *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice (Figure 10A). Consistent with results in the *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice, *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{cre/+}$ mice contained an equivalent number of B-2 B cells compared to control mice (Figure 10B). However, in contrast *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice, despite the loss of Id3, the number of PerC B-1a B cells detected in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice was equivalent to that detected in control mice. Furthermore, the amount of EO6 antibody detected in the serum was also the same (Figure 10C). These results suggest that Id3 regulates B-1a B cell number through effects in a cell type other than B cells.

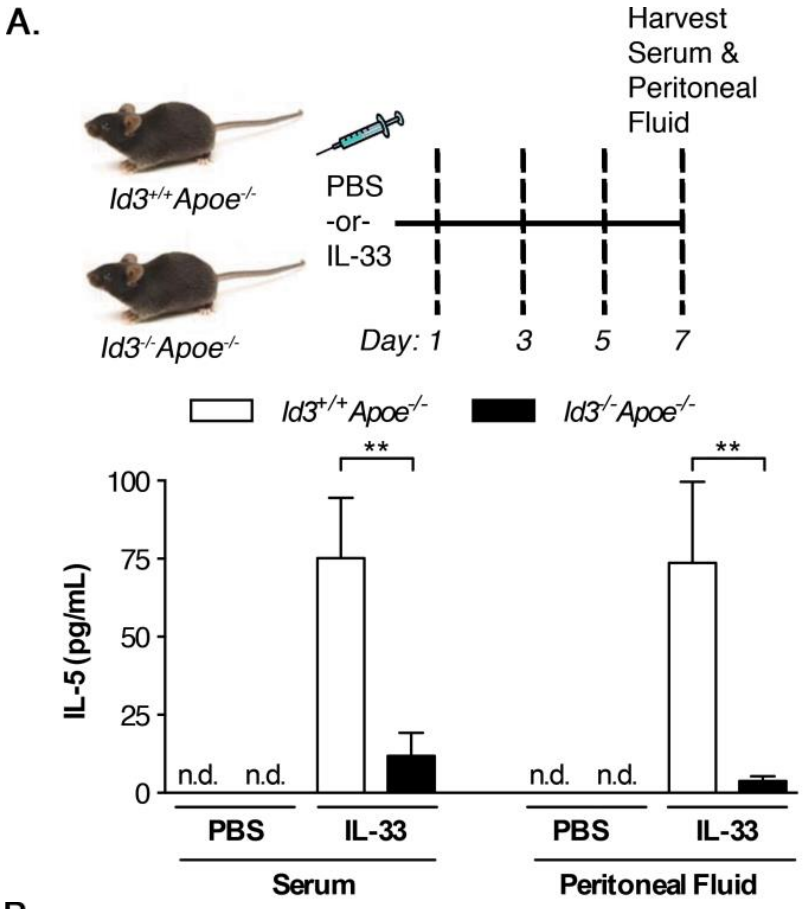
Figure 10. Generation of a B cell specific knockout of Id3 reveals that the loss of Id3 in B cells does not result in an altered number of B-1a B cells or E06 levels. **(A) Left**, Id3 and β-tubulin protein in total splenic B cells from *Id3$^{fl/fl}$Apoe$^{-/-}$ CD19$^{+/+}$, Id3$^{fl/+}$Apoe$^{-/-}$CD19$^{cre/+}$, Id3$^{fl/fl}$Apoe$^{-/-}$CD19$^{cre/+}$ and Id3$^{fl/fl}$, Apoe$^{-/-}$, CD19$^{cre/+}$* mice. Results are representative of five individual mice tested. **Right**, peritoneal lavage cells were sorted from *Id3$^{fl/fl}$Apoe$^{-/-}$CD19$^{+/+}$* (open bar) or *Id3$^{fl/fl}$Apoe$^{-/-}$ CD19$^{cre/+}$* (closed bar) mice for B-1a (CD19^{+}B220^{lo} CD5^{+}) cells as in Figure 5 and *Id3* mRNA was measured by real-time PCR and normalized to cyclophilin b (*CypB*). **(B)** Quantification of peritoneal B-2 and B-1a B cell subsets by flow cytometry in *Id3$^{fl/fl}$Apoe$^{-/-}$CD19$^{+/+}$* (n = 10) or *Id3$^{fl/fl}$Apoe$^{-/-}$CD19$^{cre/+}$* (n = 11) mice at eight weeks of age. **(C)** Serum levels of E06 in *Id3$^{fl/fl}$Apoe$^{-/-}$CD19$^{+/+}$* (open circles) or *Id3$^{fl/fl}$Apoe$^{-/-}$CD19$^{cre/+}$* (closed circles) mice were determined by ELISA. Values are the mean RLU of duplicate determinations. Abbr.: relative light unit (RLU).

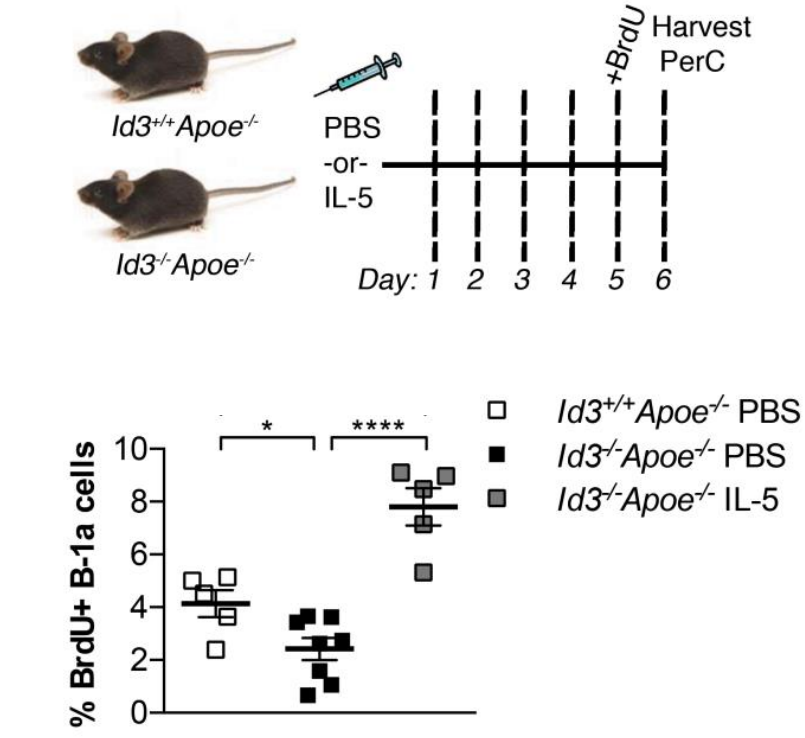

A.
Id3fl/fl Apoe-/- CD19+/+
Id3fl/+ Apoe-/- CD19cre/+
Id3fl/fl Apoe-/- CD19cre/+
Id3fl/fl Apoe-/- CD19cre/+
Id3
β–Tubulin
CD19+
CD19-
Id3/CyPB (relative units)
1.5
1.0
0.5
0.0
B-1a
B.
Id3fl/flApoe-/-CD19+/+
Id3fl/flApoe-/-CD19cre/+
PerC B cells (x10⁴)
80
60
40
20
0
B-2
B-1a
C.
E06 (RLU)
6000
4000
2000
CD19+/+
CD19cre/+

Loss of Id3 markedly attenuates IL-33-induced IL-5 production. IL-5 is an IL-33-induced Th2 cytokine that is critically important for homeostatic proliferation and survival of B-1a B cells. To determine if Id3 is necessary for IL-33 induced production of IL-5, *Id3*$^{+/+}$*Apoe*$^{-/-}$ and *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice were treated with PBS vehicle control or IL-33 and the concentration of IL-5 in the serum and peritoneal fluid was measured by an ELISA. As presented in Figure 11A, IL-33-induced abundant IL-5 in the serum and peritoneal fluid and this effect was abolished in Id3 deficient mice. IL-5 was undetectable in the serum and peritoneal fluid of PBS-treated *Apoe*$^{-/-}$ mice, consistent with previous reports[140] (Figure 11A). Since *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice had a markedly attenuated IL-33-induced IL-5 response and reduced homeostatic proliferation of B-1a B cells, we sought to determine if delivery of exogenous IL-5 could increase the proliferation of PerC B-1a B cells in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice. *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice were injected with IL-5 intraperitoneally every day for five days, and on the last day BrdU was co-injected. Consistent with earlier findings (Figure 9A), there was a smaller percentage of BrdU+ B-1a B cells in PBS control injected *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice. Moreover, exogenous IL-5 increased the proportion of BrdU+ B-1a B cells in *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice compared to PBS control (Figure 11B).

Id3 regulates NH cell production of IL-5. Previous studies have identified NH cells as the predominant source of IL-33-induced IL-5 production *in vivo* and co-culture experiments demonstrated that NH cells can directly support B-1 B cell

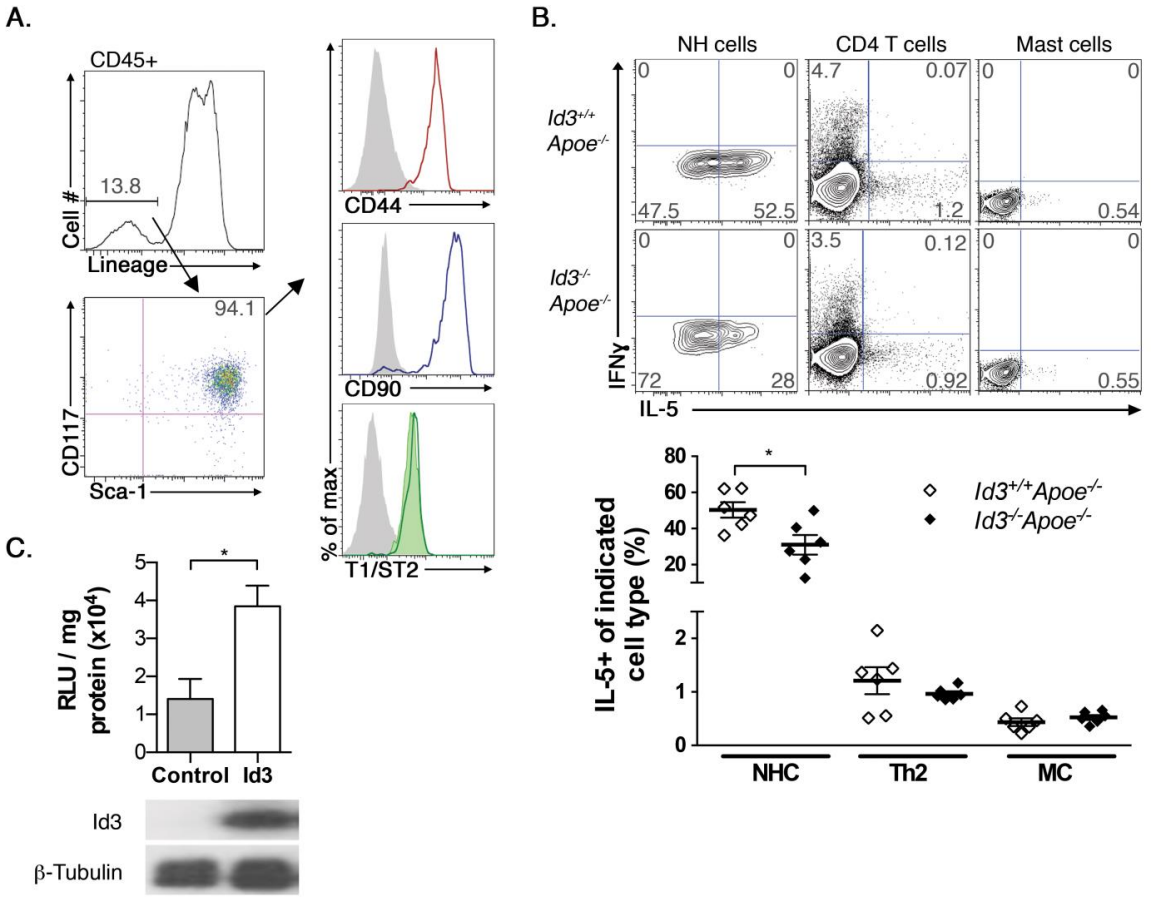
Figure 11. Id3 is necessary for IL-33 induced IL-5 protein in the serum and peritoneal fluid. **(A)** IL-33 or PBS vehicle control was administered every two days to *Id3*$^{+/+}$*Apoe*$^{-/-}$ (PBS group, n = 7; IL-33, n = 5) or *Id3*$^{-/-}$*Apoe*$^{-/-}$ (PBS group, n = 7; IL-33, n = 6) mice three times in four independent experiments. On day 7, levels of IL-5 in the serum and peritoneal fluid were determined by ELISA. Values were considered not detectable (n.d.) if below the sensitivity of the assay (≤ 1.56 pg/mL). **(B)** *Id3*$^{+/+}$*Apoe*$^{-/-}$ or *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice were administered PBS vehicle control and/or IL-5 for 5 days, including BrdU on day 5. * $p < 0.05$, ** $p < 0.001$, **** $p < 0.0001$.


A.
Harvest
Serum &
Peritoneal
Fluid
Id3+/+Apoe-/-
Id3-/-Apoe-/-
PBS
-or-
IL-33
Day: 1 3 5 7
Id3+/+Apoe-/-
Id3-/-Apoe-/-
IL-5 (pg/mL)
100
75
50
25
0
**
**
n.d.
n.d.
n.d.
n.d.
PBS
IL-33
PBS
IL-33
Serum
Peritoneal Fluid


B.
+BrdU
Harvest
PerC
Id3+/+Apoe-/-
Id3-/-Apoe-/-
PBS
-or-
IL-5
Day: 1 2 3 4 5 6
% BrdU+ B-1a cells
10
8
6
4
2
0
*
****
Id3+/+Apoe-/- PBS
Id3-/-Apoe-/- PBS
Id3-/-Apoe-/- IL-5

proliferation[125,140]. To determine if *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice had lower IL-33-induced IL-5 serum and peritoneal fluid levels due to fewer NH cells, flow cytometry was carried out to quantify NH cells in the mesentery. NH cells were identified as CD45^{+}, lineage negative (Lin^{-}: CD3ε, CD4, CD5, CD8α, CD11b, CD11c, CD19, CD45R/B220, FcεR1α, Ly6G, NK1.1, TCRβ, TCRγδ and Ter119) Sca1^{+} CD117^{+} T1/ST2^{+} CD90^{+} and CD44^{+} cells[140] (Figure 12A). In contrast to mice that are null for Id2 and lack NH cells[140], *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice contain a similar number of NH cells compared to *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice (2,566 ± 731.3 vs. 2,486 ± 515.1 respectively, n = 9) in the mesentery. We were able to detect Id3 mRNA in FACS purified NH cells by real-time PCR (not shown). The lower IL-33-induced IL-5 serum levels were not due to Id3 regulation of the IL-33 receptor, T1/ST2, on NH cells as *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice had similar expression of NH cell T1/ST2 compared to control mice (Figure 12A). There was also no difference in the concentration of IL-33 in the peritoneal fluid of *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to control mice (21.78 ± 10.01 pg/mL vs. 29.11 ± 8.18 pg/mL respectively, n = 6) and serum levels were below the sensitivity of the assay (≤ 2.39 pg/mL). To determine if Id3 regulates production of IL-5 by a specific Th2 cytokine-producing cell, flow cytometry with intracellular cytokine staining for IL-5 was performed on isolated cells cultured with IL-33. Consistent with previous reports[140], the percentage of NH cells that produced IL-5 is markedly higher than other Th2 cytokine producing cells such as polarized CD4+ T cells and bone-marrow derived mast cells (BMMC) (Figure 12B). Intracellular cytokine staining of IL-5 in IL-33 treated basophils or eosinophils was not detected (data not shown).

Figure 12. Id3 regulates IL-5 production in natural helper cells. **(A)** Representative flow cytometry for the gating strategy of FALC NH cells. Live, $CD45^+$, lin^- cells were gated for $Sca1^+$ $CD117^+$ cells. CD44, CD90 and T1/ST2 expression on $Sca1^+$ $CD117^+$ cells from *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice (open histograms) or T1/ST2 from *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice (green shaded histogram). Fluorescence-minus-one (FMO) control, grey shaded histograms. **(B)** (**Top**) Representative flow cytometry of NH cells, $CD4^+$ T cells, and mast cells (MC) from *Id3*$^{+/+}$*Apoe*$^{-/-}$ or *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice stained for intracellular IFNγ and IL-5 after IL-33 stimulation and (**bottom**) quantification of IL-5^+IFNγ$^-$ of each cell type. Results are expressed as individual mice from two independent experiments performed in duplicate. **(C)** Luciferase activity (**top**) and protein expression of Id3 and β-tubulin (**bottom**) in lymphoid cells (see methods) co-transfected with IL-5 promoter-reporter and either empty vector (control) or Id3. Data are from three independent experiments performed in duplicate. * $p < 0.05$.

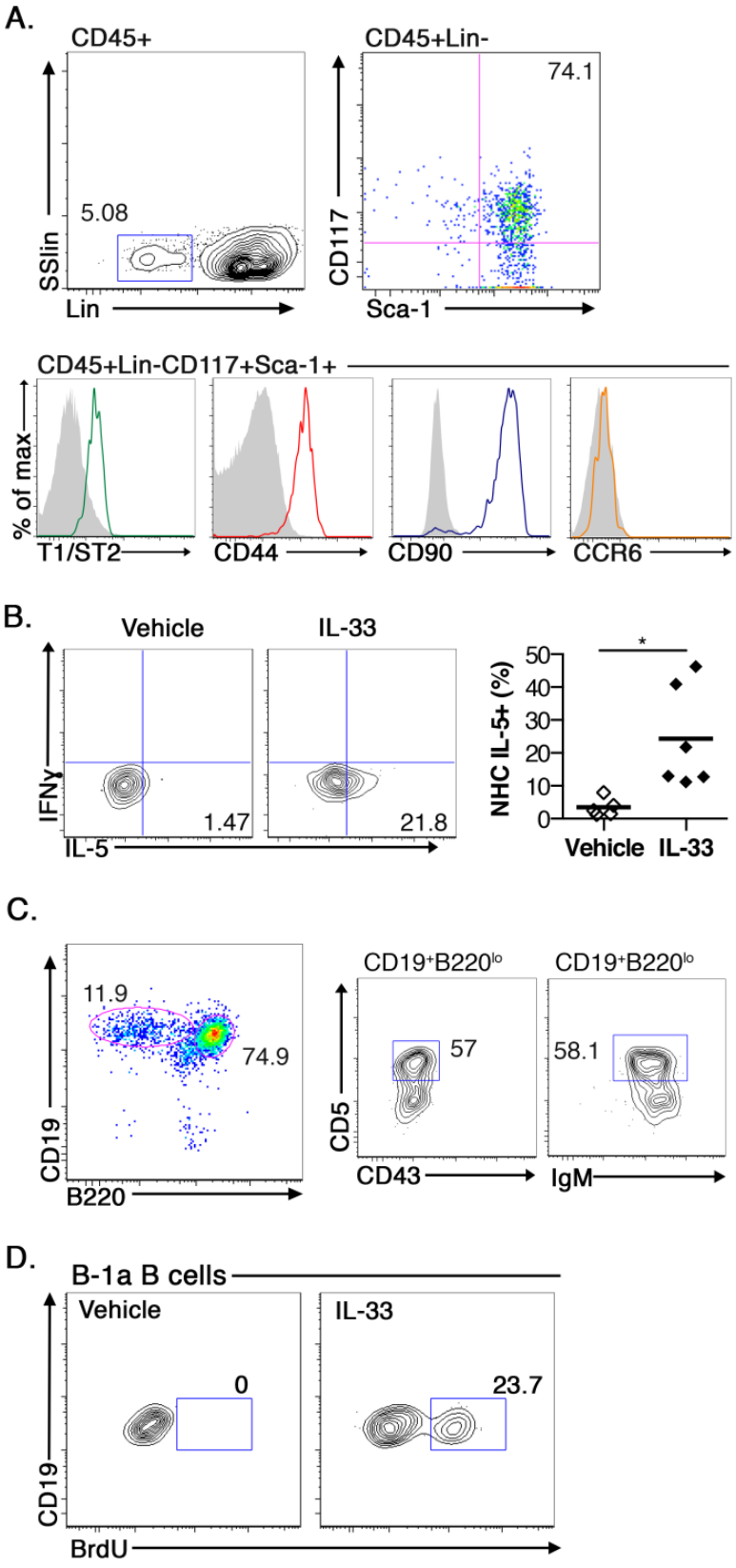

A.
CD45+
13.8
Cell #
Lineage
94.1
CD117
Sca-1
CD44
CD90
T1/ST2
% of max
B.
NH cells
CD4 T cells
Mast cells
Id3+/+ Apoe-/-
Id3-/- Apoe-/-
IFNγ
IL-5
0
0
4.7
0.07
0
0
47.5
52.5
1.2
0.54
0
0
3.5
0.12
0
0
72
28
0.92
0.55
IL-5+ of indicated cell type (%)
Id3+/+Apoe-/-
Id3-/-Apoe-/-
NHC
Th2
MC
C.
RLU / mg protein (x10⁴)
Control
Id3
Id3
β-Tubulin

Interestingly, a 50% reduction was detected in the percentage of NH cells that produce IL-5 in response to IL-33 in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to NH cells isolated from control *Id3*$^{+/+}$*Apoe*$^{-/-}$ mice (Figure 12B). Consistent with previous data[140], NH cells do not produce detectable IFNγ. Loss of Id3 did not alter IL-5 production in CD4^{+} T cells or BMMCs, other cell types known to produce IL-5. Id3 is known to both activate and repress gene expression through the regulation of promoter elements termed E-boxes[157]. The IL-5 promoter contains several E-box elements. To determine if Id3 regulates the IL-5 promoter, an IL-5 luciferase promoter-reporter, pLS-IL5, was co-transfected with control empty vector (pEF4) or Id3 (pEF4-Id3) in a lymphoid cell line. As presented in Figure 12C, transfected Id3 significantly increased IL-5 promoter activity above that induced by a control empty vector. Western blots confirm Id3 protein over-expression. Together, results demonstrate that Id3 regulates IL-5 expression in lymphoid cells and in particular, NH cells.

IL-5 producing-natural helper cells and B-1a B cells are present in the perivascular aortic adipose tissue. NH cells reside in fat associated lymphoid clusters (FALC) in the mesenteric adipose depot. The PVAT also contains immune cells, is intimately associated with the adventitial layer of the vessel wall and has been implicated in regulating atherogenesis[22,175]. B cells are known to reside in the adventitial layer of vessels and presence of B cells in the adventitia has been associated with both atherogenesis[176] and atheroprotection[25].

However, whether NH cells and B-1a B cells are present in peri-aortic adipose tissue and adventitia has not been reported. To determine if NH cells reside in peri-aortic adipose tissue, flow cytometry for NH cells was performed on cells isolated from whole aortas including the adjacent adventitia and PVAT from chow-fed, 8-10 week old *Apoe*$^{-/-}$ mice. Indeed, a population of NH cells, identified as CD45^{+} Lin^{-} Sca1^{+} CD117^{+} cells, was present in whole aortas of *Apoe*$^{-/-}$ mice (Figure 13A). Similar to the mesentery, the number of aortic NH cells was not different with the loss of Id3 (556.3 ± 46.79, n = 9 *Id3*$^{+/+}$*Apoe*$^{-/-}$ vs. 607.8 ± 149.4, n = 5 *Id3*$^{-/-}$*Apoe*$^{-/-}$). Aortic NH cells displayed surface expression of CD44, CD90 and the IL-33 receptor (T1/ST2) comparable to mesenteric NH cells. Similar to NH cells in the lung, intestines, and mesentery, these NH cells did not express C-C chemokine receptor 6 (CCR6)[139]. To determine if aortic NH cells produce IL-5 in response to IL-33 stimulation *in vivo*, *Apoe*$^{-/-}$ mice were treated with PBS vehicle control or IL-33 every two days and euthanized on day seven. Flow cytometry analysis of the entire aorta revealed IL-33-induced IL-5 expression in aortic NH cells (Figure 13B). NH cells have been demonstrated to directly support B-1 B cell proliferation[140]. To determine if B-1a B cells also reside in and around the aorta of mice prior to atheroma development, flow cytometry was performed on aortas including the peri-aortic adventitia and PVAT for B cell subsets. B-1a cells were present in the whole aorta (Figure 13C). To determine if IL-33 can induce B-1a B cell proliferation in the aorta, *Apoe*$^{-/-}$ mice were treated with PBS vehicle control or IL-33 every two days with BrdU co-injected for the final treatment and euthanized

Figure 13. Natural helper cells are present in the aorta including peri-aortic adventitia and PVAT and produce IL-5. Whole aortas containing the adventitia and PVAT were isolated from *Apoe*$^{-/-}$ mice. **(A)** Representative flow cytometry plots of NH cells (CD45^{+}, lineage negative, CD117^{+}, Sca-1^{+}). **(B)** (**Left**), IFNγ and IL-5 intracellular staining in aortic NH cells and (**right**), quantitation of the percentage of NH cells expressing IL-5 (n = 6 in each group) after vehicle or IL-33 treatment. Representative flow cytometry plots of **(C)** B cell subsets, B-2 (CD19^{+}B220^{hi}) and B-1a (CD19^{+}B220^{lo}CD5^{+}CD43^{+}IgM^{hi}) and **(D)** BrdU incorporation in aortic B-1a B cells after vehicle or IL-33 treatment. Results are representative of two independent experiments. Numbers on flow cytometry plots indicate the percentage of the population of interest. * $p < 0.05$. Abbr.: side scatter linear (SSlin).


A.
CD45+
5.08
SSlin
Lin
CD45+Lin-
74.1
CD117
Sca-1
CD45+Lin-CD117+Sca-1+
% of max
T1/ST2
CD44
CD90
CCR6
B.
Vehicle
IL-33
IFNγ
1.47
21.8
IL-5
NHC IL-5+ (%)
50
40
30
20
10
0
*
Vehicle
IL-33
C.
11.9
74.9
CD19
B220
CD19+B220lo
57
CD5
CD43
CD19+B220lo
58.1
IgM
D.
B-1a B cells
Vehicle
0
IL-33
23.7
CD19
BrdU

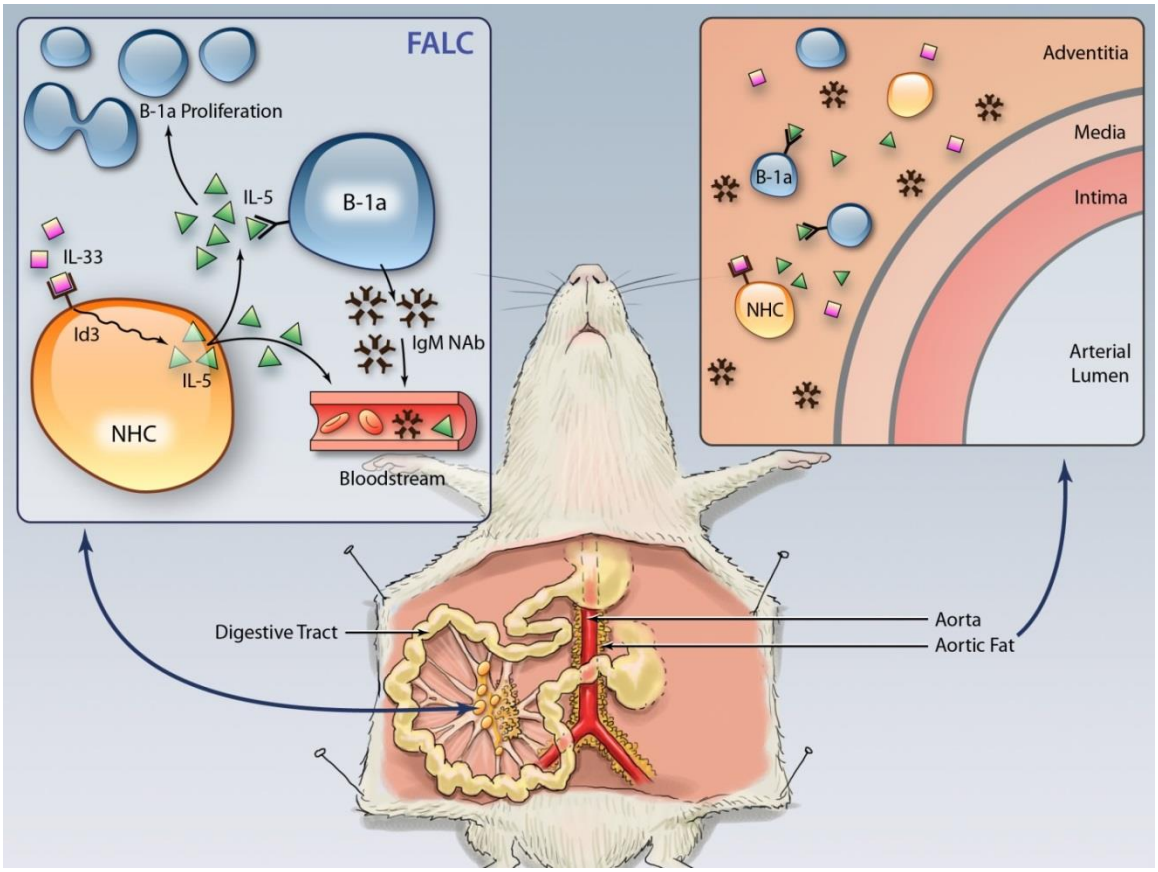
on day seven. Flow cytometry analysis of the entire aorta revealed IL-33-induced BrdU incorporation in aortic B-1a B cells (Figure 13D). This is the first demonstration that NH cells and B-1a B cells in the aortic adventitia/PVAT and provide evidence that IL-33 can induce aortic NH cells to increase local IL-5 production and B-1a B cell proliferation.

Discussion

The present study provides evidence that Id3 is a key mediator of natural immunity and the IL-33/IL-5 pathway using a genetic model of early and accelerated atherosclerosis, the *Apoe*$^{-/-}$ mouse null for Id3[25,170]. First, we identified reduced number and proliferation of B-1a B cells and lower serum levels of an IgM NAb, E06, in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice. However, when Id3 is deleted only in B cells, there is no effect on B-1a B cell number or E06 serum levels, suggesting that Id3 regulates the number of B-1a B cells by a mechanism that involves a non-B cell. Second, we demonstrated that IL-33 induced IL-5 levels are attenuated in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice, and administration of IL-5 can rescue the B-1a proliferation defect in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice. Third, we demonstrated reduced production of IL-5 in IL-33 treated NH cells isolated from *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice. We also demonstrate by flow cytometry that the aorta, including the adventitia and PVAT, contains B-1a B cells and NH cells. Aortic NH cells can be stimulated to produce the IL-5 that may maintain B-1a B cell proliferation. Thus, our data identifies Id3 as a key regulator of NH cell production of the atheroprotective cytokine IL-5 that promotes B-1a B cell proliferation (Figure 14), linking this event to natural immune protection from atherosclerosis.

Natural IgM antibodies, such as E06, and the B-1a B cells that produce these antibodies have been reported to attenuate diet-induced atherosclerosis[14,33,34,37,40,107-112]. Kyaw et.al. demonstrated a significant reduction

Figure 14. Id3 is a key regulator of NH cell IL-5 production and B-1a B cell proliferation, and NH cells may be important in innate protection from atherosclerosis. IL-33 stimulates FALC resident NH cells to produce IL-5, which is dependent on Id3. IL-5 stimulates B-1a B cell proliferation and IgM NAb production, an atheroprotective process. IL-5 and IgM NAb can enter the bloodstream. Aorta/PVAT resident NH cells can be stimulated by IL-33 to produce IL-5. This local process may stimulate resident atheroprotective B-1a cells.


FALC
B-1a Proliferation
IL-5
B-1a
IL-33
Id3
IL-5
NHC
IgM NAb
Bloodstream
Adventitia
Media
Intima
Arterial Lumen
B-1a
NHC
Digestive Tract
Aorta
Aortic Fat

in the number of peritoneal cavity B-1a B cells and levels of serum IgM antibodies that bind modified lipids after splenectomy, which was associated with enhanced atherosclerosis in *Apoe*$^{-/-}$ mice. Moreover, adoptive transfer of B-1a B cells were reported to rescue splenectomy-induced atherosclerosis, an effect dependent on the ability for B-1a B cells to secrete IgM[107]. Here we provide evidence that Id3 may be a key factor for maintaining the normal number of B-1a B cells and levels of E06 in the serum, suggesting that Id3 may be important in B-1a B cell mediated atheroprotection. However, B cell-specific deletion of Id3 did not result in a reduced number of B-1a B cells or level of E06 in the serum compared to control mice, suggesting that it is the loss of Id3 in a non-B cell that results in altered B-1a B cell homeostasis in *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice.

Results herein demonstrate that Id3 regulates IL-33-induced amounts of IL-5 in the serum and peritoneal fluid. Consistent with the known ability of IL-5 to promote homeostatic proliferation of B-1a B cells[125], administration of exogenous IL-5 rescued B-1a B cell proliferation in Id3-deficient mice. Similar to Id3[25,170], both IL-33 and IL-5 attenuate atherosclerosis[40,123,126]. Treatment with IL-33 increased serum concentration of IL-5 and oxidation-specific IgM antibodies, and reduced plaque size in *Apoe*$^{-/-}$ mice[123,126]. In addition, *Ldlr*$^{-/-}$ mice reconstituted with *Il5*$^{-/-}$ bone marrow had decreased amounts of plasma E06 antibody and increased atherosclerosis compared to controls[40]. In the present study, we provide evidence that Id3 regulates amounts of IL-5, thereby mediating this important IL-33/IL-5 atheroprotective pathway.

Id3 regulates the production of IL-5 by the recently identified innate lymphoid cell, the NH cell[140]. Importantly, Id3 is not required for the development of NH cells, unlike Id2[140]. NH cells produce large amounts of IL-5 in response to IL-33 relative to Th2 cells, basophils, invariant natural killer T (iNKT) cells, mast cells and $CD43^+$ cells[140,177,178]. Results of the present study support these findings in that IL-33 stimulated 50% of NH cells to produce IL5, while less than 2% of Th2 or mast cells produced IL-5. In this study, we did not detect any basophils and eosinophils producing IL-5 in response to IL-33. Moreover, of these cell types, only NH cells displayed reduced percentage of IL-33-stimulated IL-5 producing cells in *Apoe*$^{-/-}$ mice with deletion of Id3. Of note, differences in the intracellular staining for IL-5 in NH cells harvested from *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice compared to control mice were less marked than differences in the serum and peritoneal fluid, raising the interesting possibility that there may be an additional, yet unidentified, cell type producing IL-5 in an Id3-dependent manner. While NH cell specific deletion of Id3 would help address this question, this is not feasible as there is no lineage specific marker for NH cells. Moreover, it is more likely that measuring intracellular levels of this secreted protein underrepresents the effects of Id3 on total production of IL-5, including that which has already been secreted. Nevertheless, results clearly demonstrate that Id3 regulates NH cell production of IL-5 in response to IL-33.

While lymphocytes have long been identified in human and murine aortic adventitia[29,31], recent studies provide evidence that adventitial lymphocyte

activation, including B cells, may be important in regulating atherosclerosis[25,167,176,179-181]. Data from this study suggests that adventitial/PVAT B-1a B cells and NH cells present prior to Western diet initiation may be part of the local IL-33/IL-5 pathway poised to protect from atherogenesis. Aortic NH cells express markers identical to FALC NH cells including the IL-33 receptor. IL-33 is produced by cells in the aortic adventitia[123], which may provide a local stimulus for NH cell production of IL-5. Exogenous IL-33 induced IL-5 production by NH cells and proliferation of aortic B-1a B cells *in vivo*, suggesting that NH cell-derived IL-5 may function to support proliferation of the resident B-1a B cells. These B-1a B cells could produce E06 or other natural IgM antibodies in the adventitia/PVAT[182]. While further study is needed to more fully understand the role of innate-like lymphocytes in the aortic adventitia and surrounding PVAT, results herein demonstrate the existence of functional aortic NH cells.

The present study is the first to demonstrate Id3 regulation of the IL-33/IL-5 pathway and natural immunity. As we have clearly demonstrated that loss of Id3 leads to early atherosclerosis in mice[25,167,170], this may be an important mechanism whereby Id3 promotes innate protection from atherosclerosis. Prior studies demonstrated that loss of Id3 led to decreased B cell homing to the aorta[25,167] and increased intimal adhesion molecule expression[167] in atheroprone mouse models. In addition, it is interesting to speculate that Id3 may also provide atheroprotection by regulating Tregs. Tregs are thought to be atheroprotective[6] and Id3 has been implicated in the promotion of Treg development in

neonates[152], although consistent with previous data[152], we did not observe a difference in the frequency of Tregs with the loss of Id3. There may be other, as yet unidentified, mechanisms whereby Id3 may regulate atheroprotection. The fact that a single SNP in a single gene (*ID3)* is associated with carotid intimal medial thickness in humans would support a role for Id3 in regulating pathways in many cell types involved in atherosclerosis. Although the SNP does not result in reduced expression of Id3 protein, as seen with a knockout mouse, it does results in decreased Id3 binding to its basic helix-loop-helix partner, E12, which markedly attenuates Id3 function[170]. Therefore, identification of specific Id3-mediated atheroprotective pathways, such as the IL-33/IL-5 axis, in mice has the potential to lead to interesting hypotheses that can be tested in humans, such as whether humans with polymorphism at rs11574 have alterations in natural immunity that could be linked to premature atherosclerosis. Such findings may identify individuals amenable to novel prevention or treatment approaches.

Chapter 4

B cell specific loss of Id3 enhances number of B-1b B cells and attenuates atherosclerosis

Abstract

Objective: We have previously demonstrated that B cell-mediated attenuation of atherosclerosis in B cell-deficient mice was dependent on Id3. However, these studies were performed with a global Id3 knockout and an immune-impaired murine model of atherosclerosis. Here we sought to determine if the loss of Id3 specifically in B cells in an immune competent murine model effects atherosclerosis and identify mechanisms mediating these effects.

Approach & Results: Western diet induced atherosclerosis was significantly decreased in mice lacking Id3 in B cells compared to littermate control mice. On Western diet, B cell specific Id3 knockout mice had a greater number of B-1b B cells and higher levels of IgM and T15-Id IgM in the serum compared to control mice. Atherosclerotic lesions in B cell specific Id3 knockout mice contained fewer apoptotic cells and less macrophage content compared to controls.

Conclusion: These studies are the first to suggest that Id3 regulates B-1b B cells and that the loss of Id3 in B cells attenuates atherosclerosis potentially by enhancing apoptotic cell clearance and reducing macrophage accumulation.

Introduction

Atherosclerotic heart disease and stroke have remained the leading cause of mortality in the world[183]. Advances in immune cell research have clearly established a crucial role for inflammation in atherosclerotic plaque development in the blood vessel wall[4]. Ample studies have focused on understanding monocyte and T cells subsets in disease formation[184], and recent data provides evidence that B cells may play a subset-dependent role in atherosclerosis[2]. B-2 B cells are thought to enhance atherosclerotic disease presumably through the cytokines they produce and/or interaction with other leukocytes, and B-1 B cells are thought to protect against disease[14,185].

B-1 B cells are divided into two subtypes, B-1a and B-1b B cells[41,43,44], and while B-1a B cells have been reported to rescue enhanced atherosclerosis in splenectomized mice through cell secretion of IgM NAbs[107], the role for B-1b B cells in atherosclerosis has not been studied. B-1a B cells may spontaneously secrete IgM NAbs prior to exposure of T cell-independent type 2 (TI-2) pathogens, providing initial protection from pathogen exposure. After immunization, B-1b B cells were able to produce IgM antibody in response to antigen and provide long term protection from TI-2 pathogen exposure [72,75,186,187] together suggesting that B-1a and B-1b B cells may have different roles in protection from TI-2 pathogens. An epitope expressed by TI-2 pathogens, PC, is a molecular mimic of an oxidation-specific epitope present on oxLDL and apoptotic cells in atherosclerosis and is recognized by IgM NAbs. B-1b B cells

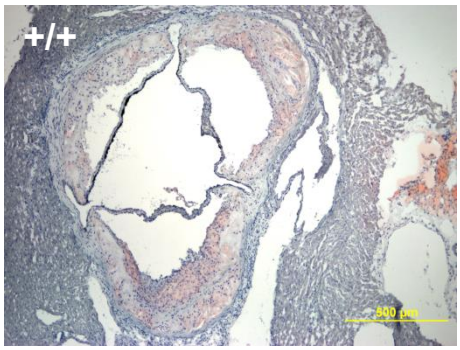
may also have a role in atherosclerosis, potentially by producing a protective antigen-specific IgM antibody response.

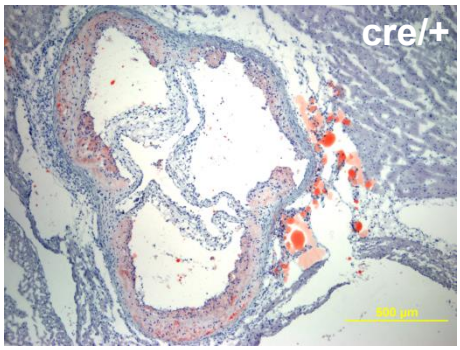
Inhibitor of DNA binding-3 (Id3) is a dominant negative transcriptional regulator in the helix-loop-helix family of transcription factors[173] and global Id3 knockout mice have accelerated atherosclerosis[25,167,170]. Moreover, Id3 was important for B cell-mediated attenuation of atherosclerosis in B cell-deficient *μMT/Apoe*$^{-/-}$ mice[25,167] and Id3 can modulate B cell homing and vessel wall adhesion molecule expression[25,167]. Together, studies suggest that a B cell specific, Id3 knockout murine model would have increased atherosclerosis. Surprisingly, here we demonstrate that the B cell specific loss of Id3 results in reduced atherosclerosis compared to littermate control mice. Furthermore, there was a greater number of B-1b B cells in the peritoneal cavity, spleen and circulation, and higher levels of T15-Id IgM in the serum of B cell specific Id3 knockout mice. Also, atherosclerotic lesions in B cell specific Id3 knockout mice contained decreased macrophage and apoptotic cell content. Together, our data suggests that Id3 is important in B-1b B cell biology and that B-1b B cells may be atheroprotective by producing T15-Id IgM which reduce macrophage accumulation and enhance apoptotic cell clearance in atherosclerotic plaques.

Results

The B cell specific loss of Id3 attenuates Western diet induced atherosclerosis. Prior studies demonstrate that the adoptive transfer of predominately B-2 B cells from the spleen of *Apoe*$^{-/-}$ mice to a B cell deficient atherosclerosis prone mouse (*μMT/Apoe*$^{-/-}$) reduces atherosclerosis compared to PBS injected control mice, while donor cells isolated from *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice cannot confer protection[25], suggesting that the loss of Id3 in B cells would aggravate atherosclerosis. To determine if the B cell specific loss of Id3 regulates atherosclerosis, *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and littermate control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice were placed on a Western diet for 16 weeks and aortic roots were harvested for oil-red-O detection of lipids for quantification of atherosclerotic lesion area. The inability to detect Id3 protein in the CD19+ splenocyte fraction in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice demonstrated B cell specific deletion of Id3 (Figure 10). Figure 15 displays reduced lesion area in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice. Cholesterol levels can effect atherogenesis[4], but there was no difference in serum lipids of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{+/+}$ compared to control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice prior to initiation of Western diet at eight weeks of age (baseline) or after 16 weeks of Western diet (Table 3).

Figure 15. B cell specific loss of Id3 attenuates atherosclerosis. Eight week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ (+/+) and litter-mate control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ (cre/+) mice were placed on a Western diet. After 16 weeks, aortic root lesion area was quantified with oil-red-O. * $p < 0.05$.


+/+


cre/+

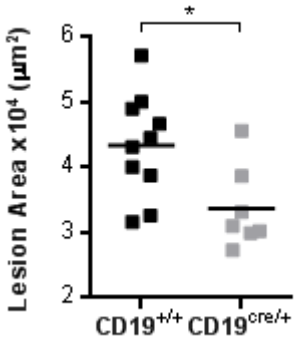

*
Lesion Area x10⁴ (µm²)
6
5
4
3
2
CD19+/+
CD19cre/+

Table 3. Average serum lipid profiles of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and litter-mate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice at baseline and after 16 weeks of Western diet

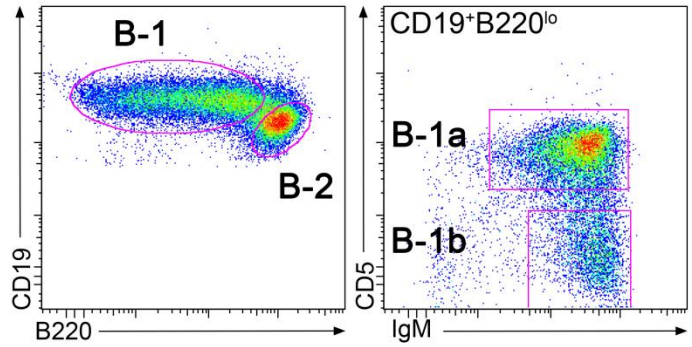
	Id3$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$	*Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$
n	10	8
Baseline		
Cholesterol	358.2 ± 34.9	370.0 ± 30.5
HDL-C	43.4 ± 1.0	47.0 ± 2.1
Non HDL-C	263.8 ± 31.8	285.9 ± 27.0
Triglycerides	218.4 ± 29.7	223.8 ± 23.1
Western Diet		
Cholesterol	1476.0 ± 65.0	1514.0 ± 100.3
HDL-C	43.6 ± 3.2	42.6 ± 4.1
Non HDL-C	NC	NC
Triglycerides	358.6 ± 23.3	387.9 ± 29.2

Values are the average ± SEM in mg/dL. No comparisons between *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ vs. *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice are statistically significant. NC: not calculated

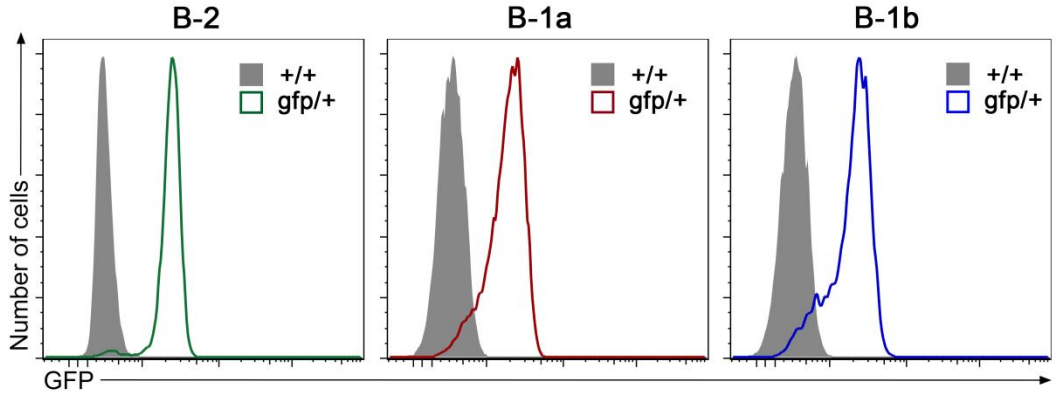
The B cell specific loss of Id3 results in a greater number of B-1b B cells and increased levels of serum IgM. Previous studies reveal that when Id3 is specifically lost in B cells in *Apoe*$^{-/-}$ mice, there are no differences in the number of PerC B-2 or B-1a B cells (Figure 10), yet it is unknown if Id3 regulates B-1b B cells in these mice. Representative flow cytometry plots for the gating strategy of B-1b B cells ($CD19^{+}B220^{lo}CD5^{-}IgM^{hi}$) are shown in Figure 16A. To determine if Id3 is expressed in B-1b B cells, Id3 promoter activation was assessed in B-1b B cells of GFP reporter (*Id3*$^{gfp/+}$) mice and compared to littermate wildtype mice (*Id3*$^{+/+}$) in the spleen and PerC by flow cytometry. Figure 16B demonstrates Id3 promoter activation expression indicated by GFP fluorescence in B-1b B cells in the PerC, similar GFP fluorescence was observed in splenic B cell subsets (not shown). For comparison, GFP fluorescence in B-2 and B-1a B cells is also displayed. To determine if Id3 is absent in B-1b B cells in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice, B-1b B cells were sorted by FACS with purification results similar to B-1a B cells in Figure 8 and Id3 transcript was detected by real-time PCR. Id3 transcript was detected in B-1b B cells purified from *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice, consistent with B-1b B cell expression of Id3 in the GFP reporter mouse (Figure 16B), but not detectable in sorted B-1b B cells from *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice (Figure 16C). To determine if the B cell specific loss of Id3 regulates the number of B-1b B cells, B-1b B cells in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ and control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice at eight weeks of age and on chow diet (defined as ‘baseline’ mice) were quantified by flow cytometry. The B cell specific loss of Id3 resulted in a marked fourfold increase in the number of B-1b B cells in the peritoneal cavity and

Figure 16: B-1b B cells express Id3. **(A)** Representative gating strategy for B-2 ($CD19^{+}B220^{hi}$), B-1a ($CD19^{+}B220^{lo}CD5^{+}IgM^{hi}$) and B-1b ($CD19^{+}B220^{lo}CD5^{-}IgM^{hi}$) B cell subsets in the PerC of C57BL/6 mice by flow cytometry. **(B)** PerC B-2, B-1a, B-1b B cells (gated as in (A)) from $Id3^{gfp/+}$ mice (open histogram) compared to littermate $Id3^{+/+}$ mice (solid grey histograms). **(C)** Peritoneal lavage cells were sorted from $Id3^{fl/fl}Apoe^{-/-}CD19^{+/+}$ ($CD19^{+/+}$, closed bar) or $Id3^{fl/fl}Apoe^{-/-}CD19^{cre/+}$ ($CD19^{cre/+}$, open bar) mice for B-1b ($CD19^{+}B220^{lo}\ CD5^{-}$) cells and Id3 transcript was measured by real-time PCR and normalized to a housekeeping gene, cyclophilin b (CypB). Abbr.: peritoneal cavity (PerC).

A.


B-1
B-2
CD19
B220
CD19+B220lo
B-1a
B-1b
CD5
IgM

B.


B-2
B-1a
B-1b
+/+
gfp/+
Number of cells
GFP

C.

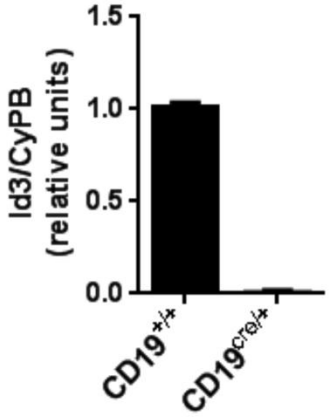
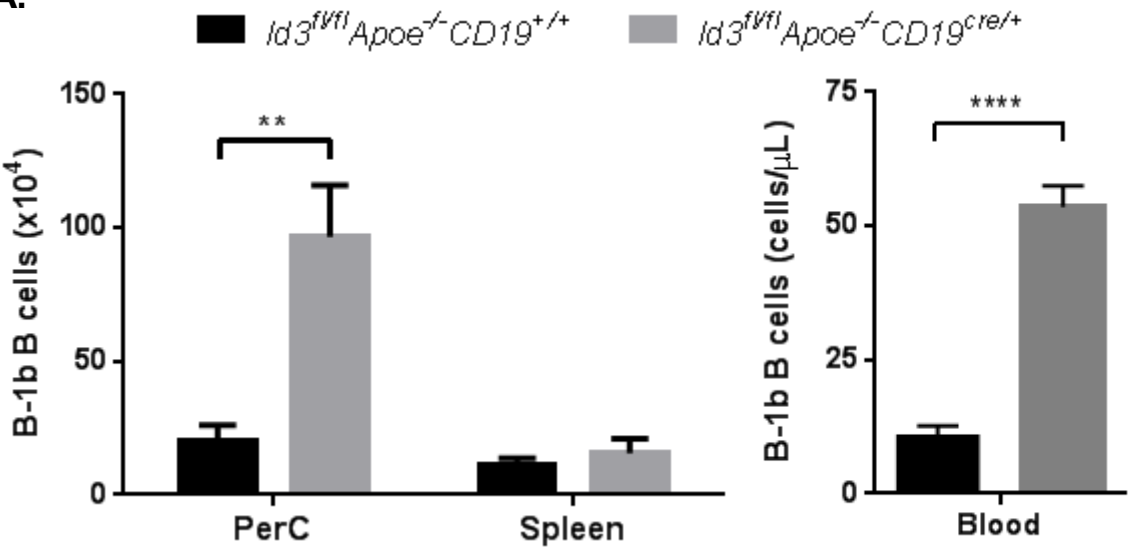
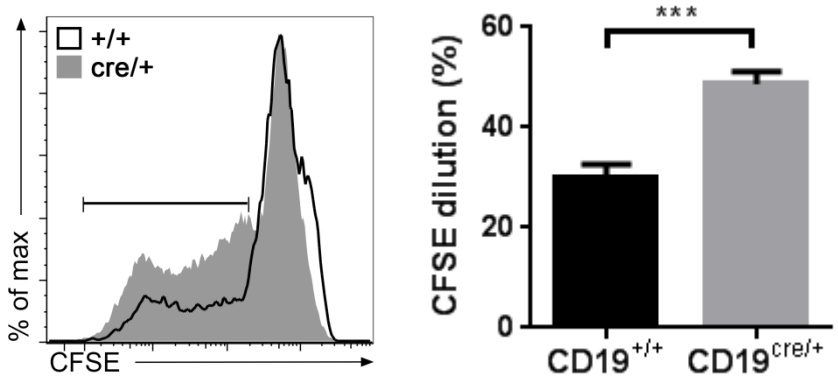

Id3/CyPB
(relative units)
1.5
1.0
0.5
0.0
CD19+/+
CD19cre/+

Figure 17. B cell specific Id3 knockout mice have a greater number of B-1b B cells. Eight to ten week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and litter-mate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{cre/+}$ mice on chow diet (baseline mice). **(A)** Quantification of B-1b B cells in the PerC, spleen (n = 10 and 13, respectively) and blood (n = 5 and 9, respectively). **(B)** (**Left**), representative flow cytometry and (**right**), quantification of CFSE labeled B-1b B cells from *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ (+/+, open histogram) and litter-mate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice (cre/+, shaded histogram) (n = 5). ** $p < 0.01$, *** $p < 0.001$.

A.


Id3fl/flApoe-/-CD19+/+
Id3fl/flApoe-/-CD19cre/+
B-1b B cells (x10^4)
150
100
50
0
**
PerC
Spleen
B-1b B cells (cells/μL)
75
50
25
0
****
Blood

B.


+/+
cre/+
% of max
CFSE
CFSE dilution (%)
60
40
20
0
***
CD19+/+
CD19cre/+

circulation and no difference was observed in the number of B-1b B cells in the spleen (Figure 17A). Next, we sought to determine if the difference in number of peritoneal B-1b B cells was due to enhanced proliferation. *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ and control mice were intraperitoneally injected with CFSE, and after seven days proliferation by CFSE dilution was determined in peritoneal B-1b B cells by flow cytometry. As shown in Figure 17B, *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice had an increased proportion of proliferating B-1b B cells compared to control mice. B-1 B cells have been reported to produce the majority of serum IgM[44]. Consistent with an increase in B-1b B cells in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice; there was also an increase in levels of IgM in the serum of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to control mice at baseline (Table 4). Serum levels of T15-Id IgM, IgG1, IgG2b, IgG2c, and IgG3 were equivalent in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to controls.

The loss of Id3 in B cells does not reduce total CD19+ B cells in the aorta. Previous data from our laboratory demonstrated that Id3 is important for B cell homing to the aorta and not to peri-aortic LNs through regulating expression of the chemokine receptor, CCR6[25,167]. To determine if the loss of Id3 in B cells results in reduced CCR6 expression, CCR6 protein on B cell subsets was determined in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice in the PerC by flow cytometry. Indeed, there was a reduced percentage of CCR6^{+} on B-2, B-1a, and B-1b B cells in B cell specific Id3 knockout mice compared to controls

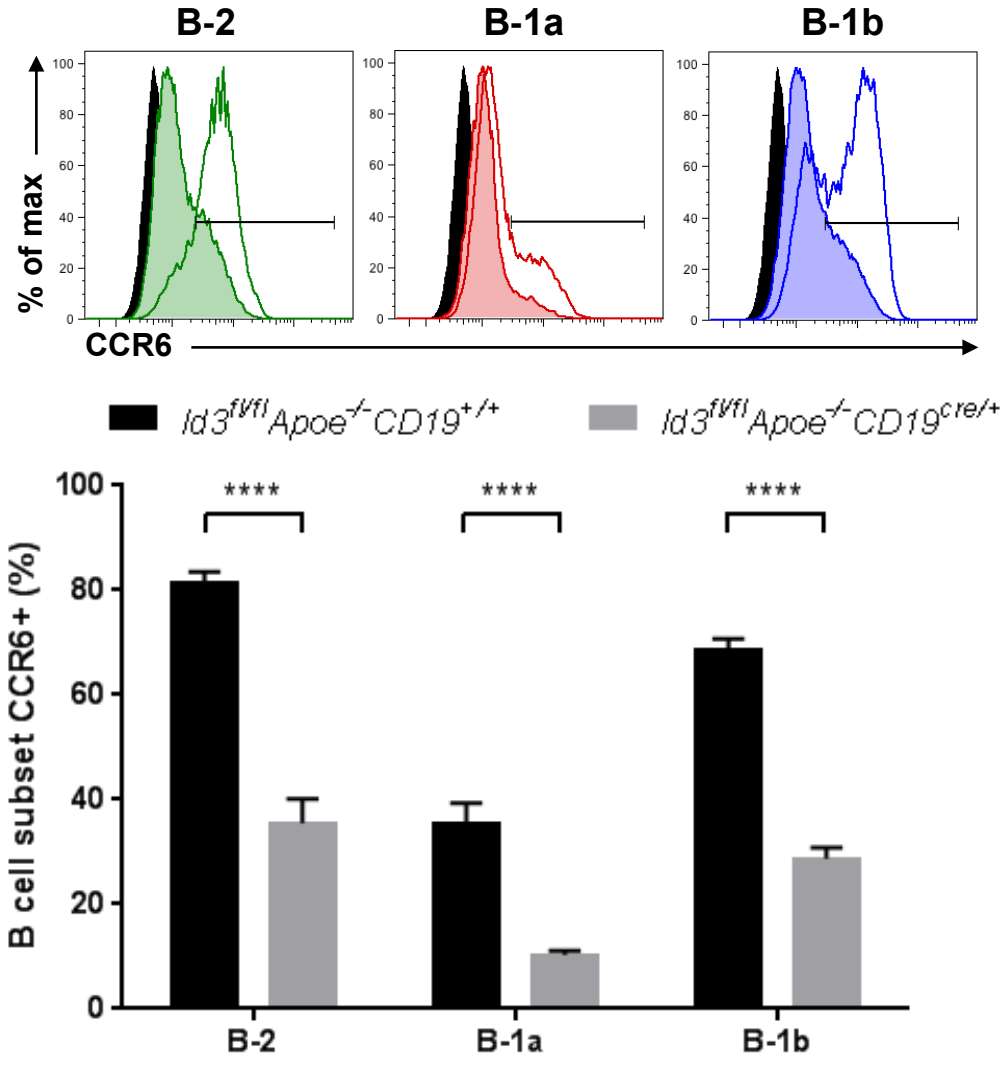
Table 4. Serum immunoglobulins in eight to ten week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and littermate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice.

	Id3$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$	*Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$	p-value
n	8	7	
IgM (µg/mL)	123.7 ± 5.7	150.1 ± 10.1^{A}	0.043
T15 IgM (RLU)	53.4 ± 3.7	59.4 ± 9.9	0.587
IgG1 (µg/mL)	295.4 ± 101.3	197.4 ± 64.1	0.466
IgG2b (µg/mL)	328.2 ± 32.1	277.5 ± 35.7	0.308
IgG2c (µg/mL)	103.1 ± 20.9	90.86 ± 17.3	0.664
IgG3 (µg/mL)	1091.0 ± 110.0	792.8 ± 188.8	0.182

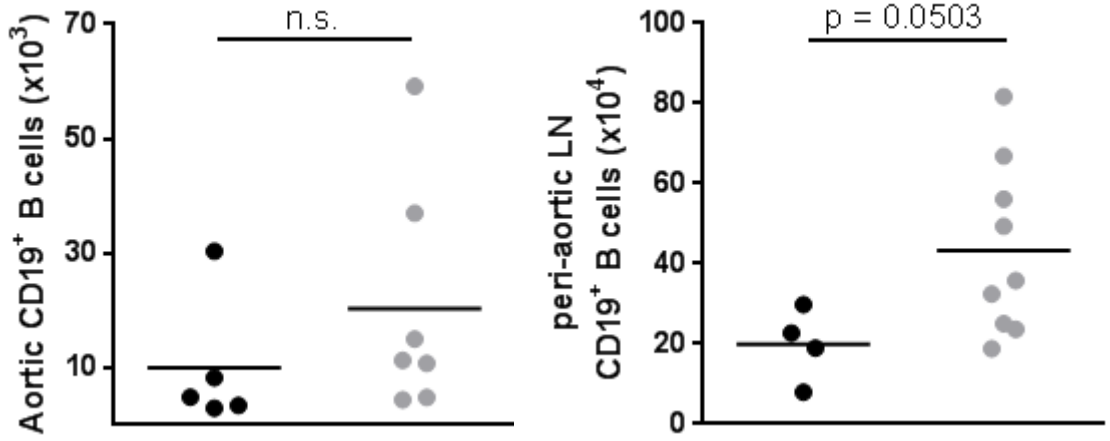
Values are average ± SEM. $^{A}p < 0.05$ *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ vs. *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$

Figure 18. Id3 regulates CCR6 expression on B cell subsets. **(A) Top,** CCR6 expression on B cells from eight to ten week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ (open histograms, n = 6) and litter-mate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ (shaded histograms, n = 9) mice compared to FMO control (solid black) in the PerC and (**bottom**) quantification by flow cytometry. **(B)** Quantification of CD19^{+} B cells in the (**left**) aorta and (**right**) peri-aortic lymph nodes (LN). **** $p < 0.0001$.

A.


B-2
B-1a
B-1b
% of max
CCR6
Id3fl/flApoe-/-CD19+/+
Id3fl/flApoe-/-CD19cre/+
B cell subset CCR6+ (%)
****
****
****
B-2
B-1a
B-1b

B.


Aortic CD19+ B cells (x10^3)
n.s.
peri-aortic LN CD19+ B cells (x10^4)
p = 0.0503

(Figure 18A). Next, to determine if there were fewer B cells in the aortas of B cell specific Id3 knockout mice, whole aortas and peri-aortic LNs from *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{+/+}$ and littermate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice at baseline were digested and B cells were quantified by flow cytometry. There was not a reduced number of CD19^{+} B cells in aortas of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ compared to *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice (Figure 18B). However, there appeared to be an increase in the number of total peri-aortic LN CD19^{+} B cells. To determine if there was an increase B-1b B cells in peri-aortic LN similar to the peritoneal cavity and circulation (Figure 17B), B-1b B cells were quantified in the peri-aortic LNs as defined in Figure 16. Indeed, a greater number of B-1b B cells was observed in the mediastinal, renal, and lumbar LNs of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ compared to littermate control mice (Figure 19).

The B cell specific loss of Id3 results in a systemic increase in B-1b B cell number and serum IgM antibodies compared to controls after Western diet. There are increased B-1b B cells and serum levels of IgM in B cell specific Id3 knockout mice at baseline, and we sought to determine if these observations are present after Western diet. As shown in Figure 20, *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice contained significantly more B-1b B cells in the spleen and circulation compared to control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice as assessed by flow cytometry. Similar to the findings at baseline (Figure 17A) there was a greater number of B-1b B cells in

Figure 19. Peri-aortic lymph nodes have a greater number of B-1b B cells. Quantification of B-1b B cells in peri-aortic lymph nodes (LN) of eight to ten week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and litter-mate *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice at baseline.

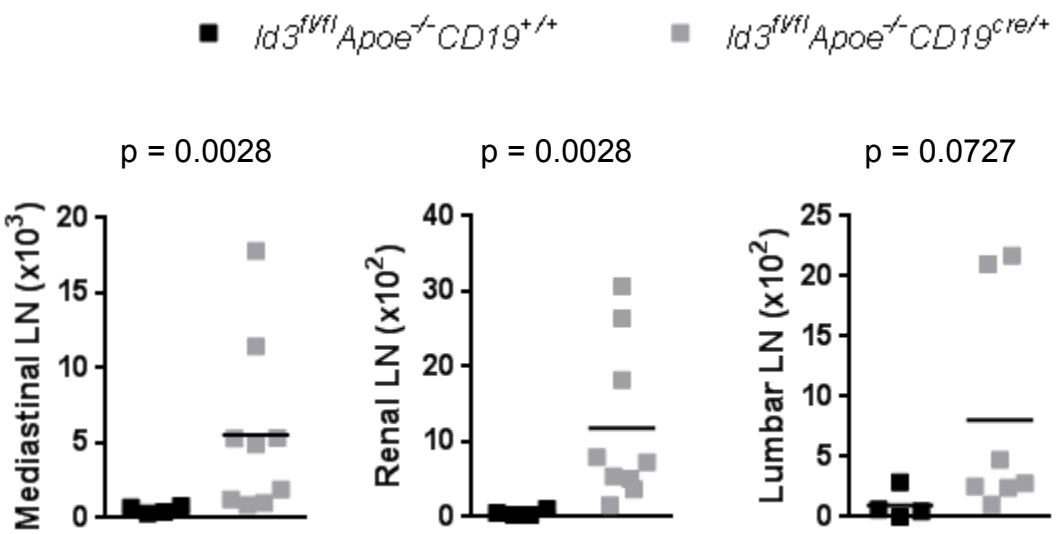
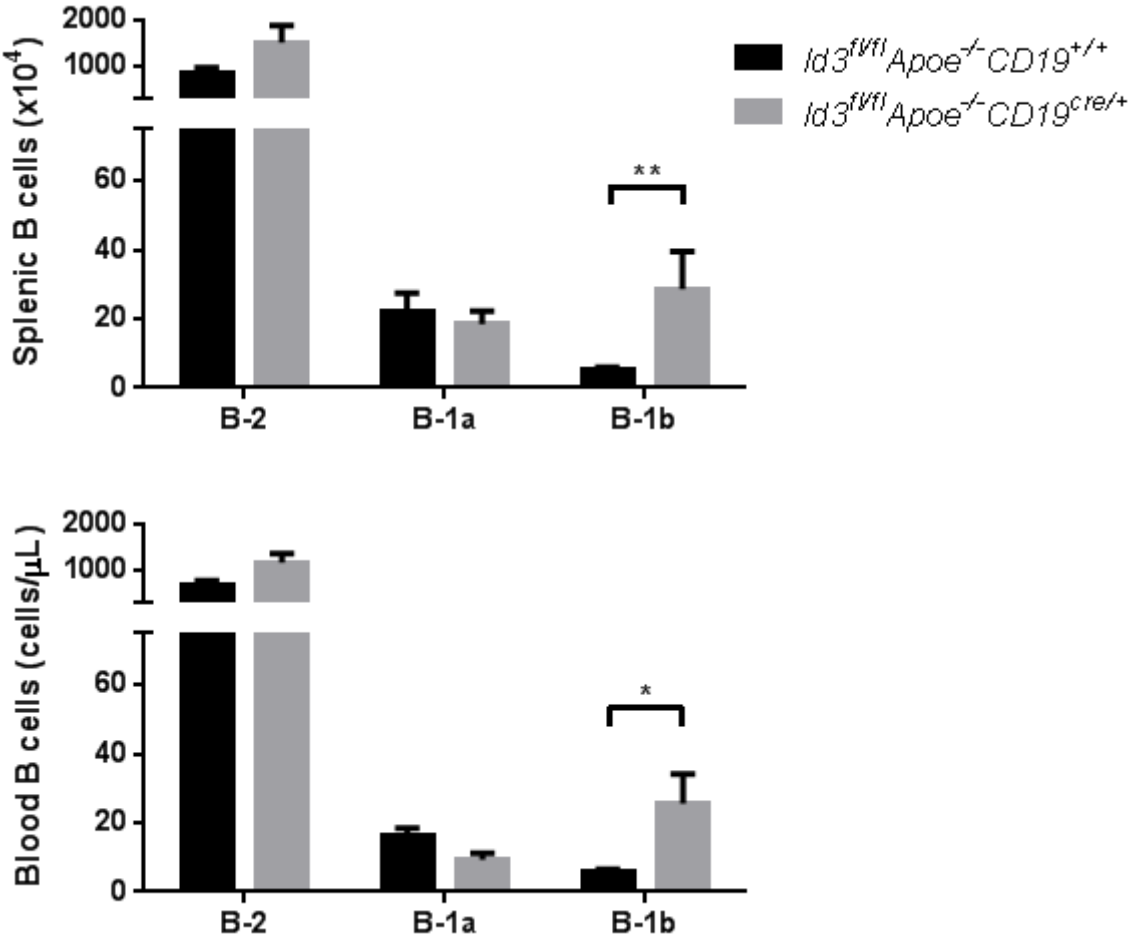

Id3fl/flApoe-/-CD19+/+
Id3fl/flApoe-/-CD19cre/+
p = 0.0028
p = 0.0028
p = 0.0727
Mediastinal LN (x10^3)
Renal LN (x10^2)
Lumbar LN (x10^2)
0
5
10
15
20
25
30
40

Figure 20. B cell specific Id3 knockout mice have a greater number of B-1b B cells systemically after Western diet. Eight week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ (n = 11) and litter-mate control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ (n = 13) mice were placed on a Western diet. After 16 weeks, the spleen and blood were harvested for flow cytometry for B cell subsets. * $p < 0.05$, ** $p < 0.01$.


Splenic B cells (x10^4)
2000
1000
60
40
20
0
B-2
B-1a
B-1b
**
Id3fl/flApoe-/-CD19+/+
Id3fl/flApoe-/-CD19cre/+
Blood B cells (cells/µL)
2000
1000
60
40
20
0
B-2
B-1a
B-1b
*

the PerC of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to controls (not shown). Consistent with findings in eight week old mice (Figure 10), there was no difference in the number of B-1a B cells in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to control mice in the PerC (not shown). There was also no difference in the number of B-2 and B-1a B cells in the spleen or circulation. To determine if the higher levels of serum IgM with the B cell specific loss of Id3 persisted after Western diet, serum antibodies were measured by ELISA. Indeed, *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{cre/+}$ mice had increased levels of IgM in the serum compared to control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice (Table 5). There was also significantly more T15-Id IgM in the serum of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to controls. Levels of IgG1, IgG2b, IgG2c, and IgG3 were equivalent after 16 weeks of Western diet, similar to baseline (Table 4).

Decreased apoptotic cells and macrophage content in atherosclerotic lesions of B cell specific Id3 knockout mice. T15-Id IgM can facilitate apoptotic cell clearance and inhibit oxLDL uptake by macrophages, which may prevent the pro-inflammatory effects of defective apoptotic cell clearance and foam cell formation, thereby attenuating atherosclerosis[16,188]. To evaluate apoptotic cell and macrophage content in atherosclerotic lesions of mice with the B cell deletion of Id3, TUNEL+ cells and Mac2 were quantified in aortic roots of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ and *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice by immunohistochemistry. There were significantly fewer TUNEL+ cells when normalized to plaque area in

atherosclerotic lesions of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to control mice (Figure 21A). Negative control (-tdt) sections did not observe any brown staining (not shown). Mac2 area normalized to plaque area was also decreased in atherosclerotic lesions of *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to controls (Figure 21B). Isotype control for Mac2 did not reveal any immunofluorescence signal (not shown).

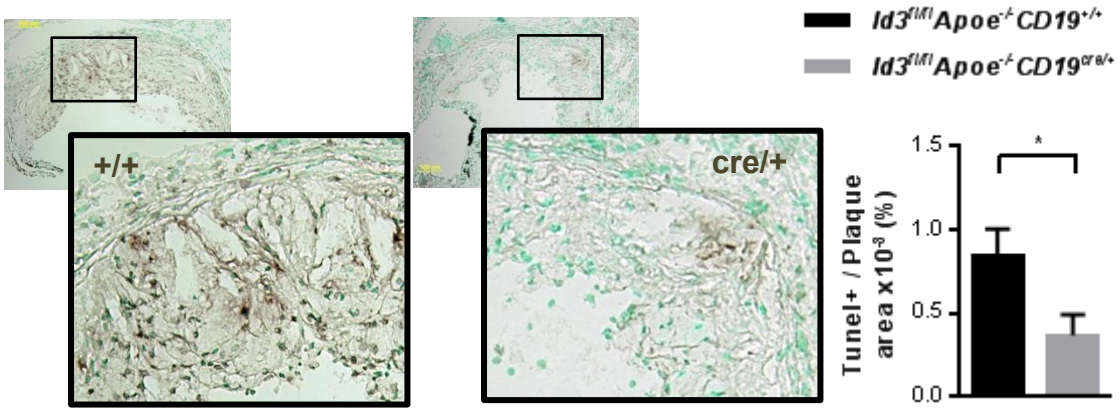
Table 5. Serum immunoglobulins in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ mice and *Id3*$^{fl/fl}$*Apoe*$^{-/-}$ *CD19*$^{cre/+}$ control mice after Western diet.

	Id3$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$	*Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$	p-value
n	9	8	
IgM (µg/mL)	102.7 ± 8.4	180.8 ± 24.0^{A}	0.014
T15 IgM (RLU)	61.5 ± 7.9	111.4 ± 18.0^{A}	0.025
IgG1 (µg/mL)	198.2 ± 66.0	164.0 ± 45.0	0.675
IgG2b (µg/mL)	384.3 ± 15.4	360.7 ± 13.9	0.278
IgG2c (µg/mL)	213.0 ± 7.6	215.6 ± 1.0	0.833
IgG3 (µg/mL)	1511.0 ± 279.6	1954.0 ± 306.4	0.301

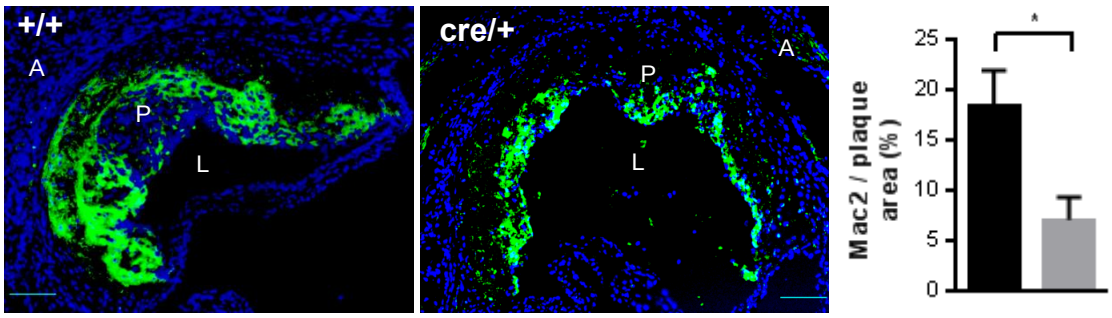
Values are average ± SEM.
$^{A}p < 0.05$ *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ vs. *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$

Figure 21. B cell specific loss of Id3 reduces lesional apoptotic cell and macrophage content. Eight week old *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{+/+}$ (+/+) and litter-mate control *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ (cre/+) mice were placed on a Western diet for 16 weeks. Aortic root lesion **(A)** TUNEL+ cells (brown nuclei) (n = 9 or 11) counterstained with methyl green and **(B)** mac2 (green) and dapi (blue) (n = 5) were determined by immunohistochemistry. Abbr.: A: adventitia, P: plaque, L: lumen. * $p < 0.05$.

A.


Id3fl/fl Apoe-/- CD19+/+
Id3fl/fl Apoe-/- CD19cre/+
+/+
cre/+
Tunel+ / Plaque area x10-3 (%)
1.5
1.0
0.5
0.0
*

B.


+/+
A
P
L
cre/+
A
P
L
Mac2 / plaque area (%)
25
20
15
10
5
0
*

Discussion

This study provides evidence that Id3 regulates the B-1b B cell pool and that deletion of Id3 in B cells decreases atherosclerosis. Western diet induced atherosclerosis is attenuated in B cell specific Id3 knockout mice and is associated with decreased apoptotic cells and reduced macrophage content in atherosclerotic plaques. Additionally, B-1b B cell number and serum levels of T15-Id IgM were enhanced in B cell specific Id3 knockout mice compared to control mice fed a Western diet, suggesting that the reduced atherosclerosis in B cell specific knockout mice may be due to increased T15-Id IgM mediating apoptotic cell clearance and reducing macrophage accumulation in atherosclerotic plaques.

In germ-free, uninfected mice most, if not all, serum IgM is thought to be spontaneously produced by B-1 B cells[44,111] yet, B-1 B cell subsets may have distinct roles during infection and potentially during Western diet conditions. IgM NAbs, including those with the T15-Id, are present in gnotobiotic, chow fed, wildtype mice and can recognize oxidation-specific epitopes on oxLDL[111] that are induced by Western diet[189,190], suggesting that IgM NAbs are present prior to any pathogenic exposure. Furthermore, T15-Id recognizes the same PC moiety on the bacterial cell wall of *S. pneumonia* as on oxLDL, identifying T15-Id antibodies and the B-1 B cells that produce them as key players in the protective humoral response to infection[75,183]. Recent evidence suggests that spontaneous production of IgM NAbs by B-1a B cells is important for early protection in *S.*

pneumonia models of infection and production of antigen specific IgM by clonal expansion of memory like B-1b B cells is important for long lasting protection[48,72,73,75,187,191]. This division of labor between B-1 B cell subsets may explain why in B cell specific Id3 knockout mice there is no difference in the levels of T15-Id IgM in the serum compared to control mice at baseline, and there are significantly higher levels of T15-Id IgM in the serum of B cell specific Id3 knockout mice compared to control mice on a Western diet. At baseline in chow fed mice, B-1a B cells may spontaneously secrete most of the T15-Id IgM found in the serum and there is no difference in the number of B-1a B cells and therefore no difference in levels of T15-Id IgM in the serum of B cell specific Id3 knockout mice compared to control mice. However, after Western diet, B-1b B cells may produce the majority of T15-Id IgM and there are more B-1b B cells in the peritoneal cavity, spleen, and circulation of B cell specific Id3 knockout mice compared to littermate controls. Moreover, levels of T15-Id IgM in the serum of B cell specific Id3 knockout mice are significantly greater than controls after Western diet. Interestingly, there are more B-1b B cells in the spleens of B cell specific Id3 knockout mice compared to controls when mice are fed a Western diet, but not at baseline. B-1b B cells may be induced to migrate from the peritoneal cavity to the spleen to secrete IgM antibodies[192]. Data suggests that under Western diet conditions, B-1b B cells may migrate to the spleen to produce T15-Id IgM in B cell specific Id3 knockout mice.

IgM NAbs have been reported to mediate clearance of apoptotic cells[108,110-112,115,193] preventing the pro-inflammatory effects of defective apoptotic cell clearance[16,63,117], which may be a primary mechanism for B-1 B cell atheroprotection. While recent evidence directly supports B-1a B cell atheroprotection mediated by secretion of IgM[107], B-1b B cells have not been independently studied. B-1 B cells in reconstituted *Rag1*$^{-/-}$*Apoe*$^{-/-}$ mice are able to produce IgM NAbs, including T15-Id IgM which recognizes oxidation-specific epitopes such as PC on apoptotic cells, and have enhanced apoptotic cell clearance compared to controls in atherosclerotic mice[111]. Furthermore, NAbs against oxidation-specific epitopes have been detected in atherosclerotic lesions[110,111] and increased plaque area is associated with a higher frequency of apoptotic cells in *sIgM.Ldlr*$^{-/-}$ mice[83]. Here we provide evidence that decreased atherosclerosis is associated with a systemic increase in B-1b B cells, increased serum T15-Id IgM, and decreased lesional apoptotic cells in B cell specific Id3 knockout mice, suggesting that the increase in T15-Id IgM may be due to the increase in B-1b B cells and that the loss of Id3 in B cells may result in enhanced apoptotic cell clearance in atherosclerotic lesions. However, it is unknown if other atherosclerotic plaque characteristics such as plaque stability, T cell content, and necrotic core area or if the stage of plaques are different in B cell specific Id3 knockout mice compared to controls. Future studies will need to be performed to determine if mice with B cell specific deletion of Id3 have an increase in antigen specific IgM in the plaque, such as T15-Id IgM, which may mediate apoptotic cell clearance in atherosclerotic lesions.

A hallmark of exacerbated atherogenesis is macrophage engulfment of oxLDL and subsequent foam cell formation that produces pro-inflammatory signals to recruit additional leukocytes, including monocytes, to the plaque[194]. Oxidation-specific IgM NAbs target epitopes including PC present on oxLDL and prevent macrophage oxLDL uptake[14] which is thought to occur in atherosclerotic plaques. In B cell specific Id3 knockout mice, reduced atherosclerosis is associated with enhanced levels of T15-Id IgM NAbs in the serum and decreased macrophage content in plaques. However, the atheroprotective effect of IgM is not thought to be due to clearance of oxidized lipids from the serum[195] and it has yet to be determined if there is increased T15-Id antibodies in lesions of B cell specific Id3 knockout mice. It has yet to be determined if another potential mechanism for atheroprotection in B cell specific Id3 knockout mice is T15-Id IgM NAb binding to oxLDL and inhibiting macrophage uptake, preventing foam cell formation and additional recruitment of monocytes to atherosclerotic plaques resulting in less macrophage accumulation.

How circulating IgM antibodies that may be produced by peripheral B-1b B cells are able to deposit into atherosclerotic plaques is currently unknown. Ischemia-reperfusion injured vasculature activates the endothelium to express neo-epitopes that natural IgM can bind and deposit into the vessel wall[196] and atherosclerotic plaques have an activated endothelium and can express neo-epitopes[108,110,116]. Oxidation specific IgM antibodies were able to target micelles to atherosclerotic plaques in *Apoe*$^{-/-}$ mice and were visualized in plaques with

magnetic resonance imaging[197], together suggesting that circulating oxidation specific antibodies may enter atherosclerotic plaques through the endothelium and deposit into lesions. In addition, IgM can bind minimally modified LDL (mmLDL) present in serum[195] and immune complex can be deposited into vasculature[198], suggesting that oxidation-specific antibodies bound as immune complex may selectively deposit into atherosclerotic plaques. Igs are not thought to be able to transit through conduits from the adventitia into the SMC layer[176,199] and therefore it is unknown if the local production of Igs can cross from the adventitia, where B cells are mostly found[5], through the SMC layer to the plaque.

There is a systemic increase in the number of B-1b B cells, levels of serum T15-Id, and a decrease in atherosclerotic plaque size, apoptotic cell content, and macrophage content in B cell specific Id3 knockout mice after Western diet, yet how these observations directly relate is currently unclear. B-1b B cells provide humoral protection to TI-2 blood borne particulate antigens[72,75,186,187] and B-1b B cells may be able to mobilize to LNs and become antibody secreting cells[200] in response to pathogens or may produce antibody in other peripheral lymphoid organs such as the spleen and peritoneal cavity[72,75,186,187]. In future studies, it will be important to determine if there is an increase in B-1b B cells locally in the aorta. B-1b B cells may be increased in peri-aortic LNs of B cell specific Id3 knockout mice and may be poised to encounter neo-antigens draining from the aorta or produce antibody in other peripheral lymphoid organs. Whether B-1b B cells in peri-aortic lymph nodes

undergo antigen dependent expansion and produce antigen specific antibodies, including those of the T15-Id, is unknown.

Prior studies from our lab demonstrate that there was reduced atherosclerosis with the adoptive transfer of splenic B cells isolated from *Apoe*$^{-/-}$ mice to *μMT/Apoe*$^{-/-}$ mice when compared to vehicle control[25]. However, donor splenic B cells from *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice could not confer atheroprotection to *μMT/Apoe*$^{-/-}$ mice, suggesting that B cell mediated atheroprotection is dependent on Id3. Presumably, Id3 would only be absent in B cells in this atherosclerotic model and present in all other cells in the recipient *μMT* mice, altogether suggesting the B cell specific loss of Id3 would result in exacerbated atherosclerosis. Surprisingly, loss of Id3 in B cells resulted in reduced atherosclerosis in *Id3*$^{fl/fl}$*Apoe*$^{-/-}$*CD19*$^{cre/+}$ mice compared to littermate control mice. Apparent discrepancies in outcomes may be explained by the different murine models of atherosclerosis. *μMT/Apoe*$^{-/-}$ mice are generated by crossing the *Apoe*$^{-/-}$ mouse with the B cell deficient, *μMT* mouse which has a point mutation in the transmembrane domain of IgM, resulting in arrested B cell development at the pre-B cell stage and complete absence of mature B cells[201]. Studies have reported that T cells from *μMT* mice produce higher levels of IFNγ and very little IL-4[202,203] and may inhibit reconstitution of B cell populations[204], implying that T cells, and maybe even other cells of the immune system, in *μMT/Apoe*$^{-/-}$ mice have altered function compared to T cells that would be present in B cell specific, Id3 knockout *Apoe*$^{-/-}$ mice. Thus, the Id3-dependent B cell mediated

atheroprotection observed in *μMT/Apoe*$^{-/-}$ mice may be specific to the immune system of that model.

B cell atheroprotection mediated by Id3 in the *μMT/Apoe*$^{-/-}$ murine model was also associated with Id3-dependent B cell homing to the aorta. In addition, the global loss of Id3 resulted in enhanced atherosclerosis, fewer aortic B cells, and decreased B cell expression of the homing chemokine receptor, CCR6[25,167], suggesting that Id3 is necessary for atheroprotective B cell homing to the aorta. Notably, the global Id3 knockout model would result in the loss of Id3 in other cells important in atherosclerosis such as T cells, which Id3 can regulate[148,152,155,162]. Aortas of CCR6-null mice contained fewer B cells and B cell mediated atheroprotection in the *μMT/Apoe*$^{-/-}$ murine model was CCR6 dependent[25], providing evidence that Id3 regulates the expression of CCR6 on B cells and B cell homing to the aorta in protection from atherosclerosis. These studies would predict that mice with the B cell specific loss of Id3 would have reduced B cell expression of CCR6 and fewer B cells in the aorta. However, while all B cell subsets observed less CCR6 expression, there did not appear to be fewer B cells in the aortas of B cell specific Id3 knockout mice implying that in this model, Id3 can regulate expression of CCR6 but that reduced CCR6 expression in B cells may not be sufficient to reduce B cell number in the aorta. Data suggests that the loss of Id3 in B cells and an aberrant immune system, potentially T cell mediated, in *μMT* and global Id3 knockout mice may account for the differences observed in Id3 regulation of B cells and atherosclerosis in the

global Id3 knockout and *μMT/Apoe*$^{-/-}$ model compared to the B cell specific Id3 knockout model.

While many questions remain, data from this study is the first to implicate a potential atheroprotective role for B-1b B cells, which may be through production of T15-Id IgMs that clear apoptotic cells and prevent macrophage accumulation in atherosclerotic lesions. Immunization with *S. pneumonia* protects from atherosclerosis through the T15-Id response[37,40] and B-1b B cells may play a key role in immunization by providing long lasting protection through antigen specific, memory B-1b B cells[73,75]. As such, enhancing B-1b B cell proliferation and memory formation may benefit immunization strategies to protect from atherosclerosis[205].

Chapter 5

General Discussion and Future Directions

Atherosclerosis can be described as a polygenic disorder with chronic inflammation of the arterial blood vessel wall, underscoring the complexity of the disease with contributing factors including the environment and genetics that regulate vascular hemodynamics, lipid metabolism, vascular wall cells, and the immune system. Despite advancements in high dose statin therapy to limit hyperlipidemia, cardiovascular events and atherosclerotic burden remain high[206]. Abundant research has focused on the role for immune cells in murine models of atherosclerosis yet, applying discoveries in mice to humans remains challenging[8]. Targeting leukocytes for therapy is complicated due to the opposing roles of subtypes within subsets of immune cells[6]. However, with the promising advancement of whole systems technologies such as systems genetics[207] and mass cytometry where pathway alterations can be observed in many cell types at once[208], the daunting task of managing such a complex disease by understanding the various contributions of different leukocytes in response to therapy in individuals with a particular genetic makeup may become a reality. In particular, as opposing roles for B cell subsets has emerged in murine atherosclerosis[2], recent efforts to standardize human B cell phenotypic profiling[209,210] may help identify the unknown phenotypic differences of B cell subsets in human atherosclerosis.

Id3 is an important regulator of natural immunity in atherosclerosis

Immunization strategies to inhibit atherosclerosis are thought to modulate anti-PC IgM NAbs[37,205,211] potentially through IL-5 dependent B-1 B cell proliferation in mice[40]. In humans, plasma levels of IL-5 correlate with levels of anti-oxLDL IgM, which are negatively associated with measures of atherosclerosis[101,212-216]. Additionally, IL-5 production and levels of anti-PC IgM in the serum are dependent on Id3 and Id3-deficient mice have a defective humoral response to TI type 2 antigens[154], which includes PC, suggesting that Id3 may be an important factor in immunization strategies against atherosclerosis. In addition, the *ID3* SNP at rs11574 results in attenuated protein function and is associated with increased atherosclerosis in humans[170] altogether suggesting that the *ID3* SNP may be a biomarker for those with a defective IL-5 pathway and unresponsive to immunization. An association of patients with the *ID3* risk allele and decreased levels of IL-5 and anti-PC IgM in the serum would support this intriguing hypothesis. Moreover, this ID3 SNP may be a biomarker for humans with defective natural immunity leading to premature atherosclerosis and may identify individuals amenable to alternative prevention or treatment approaches. Currently, clinical trials for vaccination against atherosclerosis are still in infant stages[217].

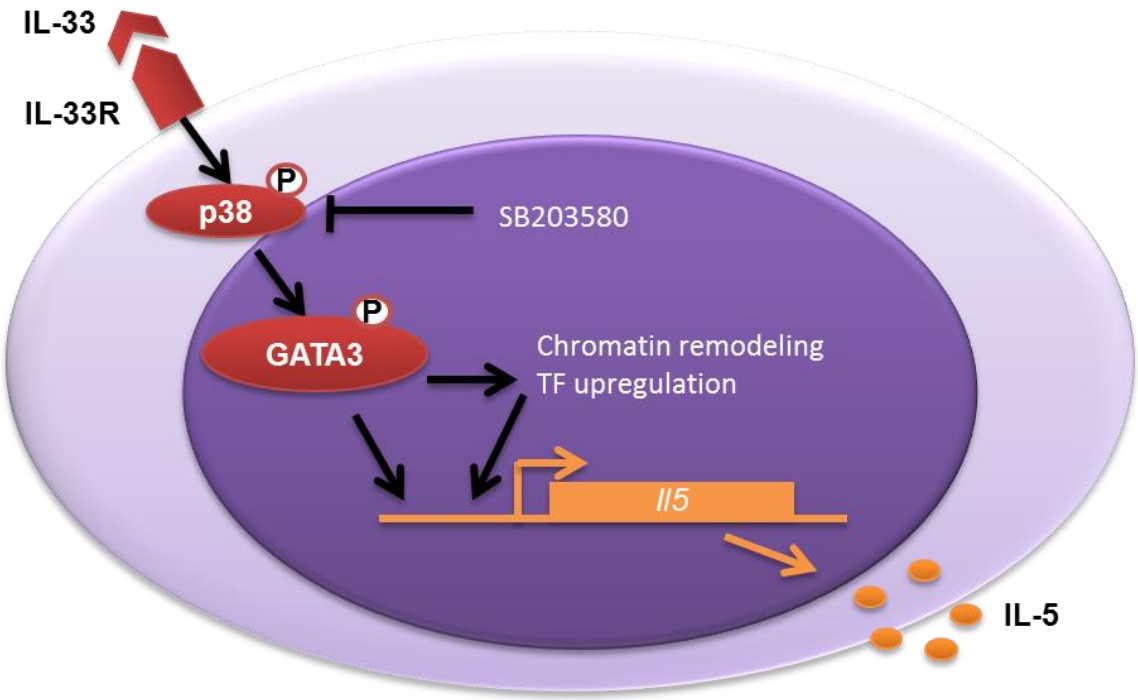
Id3 regulates production of the atheroprotective cytokine, IL-5, by NH cells, which are found in the PVAT/adventitia and can be stimulated to produce IL-5, implicating a local role in addition to a systemic role for this cell type in atherosclerosis. Importantly, circulating NH cells have been detected in humans and can produce IL-5 in response to IL-33[130], raising the possibility to study an

association between circulating NH cells and burden of atherosclerosis or stenosis in the coronary artery measured by intravascular ultrasound. Current approaches to study the role of NH cells in murine models of atherosclerosis may be difficult due to the lack of a lineage specific marker for lineage depletion studies and the number of mice needed to pool for sufficient numbers for adoptive transfer studies. Nonetheless, the challenging task of understanding the role of NH cells in atherosclerosis is worthwhile and may become more feasible as new model systems are developed and if a lineage specific marker is identified.

Id3 may be a key factor in IL-5 production by NH cells

The mechanism by which Id3 regulates IL-5 production in NH cells has yet to be explored, but insight may be gained from the observation that Id3 regulation of IL-33-induced IL-5 production is specific to NH cells and not Th2 T cells or BMMCs. A recent study found that in NH cells, IL-33 induces the MAPK pathway, similar to Th2 T cells[218], to promote IL-5 transcription (Figure 22). Furthermore, IL-33 induced phosphorylation of p38 MAPK which was important for phosphorylation and increased expression of the transcription factor GATA3 allowed for binding of GATA3 to the *il5* promoter and activation of transcription[143,144,218]. IL-33 also induced protein expression of transcription factors highly expressed by Th2 T cells important for cytokine production, Gfi-1, c-Maf, and NFATc1, in NH cells. Notably, the MAPK pathway was activated and transcription factors were expressed at low levels in unstimulated NH cells but

Figure 22. IL-33 induction of the MAPK pathway and production of IL-5. IL-33 receptor engagement induces p38 MAPK activation and phosphorylation of GATA3, which then bound to the *il5* promoter in NH cells. SB203580, a p38 inhibitor, blocked this pathway. IL-33 was also able to induce acetylation of histones at the IL-5 promoter and transcription factors important for Th2 cytokine production.


IL-33
IL-33R
P
p38
SB203580
P
GATA3
Chromatin remodeling
TF upregulation
Il5
IL-5

not in other IL-5 producing cells such as BMMCs. IL-33 could rapidly activate the MAPK pathway and upregulate transcription factors important in IL-5 production[218], suggesting that the pathways for abundant cytokine expression by NH cells is in a low level state and can be rapidly induced. Consistent with this notion, Id3 is a transcription factor poised for rapid upregulation[219,220] and can be induced by the MAPK pathway[221,222].

As antibodies against phosphorylation specific kinases and intracellular transcription factors become readily available for flow cytometry, future studies exploring the Id3 dependent pathways important for NH cell IL-5 production that are different in Th2 T cells or BMMCs may become more feasible. Mesenteric cells could be isolated from *Id3*$^{-/-}$*Apoe*$^{-/-}$ and control *Apoe*$^{-/-}$ mice and flow cytometry could be performed to identify NH cells and intracellular staining of GATA3. Interestingly, Id3 inhibits expression of GATA3 in T cells[152], suggesting that Id3 dependent NH cell IL-5 production is by a mechanism other than inhibiting GATA3 expression or that Id3 actually induces GATA3 expression in NH cells. Therefore, it would be predicted that in NH cell isolated from Id3-deficient mice, GATA3 expression would be diminished. In addition, only 5,000 NH cells cultured with IL-2 were required for detection of transcript or protein levels of transcription factors or phosphorylation of kinases, suggesting it may be feasible to study how Id3 may regulate IL-5 production by NH cells *in vitro*. In our hands, roughly 20,000 cells can be acquired from pooling five, 24 week old mice by FACS. Initial experiments would include culturing 5,000 NH cells isolated from *Apoe*$^{-/-}$ mice and determining if varying concentrations (0 – 30 μM) of the p38

inhibitor, SB203580, in the presence of IL-33 reduces Id3 expression by real-time PCR and/or Western blotting similar to conditions previously described[218]. If there was reduced Id3 expression, results would indicate that Id3 is downstream of the MAPK pathway and that Id3 may regulate GATA3 transcription. In this assay, IL-5 in NH cells could be measured by intracellular cytokine detection by flow cytometry and in the supernatant by ELISA. It would be expected that with the addition of the p38 inhibitor, there would be decreased levels of IL-5 compared to DMSO. If there was not reduced expression, earlier time points may need to be explored since induction of the MAPK pathway occurred within 30 minutes[218]. Additionally, if Id3 was downstream of p38, then levels of other transcription factors important for production of IL-5 such as Gfi-1, c-Maf, and NFATc1 could be measured in nuclear extracts of NH cells isolated from Id3-deficient and control mice by western blotting after culturing with IL-33. It would be important to compare NH cells to Th2 T cells or BMMCs cells derived from the same mice, to determine if there was differential regulation of transcription factor expression with the loss of Id3 in NH cells but not in Th2 T cells or BMMCs.

Id3 may regulate acetylation of histone H3 at Lys9 and/or Lys14 at the *il5* promoter, which can be induced by IL-33 in NH cells[218]. E2A, an E-protein binding partner for Id3, has been reported to interact with the transcriptional co-activator, p300/CBP, which can then recruit histone acetyltransferases and RNA polymerase II[223,224]. The *e2a* gene encodes two alternatively splice proteins, E12 and E47, but the role for E-proteins has been understudied in ILC lineages. Studies suggest that E2A can restore group 1 and 2 ILC populations in Id2-

deficient mice[225], but it is unknown if E-proteins or other bHLHs can directly regulate transcription by binding to the promoter of *il5*. In other IL-5 expressing lineages, E-proteins are thought to be upstream of factors regulating the IL-5 promoter[173]. Currently, we have *E12*$^{-/-}$ and *E47*$^{-/-}$ mice in the laboratory and future experiments could be to determine if, similar to Id2, either of these proteins are important for group 2 ILC lineage development or if they regulate group 2 ILC function, similar to Id3.

Id3 may regulate B-1b B cells and atherosclerosis

Id3 is important in atherosclerosis potentially by regulating NH cells, smooth muscle cells, B-2 cells[25,167,219,226-228], and potentially B-1b B cells. While the existence of B-1b B cells has been known for three decades[42], only recent evidence suggests that B-1b B cells may have a distinct role from B-1a B cells in immunity[44]. In B cell specific Id3 knockout mice there is a greater number of B-1b B cells and there is no change in the number of B-1a B cells compared to control mice, supporting the concept that B-1a and B-1b B cells are distinctly functioning B-1 B cell subsets. While attenuated atherosclerosis in Western diet fed B cell specific Id3 knockout mice was associated with increased B-1b B cells and T15-Id IgM in the serum compared to control mice, it is unknown if there is increased T15-Id IgM in atherosclerotic lesions. Numerous labs have detected IgM in atherosclerotic plaques by immunohistochemistry including immunofluorescence[83,107,111] and a reduction in plaque IgM content caused by splenectomy could be rescued by the adoptive transfer of B-1a B cells[107].

Furthermore, T15-Id antibodies and other oxidation-specific natural antibodies could be detected in atherosclerotic plaques[110,111,229]. Atherosclerotic lesions of *sIgM.Apoe*$^{-/-}$ or *Rag1*$^{-/-}$*Ldlr*$^{-/-}$ mice served as negative controls in addition to isotype controls[83,111]. To assess if increased serum T15-Id IgM is associated with increased T15-Id in atherosclerotic plaques, frozen sections of aortic roots from 16 week Western diet fed B cell specific Id3 knockout mice and controls could be stained with biotinylated AB1-2, an anti-T15 Id Ab, and compared to control non-immune biotinylated IgG followed by an avidin-biotin–AP complex and phosphatase substrate. We would predict increased T15-Id staining per plaque area with the B cell specific Id3 knockout compared to controls, suggesting that the increased T15-Id mediates enhanced apoptotic cell clearance and reduced macrophage accumulation. We observed increased IgM in the serum of B cell specific Id3 knockout mice but initial attempts to quantify total IgM in atherosclerotic plaques were inconclusive and immunohistochemistry may not be a sensitive enough assay to quantify immunoglobulins in the plaque. An alternative approach to quantifying IgM and T15-Id antibodies may be to elute antibodies from aortas in Western diet fed mice as previously described[230].

Opsonization of apoptotic cells mediated by IgM and T15-Id NAbs requires cooperation with complement factor C1q in activation of the classical complement cascade[63,115,117,193,231] therefore, if we observed increased T15-Id in atherosclerotic plaque, we would also predict that there would be increased C1q deposition. *C1qa.Ldlr*$^{-/-}$ and *sIgM.Ldlr*$^{-/-}$ have increased atherosclerosis compared to control mice. Interestingly, *C1qa.sIgM.Ldlr*$^{-/-}$ mice fed a Western diet did not

have exacerbated atherosclerosis relative to *C1qa.Ldlr*$^{-/-}$ or *sIgM.Ldlr*$^{-/-}$ mice, suggesting that the deletion of C1q and IgM does not have an additive effect on atherosclerosis[83]. However, atherosclerotic lesions of *sIgM.Ldlr*$^{-/-}$ mice did not have less C3 or C5b-9 deposition, which are downstream of both the classic and alternative complement pathways, suggesting that the protective effect of IgM may be independent of complement activation in the arterial wall. Furthermore, there was not an increase in apoptotic cell content in atherosclerotic plaques of *C1aq.sIgM.Ldlr*$^{-/-}$ mice relative to *sIgM.Ldlr*$^{-/-}$ or *C1qa.Ldlr*$^{-/-}$, raising the possibility of compensatory mechanisms in the triple knockout mouse. It is currently unclear how IgM may mediate apoptotic cell clearance, termed 'efferocytosis' *in vivo*, yet, it would still be important to detect T15-Id Ab, and C1q, C3, or C5b-9 deposition by confocal microscopy in atherosclerotic plaques of B cell specific Id3 knockout mice and controls. We would expect increased T15-Id Ab and C1q if apoptotic cell clearance is mediated by the classical pathway, and increased C3 or C5b-9 detection if enhanced apoptotic cell clearance is mediated by complement through activation of either the classical or alternative pathway in B cell specific Id3 knockout mice compared to control mice. The window of protective efferocytosis may be earlier in Western diet induced atherosclerotic plaque formation in this cohort of mice or, complement recruitment and apoptotic cell clearance happens so rapidly, and we may not be able to observe differences in mediators of this pathway but only the end result of fewer apoptotic cells. Nonetheless, results of these studies would help to elucidate the mechanism of IgM mediated atheroprotection *in vivo*.

It is currently unknown if there are increased B-1b B cells in the aortic adventitia of B cell specific Id3 knockout mice and if they are producing T15-Id IgM, providing evidence for a local role of B-1b B cells after Western diet. Using a combined approach of quantifying B-1b B cells by flow cytometry and enumerating anti-T15-Id IgM antibody secreting cells in the stromal vascular fraction of the aorta by ELISPOT, we would anticipate increased numbers of B-1b B cells and anti-T15-Id IgM antibody secreting cells in aortas of B cell specific Id3 knockout mice compared to control mice after Western diet. However, while focus has been on the T15-Id antibodies, there are many other pattern recognition receptors (PRRs) that can mediate apoptotic cell clearance and inhibit macrophage oxLDL uptake, contributing to innate immune protection from atherosclerosis. MDA, oxidized cardiolipin, and 4HNE are all thought to be present on oxLDL and apoptotic cells, and PRRs including other NAbs, TLRs, scavenger receptors, and soluble receptors, can target these oxidation-specific epitopes. It is currently unknown if B-1b B cell TLR expression or production of other natural antibodies are important in atherosclerosis. Furthermore, if the B cell specific loss of Id3 affects expression of PRRs on phagocytes, enhanced phagocyte engulfment of apoptotic cells may also result in fewer apoptotic cells in atherosclerotic plaques of B cell specific Id3 knockout mice. Recent data suggests that B cells may induce monocyte mobilization to the myocardium by producing CCL7 in a murine model of myocardial infarction[232] and B cells can reduce macrophage content in plaques potentially through regulating VCAM-1 expression[167]. It is also currently unknown if B-1b B cells can inhibit atherogenic

monocyte ($Ly6c^{hi}$) recruitment to the plaque, potentially through cytokine production, attributing to the reduced macrophage content in atherosclerotic plaques of B cell specific Id3 knockout mice.

B-1b B cells may also have systemic effects that could influence apoptotic cell clearance and macrophage accumulation in atherosclerotic plaques. Interestingly, there appear to be increased B-1b B cells in draining lymph nodes of the aortas of B cell specific Id3 knockout mice, and if this local environment triggers B-1b B cells to enhance pathogenic monocyte egress from aortas to lymph nodes, which is attributed as an atheroprotective event[233], this may be an additional mechanism for reduced macrophage content in atherosclerotic plaques of B cell specific Id3 knockout mice. An experiment to test this hypothesis would be to transplant aortas as previously described[234] from $CD45.1^{+}$ *Apoe*$^{-/-}$ mice to B cell specific Id3 knockout mice or control mice, with the anticipated result of increased pathogenic $CD45.1^{+}$ monocyte egress to draining lymph nodes in B cell specific Id3 knockout mice compared to controls. While we see a trend in an increase in monocytes in the draining lymph nodes of aortas of B cell specific Id3 knockout mice at baseline (data not provided), however which subset and if this affects atherogenesis is unknown. CCR7 may mediate pathogenic monocyte egress from the aorta to nearby lymphatics[234], it would be interesting to determine if B-1b B cells in aortic lymph nodes produce the ligands for CCR7, CCL19 and CCL21[235]. In addition, monocyte migration to B-1b conditioned media in a transwell assay could also help to the hypothesis that B-1b B cells may induce $Ly6c^{hi}$ monocyte migration. In total, if there were

fewer circulating Ly6c^{hi} monocytes and less recruitment of these monocytes to the plaque, or if B-1b B cells trigger monocyte egress from atherosclerotic plaques, data would provide the first evidence that B-1b B cells may regulate monocyte mobilization in atheroprotection. In addition, apoptotic cells can activate B cells to produce the anti-inflammatory and atheroprotective cytokine, IL-10[236,237]. IL-10 is thought to be atheroprotective by inhibiting macrophage activation and death, and the production of pro-inflammatory cytokines, resulting in inhibition of foam cell formation and apoptosis[238-240]. CD5^{+} B cells have been reported to produce IL-10, yet it is unclear if B-1b B cells can be stimulated to produce IL-10. A simple experiment to test this would be to harvest peritoneal cavity B-1b B cells in B cell specific Id3 knockout and control mice and determine IL-10 intracellular cytokine staining by flow cytometry after stimulation using methods previously described[241]. Additionally, levels of IL-10 in serum of mice could be measured by ELISA in B cell specific Id3 knockout and control mice fed a Western diet. It would be anticipated that there would be increased levels of IL-10 in the serum and/or atherosclerotic plaques of B cell specific Id3 knockout compared to controls, altogether indicating that the loss of Id3 in B cells is atheroprotective by enhancing anti-inflammatory IL-10 production by B-1b B cells, resulting in fewer apoptotic cells and less macrophage content in atherosclerotic lesions.

While the current study suggests that B-1b B cells may be atheroprotective, this, along with the hypothesis that B-1b B cell atheroprotection is IgM mediated remains to be directly tested. FACS purified 100,000 PerC B-1b

B cells from B cell specific Id3 knockout mice, littermate controls, and *sIgM/Apoe*$^{-/-}$ mice could be adoptively transferred to *Rag/Apoe*$^{-/-}$ mice. Recipients, along with mice receiving a PBS vehicle control would be then placed on Western diet for 16 weeks. 100,000 B-1b B cells adoptively transferred to *Rag*$^{-/-}$ mice are able to produce IgM and provide protection from pathogens[186,187]. Additionally, only 30,000 B-1a B cells adoptively transferred to splenectomized mice were able to rescue splenectomy aggravated atherosclerosis. This study will also directly test if the adoptive transfer of B-1b B cells results in fewer apoptotic cells and macrophage content in atherosclerotic lesions and furthermore, if this is dependent on IgM.

Id3 regulates B-1b B cell number

B cell specific Id3 knockout mice have a fourfold increase in the number of B-1b B cells in the peritoneal cavity and circulation yet, it is largely unknown how Id3 may regulate B-1b B cell number. *In vivo* CFSE labeling experiments suggest that Id3 may regulate B-1b B cell proliferation however, future studies will need to be performed to determine if BrdU incorporation is increased in B-1b B cells in B cell specific Id3 knockout mice. BrdU labeling could be determined in B-1b B cells over a time period of days *in vivo* and *in vitro* without stimulus to determine if the loss of Id3 in B cells regulates self-renewal of B-1b B cells. If Id3-deficient B-1b B cells are able to maintain themselves in culture without stimulus in greater numbers relative to wildtype, results would suggest that Id3 may control survival, and/or self-renewal depending on the status of BrdU incorporation, of B-1b B

cells. IL-5, CD40L +IL-4, and LPS are thought to induce proliferation of B-1b B cells[123-125,242] and *in vitro* experiments of B-1b B cells harvested from B cell specific Id3 knockout mice with these stimuli in conjunction with BrdU labeling may reveal if Id3 regulates B-1b B cell proliferation. Furthermore, if there is a difference in proliferation, B-1b B cells could be labeled with DRAQ5 to determine which stage of the cell cycle is altered. It will also be important to determine if Id3 regulates the expression of the IL-5 receptor on B-1b B cells by flow cytometry. Prior studies reveal that Id3 may be downstream of BCR cross-linking, resulting in defective mature B-2 B cell proliferation and clonal expansion in global Id3 deficient mice[154]. However, B-1 B cells do not proliferate in response to BCR crosslinking alone and B-2 and B-1 B cells have different regulation of the cell cycle G1/S checkpoint by cyclin D2 and D3[243]. If prior studies reveal that Id3 regulates self-renewal or proliferation of B-1b B cells at the G1/S checkpoint, future studies could be to explore if Id3 regulates expression of cyclin D2 and D3 in B-1b cells or other known cell cycle genes regulated by Id3 such as p21[219]. A candidate approach in combination with a cell cycle array would provide potential targets of Id3. PD-L1 BCR co-engagement suppresses B-1b B cell proliferation but not survival, and PD-L1 expression may be another target of Id3 in B-1b B cells[72]. Alternatively, there may be increased number of B-1b B cells in B cell specific Id3 knockout mice due to decreased apoptosis. Annexin V can be detected on apoptotic cells by flow cytometry, which can easily be performed on peritoneal cavity B-1b B cells.

B-1b B cells may also be increased in B cell specific Id3 knockout mice due to increased B-1b B cell homing to the peritoneal cavity, spleen and lymph nodes. CCR7 and CXCR5 are important for B-1b B cell homing to the PerC[244-246] and TLR stimulation can induce migration B-1 B cells out of the PerC[247,248]. B-1b B cell homing to the spleen and peri-aortic lymph nodes is largely unknown[249]. It will be important to determine if Id3 regulates expression of the receptors CCR7 and CXCR5 on B-1b B cells or secretion of their ligands. Additionally, there may be a combination of proliferation, survival, apoptosis, and homing regulating increased number of B-1b B cells in B cell specific Id3 knockout mice.

Natural immunity is emerging as a protective process in atherosclerosis and we provide evidence that Id3 may be a key factor in regulating B-1 B cell subsets. Natural IgM antibodies that recognize oxidative epitopes on LDL or phospholipids prevent atherosclerosis and are thought to be produced by the atheroprotective B cell subset, the B-1a B cell. We identify Id3 as an important regulator of B-1a B cell homeostasis through NH cell production of IL-5. In the B cell specific Id3 knockout mouse, B-1a B cell number was unchanged, but we did observe an increase in the number of B-1b B cells and levels of IgM in the serum in baseline mice. Moreover, this phenotype persisted and was associated with attenuated atherosclerosis in Western diet fed mice. As B-1b B cells can undergo antigen dependent clonal expansion in response to TI-2 antigens[75,186,187], it would be very interesting to determine if B-1b B cells deficient in Id3 have the enhanced capacity to expand and produce antigen specific antibodies in response to oxidation-specific epitopes such as PC on POVPC that are important in

atherosclerosis[14,250]. If B-1b B cells were able to undergo enhanced antigen dependent expansion with the loss of Id3, this event would link the increase in B-1b B cell number and protection from atherosclerosis in B cell deficient Id3 knockout mice and potentially identify a novel role for B-1b B cells in atherosclerosis.

Chapter 6

Literature Cited

1 Roger, V. L. *et al.* Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation* **125**, e2-e220, doi:10.1161/CIR.0b013e31823ac046 (2012).

2 Perry, H. M., Bender, T. P. & McNamara, C. A. B cell subsets in atherosclerosis. *Front Immunol* **3**, 373, doi:10.3389/fimmu.2012.00373 (2012).

3 Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868-874, doi:10.1038/nature01323 (2002).

4 Libby, P. Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol* **32**, 2045-2051, doi:10.1161/ATVBAHA.108.179705 (2012).

5 Hansson, G. K. & Hermansson, A. The immune system in atherosclerosis. *Nat Immunol* **12**, 204-212, doi:10.1038/ni.2001 (2011).

6 Lahoute, C., Herbin, O., Mallat, Z. & Tedgui, A. Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nature reviews. Cardiology* **8**, 348-358, doi:10.1038/nrcardio.2011.62 (2011).

7 Keaney, J. F., Jr. Immune modulation of atherosclerosis. *Circulation* **124**, e559-560, doi:10.1161/CIRCULATIONAHA.111.074096 (2011).

8 Libby, P., Ridker, P. M. & Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature* **473**, 317-325, doi:10.1038/nature10146 (2011).

9 Weber, C. & Noels, H. Atherosclerosis: current pathogenesis and therapeutic options. *Nature medicine* **17**, 1410-1422, doi:10.1038/nm.2538 (2011).

10 Lusis, A. J. Atherosclerosis. *Nature* **407**, 233-241, doi:10.1038/35025203 (2000).

11 Hansson, G. K., Robertson, A. K. & Soderberg-Naucler, C. Inflammation and atherosclerosis. *Annual review of pathology* **1**, 297-329, doi:10.1146/annurev.pathol.1.110304.100100 (2006).

12 Chou, M. Y. *et al.* Oxidation-specific epitopes are important targets of innate immunity. *J Intern Med* **263**, 479-488, doi:10.1111/j.1365-2796.2008.01968.x (2008).

13 Steinberg, D. & Witztum, J. L. Oxidized low-density lipoprotein and atherosclerosis. *Arterioscler Thromb Vasc Biol* **30**, 2311-2316, doi:10.1161/ATVBAHA.108.179697 (2010).

14 Miller, Y. I. *et al.* Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res* **108**, 235-248, doi:10.1161/CIRCRESAHA.110.223875 (2011).

15 Weber, C., Zernecke, A. & Libby, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nature reviews. Immunology* **8**, 802-815, doi:10.1038/nri2415 (2008).

16 Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nature reviews. Immunology* **10**, 36-46, doi:10.1038/nri2675 (2010).

17 Murray, P. J. & Wynn, T. A. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology* **11**, 723-737, doi:10.1038/nri3073 (2011).

18 Galkina, E. & Ley, K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* **27**, 165-197, doi:10.1146/annurev.immunol.021908.132620 (2009).

19 Van Vre, E. A., Ait-Oufella, H., Tedgui, A. & Mallat, Z. Apoptotic cell death and efferocytosis in atherosclerosis. *Arterioscler Thromb Vasc Biol* **32**, 887-893, doi:10.1161/ATVBAHA.111.224873 (2012).

20 Sixt, M. *et al.* The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* **22**, 19-29, doi:10.1016/j.immuni.2004.11.013 (2005).

21 Nolte, M. A. *et al.* A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *J Exp Med* **198**, 505-512, doi:10.1084/jem.20021801 (2003).

22 Campbell, K. A. *et al.* Lymphocytes and the adventitial immune response in atherosclerosis. *Circ Res* **110**, 889-900, doi:10.1161/CIRCRESAHA.111.263186 (2012).

23 Weih, F., Gräbner, R., Hu, D., Beer, M. & Habenicht, A. J. Control of Dichotomic Innate and Adaptive Immune Responses by Artery Tertiary Lymphoid Organs in Atherosclerosis. *Frontiers in physiology* **3**, 226, doi:10.3389/fphys.2012.00226 (2012).

24 Galkina, E. *et al.* Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med* **203**, 1273-1282, doi:10.1084/jem.20052205 (2006).

25 Doran, A. C. *et al.* B-cell aortic homing and atheroprotection depend on Id3. *Circ Res* **110**, e1-12, doi:10.1161/CIRCRESAHA.111.256438 (2012).

26 Jongstra-Bilen, J. *et al.* Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis. *J Exp Med* **203**, 2073-2083, doi:10.1084/jem.20060245 (2006).

27 Allbutt, T. C. *Diseases of the arteries, including angina pectoris*. Vol. 1 (Macmillan, 1915).

28 Gerlis, L. M. The significance of adventitial infiltrations in coronary atherosclerosis. *Br Heart J* **18**, 166-172, doi:10.1136/hrt.18.2.166 (1956).

29 Schwartz, C. J. & Mitchell, J. R. Cellular infiltration of the human arterial adventitia associated with atheromatous plaques. *Circulation* **26**, 73-78 (1962).

30 Parums, D. V., Dunn, D. C., Dixon, A. K. & Mitchinson, M. J. Characterization of inflammatory cells in a patient with chronic periaortitis. *The American journal of cardiovascular pathology* **3**, 121-129 (1990).

31 Zhou, X. & Hansson, G. K. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. *Scand J Immunol* **50**, 25-30 (1999).

32 Houtkamp, M. A., de Boer, O. J., van der Loos, C. M., van der Wal, A. C. & Becker, A. E. Adventitial infiltrates associated with advanced

atherosclerotic plaques: structural organization suggests generation of local humoral immune responses. *The Journal of pathology* **193**, 263-269, doi:10.1002/1096-9896(2000)9999:9999<::AID-PATH774>3.0.CO;2-N (2001).

33 Palinski, W., Miller, E. & Witztum, J. L. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A* **92**, 821-825 (1995).

34 Freigang, S., Hörkkö, S., Miller, E., Witztum, J. L. & Palinski, W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. *Arterioscler Thromb Vasc Biol* **18**, 1972-1982 (1998).

35 Nicoletti, A., Kaveri, S., Caligiuri, G., Bariéty, J. & Hansson, G. K. Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Invest* **102**, 910-918, doi:10.1172/JCI119892 (1998).

36 Zhou, X., Caligiuri, G., Hamsten, A., Lefvert, A. K. & Hansson, G. K. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler Thromb Vasc Biol* **21**, 108-114 (2001).

37 Binder, C. J. *et al.* Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med* **9**, 736-743, doi:10.1038/nm876 (2003).

38 Faria-Neto, J. R. *et al.* Passive immunization with monoclonal IgM antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein E-null mice. *Atherosclerosis* **189**, 83-90, doi:10.1016/j.atherosclerosis.2005.11.033 (2006).

39 Ameli, S. *et al.* Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol* **16**, 1074-1079 (1996).

40 Binder, C. J. *et al.* IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest* **114**, 427-437, doi:10.1172/JCI20479 (2004).

41 Montecino-Rodriguez, E. & Dorshkind, K. B-1 B cell development in the fetus and adult. *Immunity* **36**, 13-21, doi:10.1016/j.immuni.2011.11.017 (2012).

42 Kantor, A. B. & Herzenberg, L. A. Origin of murine B cell lineages. *Annu Rev Immunol* **11**, 501-538, doi:10.1146/annurev.iy.11.040193.002441 (1993).

43 Herzenberg, L. A. & Tung, J. W. B cell lineages: documented at last! *Nat Immunol* **7**, 225-226, doi:10.1038/ni0306-225 (2006).

44 Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* **11**, 34-46, doi:10.1038/nri2901 (2011).

45 Rothstein, T. L. Cutting edge commentary: two B-1 or not to be one. *J Immunol* **168**, 4257-4261 (2002).

46 Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* **381**, 751-758, doi:10.1038/381751a0 (1996).

47 Fairfax, K. A., Kallies, A., Nutt, S. L. & Tarlinton, D. M. Plasma cell development: from B-cell subsets to long-term survival niches. *Seminars in immunology* **20**, 49-58, doi:10.1016/j.smim.2007.12.002 (2008).

48 Tarlinton, D. B-cell memory: are subsets necessary? *Nat Rev Immunol* **6**, 785-790, doi:10.1038/nri1938 (2006).

49 Allen, C. D., Okada, T. & Cyster, J. G. Germinal-center organization and cellular dynamics. *Immunity* **27**, 190-202, doi:10.1016/j.immuni.2007.07.009 (2007).

50 Pillai, S., Cariappa, A. & Moran, S. T. Marginal zone B cells. *Annu Rev Immunol* **23**, 161-196, doi:10.1146/annurev.immunol.23.021704.115728 (2005).

51 Martin, F. & Kearney, J. F. Marginal-zone B cells. *Nat Rev Immunol* **2**, 323-335, doi:10.1038/nri799 (2002).

52 Barber, C. L., Montecino-Rodriguez, E. & Dorshkind, K. Reduced production of B-1-specified common lymphoid progenitors results in

diminished potential of adult marrow to generate B-1 cells. *Proc Natl Acad Sci U S A* **108**, 13700-13704, doi:10.1073/pnas.1107172108 (2011).

53 Montecino-Rodriguez, E., Leathers, H. & Dorshkind, K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol* **7**, 293-301, doi:10.1038/ni1301 (2006).

54 Ghosn, E. E., Sadate-Ngatchou, P., Yang, Y., Herzenberg, L. A. & Herzenberg, L. A. Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen. *Proc Natl Acad Sci U S A* **108**, 2879-2884, doi:10.1073/pnas.1019764108 (2011).

55 Esplin, B. L., Welner, R. S., Zhang, Q., Borghesi, L. A. & Kincade, P. W. A differentiation pathway for B1 cells in adult bone marrow. *Proc Natl Acad Sci U S A* **106**, 5773-5778, doi:10.1073/pnas.0811632106 (2009).

56 Holodick, N. E., Repetny, K., Zhong, X. & Rothstein, T. L. Adult BM generates CD5+ B1 cells containing abundant N-region additions. *European journal of immunology* **39**, 2383-2394, doi:10.1002/eji.200838920 (2009).

57 Hardy, R. R. B-1 B cell development. *J Immunol* **177**, 2749-2754 (2006).

58 Tumang, J. R., Hastings, W. D., Bai, C. & Rothstein, T. L. Peritoneal and splenic B-1 cells are separable by phenotypic, functional, and transcriptomic characteristics. *European journal of immunology* **34**, 2158-2167, doi:10.1002/eji.200424819 (2004).

59 Forster, I. *et al.* CD5 B cells in the mouse. *Current topics in microbiology and immunology* **173**, 247-251 (1991).

60 Mond, J. J., Lees, A. & Snapper, C. M. T cell-independent antigens type 2. *Annu Rev Immunol* **13**, 655-692, doi:10.1146/annurev.iy.13.040195.003255 (1995).

61 Mond, J. J., Vos, Q., Lees, A. & Snapper, C. M. T cell independent antigens. *Current opinion in immunology* **7**, 349-354 (1995).

62 Baumgarth, N. *et al.* Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* **96**, 2250-2255 (1999).

63 Ehrenstein, M. R. & Notley, C. A. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* **10**, 778-786, doi:10.1038/nri2849 (2010).

64 Hardy, R. R., Carmack, C. E., Shinton, S. A., Riblet, R. J. & Hayakawa, K. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J Immunol* **142**, 3643-3651 (1989).

65 Mercolino, T. J. *et al.* Restricted immunoglobulin variable region gene usage by normal Ly-1 (CD5+) B cells that recognize phosphatidyl choline. *J Exp Med* **169**, 1869-1877 (1989).

66 Pennell, C. A. *et al.* Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. *European journal of immunology* **19**, 1289-1295, doi:10.1002/eji.1830190721 (1989).

67 Rowley, B., Tang, L., Shinton, S., Hayakawa, K. & Hardy, R. R. Autoreactive B-1 B cells: constraints on natural autoantibody B cell

antigen receptors. *Journal of autoimmunity* **29**, 236-245, doi:10.1016/j.jaut.2007.07.020 (2007).

68 Wang, H. & Clarke, S. H. Positive selection focuses the VH12 B-cell repertoire towards a single B1 specificity with survival function. *Immunological reviews* **197**, 51-59 (2004).

69 Pillai, S. & Cariappa, A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* **9**, 767-777, doi:10.1038/nri2656 (2009).

70 Hardy, R. R. & Hayakawa, K. B cell development pathways. *Annu Rev Immunol* **19**, 595-621, doi:10.1146/annurev.immunol.19.1.595 (2001).

71 Hsu, M. C., Toellner, K. M., Vinuesa, C. G. & Maclennan, I. C. B cell clones that sustain long-term plasmablast growth in T-independent extrafollicular antibody responses. *Proc Natl Acad Sci U S A* **103**, 5905-5910, doi:10.1073/pnas.0601502103 (2006).

72 Haas, K. M. Programmed cell death 1 suppresses B-1b cell expansion and long-lived IgG production in response to T cell-independent type 2 antigens. *J Immunol* **187**, 5183-5195, doi:10.4049/jimmunol.1101990 (2011).

73 Foote, J. B. & Kearney, J. F. Generation of B cell memory to the bacterial polysaccharide alpha-1,3 dextran. *J Immunol* **183**, 6359-6368, doi:10.4049/jimmunol.0902473 (2009).

74 Obukhanych, T. V. & Nussenzweig, M. C. T-independent type II immune responses generate memory B cells. *J Exp Med* **203**, 305-310, doi:10.1084/jem.20052036 (2006).

75 Alugupalli, K. R. A distinct role for B1b lymphocytes in T cell-independent immunity. *Current topics in microbiology and immunology* **319**, 105-130 (2008).

76 Mauri, C. & Bosma, A. Immune regulatory function of B cells. *Annu Rev Immunol* **30**, 221-241, doi:10.1146/annurev-immunol-020711-074934 (2012).

77 Lund, F. E. & Randall, T. D. Effector and regulatory B cells: modulators of CD4(+) T cell immunity. *Nat Rev Immunol* **10**, 236-247, doi:10.1038/nri2729 (2010).

78 Klinker, M. W. & Lundy, S. K. Multiple mechanisms of immune suppression by B lymphocytes. *Mol Med* **18**, 123-137, doi:10.2119/molmed.2011.00333 (2012).

79 Mauri, C. & Blair, P. A. Regulatory B cells in autoimmunity: developments and controversies. *Nature reviews. Rheumatology* **6**, 636-643, doi:10.1038/nrrheum.2010.140 (2010).

80 Bouaziz, J. D., Yanaba, K. & Tedder, T. F. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunological reviews* **224**, 201-214, doi:10.1111/j.1600-065X.2008.00661.x (2008).

81 Caligiuri, G., Nicoletti, A., Poirier, B. & Hansson, G. K. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest* **109**, 745-753, doi:10.1172/JCI7272 (2002).

82 Major, A. S., Fazio, S. & Linton, M. F. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol* **22**, 1892-1898 (2002).

83 Lewis, M. J. *et al.* Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* **120**, 417-426, doi:10.1161/CIRCULATIONAHA.109.868158 (2009).

84 Kyaw, T. *et al.* Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol* **185**, 4410-4419, doi:10.4049/jimmunol.1000033 (2010).

85 Ait-Oufella, H. *et al.* B cell depletion reduces the development of atherosclerosis in mice. *J Exp Med* **207**, 1579-1587, doi:10.1084/jem.20100155 (2010).

86 Kyaw, T. *et al.* BAFF Receptor mAb Treatment Ameliorates Development and Progression of Atherosclerosis in Hyperlipidemic ApoE(-/-) Mice. *PLoS One* **8**, e60430, doi:10.1371/journal.pone.0060430 (2013).

87 Sage, A. P. *et al.* BAFF Receptor Deficiency Reduces the Development of Atherosclerosis in Mice--Brief Report. *Arterioscler Thromb Vasc Biol* **32**, 1573-1576, doi:10.1161/ATVBAHA.111.244731 (2012).

88 Kyaw, T. *et al.* Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS ONE* **7**, e29371, doi:10.1371/journal.pone.0029371 (2012).

89 Mackay, F. & Browning, J. L. BAFF: a fundamental survival factor for B cells. *Nat Rev Immunol* **2**, 465-475, doi:10.1038/nri844 (2002).

90 Sasaki, Y., Casola, S., Kutok, J. L., Rajewsky, K. & Schmidt-Supprian, M. TNF Family Member B Cell-Activating Factor (BAFF) Receptor-Dependent and -Independent Roles for BAFF in B Cell Physiology. *The Journal of Immunology* **173**, 2245-2252 (2004).

91 Danzaki, K. *et al.* Interleukin-17A deficiency accelerates unstable atherosclerotic plaque formation in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **32**, 273-280, doi:10.1161/ATVBAHA.111.229997 (2012).

92 Taleb, S. *et al.* Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J Exp Med* **206**, 2067-2077, doi:10.1084/jem.20090545 (2009).

93 Erbel, C. *et al.* Inhibition of IL-17A attenuates atherosclerotic lesion development in apoE-deficient mice. *J Immunol* **183**, 8167-8175, doi:10.4049/jimmunol.0901126 (2009).

94 Smith, E. *et al.* Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **121**, 1746-1755, doi:10.1161/CIRCULATIONAHA.109.924886 (2010).

95 Gao, Q. *et al.* A critical function of Th17 proinflammatory cells in the development of atherosclerotic plaque in mice. *J Immunol* **185**, 5820-5827, doi:10.4049/jimmunol.1000116 (2010).

96 van Es, T. *et al.* Attenuated atherosclerosis upon IL-17R signaling disruption in LDLr deficient mice. *Biochemical and biophysical research communications* **388**, 261-265, doi:10.1016/j.bbrc.2009.07.152 (2009).

97 Butcher, M. J., Gjurich, B. N., Phillips, T. & Galkina, E. V. The IL-17A/IL-17RA axis plays a proatherogenic role via the regulation of aortic myeloid cell recruitment. *Circ Res* **110**, 675-687, doi:10.1161/CIRCRESAHA.111.261784 (2012).

98 Dumitriu, I. E. & Kaski, J. C. The role of T and B cells in atherosclerosis: potential clinical implications. *Current pharmaceutical design* **17**, 4159-4171 (2011).

99 Taleb, S., Tedgui, A. & Mallat, Z. Adaptive T cell immune responses and atherogenesis. *Current opinion in pharmacology* **10**, 197-202, doi:10.1016/j.coph.2010.02.003 (2010).

100 Zhou, X. CD4+ T cells in atherosclerosis. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **57**, 287-291 (2003).

101 Tsimikas, S. *et al.* Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *J Lipid Res* **48**, 425-433, doi:10.1194/jlr.M600361-JLR200 (2007).

102 Hernandez-Vargas, P. *et al.* Fcgamma receptor deficiency confers protection against atherosclerosis in apolipoprotein E knockout mice. *Circ Res* **99**, 1188-1196, doi:10.1161/01.RES.0000250556.07796.6c (2006).

103 Mendez-Fernandez, Y. V. *et al.* The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. *Atherosclerosis* **214**, 73-80, doi:10.1016/j.atherosclerosis.2010.10.018 (2011).

104 Kelly, J. A. *et al.* Inhibition of arterial lesion progression in CD16-deficient mice: evidence for altered immunity and the role of IL-10. *Cardiovascular research* **85**, 224-231, doi:10.1093/cvr/cvp300 (2010).

105 Wang, J. *et al.* IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J Clin Invest* **121**, 3564-3577, doi:10.1172/JCI46028 (2011).

106 Binder, C. J. *et al.* The role of natural antibodies in atherogenesis. *J Lipid Res* **46**, 1353-1363, doi:10.1194/jlr.R500005-JLR200 (2005).

107 Kyaw, T. *et al.* B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res* **109**, 830-840, doi:10.1161/CIRCRESAHA.111.248542 (2011).

108 Chang, M. K. *et al.* Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A* **96**, 6353-6358 (1999).

109 Hörkkö, S. *et al.* Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest* **103**, 117-128, doi:10.1172/JCI4533 (1999).

110 Shaw, P. X. *et al.* Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* **105**, 1731-1740, doi:10.1172/JCI8472 (2000).

111 Chou, M. Y. *et al.* Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* **119**, 1335-1349, doi:10.1172/JCI36800 (2009).

112 Chang, M. K. *et al.* Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *J Exp Med* **200**, 1359-1370, doi:10.1084/jem.20031763 (2004).

113 Palinski, W. *et al.* Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest* **98**, 800-814, doi:10.1172/JCI118853 (1996).

114 Bird, D. A. *et al.* Receptors for oxidized low-density lipoprotein on elicited mouse peritoneal macrophages can recognize both the modified lipid moieties and the modified protein moieties: implications with respect to macrophage recognition of apoptotic cells. *Proc Natl Acad Sci U S A* **96**, 6347-6352 (1999).

115 Chen, Y., Park, Y. B., Patel, E. & Silverman, G. J. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* **182**, 6031-6043, doi:10.4049/jimmunol.0804191 (2009).

116 Huber, J. *et al.* Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arterioscler Thromb Vasc Biol* **22**, 101-107 (2002).

117 Grönwall, C., Vas, J. & Silverman, G. J. Protective Roles of Natural IgM Antibodies. *Front Immunol* **3**, 66, doi:10.3389/fimmu.2012.00066 (2012).

118 Seimon, T. & Tabas, I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res* **50 Suppl**, S382-387, doi:10.1194/jlr.R800032-JLR200 (2009).

119 Holodick, N. E., Tumang, J. R. & Rothstein, T. L. Immunoglobulin secretion by B1 cells: differential intensity and IRF4-dependence of spontaneous IgM secretion by peritoneal and splenic B1 cells. *European journal of immunology* **40**, 3007-3016, doi:10.1002/eji.201040545 (2010).

120 O'Garra, A. *et al.* Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *European journal of immunology* **22**, 711-717, doi:10.1002/eji.1830220314 (1992).

121 Viau, M. & Zouali, M. B-lymphocytes, innate immunity, and autoimmunity. *Clin Immunol* **114**, 17-26, doi:10.1016/j.clim.2004.08.019 (2005).

122 Lichtman, A. H., Binder, C. J., Tsimikas, S. & Witztum, J. L. Adaptive immunity in atherogenesis: new insights and therapeutic approaches. *J Clin Invest* **123**, 27-36, doi:10.1172/JCI63108 (2013).

123 Miller, A. M. *et al.* IL-33 reduces the development of atherosclerosis. *J Exp Med* **205**, 339-346, doi:10.1084/jem.20071868 (2008).

124 Erickson, L. D., Foy, T. M. & Waldschmidt, T. J. Murine B1 B cells require IL-5 for optimal T cell-dependent activation. *J Immunol* **166**, 1531-1539 (2001).

125 Moon, B. G., Takaki, S., Miyake, K. & Takatsu, K. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. *J Immunol* **172**, 6020-6029 (2004).

126 Miller, A. M. & Liew, F. Y. The IL-33/ST2 pathway--A new therapeutic target in cardiovascular disease. *Pharmacology & therapeutics* **131**, 179-186, doi:10.1016/j.pharmthera.2011.02.005 (2011).

127 Spits, H. & Di Santo, J. P. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol* **12**, 21-27, doi:10.1038/ni.1962 (2011).

128 Monticelli, L. A., Sonnenberg, G. F. & Artis, D. Innate lymphoid cells: critical regulators of allergic inflammation and tissue repair in the lung. *Current opinion in immunology* **24**, 284-289, doi:10.1016/j.coi.2012.03.012 (2012).

129 Mjosberg, J., Bernink, J., Peters, C. & Spits, H. Transcriptional control of innate lymphoid cells. *European journal of immunology* **42**, 1916-1923, doi:10.1002/eji.201242639 (2012).

130 Mjosberg, J. M. *et al.* Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* **12**, 1055-1062, doi:10.1038/ni.2104 (2011).

131 Monticelli, L. A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* **12**, 1045-1054, doi:10.1031/ni.2131 (2011).

132 Mjosberg, J. & Spits, H. Type 2 innate lymphoid cells-new members of the "type 2 franchise" that mediate allergic airway inflammation. *European journal of immunology* **42**, 1093-1096, doi:10.1002/eji.201242549 (2012).

133 Tait Wojno, E. D. & Artis, D. Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine. *Cell host & microbe* **12**, 445-457, doi:10.1016/j.chom.2012.10.003 (2012).

134 Koyasu, S. & Moro, K. Role of innate lymphocytes in infection and inflammation. *Front Immunol* **3**, 101, doi:10.3389/fimmu.2012.00101 (2012).

135 Koyasu, S. & Moro, K. Natural "helper" cells in the lung: good or bad help? *Immunity* **36**, 317-319, doi:10.1016/j.immuni.2012.03.001 (2012).

136 Koyasu, S., Moro, K., Tanabe, M. & Takeuchi, T. Natural helper cells: a new player in the innate immune response against helminth infection. *Adv Immunol* **108**, 21-44, doi:10.1016/B978-0-12-380995-7.00002-1 (2010).

137 Chang, Y. J. *et al.* Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol* **12**, 631-638, doi:10.1038/ni.2045 (2011).

138 Halim, T. Y., Krauss, R. H., Sun, A. C. & Takei, F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* **36**, 451-463, doi:10.1016/j.immuni.2011.12.020 (2012).

139 Halim, T. Y. *et al.* Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity* **37**, 463-474, doi:10.1016/j.immuni.2012.06.012 (2012).

140 Moro, K. *et al.* Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463**, 540-544, doi:10.1038/nature08636 (2010).

141 Fallon, P. G. *et al.* Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med* **203**, 1105-1116, doi:10.1084/jem.20051615 (2006).

142 Yang, Q., Saenz, S. A., Zlotoff, D. A., Artis, D. & Bhandoola, A. Cutting edge: Natural helper cells derive from lymphoid progenitors. *J Immunol* **187**, 5505-5509, doi:10.4049/jimmunol.1102039 (2011).

143 Hoyler, T. *et al.* The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity* **37**, 634-648, doi:10.1016/j.immuni.2012.06.020 (2012).

144 Mjosberg, J. *et al.* The Transcription Factor GATA3 Is Essential for the Function of Human Type 2 Innate Lymphoid Cells. *Immunity* **37**, 649-659, doi:10.1016/j.immuni.2012.08.015 (2012).

145 Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. & Weintraub, H. The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49-59 (1990).

146 Jones, S. An overview of the basic helix-loop-helix proteins. *Genome Biology* **5**, 226 (2004).

147 Ruzinova, M. B. & Benezra, R. Id proteins in development, cell cycle and cancer. *Trends in Cell Biology* **13**, 410-418 (2003).

148 Ji, Y. *et al.* Repression of the DNA-binding inhibitor Id3 by Blimp-1 limits the formation of memory CD8+ T cells. *Nat Immunol* **12**, 1230-1237, doi:10.1038/ni.2153 (2011).

149 Jones-Mason, M. E. *et al.* E protein transcription factors are required for the development of CD4(+) lineage T cells. *Immunity* **36**, 348-361, doi:10.1016/j.immuni.2012.02.010 (2012).

150 Lauritsen, J. P. *et al.* Marked induction of the helix-loop-helix protein Id3 promotes the gammadelta T cell fate and renders their functional maturation Notch independent. *Immunity* **31**, 565-575, doi:10.1016/j.immuni.2009.07.010 (2009).

151 Li, J. *et al.* Mutation of inhibitory helix-loop-helix protein Id3 causes gammadelta T-cell lymphoma in mice. *Blood* **116**, 5615-5621, doi:10.1182/blood-2010-03-274506 (2010).

152 Maruyama, T. *et al.* Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. *Nat Immunol* **12**, 86-95, doi:10.1038/ni.1965 (2011).

153 Miyazaki, M. *et al.* The opposing roles of the transcription factor E2A and its antagonist Id3 that orchestrate and enforce the naive fate of T cells. *Nat Immunol* **12**, 992-1001, doi:10.1038/ni.2086 (2011).

154 Pan, L., Sato, S., Frederick, J. P., Sun, X. H. & Zhuang, Y. Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. *Mol Cell Biol* **19**, 5969-5980 (1999).

155 Yang, C. Y. *et al.* The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nat Immunol* **12**, 1221-1229, doi:10.1038/ni.2158 (2011).

156 Lin, Y. C. *et al.* A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat Immunol* **11**, 635-643, doi:10.1038/ni.1891 (2010).

157 Murre, C. Helix-loop-helix proteins and lymphocyte development. *Nat Immunol* **6**, 1079-1086, doi:10.1038/ni1260 (2005).

158 Langlands, K., Yin, X., Anand, G. & Prochownik, E. V. Differential Interactions of Id Proteins with Basic-Helix-Loop-Helix Transcription Factors. *Journal of Biological Chemistry* **272**, 19785-19793, doi:10.1074/jbc.272.32.19785 (1997).

159 Norton, J. D., Deed, R. W., Craggs, G. & Sablitzky, F. Id helix--loop--helix proteins in cell growth and differentiation. *Trends in Cell Biology* **8**, 58-65 (1998).

160 Quong, M. W. *et al.* Receptor Editing and Marginal Zone B Cell Development Are Regulated by the Helix-Loop-Helix Protein, E2A. *J. Exp. Med.* **199**, 1101-1112, doi:10.1084/jem.20031180 (2004).

161 Forrest, S. T., Barringhaus, K. G., Perlegas, D., Hammarskjold, M.-L. & McNamara, C. A. Intron Retention Generates a Novel Id3 Isoform That Inhibits Vascular Lesion Formation. *Journal of Biological Chemistry* **279**, 32897-32903, doi:10.1074/jbc.M404882200 (2004).

162 Guo, Z. *et al.* Modeling Sjogren's syndrome with Id3 conditional knockout mice. *Immunology letters* **135**, 34-42, doi:10.1016/j.imlet.2010.09.009 (2011).

163 Hayakawa, I., Tedder, T. F. & Zhuang, Y. B-lymphocyte depletion ameliorates Sjögren's syndrome in Id3 knockout mice. *Immunology* **122**, 73-79, doi:10.1111/j.1365-2567.2007.02614.x (2007).

164 Kee, B. L., Rivera, R. R. & Murre, C. Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-[beta]. *Nat Immunol* **2**, 242-247 (2001).

165 Sayegh, C. E., Quong, M. W., Agata, Y. & Murre, C. E-proteins directly regulate expression of activation-induced deaminase in mature B cells. *Nat Immunol* **4**, 586-593, doi:10.1038/ni923 (2003).

166 Honjo, T., Kinoshita, K. & Muramatsu, M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu Rev Immunol* **20**, 165-196, doi:10.1146/annurev.immunol.20.090501.112049 (2002).

167 Lipinski, M. J. *et al.* Loss of id3 increases vcam-1 expression, macrophage accumulation, and atherogenesis in ldlr-/- mice. *Arterioscler Thromb Vasc Biol* **32**, 2855-2861, doi:10.1161/ATVBAHA.112.300352 (2012).

168 Dyer, K. D. *et al.* Defective eosinophil hematopoiesis ex vivo in inbred Rocky Mountain White (IRW) mice. *Journal of leukocyte biology* **90**, 1101-1109, doi:10.1189/jlb.0211059 (2011).

169 Stolarski, B., Kurowska-Stolarska, M., Kewin, P., Xu, D. & Liew, F. Y. IL-33 exacerbates eosinophil-mediated airway inflammation. *J Immunol* **185**, 3472-3480, doi:10.4049/jimmunol.1000730 (2010).

170 Doran, A. C. *et al.* Id3 is a novel atheroprotective factor containing a functionally significant single-nucleotide polymorphism associated with intima-media thickness in humans. *Circ Res* **106**, 1303-1311, doi:10.1161/CIRCRESAHA.109.210294 (2010).

171 Boullier, A. *et al.* Phosphocholine as a pattern recognition ligand for CD36. *J Lipid Res* **46**, 969-976, doi:10.1194/jlr.M400496-JLR200 (2005).

172 Kopf, M. *et al.* IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* **4**, 15-24 (1996).

173 Kee, B. L. E and ID proteins branch out. *Nat Rev Immunol* **9**, 175-184, doi:10.1038/nri2507 (2009).

174 Fahl, S. P., Crittenden, R. B., Allman, D. & Bender, T. P. c-Myb is required for pro-B cell differentiation. *J Immunol* **183**, 5582-5592, doi:10.4049/jimmunol.0901187 (2009).

175 Rajsheker, S. *et al.* Crosstalk between perivascular adipose tissue and blood vessels. *Current opinion in pharmacology* **10**, 191-196, doi:10.1016/j.coph.2009.11.005 (2010).

176 Grabner, R. *et al.* Lymphotoxin beta receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged ApoE-/- mice. *J Exp Med* **206**, 233-248, doi:10.1084/jem.20080752 (2009).

177 Smithgall, M. D. *et al.* IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* **20**, 1019-1030, doi:10.1093/intimm/dxn060 (2008).

178 Allakhverdi, Z., Smith, D. E., Comeau, M. R. & Delespesse, G. Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol* **179**, 2051-2054 (2007).

179 Hamze, M. *et al.* Characterization of Resident B Cells of Vascular Walls in Human Atherosclerotic Patients. *J Immunol*, doi:10.4049/jimmunol.1202870 (2013).

180 Koltsova, E. K. *et al.* Dynamic T cell-APC interactions sustain chronic inflammation in atherosclerosis. *J Clin Invest* **122**, 3114-3126, doi:10.1172/JCI61758 (2012).

181 Huan, T. *et al.* A systems biology framework identifies molecular underpinnings of coronary heart disease. *Arterioscler Thromb Vasc Biol* **33**, 1427-1434, doi:10.1161/ATVBAHA.112.300112 (2013).

182 Welch, C. L. *et al.* Localization of atherosclerosis susceptibility loci to chromosomes 4 and 6 using the Ldlr knockout mouse model. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 7946-7951, doi:10.1073/pnas.141239098 (2001).

183 Witztum, J. L. & Lichtman, A. H. The Influence of Innate and Adaptive Immune Responses on Atherosclerosis. *Annual review of pathology*, doi:10.1146/annurev-pathol-020712-163936 (2013).

184 Swirski, F. K. & Nahrendorf, M. Leukocyte Behavior in Atherosclerosis, Myocardial Infarction, and Heart Failure. *Science* **339**, 161-166, doi:10.1126/science.1230719 (2013).

185 Kyaw, T., Tipping, P., Toh, B. H. & Bobik, A. Current understanding of the role of B cell subsets and intimal and adventitial B cells in atherosclerosis. *Curr Opin Lipidol* **22**, 373-379, doi:10.1097/MOL.0b013e32834adaf3 (2011).

186 Alugupalli, K. R. *et al.* B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* **21**, 379-390, doi:10.1016/j.immuni.2004.06.019 (2004).

187 Haas, K. M., Poe, J. C., Steeber, D. A. & Tedder, T. F. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity* **23**, 7-18, doi:10.1016/j.immuni.2005.04.011 (2005).

188 Binder, C. J. & Silverman, G. J. Natural antibodies and the autoimmunity of atherosclerosis. *Springer Semin Immunopathol* **26**, 385-404, doi:10.1007/s00281-004-0185-z (2005).

189 Stoletov, K. *et al.* Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. *Circ Res* **104**, 952-960, doi:10.1161/CIRCRESAHA.108.189803 (2009).

190 Fang, L. *et al.* Oxidized cholesteryl esters and phospholipids in zebrafish larvae fed a high cholesterol diet: macrophage binding and activation. *The Journal of biological chemistry* **285**, 32343-32351, doi:10.1074/jbc.M110.137257 (2010).

191 Alugupalli, K. R. & Gerstein, R. M. Divide and conquer: division of labor by B-1 B cells. *Immunity* **23**, 1-2, doi:10.1016/j.immuni.2005.07.001 (2005).

192 Kawahara, T., Ohdan, H., Zhao, G., Yang, Y. G. & Sykes, M. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. *J Immunol* **171**, 5406-5414 (2003).

193 Ogden, C. A., Kowalewski, R., Peng, Y., Montenegro, V. & Elkon, K. B. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. *Autoimmunity* **38**, 259-264 (2005).

194 Moore, K. J., Sheedy, F. J. & Fisher, E. A. Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol* **13**, 709-721, doi:10.1038/nri3520 (2013).

195 Reardon, C. A. *et al.* Autoantibodies to OxLDL fail to alter the clearance of injected OxLDL in apolipoprotein E-deficient mice. *J Lipid Res* **45**, 1347-1354, doi:10.1194/jlr.M400075-JLR200 (2004).

196 Chan, R. K. *et al.* Ischaemia-reperfusion is an event triggered by immune complexes and complement. *The British journal of surgery* **90**, 1470-1478, doi:10.1002/bjs.4408 (2003).

197 Briley-Saebo, K. C. *et al.* Targeted molecular probes for imaging atherosclerotic lesions with magnetic resonance using antibodies that recognize oxidation-specific epitopes. *Circulation* **117**, 3206-3215, doi:10.1161/CIRCULATIONAHA.107.757120 (2008).

198 Liu, Z. & Davidson, A. Taming lupus-a new understanding of pathogenesis is leading to clinical advances. *Nat Med* **18**, 871-882, doi:10.1038/nm.2752 (2012).

199 Weih, F., Grabner, R., Hu, D., Beer, M. & Habenicht, A. J. Control of dichotomic innate and adaptive immune responses by artery tertiary lymphoid organs in atherosclerosis. *Frontiers in physiology* **3**, 226, doi:10.3389/fphys.2012.00226 (2012).

200 Watanabe, N. *et al.* Migration and differentiation of autoreactive B-1 cells induced by activated gamma/delta T cells in antierythrocyte immunoglobulin transgenic mice. *J Exp Med* **192**, 1577-1586 (2000).

201 Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* **350**, 423-426, doi:10.1038/350423a0 (1991).

202 Moulin, V. *et al.* B lymphocytes regulate dendritic cell (DC) function in vivo: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. *J Exp Med* **192**, 475-482 (2000).

203 Rivera, A., Chen, C. C., Ron, N., Dougherty, J. P. & Ron, Y. Role of B cells as antigen-presenting cells in vivo revisited: antigen-specific B cells are essential for T cell expansion in lymph nodes and for systemic T cell responses to low antigen concentrations. *Int Immunol* **13**, 1583-1593 (2001).

204 Baumgarth, N., Jager, G. C., Herman, O. C. & Herzenberg, L. A. CD4+ T cells derived from B cell-deficient mice inhibit the establishment of peripheral B cell pools. *Proc Natl Acad Sci U S A* **97**, 4766-4771 (2000).

205 Nilsson, J., Wigren, M. & Shah, P. K. Vaccines against atherosclerosis. *Expert review of vaccines* **12**, 311-321, doi:10.1586/erv.13.4 (2013).

206 Libby, P. & Theroux, P. Pathophysiology of coronary artery disease. *Circulation* **111**, 3481-3488, doi:10.1161/CIRCULATIONAHA.105.537878 (2005).

207 Nadeau, J. H. & Dudley, A. M. Genetics. Systems genetics. *Science* **331**, 1015-1016, doi:10.1126/science.1203869 (2011).

208 Bendall, S. C. *et al.* Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* **332**, 687-696, doi:10.1126/science.1198704 (2011).

209 Maecker, H. T., McCoy, J. P. & Nussenblatt, R. Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol* **12**, 191-200, doi:10.1038/nri3158 (2012).

210 Kaminski, D. A., Wei, C., Qian, Y., Rosenberg, A. F. & Sanz, I. Advances in human B cell phenotypic profiling. *Front Immunol* **3**, 302, doi:10.3389/fimmu.2012.00302 (2012).

211 Caligiuri, G. *et al.* Phosphorylcholine-targeting immunization reduces atherosclerosis. *Journal of the American College of Cardiology* **50**, 540-546, doi:10.1016/j.jacc.2006.11.054 (2007).

212 Sampi, M. *et al.* Plasma interleukin-5 levels are related to antibodies binding to oxidized low-density lipoprotein and to decreased subclinical atherosclerosis. *Journal of the American College of Cardiology* **52**, 1370-1378, doi:10.1016/j.jacc.2008.06.047 (2008).

213 Frostegard, J. Immunity, atherosclerosis and cardiovascular disease. *BMC medicine* **11**, 117, doi:10.1186/1741-7015-11-117 (2013).

214 Caidahl, K. *et al.* IgM-phosphorylcholine autoantibodies and outcome in acute coronary syndromes. *International journal of cardiology* **167**, 464-469, doi:10.1016/j.ijcard.2012.01.018 (2013).

215 Tsimikas, S., Palinski, W. & Witztum, J. L. Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized

LDL in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol* **21**, 95-100 (2001).

216 Fiskesund, R. *et al.* IgM phosphorylcholine antibodies inhibit cell death and constitute a strong protection marker for atherosclerosis development, particularly in combination with other auto-antibodies against modified LDL. *Results in Immunology* **2**, 13-18, doi:10.1016/j.rinim.2012.01.001 (2012).

217 Libby, P., Lichtman, A. H. & Hansson, G. K. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* **38**, 1092-1104, doi:10.1016/j.immuni.2013.06.009 (2013).

218 Furusawa, J. *et al.* Critical role of p38 and GATA3 in natural helper cell function. *J Immunol* **191**, 1818-1826, doi:10.4049/jimmunol.1300379 (2013).

219 Forrest, S. T., Taylor, A. M., Sarembock, I. J., Perlegas, D. & McNamara, C. A. Phosphorylation regulates Id3 function in vascular smooth muscle cells. *Circ Res* **95**, 557-559, doi:10.1161/01.RES.0000142735.67542.5a (2004).

220 Kersten, C. *et al.* BMP-6 inhibits human bone marrow B lymphopoiesis--upregulation of Id1 and Id3. *Experimental hematology* **34**, 72-81, doi:10.1016/j.exphem.2005.09.010 (2006).

221 Fiori, J. L., Billings, P. C., de la Pena, L. S., Kaplan, F. S. & Shore, E. M. Dysregulation of the BMP-p38 MAPK signaling pathway in cells from patients with fibrodysplasia ossificans progressiva (FOP). *Journal of bone*

and mineral research : the official journal of the American Society for Bone and Mineral Research **21**, 902-909, doi:10.1359/jbmr.060215 (2006).

222 Bain, G. *et al.* Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. *Nat Immunol* **2**, 165-171, doi:10.1038/84273 (2001).

223 Sakamoto, S. *et al.* E2A and CBP/p300 act in synergy to promote chromatin accessibility of the immunoglobulin kappa locus. *J Immunol* **188**, 5547-5560, doi:10.4049/jimmunol.1002346 (2012).

224 Bayly, R. *et al.* E2A-PBX1 interacts directly with the KIX domain of CBP/p300 in the induction of proliferation in primary hematopoietic cells. *The Journal of biological chemistry* **279**, 55362-55371, doi:10.1074/jbc.M408654200 (2004).

225 Boos, M. D., Yokota, Y., Eberl, G. & Kee, B. L. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *J Exp Med* **204**, 1119-1130, doi:10.1084/jem.20061959 (2007).

226 Deliri, H. *et al.* Increased 12/15-lipoxygenase enhances cell growth, fibronectin deposition, and neointimal formation in response to carotid injury. *Arterioscler Thromb Vasc Biol* **31**, 110-116, doi:10.1161/ATVBAHA.110.212068 (2011).

227 Taylor, A. M. *et al.* The effects of leukocyte-type 12/15-lipoxygenase on Id3-mediated vascular smooth muscle cell growth. *Arterioscler Thromb*

Vasc Biol **25**, 2069-2074, doi:10.1161/01.ATV.0000178992.40088.f2 (2005).

228 Taylor, A. M. *et al.* Hyperlipemia and oxidation of LDL induce vascular smooth muscle cell growth: an effect mediated by the HLH factor Id3. *Journal of vascular research* **43**, 123-130, doi:10.1159/000090131 (2006).

229 Shaw, P. X., Goodyear, C. S., Chang, M. K., Witztum, J. L. & Silverman, G. J. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells. *J Immunol* **170**, 6151-6157 (2003).

230 Yla-Herttuala, S. *et al.* Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association* **14**, 32-40 (1994).

231 Chen, Y. *et al.* Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol* **183**, 1346-1359, doi:10.4049/jimmunol.0900948 (2009).

232 Zouggari, Y. *et al.* B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. *Nat Med* **19**, 1273-1280, doi:10.1038/nm.3284 (2013).

233 Randolph, G. J. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. *Curr Opin Lipidol* **19**, 462-468, doi:10.1097/MOL.0b013e32830d5f09 (2008).

234 Trogan, E. *et al.* Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci U S A* **103**, 3781-3786, doi:10.1073/pnas.0511043103 (2006).

235 Forster, R., Davalos-Misslitz, A. C. & Rot, A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* **8**, 362-371, doi:10.1038/nri2297 (2008).

236 Chung, E. Y. *et al.* Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. *Immunity* **27**, 952-964, doi:10.1016/j.immuni.2007.11.014 (2007).

237 Gray, M., Miles, K., Salter, D., Gray, D. & Savill, J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* **104**, 14080-14085, doi:10.1073/pnas.0700326104 (2007).

238 Han, X., Kitamoto, S., Wang, H. & Boisvert, W. A. Interleukin-10 overexpression in macrophages suppresses atherosclerosis in hyperlipidemic mice. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 2869-2880, doi:10.1096/fj.09-148155 (2010).

239 Caligiuri, G. *et al.* Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med* **9**, 10-17 (2003).

240 Mallat, Z. *et al.* Protective role of interleukin-10 in atherosclerosis. *Circ Res* **85**, e17-24 (1999).

241 Matsushita, T. & Tedder, T. F. Identifying regulatory B cells (B10 cells) that produce IL-10 in mice. *Methods Mol Biol* **677**, 99-111, doi:10.1007/978-1-60761-869-0_7 (2011).

242 Mori, S. *et al.* Effect of c-fos overexpression on development and proliferation of peritoneal B cells. *Int Immunol* **16**, 1477-1486, doi:10.1093/intimm/dxh149 (2004).

243 Piatelli, M., Tanguay, D., Rothstein, T. & Chiles, T. Cell cycle control mechanisms in B-1 and B-2 lymphoid subsets. *Immunologic research* **27**, 31-52, doi:10.1385/IR:27:1:31 (2003).

244 Hopken, U. E., Achtman, A. H., Kruger, K. & Lipp, M. Distinct and overlapping roles of CXCR5 and CCR7 in B-1 cell homing and early immunity against bacterial pathogens. *Journal of leukocyte biology* **76**, 709-718, doi:10.1189/jlb.1203643 (2004).

245 Muller, G., Hopken, U. E. & Lipp, M. The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity. *Immunological reviews* **195**, 117-135 (2003).

246 Ansel, K. M., Harris, R. B. & Cyster, J. G. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* **16**, 67-76 (2002).

247 Ha, S. A. *et al.* Regulation of B1 cell migration by signals through Toll-like receptors. *J Exp Med* **203**, 2541-2550, doi:10.1084/jem.20061041 (2006).

248 Moon, H., Lee, J. G., Shin, S. H. & Kim, T. J. LPS-induced migration of peritoneal B-1 cells is associated with upregulation of CXCR4 and increased migratory sensitivity to CXCL12. *Journal of Korean medical science* **27**, 27-35, doi:10.3346/jkms.2012.27.1.27 (2012).

249 Cyster, J. G. B cell follicles and antigen encounters of the third kind. *Nat Immunol* **11**, 989-996, doi:10.1038/ni.1946 (2010).

250 Lee, S. *et al.* Role of phospholipid oxidation products in atherosclerosis. *Circ Res* **111**, 778-799, doi:10.1161/CIRCRESAHA.111.256859 (2012).