Role of B1a and B2 B lymphocytes in atherosclerosis

By

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PART A: General Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals and 1 unpublished publications. The core theme of the thesis is the role of B cells in atherosclerosis. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Medicine, Monash Medical Centre under the supervision of Professor Ban-Hock Toh, Professor Alexander Bobik and Dr. Tin Soe Kyaw.

[The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.]

In the case of Chapters 2-5 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis	Published	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 45%
3	B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions	Published	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 45%
4	Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE-/- mice attenuates atherosclerosis by potently ameliorating arterial inflammation	Published	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 45%
5	TNFα derived from B2 lymphocytes promotes development of inflammation, apoptosis and necrotic cores characteristic of unstable, rupture-prone atherosclerotic lesions	Returned for revision	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 85%

[* For example, 'published'/ 'in press'/ 'accepted'/ 'returned for revision']

I have / have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: …… **Date:** ……

Abstract

B cells constitute a major component of the immune system. They have significant roles in many immune diseases. There are multiple subtypes of B cells. B1a and conventional B2 cells are the two main subsets. B1a cells are the primary producers of natural IgM antibodies while B2 cells are the primary producers of IgG antibodies. IgM and IgG antibodies are believed to protect and aggravate atherosclerosis respectively. Atherosclerosis is a chronic inflammatory disease that is characterised by focal accumulation of lipids and immune cells in the walls of medium and large arteries. It results in cardiovascular diseases which are the leading causes of death from heart attacks and strokes in the world. Current treatment is restricted to lipid-lowering statins which are not sufficient in preventing and remitting atherosclerosis. The differential roles of IgM and IgG antibodies in atherosclerosis suggest that B1a cells are atheroprotective and B2 cells are atherogenic.

In this thesis, an atheroprotective effect of B1a cells was supported by increased atherosclerosis in $ApoE^{-/-}$ mice with diminished B1a cells after splenectomy. The aggravation of atherosclerosis in splenectomised $ApoE^{-/-}$ mice was markedly repressed by the reconstitution of B1a cells by adoptive transfer. On the other hand, an atherogenic effect of B2 cells was underscored by the exacerbation of atherosclerosis following their adoptive transfer into lymphocyte-deficient $ApoE^{-/-}$ $Rag2^{-/-}\gamma c^{-/-}$ mice and B cell-deficient $ApoE^{-/-}\mu MT$ mice. Moreover, $ApoE^{-/-}BAFF\text{-}R^{-/-}$ mice that were selectively deficient in B2 cells, but not B1a cells, had reduced atherosclerosis. These studies clearly indicate that B1a cells protect against atherosclerosis whereas B2 cells exacerbate atherosclerosis.

Notably, IgM and TNFα deficiencies in B1a cells and B2 cells prevented them from suppressing and aggravating atherosclerosis respectively. It is likely that B1a cells suppress atherosclerosis by producing natural IgM antibodies which are deposited within atherosclerotic lesions where they take part in the removal of apoptotic cells and oxidized lipids to modulate inflammation. Conversely, B2 cells may aggravate atherosclerosis by producing TNFα which is a potent inducer of inflammation and apoptosis. These mechanistic actions of B1a cells and B2 cells can be exploited to develop new and improved therapies against atherosclerosis.

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I wish to express gratitude to my supervisors – Professor Ban-Hock Toh (Department of Medicine, Monash Medical Centre), Professor Alexander Bobik (BakerIDI Heart and Diabetes Institute) and Dr. Tin Kyaw (BakerIDI Heart and Diabetes Institute) for supporting me in my studies. I have learned and benefited much from their ideas and insights. I am also indebted to Associate Professor Peter Tipping (Department of Medicine, Monash Medical Centre) for his guidance and help in acquiring the experiment models that I needed for my project.

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List of Publications

Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P[2], Bobik A[2], Toh BH[2]. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol*. 2010; 185(7):4410-9

Kyaw T[1], Tay C[1], Krishnamurthi S[1], Kanellakis P, Agrotis A, Tipping P[2], Bobik A[2], Toh BH[2]. B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res*. 2011; 109(8):830-40

Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, Tipping P[2], Bobik A[2], Toh BH[2]. Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS One*. 2012; 7(1):e29371

Kyaw T, Winship A, Tay C, Kanellakis P, Hosseini H, Cao A, Li P, Tipping P[2], Bobik A[2], Toh BH[2]. Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation*. 2013; 127(9):1028-39

Kyaw T, Cui P, Tay C, Kanellakis P, Hosseini H, Liu E, Rolink AG, Tipping P[2], Bobik A[2], Toh BH[2]. BAFF receptor mAb treatment ameliorates development and progression of atherosclerosis in hyperlipidemic ApoE(-/-) mice. *PLoS One*. 2013; 8(4):e60430.

Koulis C[1], Chen YC[1], Hausding C, Ahrens I, Kyaw TS, Tay C, Allen T, Jandeleit-Dahm K, Sweet MJ, Akira S, Bobik A, Peter K[2], Agrotis A[2]. Protective Role for Toll-Like Receptor-9 in the Development of Atherosclerosis in Apolipoprotein E-Deficient Mice. *Arterioscler Thromb Vasc Biol.* 2014. [Epub ahead of print]

1-equal contribution; 2-equal contribution

List of Conferences

Tay C, Kyaw T, Winship A, Kanellakis P, Hosseini H, Cao A, Li P, Tipping P, Bobik A, Toh BH. (2012) Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. Australian Vascular Biology Society 20th National Scientific Conference (Oral Presentation)

Tay C, Kyaw T, Winship A, Kanellakis P, Hosseini H, Cao A, Li P, Tipping P, Bobik A, Toh BH. (2012) Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. The Inflammation Conference 2012 'Inflammation – the key to much pathology' (Oral Presentation)

Abbreviations

ABCA	ATP-binding Cassette Transporter A-1
Ab	Antibody
ACAT	Acyl Coenzyme A Acylcholesterol Transferase
ADCC	Antibody Dependent Cellular Cytotoxicity
APC	Antigen Presenting Cell
ApoE	Apolipoprotein E
ApoB-100	Apolipoprotein B-100
Apobec1	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide
ATLO	Aortic Tertiary Lymphoid Organ
BAFF	B cell Activation Factor
BAFF-R	B cell Activation Factor Receptor
BCR	B cell receptor
Be	B effector
BLyS	B-lymphocyte Stimulator
BM	Bone marrow
CCR	CC Chemokine Receptor
CDL	Complement Dependent Lysis
CVD	Cardiovascular Disease
DC	Dendritic cell
FACS	Fluorescence Activated Cell Sorter
FDC	Follicular Dendritic cell
Fc	Constant region of immunoglobulin
FO	Follicular
FoxP3	Foxhead Box P3

GC	Germinal Center
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HMGB1	High Mobility Group Box Protein-1
HSP	Heat Shock Protein
HSC	Hematopoietic Stem Cells
ICAM-1	Intercellular Adhesion Molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LDL	Low-density Lipoprotein
LDLR	Low-density Lipoprotein Receptor
LFA-1	Lymphocyte Function-associated Antigen 1
MAPK	Mitogen Activated Protein Kinase
mAb	Monoclonal Antibody
mRNA	Messenger Ribonucleic acid
MDA	Malondialdehyde
MBL	Mannose Binding Lectin
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
MZ	Marginal Zone
NK	Natural Killer
OxLDL	Oxidized Low-density Lipoprotein
PALS	Periarteriolar Lymphoid Sheath
PCR	Polymerase Chain Reaction

PRR	Pattern Recognition Receptor
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
SCA-1	Scavenger Receptor-A
SLE	Systemic Lupus Erythematosus
SMC	Smooth Muscle Cell
SOCS	Suppressor of Cytokine Signalling
TGF	Transforming Growth Factor
Th	T-helper
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
Treg	T regulatory
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen 4

Chapter 1 Introduction

1.1 Global impact of cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death from heart attack and strokes. According to latest estimates, the number of deaths per year from CVD stands at an astonishing 17.3 million worldwide. This is expected to reach 23.6 million by the year 2030 (Smith, Collins et al. 2012). CVD is prevalent in most developed countries. The sheer magnitude of CVD is evident in the United States where it still accounts for nearly 35% of deaths even after the country recorded a marked reduction of 30.6% in cardiovascular mortality, largely because of improvements in healthcare, from 1998 to 2008 (Roger, Go et al. 2012). A more disturbing statistic is that 33% of CVD related deaths strike below the average life expectancy of 77.9 years. In addition, the cost of CVD burden rose by more than $11 billion from 2007 to 2008 (Roger, Go et al. 2012). The same holds true in Australia given that CVD affects 17% of the population and costs $4-6 billion annually (Bourchier M 2011).

The full brunt of CVD is still to come. This prospect hangs in the backdrop of a much larger CVD pandemic that looms on the horizon of low- and middle-income countries. At present, these countries sustain 80-90% of global CVD mortality (Smith, Collins et al. 2012). The impending crisis is primarily driven by rapid development and industrialization which lead to unhealthy lifestyles and increased exposure to risk factors such as smoking.

Indeed, massive efforts are required to dampen the impact of CVD. CVD is mainly caused by atherosclerosis. Existing medical strategies are restricted to surgical angioplasty/stent intervention and the administration of lipid-lowering statins and

anti-thrombotics. None of these offer sufficient remission and protection from CVD. From a therapeutic standpoint, the design of better treatments is only tangible with a sound understanding of the pathologies underlying atherosclerosis.

1.2 Atherosclerosis

Atherosclerosis is a chronic inflammatory disorder that is associated with elevated plasma cholesterol. It begins with the deposition of lipids in the arterial wall. It is a highly focal disease that occurs mainly at sites of arterial curvature and bifurcations where turbulent/oscillatory blood flow results in low hemodynamic shear stress. Shear stress refers to the tangential force of blood flow on the vascular wall (Malek, Alper et al. 1999, VanderLaan, Reardon et al. 2004). Over time, atherosclerotic plaques containing a plethora of immune cells and mediators develop. As plaques grow in size and complexity, they encroach on the vessel lumen which eventually becomes narrower. This limits blood flow to major organs resulting in ischaemia. In the event of a plaque rupture, thrombotic occlusion of arteries can give rise to myocardial infarction and stroke with a fatal outcome (Hansson and Hermansson 2011).

Macrophages and dendritic cells (DC) take part in all stages of atherosclerosis. They are present even in healthy non-atherosclerotic aorta, especially in the intimal and adventitial layers of atherosclerosis-prone regions (e.g. arterial branch points/bifurcations) (Millonig, Niederegger et al. 2001, Jongstra-Bilen, Haidari et al. 2006). Hence, they are well poised to initiate atherosclerosis. They become foam cells after ingesting lipids and are principally responsible for the cytokine and chemokine milieu that instigates plaque inflammation (Moore, Sheedy et al. 2013). The atherogenic functions of macrophages and DCs are amplified by adaptive immune

responses during the development and progression of atherosclerosis. Two examples that will be described in detail are the priming of macrophages by CD4 T-helper 1 cells and antibodies of the IgG subclass. There is substantial evidence of highly immunogenic oxidized low-density lipoproteins as the chief stimulating factors behind these processes (Stemme, Faber et al. 1995, Glass and Witztum 2001). Other immune cells that are known to be atherogenic include CD8 T cells, Natural Killer T cells and Natural Killer cells (Hansson, Robertson et al. 2006).

New insights have recently emerged from the study of B cells in atherosclerosis. B cells are well reputed for their arsenal of cellular and humoral immune functions. They constantly survey for neo-antigens and actively participate in coordinating immune mechanisms through the secretion of cytokines, production of antibodies and activation of CD4 T-helper cells by antigen presentation. There are multiple subtypes of B cells. Each has a specialized set of actions that facilitate immune activities under defined settings. The focus of this review is on the subset-specific roles of B cells in atherosclerosis and the benefits of exploiting them for designing therapies against atherosclerosis.

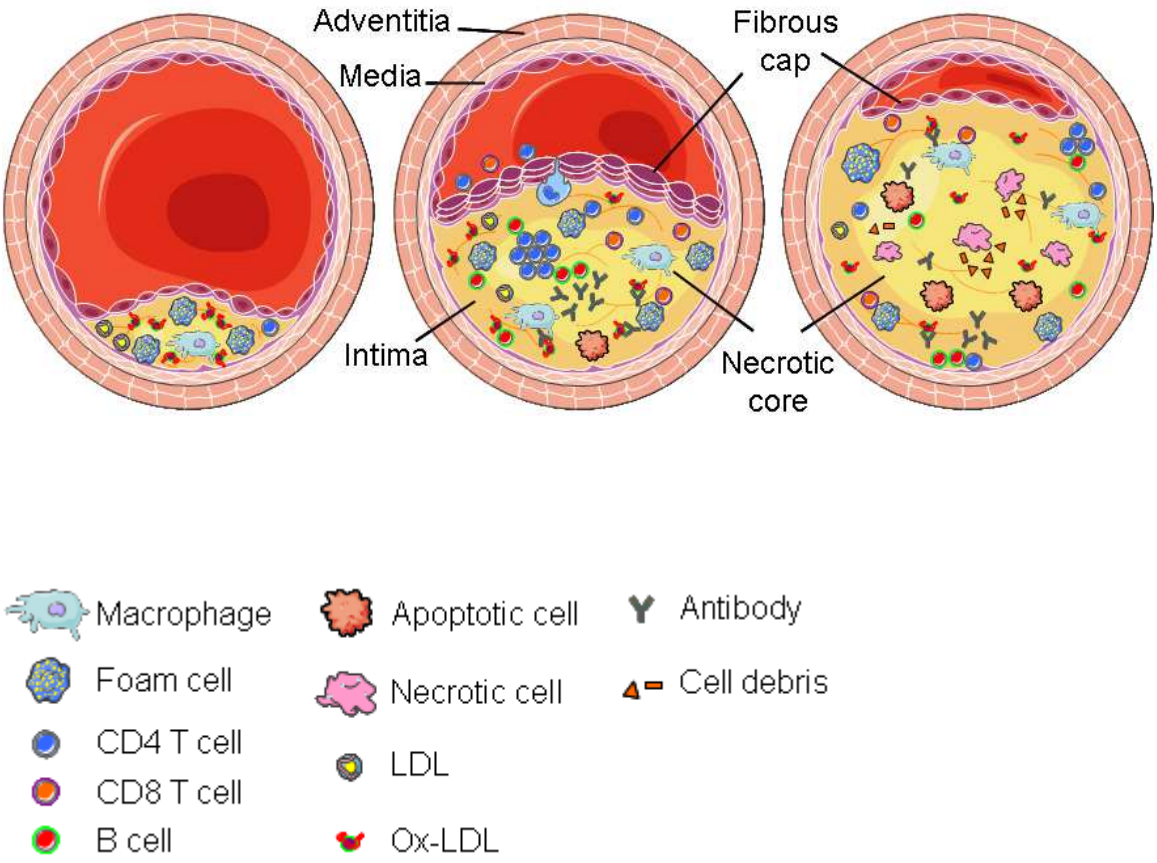

Figure 1 : LDL particles that are trapped within the arterial wall are rapidly denatured and oxidized by free radicals and enzymes. The resultant oxidized LDL (oxLDL) and lipid peroxides stimulate endothelial cells to express leukocyte adhesion and chemoattractant molecules which recruit immune cells such as T cells and B cells into the intimal layer. The ingestion of oxLDL and aberrant LDL transforms macrophages into foam cells. Lipid-laden foam cells aggregate to form a fatty streak. Overtime, more immune cells participate to induce cytotoxicity against foam cells and secrete inflammatory cytokines. The death of foam cells and other cells results in the formation of a necrotic core that contains cholesterol, lipid droplets and cell debris. oxLDL also stimulates endothelial cells to produce platelet-derived growth factor which stimulates smooth muscle cells (SMC) to migrate from the media to the intima where they proliferate and produce collagen. A fibrous cap rich in SMCs and collagen eventually forms. Progression of atherosclerosis is evidenced by the enlargement of necrotic core and thinning of fibrous cap.

1.3 Low-density lipoprotein

Low-density lipoprotein (LDL) is a spherical complex that transports triglycerides and cholesterol esters to tissues throughout the body. It is encapsulated by a phospholipid monolayer embedded with cholesterol and a single chain of apolipoprotein B-100 (ApoB-100) (Matsuura, Kobayashi et al. 2006). Circulating LDL routinely enters the intimal layer of arteries and returns to the bloodstream via transmural flow. However, there is a tendency for LDL, which carries a net positive charge, to be retained by negatively charged proteoglycan sugars in the subendothelial space of arteries (Skalen, Gustafsson et al. 2002, Matsuura, Hughes et al. 2008). This is particularly pervasive under hypertensive and hypercholesterolemic conditions which promote the influx of LDL. When exposed to metabolic enzymes (e.g. lipoxygenases and NADPH oxidase) within the arterial intima, LDL is rapidly denatured and oxidized by reactive oxygen species (e.g. superoxide anion and hydrogen peroxide) (Stocker and Keaney 2004). There are copious derivatives of oxidized LDL (oxLDL) ranging from minimally to highly oxidized LDL. LDL is susceptible to oxidation at the polyunsaturated fatty acid chains of esterified cholesterol and triglycerides and the sterol group of cholesterol (Matsuura, Kobayashi et al. 2006, Matsuura, Hughes et al. 2008). When these LDL components are attacked by free radicals, a cascade of reactions occurs and further oxidative modifications ensue.

1.3.1 Minimally-oxidized LDL

Oxidation of LDL is considered minimal when only the fatty acyl chains are modified without any major effect on the native structure of ApoB-100 (Navab, Ananthramaiah et al. 2004, Itabe, Obama et al. 2011). Mild oxidation of LDL does not affect recognition by the LDL-receptor (LDLR) (Stocker and Keaney 2004, Itabe, Obama et al. 2011). Mildly oxidized LDL stimulates the production of cAMP in endothelial

cells. In doing so, it increases the expression of several inflammatory genes of which P-selectin, monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (MCSF) have been identified in vivo (Berliner, Territo et al. 1990, Cushing, Berliner et al. 1990, Rajavashisth, Andalibi et al. 1990, Liao, Berliner et al. 1991, Parhami, Fang et al. 1993). Together, these molecules guide the trafficking of blood borne monocytes into atherosclerotic lesions. Upon contact with P-selectin, monocytes tether along the endothelium and migrate chemotactically from sensing MCP-1 into the arterial intima where MCSF mediates the differentiation of the monocytes into macrophages (Stocker and Keaney 2004, Hansson 2005).

1.3.2 Highly-oxidized LDL

LDL is extensively modified when its oxidized lipid components disintegrate into smaller molecules comprising mostly ketones and aldehydes. These lipid byproducts can form covalent cross-links with other lipids as well as protein adducts with ApoB-100 (Itabe, Obama et al. 2011). One example is the association between malondialdehyde (MDA) and the ε-amino ends of exposed lysine and histidine residues on ApoB-100 (Palinski, Rosenfeld et al. 1989, Stocker and Keaney 2004). The resultant Schiff bases are also very reactive as they bring about further lipid and peptide conjugation (Matsuura, Kobayashi et al. 2006). Consequently, ApoB-100 loses its conformation and breaks down into secondary fragments that can fuse with the phosphorylcholine headgroups of LDL phospholipids and reconfigure them to reveal cryptic epitopes (Binder, Chang et al. 2002, Friedman, Horkko et al. 2002). Unlike mildly oxidized LDL, highly oxidized LDL is recognized by scavenger receptors, in particular, scavenger receptor A (SR-A) and CD36. Scavenger receptors are regulated independently of cholesterol levels in cells. As such, they are the

primary means of oxLDL uptake and accumulation in lipid-laden foam cells (Krieger 1997, Stocker and Keaney 2004, Matsuura, Kobayashi et al. 2006).

1.3.3 OxLDL as an immunogen

The oxidation of LDL creates a cocktail of immunogenic autoantigens. This is reflected by the increased presence of oxLDL-binding immunoglobulins in the blood sera of mice and humans with established atherosclerosis (Salonen, Yla-Herttuala et al. 1992, Palinski, Tangirala et al. 1995). Enormous effort has been devoted to elucidate the immune mechanisms induced by specific oxLDL epitopes. Some are ascertained to attenuate whereas others aggravate atherosclerosis. This is likely due to the types of immune cell that are involved and the interplay between them. In the context of B cell responses, IgM antibodies are linked to the suppression of atherosclerosis while IgG antibodies are associated with the aggravation of atherosclerosis (Shoenfeld, Wu et al. 2004). This dogma has kindled a keen interest to develop vaccines against atherosclerosis.

Since oxLDL particles are mostly confined within atherosclerotic lesions, this poses the question about their transfer to peripheral lymphoid organs where they activate immune cells. The heterogeneity of DCs offers some interesting clues. Tissue-resident DCs regularly screen for aberrant antigens especially when recruited to inflammatory regions. After engulfing the aberrant molecules, DCs become specialized at processing and presenting them for T-cell engagement. Some may also migrate to draining lymph nodes to transfer the antigens to other DCs and even B cells (Carbone, Belz et al. 2004). Alternatively, antigens from inflamed tissues can directly access draining lymph nodes through lymphatic conduits (Harwood and Batista 2009). These

could be the means which B cells and other immune cells gain contact with oxLDL antigens in lymphoid organs.

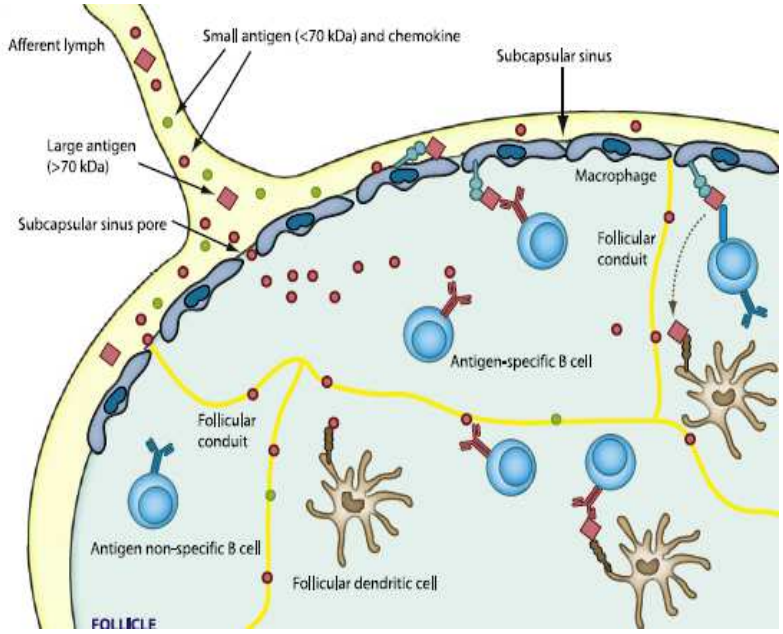

Figure 2 : OxLDL antigens may travel directly from atherosclerotic lesions via the lymphatic conduit to draining lymph nodes where they encounter and activate antigen-specific B2 cells. Upon contact, activated B2 cells may mobilise cognate CD4 T cells and other inflammatory immune cells. This process of activation is likely to take place during early atherosclerosis to kick start atherogenic inflammatory immune responses. Picture adapted from (Harwood and Batista 2009).

1.4 Natural IgM and B1a cells

The mechanistic actions of oxLDL-specific IgM in atherosclerosis have been under the spotlight for many years. This is attributed to increasing evidence of oxLDL-IgM restricting the development of atherosclerosis through various immunosuppressive ways at the local and systemic levels. Given its importance, measurements of oxLDL-IgM are routinely included in studies to document variations related to the pathology of atherosclerosis. For instance, increased oxLDL-IgM is deemed to modulate inflammation in atherosclerotic plaques by deterring necrotic cell death, reducing the accumulation of foam cells by preventing the uptake of oxLDL by plaque macrophages and promoting the removal of circulating oxLDL (Shaw, Horkko et al.

2000, Rose and Afanasyeva 2003). These and other IgM-mediated atheroprotective effects will be described below.

1.4.1 Natural IgM properties

Atheroprotective IgM antibodies mainly belong to a class of naturally occurring IgM antibodies, termed natural IgM. The variable regions of natural IgM antibodies are germline encoded and highly conserved (Dorshkind and Montecino-Rodriguez 2007). They are believed to evolve under selective pressures from endogenous neo-antigens such as apoptotic ligands and commensal pathogens (Baumgarth, Tung et al. 2005, Rapaka, Ricks et al. 2010). As a consequence, natural IgM antibodies are poly-specific and recognize a broad range of molecules (e.g. phospholipids and glycoproteins) with common structural moieties. Natural IgM antibodies do not undergo somatic hypermutation and affinity maturation (Kantor, Merrill et al. 1997, Herzenberg, Baumgarth et al. 2000, Gronwall, Vas et al. 2012). The ensuing drawbacks are a limited array of antigenic targets and low antigen-binding affinity. Nevertheless, an inherent property compels IgM antibodies to assemble into pentamers of about 970kDa, and at times even hexamers, to enhance overall antigen-binding avidity (Ehrenstein and Notley 2010, Kaveri, Silverman et al. 2012). In essence, natural IgM antibodies are configured to interact promptly and simultaneously with multiple antigens.

1.4.2 Natural-IgM producing B1a cells

In general, B cells are classified as B1 cells (IgM^{hi} $B220^{lo}$ IgD^{lo} $CD23^{-}$) or conventional B2 cells (IgM^{lo} $B220^{hi}$ IgD^{hi} $CD23^{+}$) (Fagarasan, Watanabe et al. 2000). B1 cells are a minor subset of B cells consisting of three subtypes namely, B1a, B1b and B1c (Hastings, Gurdak et al. 2006). In contrast to B2 cells, which represent the major population of B cells, B1 cells are long-lived and self-renewing (Hayakawa and

Hardy 1988, Karras, Wang et al. 1997, Berland and Wortis 2002). They are absent in peripheral lymph nodes and only account for about 5% of B cells in the spleen (Kroese, Ammerlaan et al. 1992, Berland and Wortis 2002). Much of the current knowledge on B1 cells is derived from murine models. Human B1 cells have only recently been characterized as $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ cells (Griffin, Holodick et al. 2011). In mice, the genesis of B1 cells stems from fetal hematopoietic stem cells (HSCs) in the fetal liver and omentum. The $Lin^{-}CD45R^{-/lo}CD19^{+}$ B1 precursors decline markedly postnatal and scarcely reside in the adult bone marrow (Fagarasan, Watanabe et al. 2000, Montecino-Rodriguez and Dorshkind 2006). Nonetheless, they remain central to the homeostatic maintenance of B1 cells throughout life. Maturing B1 cells that bind strongly to self and non-self antigens are positively selected while those that exhibit weak binding do not prevail (Casola, Otipoby et al. 2004, Hardy 2006). The current paradigm suggests that B1 cells in young mice provide a first line of defense against common pathogens. As the mice grow older, antigen experience becomes increasingly involved in shaping the B1 repertoire.

B1a cells are the principal source of natural IgM antibodies. Positive expression of CD5 and CD11b distinguishes B1a cells from other B1 cells (B1b, $CD5^{+}$ $CD11b^{-}$ and B1c, $CD5^{+}$ $CD11b^{-}$) (Hastings, Gurdak et al. 2006). The main sites of residence for B1a cells are the pleural and peritoneal cavities. Within these compartments, B1a cells comprise 10-15% of B cells (Marcos, Huetz et al. 1989, Kroese, Ammerlaan et al. 1992). B1a cells constitutively produce natural IgM without T-cell help and supply half of the entire IgM pool (Thurnheer, Zuercher et al. 2003). The near normal levels of IgM in mice that were reared in germ-free environment bore testament to the spontaneity of IgM production by B1a cells (Coutinho, Kazatchkine et al. 1995). This

was further exemplified by the almost complete restoration of IgM in immune-deficient Rag1$^{-/-}$ mice following the adoptive transfer of B1a cells (Binder, Shaw et al. 2005, Chou, Fogelstrand et al. 2009). When B1a cells encounter cognate antigens, they expand and generate increased amounts of clonotypic IgM (Hardy 2006, Gronwall, Vas et al. 2012). This may explain the correlation between oxLDL-IgM and atherosclerosis. Taken together, B1a cells constitute a vital branch of the innate immune system by maintaining an umbrella of natural IgM antibodies to shield against invading microbes.

1.5 Atheroprotective functions of Natural IgM

The pathogenesis of atherosclerosis has been extensively studied from the initiation of fatty streak to the development and progression of mature plaque into vulnerable plaque. However, the events that propel the transition from one disease stage to another still need further investigation. This particularly applies to the interactions among cells in the atherosclerotic plaque and their effects on atherogenesis. One such area pertains to the clearance of apoptotic plaque cells, a process known as efferocytosis, which is severely impaired in advanced atherosclerotic plaques (Schrijvers, De Meyer et al. 2007).

1.5.1 Efferocytosis in atherosclerotic lesions

Besides phagocytosing oxLDL, plaque macrophages have a secondary role in removing dying cells since they are the main professional efferocytes in plaques (Li, Sun et al. 2009, Hansson 2012). This takes place efficiently during early atherosclerosis but subsides in the later phases when there is a high level of inflammatory and oxidative stress (Yvan-Charvet, Pagler et al. 2010, Moore and Tabas 2011). In advanced plaques, the frequency of necrotic cells increases due to

inefficient efferocytosis. The uncontrolled leakage of toxic intracellular factors (e.g. High mobility group box protein 1; HMGB1 and heat shock proteins; HSP) from necrotic cells can inflict profound damage on atherosclerotic lesions (Moore, Sheedy et al. 2013). Our previous study showed that HMGB1 was atherogenic and contrived the recruitment of macrophages and stimulation of inflammatory mediators in plaques (Kanellakis, Agrotis et al. 2011). In time, the atherosclerotic plaque becomes more inflamed and unstable as manifested by the enlargement of necrotic core and the thinning of fibrous cap. The exact reason behind the impairment of efferocytosis remains elusive. Initial work suggests that it could be multifactorial. Potential mechanisms include a decline in the number of functional efferocytes as a result of oxLDL-induced macrophage death and reduced recognition of apoptotic cells due to the disruption of efferocytic ligands (e.g phosphatidylserine) and receptors (e.g. MerTK) in advanced plaques (Thorp, Cui et al. 2008, Seimon and Tabas 2009, Moore and Tabas 2011).

1.5.2 Natural IgM-mediated efferocytosis in atherosclerotic lesions

Lewis and colleagues reported that the loss of natural IgM by gene deletion of secretory IgM (IgM) accentuated atherosclerosis in $LDLR^{-/-}$ mice (Lewis, Malik et al. 2009). It also appeared that plaque apoptosis was significantly elevated in the absence of IgM. Although it could be argued that this was a mere reflection of the increase in plaque size in $sIgM^{-/-}LDLR^{-/-}$ mice compared to $LDLR^{-/-}$ mice, the result strongly suggested defective removal of apoptotic cells in mature atherosclerotic plaques that lack IgM. This is an important finding with relevant implications of natural IgM serving as scavengers of apoptotic cells in atherosclerotic plaques.

The opsonization of only apoptotic cells but not viable cells is a notable trait of natural IgM (Shaw, Goodyear et al. 2003). Apoptotic cells display an assortment of 'gobble me' ligands which are readily recognized by natural IgM. Amongst them are phosphatidylserine and phosphorylcholine of oxidized phospholipids (Gronwall, Vas et al. 2012). Phosphatidylserine is a membrane phospholipid that is located on the cytosolic side in healthy cells but becomes exposed on the outer surface of apoptotic cells (Peng, Kowalewski et al. 2005). Of note, the cross-reactivity of certain natural IgM clones (e.g. T15/E06) against oxLDL and apoptotic cells may be explained by the phosphorylcholine group that is shared between the two. This extends to other apoptotic determinants bearing oxLDL motifs such as MDA-lysine. The dual specificity of natural IgM was verified by competition assays demonstrating that IgM binding of apoptotic cells decreases markedly in the presence of oxLDL or MDA-LDL but not native LDL antigens (Chang, Binder et al. 2004). For this reason, apoptotic markers and oxLDL antigens are partner candidates for the intrinsic selection of natural IgM.

1.5.3 Combined role of Natural IgM and complement proteins in efferocytosis

More studies are needed to reveal the downstream signals of IgM-assisted efferocytosis. The complement system is necessary for completion of the apoptotic cell : efferocyte synapse (Peng, Kowalewski et al. 2005, Gronwall, Vas et al. 2012). This was apparent when the introduction of IgM antibodies improved efferocytosis in concert with increased deposition of the complement protein, C3, on apoptotic cells in $sIgM^{-/-}$ mice but not $sIgM^{-/-}C1q^{-/-}$ mice (Ogden, Kowalewski et al. 2005). Perhaps, the same should be attempted on atherosclerosis-prone mice ($LDLR^{-/-}$ or $ApoE^{-/-}$) given that the atherosclerotic plaques in $C1q^{-/-}$ $sIgM^{-/-}LDLR^{-/-}$ and $sIgM^{-/-}LDLR^{-/-}$ mice had been found to be of similar size but larger compared to the plaques in $C1q^{-/-}LDLR^{-/-}$

and LDLR$^{-/-}$ mice on high fat diet (Lewis, Malik et al. 2009). This can provide clarification on C1q as a co-factor for IgM to initiate efferocytosis in atherosclerotic plaques. One other member of the complement system that is worth addressing is mannose-binding lectin (MBL), a protein analogous to C1q. IgM antibodies contain mannose-anchor sites for MBL to swamp IgM-bound apoptotic cells and prime them for engulfment by phagocytes (Chen, Khanna et al. 2009, Gronwall, Vas et al. 2012).

Although C1q and MBL, by themselves, conduct critical roles in the clearance of apoptotic cells, they mostly act on cells in late apoptotic stages (Nauta, Raaschou-Jensen et al. 2003, Gronwall, Vas et al. 2012). On the other hand, IgM antibodies attach to early- as well as late-stage apoptotic cells and provide a platform for C1q and MBL to join and summon efferocytosis (Chen, Khanna et al. 2009). Hence, the importance of IgM for optimal efferocytosis outweighs that of C1q and MBL. At present, it is not known whether effective efferocytosis necessitates late complement proteins (e.g. C3b and C4). Additional experiments have to precisely verify the degree of activation required of the complement cascade. By dissecting the dependence of IgM on each complement pathway – classic and lectin – weaknesses in the efferocytic system in advanced atherosclerotic plaques may come to light and offer avenues to prevent plaque destabilization.

1.5.4 Anti-inflammatory role of Natural IgM and complement proteins

The alliance between natural IgM and complement proteins against apoptotic cells may offer more atheroprotective benefits. Support for an anti-inflammatory effect surfaced when infusion of apoptotic cells inhibited the activation of macrophages and DCs by poly:IC in naïve mice. There was even greater suppression following the combined infusion of natural IgM and apoptotic cells (Chen, Khanna et al. 2009).

Decreased levels of pro-inflammatory cytokines and chemokines were also recorded in blood. Based on these observations, it is reasonable to speculate that the binding of IgM antibodies to apoptotic cells prevents phagocytes from exerting unwarranted inflammation and instead beckon for inflammation resolution. It is plausible that anti-apoptotic IgM in combination with C1q/MBL allow apoptotic cells to form stable connections with phagocytes and therein turn off inflammatory pathways (reviewed in (Gronwall, Vas et al. 2012)). In vitro tests have indicated that the blockade of mitogen activated protein kinase (MAPK) signal transduction is a potential immune modulatory action of the anti-apoptotic natural IgM and C1q/MBL complex (Gronwall, Chen et al. 2012, Gronwall, Vas et al. 2012). Questions remain on the types of receptor involved and the possible stimulation of ancillary regulatory responses (e.g. suppressor of cytokine signaling; SOCS) that favor the inhibition of MAPK.

The structural biology of anti-apoptotic IgM and C1q/MBL could soon be an area of interest too. The binding of IgM to apoptotic ligands sets off allosteric reconfiguration of the μ-constant region for C1q or MBL to access and attach (Gronwall, Vas et al. 2012). Due to the high ionic and pH sensitivity of IgM, drastic variations in the physical environment within advanced atherosclerotic plaques may interfere with the conformational properties of IgM and hinder its association with complement proteins. This may explain the waning of efferocytosis as atherosclerosis progresses. In view of the IgM structure : function relationship and the premise that natural IgM is germline encoded, individuals may be more resistant or susceptible to rapid progression of atherosclerosis depending on the specific modules in IgM structure that are crucial for maintaining the efferocytic function of IgM. Identification of these key

domains in IgM could pave the way for screening tests to detect vulnerable individuals as well as therapeutic strategies to compensate the efferocytic inefficiencies due to anti-apoptotic IgM failing to sustain during advanced atherosclerosis.

1.6 Association between Natural IgM and Th2 responses

Natural IgM can be instrumental in regulating inflammatory responses by steering the differentiation of effector CD4 T cells toward Th2. This was attested in mice that were immunized with antigenic targets of natural IgM; mice displayed Th2 immunity even when the accompanying adjuvant was Th1 biased (Binder, Hartvigsen et al. 2004). Moreover, while the production of IFNγ, a Th1 cytokine, was unaltered in $sIgM^{-/-}$ mice, production of the Th2 cytokines, IL-5 and IL-9, was strikingly reduced (Rapaka, Ricks et al. 2010). It is conceivable that antigens bound by natural IgM induce antigen presenting cells (APC) to coerce CD4 T cells into becoming Th2 cells. There are several possible ways for this to occur. Complement and pattern recognition receptor signaling pathways in APCs are just two examples. A more direct signal transducer is the Fcα/μ receptor which recognizes specifically antibodies of the IgM isotype. The resultant effect likely culminates in the presentation of antigens along with accessory molecules that activate the transcription factor GATA3 in cognate CD4 T cells. GATA3 stimulates Th2 characteristics by downregulating the expression of IFNγ and upregulating the expression of IL-4 and IL-5 (Ho and Glimcher 2002). Alternatively, IgM-bound antigens may incite the trafficking of APCs to secondary lymphoid organs to induce Th2 responses as reported previously (Rapaka, Ricks et al. 2010). This potentially involves the expression of specific chemokines and chemokine receptors.

Two studies have shed light on IgM-mediated Th2 polarization in atherosclerosis. The first demonstrated that hypercholesterolemic ApoE$^{-/-}$ mice with established atherosclerosis displayed increased oxLDL-IgM levels in the blood with a concomitant switch from Th1 to Th2 (Zhou, Paulsson et al. 1998). However, it remains to be determined whether the dominance of Th2 over Th1 intervenes or contributes to the progression of atherosclerosis. Thus far, the Th2 arm is believed to exacerbate advanced atherogenesis as evidenced by a decrease in the size of mature plaques in ApoE$^{-/-}$IL-4$^{-/-}$ mice compared to ApoE$^{-/-}$ mice (Davenport and Tipping 2003). Furthermore, there are indications that suggest Th2 cells are detrimental to the architecture of established lesions. Th2 cytokines were shown to promote the formation of aortic aneurysms by stimulating elastolytic enzymes that break down elastic tissue essential for maintaining arterial wall integrity (Shimizu, Shichiri et al. 2004). Likewise, Th2 cells may undermine lesion stability by causing the degradation of extracellular matrix in lesions. On a more positive note, the mitigation of Th1 in favor of Th2 suppresses early atherosclerosis development. This was reported in the second study which examined LDLR$^{-/-}$ mice deficient in the Th1 transcription factor, T-bet. LDLR$^{-/-}$T-bet$^{-/-}$ mice exhibited reduced atherosclerosis coupled with increased Th2 responses and natural IgM (Buono, Binder et al. 2005). A detailed knowledge of the benefits and drawbacks of Th2 cells and natural IgM is paramount to achieve the best outcome for employing Th2- and natural IgM-based therapies to either stem the growth of atherosclerotic lesions or prevent them from becoming unstable.

1.6.1 IL-10

An alternative explanation for the relationship between natural IgM and Th2 cells is the production of IL-10 by B1a cells. Not only are B1a cells the major source of natural IgM, a subpopulation also constitutively secrete IL-10 (O'Garra and Howard

1992, Garaud, Le Dantec et al. 2009). IL-10 is a potent Th2 cytokine that modulates inflammation. It serves as a critical autocrine factor for B1a cells to thrive and proliferate (Nisitani, Tsubata et al. 1995). The introduction of an IL-10 neutralizing antibody into mice selectively depleted B1a cells but not B2 cells (Ishida, Hastings et al. 1992). B1a cells are the largest producers of IL-10 amongst all B cells in the peritoneum (O'Garra, Chang et al. 1992). As a gauge, peritoneal cells produce higher levels of IL-10 than splenic cells. Although this is not an exact measure and comparison of B1a cells to other specialized IL-10 producing cells such as T-regulatory (Treg) cells, it highlights the ability of B1a cells to secrete IL-10 in considerable quantities that may influence immune activities.

One probable scenario involves B1a-derived IL-10 sustaining immune-regulatory responses by maintaining the expression of FoxP3 in Treg cells. This is adjudged to be one of the manners which IL-10 represses autoimmunity in Lyn kinase-deficient mice (Scapini, Lamagna et al. 2011). Perhaps, the next challenge is to ascertain the conditions that underlie the dominance of IL-10 derived from B1a cells and Treg cells. Treg-derived IL-10 is expected to precede B1a-derived IL-10 in most inflammatory settings. However, the latter may be more relevant at sites where $FoxP3^{+}IL\text{-}10^{-}$ Treg cells in particular, and $FoxP3^{+}IL\text{-}10^{+}$ Treg cells, but not $FoxP3^{-}IL\text{-}10^{+}$ Treg cells, dictate anti-inflammatory processes. For example, the lungs, liver and secondary lymphoid organs are the main residences of $FoxP3^{+}IL\text{-}10^{-}$ Treg cells which can secrete other regulatory cytokines such as Transforming Growth Factor beta (TGF-β) (Maynard, Harrington et al. 2007). Within these regions, B1a cells may be the only source of IL-10, and despite their low numbers, ensure the long-term provision of IL-10-independent Treg function that is critical to immune homeostasis.

Treg cells are known to be atheroprotective. Much clinical benefit can be gained from harnessing B1a-derived IL-10 to amplify the effectiveness of Treg cells in circumventing atherosclerosis.

1.6.2 IL-5

The link between B1a cells and Th2 cells is underpinned by another cytokine, IL-5. Mice rendered deficient in IL-5 by gene knockout or administration of IL-5 depleting antibody had reduced numbers of B1a cells (Kopf, Brombacher et al. 1996, Moon, Takaki et al. 2004). The same effect was attained in mice that were devoid of the α-chain of IL-5 receptor (IL-5Rα), a receptor which is constantly expressed in B1a cells (Yoshida, Ikuta et al. 1996). Conversely, mice that over express IL-5 have increased B1a cells and serum IgM (Tominaga, Takaki et al. 1991, Katoh, Bendig et al. 1993). The signals and events triggered by IL-5 are still not completely uncovered. For instance, IL-5 is only crucial for the survival of B1a cells but not B2 cells. The number of B2 cells is not affected in the absence of IL-5 and IL-5Rα (Moon, Takaki et al. 2004). However, IL-5 appears to play a minor role in the differentiation of B2 cells into antibody-secreting plasma cells (Yoshida, Ikuta et al. 1996). IL-5Rα$^{-/-}$ mice have decreased IgG1 but normal IgG2a levels after treatment with Lipopolysaccharide, an indication that they are inclined toward eliciting Th1 responses (Moon, Takaki et al. 2004). This is consistent with the release of IL-5 by activated Th2 cells.

The secretion of IL-5 by Th2 cells is believed to bridge innate and adaptive immune responses. Intriguingly, this notion surfaced from a study that examined the role of IL-5 in atherosclerosis. Immunization of LDLR$^{-/-}$ mice with MDA-LDL was found to ameliorate atherosclerosis (Freigang, Horkko et al. 1998, Binder, Hartvigsen et al.

2004). It was proposed that the activation and expansion of Th2 cells against MDA-LDL may have initiated the protective response. Indeed, MDA-LDL-specific Th2 cells produced large quantities of IL-5, even more so compared to other prominent Th2 cytokines namely IL-4, IL-10 and IL-13. They could be the reason for the increase in IL-5 in the serum of MDA-LDL immunized mice (Binder, Hartvigsen et al. 2004). In vitro and in vivo experiments showed that the increase in IL-5 spontaneously activated B1a cells to secrete increased amounts of atheroprotective oxLDL-binding natural IgM (Binder, Hartvigsen et al. 2004).

Similar observations were made in $ApoE^{-/-}$ mice that were treated with IL-33, a member of the IL-1 family and a Th2-inducing cytokine (Miller, Xu et al. 2008). Mice that were given IL-33 had significant increases in IL-4, IL-5 and IL-13 in the blood and lymph nodes. Again, the increase in IL-5 was the most pronounced and was accompanied by higher plasma titers of oxLDL-specific IgM and reduced atherosclerosis. However, the inclusion of an anti-IL-5 blocking antibody in the treatment abrogated the atheroprotective effects of IL-33, underlining the importance of IL-5 in IL-33-mediated atheroprotection (Miller, Xu et al. 2008).

These findings portray a classic example of Th2 cells leveraging on the T-independent humoral system through IL-5 for a more efficient response against oxLDL. Further validation of the protective role of IL-5 in atherosclerosis was gained from $LDLR^{-/-}$ mice that underwent bone marrow transplant from $IL\text{-}5^{-/-}$ mice. $LDLR^{-/-}$ recipients of $IL\text{-}5^{-/-}$ bone marrow cells had significantly lower oxLDL-natural IgM levels and larger atherosclerotic lesions compared to $LDLR^{-/-}$ recipients of $IL\text{-}5^{+/+}$ bone marrow cells (Binder, Hartvigsen et al. 2004). Confidence in the atheroprotective feature of IL-5

was also bolstered when IL-5 levels were shown to correlate positively with oxLDL-specific IgM levels and negatively with atherosclerosis in humans (Sampi, Ukkola et al. 2008).

Hence forth, added emphasis on exploiting IL-5 to limit atherosclerosis is greatly desired. The two most appealing advantages of using an IL-5 agonist over MDA-LDL immunization hinge on its highly targeted action specifically on B1a cells. B2 cells and their T-dependent functions, such as MDA-LDL-specific IgG2a production which is potentially atherogenic, are largely spared (Binder, Hartvigsen et al. 2004, Moon, Takaki et al. 2004). Moreover, stimulation of MDA-LDL-specific Th2 cells, despite their beneficial properties in early atherosclerosis, can be bypassed to lessen the chance of atherosclerotic plaques becoming prone to rupture as mentioned above. The elevation of natural IgM from injecting IL-5 into the peritoneum of mice offers much promise (Binder, Hartvigsen et al. 2004). This approach certainly needs to be further examined in $LDLR^{-/-}$ and $ApoE^{-/-}$ mice to assess its efficacy in preventing the development of atherosclerosis as well as its potential in intervening the progression of atherosclerosis.

1.7 B1a cells are atheroprotective

Heretofore, B1a cells are only implicated by their effector functions to be atheroprotective. There is as yet no direct evidence of them protecting against atherosclerosis. Until such verification is made, B1a cells will not be as resourceful as they can for the pursuit of therapies to retard atherosclerosis. To resolve this outstanding issue, in vivo models are required to corroborate that B1a cells impede

the development of atherosclerosis and assess the extent that they do so under normal and adverse (e.g. hypercholesterolemia) circumstances.

The spleen is pivotal to the maintenance of B1a cells. B1a cell numbers were markedly diminished in Hox11$^{-/-}$ mice that were congenitally asplenic as well as wild type mice that had undergone splenectomy (Wardemann, Boehm et al. 2002). Specific B1a progenitors are hitherto not identified in the spleen. To date, speculation remains on antigen exposure influencing the designation of splenic B cell precursors, termed transitional B cells, to progress down the B1a lineage. The present theory postulates that strong and weak B cell receptor (BCR) signaling respectively causes survival and death of B1a cells (Casola 2007). If true, this explains the preferential selection of B1a cells in mice that transgenically express BCR with high affinity for particular self-antigens (Wen, Brill-Dashoff et al. 2005). Access to survival factors may be another reason for B1a cells to depend on the spleen. To explore this, studies can begin by identifying the receptors on B1a cells that are crucial for B1a cells to persist over prolonged periods.

The feasibility of using splenectomy to examine the atheroprotective functions of B1a cells received a huge boost when splenectomised ApoE$^{-/-}$ mice were shown to sustain more atherosclerosis than sham-operated ApoE$^{-/-}$ mice (Caligiuri, Nicoletti et al. 2002, Rezende, Neto et al. 2011). A more encouraging result was the almost complete alleviation of atherosclerosis in splenectomised ApoE$^{-/-}$ mice that were adoptively transferred with splenic B cells but not T cells. Along with the reduction in atherosclerosis, an increase in MDA-LDL-specific IgM was also noted in the B cell-transferred mice (Caligiuri, Nicoletti et al. 2002). Nevertheless, it was not known

whether the amelioration of atherosclerosis was brought about by the inclusion of B1a cells in the unfractionated population of transferred B cells. This prompted a study to delineate the effects of reconstituting splenectomised $ApoE^{-/-}$ mice with peritoneal B1a cells and splenic B2 cells. Indeed, atherosclerosis was significantly suppressed in mice that received B1a cells but not in mice that received B2 cells. Moreover, the protective effect relied on the secretion of IgM by B1a cells (Kyaw, Tay et al. 2011). Perhaps, the capacity of B1a cells to regress established atherosclerosis can be considered in future studies.

In spite of their small numbers, B1a cells prove to be rather potent in protecting against atherosclerosis. Here, the function of B1a cells takes precedence over the absolute size of the B1a population. This brings forth other minor B cell subsets that have similar properties to B1a cells. For instance, marginal zone B cells and B regulatory cells release IgM and IL-10 respectively upon activation and may also be atheroprotective. By unravelling atheroprotective attributes of each B cell subset, more ideas can be formulated to exploit the cells to counteract atherosclerosis without severely impairing the immune system.

1.8 Conventional B2 cells

Conventional B2 cells are integral to adaptive immune responses. Random gene rearrangement and non-template N-nucleotide addition confer the B2 cell population with a highly polymorphic BCR repertoire. B2 cells produce responses that are highly restricted to their target antigens. They secrete antibodies that are antigen-specific and activate CD4 T cells with corresponding antigen-specificity to mount concerted and sustained actions against the antigens. On the downside, the immune commitments

that B2 cells shoulder render B2 cells capable of orchestrating many inflammatory disorders. rituximab, a chimeric anti-CD20 monoclonal antibody which was developed to treat B cell chronic lymphocytic leukemia in the mid 1990s, and other B cell-based therapies (e.g. belimumab) have been used to alleviate autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Tsokos 2004, Sfikakis, Boletis et al. 2005). Much of the success owes to the depletion of pro-inflammatory B2 cells as shown in experimental animals (Hamaguchi, Uchida et al. 2005, Yanaba, Hamaguchi et al. 2007, Xiu, Wong et al. 2008). This strongly appeals for the design of B2 cell-centric immunotherapy, an undertaking that is only achievable upon understanding the precise mechanistic actions of B2 cells in each disease. The ultimate aim is to nullify only the key responses of B2 cells that are damaging rather than the broad stroke approach of deleting the entire population of B cells.

1.9 Development and maturation of B2 cells

The lymphopoiesis of B2 cells depends entirely on adult HSCs in the bone marrow. Once they have acquired the essential characteristics and gone through stringent selection that excludes auto-reactive B2 cells, mature B2 cells begin patrolling the periphery to carry out their immune functions. The peripheral pool of B2 cells is continually screened in the spleen to ensure constant vigilance against foreign antigens and tolerance to self-antigens. This process is organized into three transitional stages – T1, T2 and T3 (Loder, Mutschler et al. 1999). T1 B2 cells are the earliest B2 immigrants in the spleen. They enter the red pulp of the spleen and migrate to the outer periarteriolar lymphoid sheath (PALS) at the T cell : B cell interface. T2 and T3 B2 cells co-localize in the splenic follicle (Chung, Silverman et al. 2003).

The fate of transitional B2 cells depends on the level of BCR signal strength received during engagement with self-antigens in the PALS. Strong signals lead to either anergy or clonal deletion, intermediate signals favor differentiation into $IgM^{hi}IgD^{lo}CD21^{hi}CD23^{lo}$ marginal zone (MZ) B2 cells that subsequently occupy the MZ compartments of the spleen and weak signals generate $IgM^{lo}IgD^{hi}CD21^{lo}CD23^{hi}$ Follicular (FO) B2 cells (Amano, Baumgarth et al. 1998, Wen, Brill-Dashoff et al. 2005).

MZ B2 cells bear resemblance to B1a cells in terms of their ability to self-renew and respond T-independently with antibodies of the IgM and IgG3 isotypes against blood-borne antigens (Martin and Kearney 2000, Martin, Oliver et al. 2001, Hardy 2006). MZ B2 cells are mostly confined within the splenic MZ. After capturing antigens, however, MZ B2 cells shuttle from the MZ to the FO zone to transfer the captured antigens to follicular DCs (Ferguson, Youd et al. 2004, Cinamon, Zachariah et al. 2008, Arnon, Horton et al. 2013). MZ B2 cells are retained in the MZ through the integrins – Lymphocyte function-associated antigen-1 (LFA-1) and Very late antigen-4 (VLA-4) and their ligands – Intercellular adhesion molecule-1 (ICAM-1) and Vascular cell adhesion molecule-1 (VCAM-1). Dual treatment with anti-LFA-1 and anti-VLA-4 antibodies will displace MZ B2 cells from the splenic MZ (Lu and Cyster 2002).

FO B2 cells are the majority of conventional B2 cells. FO B2 cells undergo further maturation to become efficient in collaborating with CD4 T helper cells (Chung, Sater et al. 2002). Unlike MZ B2 cells, FO B2 cells are free to circulate between the blood

and the lymphatic system (Hardy, Hayakawa et al. 1983, Cariappa, Chase et al. 2007). Presently, there is no known interaction between MZ B2 cells and FO B2 cells.

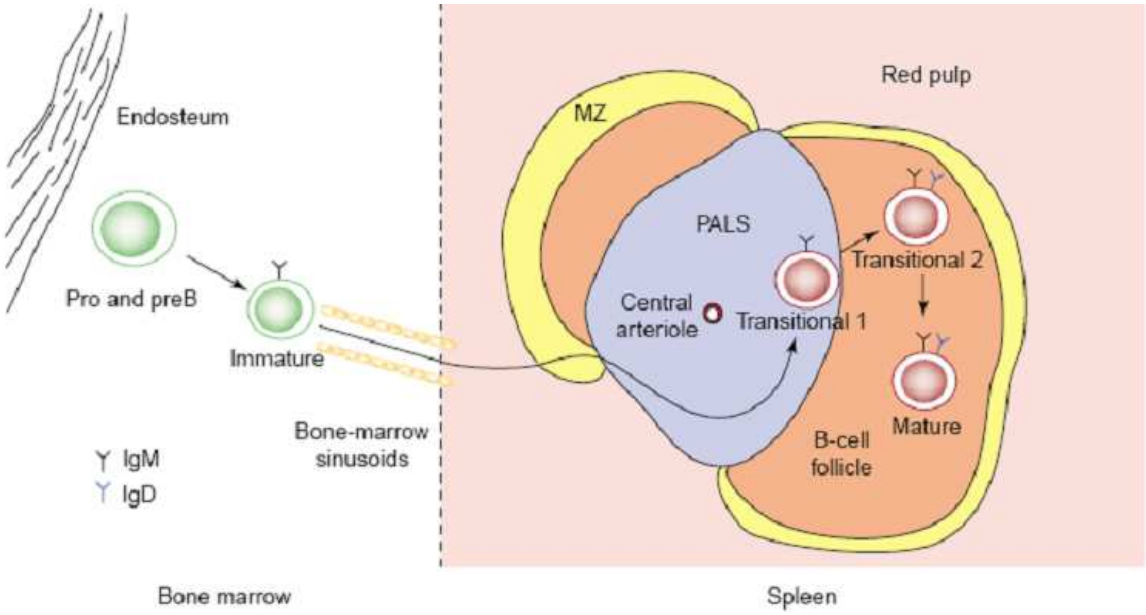

Figure 3 : After emerging from the bone marrow, B cells undergo further selection in the spleen. During maturation in the spleen, B cells are sorted into two transitional stages, namely Transitional 1 and Transitional 2. They are exposed to antigens by follicular dendritic cells and are selected according to the strength of BCR : antigen binding. This ensures efficient B cell self-tolerance and immunity. Picture adapted from (Chung, Silverman et al. 2003).

1.10 B2 cells are atherogenic

Recent studies indicated that B2 cells play a major atherogenic role in atherosclerosis alone and possibly also synergistically with other cells. The earliest lead came about when the depletion of B2 cells in ApoE$^{-/-}$ mice significantly suppressed the development and progression of atherosclerosis (Ait-Oufella, Herbin et al. 2010, Kyaw, Tay et al. 2010). In addition, lymphocyte- and B cell-deficient ApoE$^{-/-}$ mice had increased atherosclerosis after receiving adoptive transfers of B2 cells (Kyaw, Tay et al. 2010). An important extension to these findings leveraged on ApoE-/- mice that were genetically deficient in the receptor for B-cell activating factor (BAFF-R). BAFF is a ligand of the tumor necrosis factor superfamily. The association of BAFF

and BAFF-R is crucial for transducing survival and maturation signals in B2 cells but not B1a cells (Rauch, Tussiwand et al. 2009). Hence, ApoE$^{-/-}$BAFF-R$^{-/-}$ mice have diminished B2 and normal B1a populations. Indeed, the hypothesis on anti-atherogenic B1a cells and pro-atherogenic B2 cells was strengthened by ApoE$^{-/-}$ BAFF-R$^{-/-}$ mice presenting marked reduction in atherosclerosis compared to control ApoE$^{-/-}$ mice after eight weeks of high fat diet (Kyaw, Tay et al. 2012). A similar outcome was observed in chimeric LDLR$^{-/-}$ mice that were selectively deprived of BAFF-R in B2 cells (Sage, Tsiantoulas et al. 2012). It is clear that B2 cells are viable therapeutic targets for combating atherosclerosis. The next phase of work ought to unveil the atherogenic functions of B2 cells.

1.11 IgG antibody production by B2 cells

IgG is the hallmark isotype of the immunoglobulins produced by B2 cells during an immune reaction. There are multiple IgG subclasses, for example in the mouse – IgG1, IgG2a, IgG2b and IgG3. Each is distinguished by the constant region on the γ heavy chain ($F_c\gamma$). The same applies to the $F_c\gamma$ receptors (e.g. $F_c\gamma$RI, $F_c\gamma$RIIa and $F_c\gamma$RIIb) which differ by the strength of binding to IgG molecules (Ravetch and Bolland 2001, Nimmerjahn and Ravetch 2006). The overall impact of IgG antibodies on atherosclerosis remains controversial with several studies providing distinct views (Schiopu, Bengtsson et al. 2004, Kelly, Griffin et al. 2010). Although oxLDL-IgG antibodies are detectable in the blood of healthy subjects, they are found at much higher levels in atherosclerotic mice and patients with CVD (Tsimikas, Palinski et al. 2001, Fredrikson, Hedblad et al. 2003, Sjogren, Fredrikson et al. 2008). Deposition of oxLDL-IgG within atherosclerotic lesions is also evident (Yla-Herttuala, Palinski et al. 1994). Despite these indications, there are claims that oxLDL-specific IgG

antibodies are mere biomarkers of atherosclerosis and are only suitable for diagnostic and prognostic purposes. This calls for a thorough review of the behaviour of oxLDL-IgG antibodies in atherosclerosis.

1.11.1 Role of oxLDL-IgG in atherosclerosis

Increasing oxLDL-IgG titers through vaccination has shown to reduce atherosclerosis in animal models (Schiopu, Bengtsson et al. 2004, Witztum and Lichtman 2013). In one case, two groups of Watanabe heritable hyperlipidemic rabbits were immunized with MDA-LDL with one beginning at the age of 6 weeks and the other at 6 months for 6.5 months (Palinski, Miller et al. 1995). Although oxLDL-IgG levels were markedly elevated in all immunized rabbits, atherosclerosis was reduced only in the group that began vaccination at 6 months old and not in the younger group of rabbits. oxLDL-IgA was also increased albeit modestly compared to oxLDL-IgG whereas oxLDL-IgM was unaltered by MDA-LDL immunization. This suggests a late-stage atheroprotective role for oxLDL-IgG antibodies. A similar result was obtained in a separate study that examined the effect of immunizing high fat diet-fed White New Zealand rabbits with MDA-LDL (Ameli, Hultgardh-Nilsson et al. 1996). Following the success in rabbits, confirmation of the atheroprotective effect of increasing oxLDL-IgG levels was attained in $LDLR^{-/-}$ and $ApoE^{-/-}$ mice (Freigang, Horkko et al. 1998, Nicoletti, Kaveri et al. 1998, Zhou, Caligiuri et al. 2001, Fredrikson, Soderberg et al. 2003).

The increase in oxLDL-IgG in rabbits with established atherosclerosis may have facilitated ongoing regulatory processes that were still inactive in the young non-atherosclerotic rabbits. A second plausibility points to MDA-LDL immunization increasing a specific IgG subtype (e.g. IgG1) against oxLDL that keeps

atherosclerosis in check. This resonates with the progressive dominance of oxLDL-IgG1 over oxLDL-IgG2a as atherosclerosis develops in hypercholesterolemic mice (Zhou, Paulsson et al. 1998). IgG1 antibodies can bind strongly to $F_c\gamma$Rs. One advantage of this is the rapid sequestration of IgG1 immune complexes by macrophages. On this account, oxLDL-IgG1 antibodies may reduce the retention of oxLDL particles that can otherwise persist to fuel inflammatory processes in the circulation and atherosclerotic lesions at the expense of increased foam cell formation. The antibodies also create a competitive environment for macrophage pattern recognition receptors (PRR) and toll-like receptors (TLR) to bind to oxLDL. Ligation of oxLDL to certain PRR and TLR (e.g. CD14 and TLR4) on macrophages not only enhances inflammation and ingestion of oxLDL but also hampers efferocytosis (Miller, Viriyakosol et al. 2003). These effects may be buffered in the presence of oxLDL-IgG1. The reduction of atherosclerosis after passive immunization with oxLDL-IgG1 in mice further underscores the atheroprotective facet of oxLDL-IgG1 antibodies and their potential for future therapeutic applications (Schiopu, Bengtsson et al. 2004).

A third reason that has to be taken into consideration refers to the types of oxLDL antigen targeted by IgG antibodies. Some oxLDL antigens may be atherogenic while others atheroprotective. Three apoB-100 peptides (p2, p45 and p210) have been identified to protect against atherosclerosis (Freigang, Horkko et al. 1998). Apobec-1$^{-/-}$LDLR$^{-/-}$ mice that were passively immunized with human IgG against MDA-modified apoB-100 p45 showed a significant reduction in atherosclerosis (Schiopu, Frendeus et al. 2007).

1.11.2 Role of $F_c\gamma$ receptors in atherosclerosis

On the contrary, most $F_c\gamma$ receptors are highly atherogenic. They are expressed throughout the medial and intimal regions of human atherosclerotic lesions (Ratcliffe, Kennedy et al. 2001). In mice, the mRNA expression levels of $F_c\gamma$RI, $F_c\gamma$RIIb and $F_c\gamma$RIIIa in the aorta were found to be significantly increased in ApoE$^{-/-}$ mice compared to wild type mice on high fat diet (Hernandez-Vargas, Ortiz-Munoz et al. 2006). Moreover, atherosclerosis was more than halved by gene knockout of the common γ-chain of $F_c\gamma$ in ApoE$^{-/-}$ mice (Hernandez-Vargas, Ortiz-Munoz et al. 2006). The atherosclerotic lesions in ApoE$^{-/-}\gamma^{-/-}$ mice contained less macrophages as well as T-cells. It is also important to note that ApoE$^{-/-}\gamma^{-/-}$ mice express only the inhibitory $F_c\gamma$RIIb but not the activating $F_c\gamma$RI and $F_c\gamma$RIIIa (Hernandez-Vargas, Ortiz-Munoz et al. 2006). This is in line with two other reports of increased atherosclerosis in LDLR$^{-/-}$ and ApoE$^{-/-}$ mice deficient in $F_c\gamma$RIIb (Zhao, Wigren et al. 2010, Mendez-Fernandez, Stevenson et al. 2011). In contrast, LDLR$^{-/-}$ mice that lacked $F_c\gamma$RIII showed decreased atherosclerosis (Kelly, Griffin et al. 2010). The reasons behind these observations are still unknown. There are suggestions of $F_c\gamma$RI / $F_c\gamma$RIIIa and $F_c\gamma$RIIb regulating the expression of Th1 and Th2 cytokines in immune cells respectively (Pricop, Redecha et al. 2001, Kelly, Griffin et al. 2010). Additionally, $F_c\gamma$RI and $F_c\gamma$RIIIa may anchor circulating leukocytes to oxLDL:IgG immune complexes that are increasingly deposited on the vascular endothelium during the development of atherosclerosis (Nagarajan 2007). This can trigger the production of pro-inflammatory chemokines (e.g. MCP-1) that mediate leukocyte infiltration into atherosclerotic lesions as witnessed in ApoE$^{-/-}$ mice in comparison to ApoE$^{-/-}\gamma^{-/-}$ mice (Hernandez-Vargas, Ortiz-Munoz et al. 2006). More specifically, engagement of $F_c\gamma$RI and

$F_c\gamma$RIIIa on monocytes can tip the balance of lesional macrophages toward the pro-inflammatory M1 subtype in lieu of the anti-inflammatory M2 subtype. This potentiality arose when bone marrow transplantation from $ApoE^{-/-}\gamma^{-/-}$ into $ApoE^{-/-}$ mice resulted in smaller and more stable atherosclerotic lesions that contained a lower M1 : M2 ratio (Mallavia, Oguiza et al. 2013).

Perhaps, one way to resolve the complex role of oxLDL-IgG in atherosclerosis is to reconstitute a B cell-deficient model with B2 cells that are engineered to apoptose on differentiation to plasma cells.

1.12 Cytokine production by B2 cells

In addition to their trademark of producing IgG antibodies, B2 cells can react to immunogens by secreting cytokines. The secretion of cytokines empowers B2 cells with the ability to launch multiple responses systematically rather than sequentially against a range of targets. It also enables B2 cells to complement the responses that are mediated by other immune cells. In general, cytokine-producing B2 cells fall into two categories – B effector 1 (Be-1) and B effector 2 (Be-2), based on the cytokines that they produce. Be-1 cells mainly secrete pro-inflammatory cytokines (e.g. IFNγ and TNFα) while Be-2 cells secrete anti-inflammatory cytokines (e.g. IL-4, IL-10 and TGF-β) (Lund 2008). This coincides with the designation of Th1 and Th2 cells for CD4+ T cells. Presently, it is understood that Th1 and Th2 cells stimulate B2 cells to assume Be-1 and Be-2 roles respectively. Evidence for this came from the dominance of Be-1 cells in Th1 primed mice and Be-2 cells in Th2 primed mice (Harris, Haynes et al. 2000). Human and mouse studies also showed that Th1 and Th2 activities are further reinforced by Be-1 and Be-2 cells in a reciprocal manner (Schultze, Michalak

et al. 1999, Harris, Haynes et al. 2000, Shirota, Sano et al. 2002, Wagner, Poeck et al. 2004, Gagro, Servis et al. 2006). The bilateral relationship between Be-1/Be-2 and Th1/Th2 serves as a central node that consolidates Type 1/2 immunity for maximal effect on target antigens or allergens.

Be-1 cells are alleged culprits of the Type 1 immune responses that drive atherosclerosis. This was conceived from the peri-adventitial localization of B cells and pro-inflammatory cytokines (TNFα and IL-6) in atherosclerotic lesions of $ApoE^{-/-}$ mice (Zhou and Hansson 1999). Moreover, the antibody-independent remission of autoimmune disorders in rituximab-treated patients suggests an important role for Be-1 cells in promoting pathological inflammation, a characteristic of atherosclerosis (Tsokos 2004, Ramos-Casals, Soto et al. 2009). In this regard, TNFα- and IFNγ-producing Be-1 cells are well geared to exact atherogenic effects due to their versatility in stimulating T cells and macrophages within lymphoid tissues and at inflammatory sites. Here, I outline the functions of these cells and their potential to aggravate atherosclerosis.

1.12.1 Role of TNFα-producing B2 cells in lymphoid organs

It is imperative that B2 cells produce TNFα for the immune system to stay vigilant. B2-derived TNFα is fundamental for the development and maintenance of key structures in secondary lymphoid organs (Gonzalez, Mackay et al. 1998, Endres, Alimzhanov et al. 1999, Ngo, Cornall et al. 2001, Tumanov, Grivennikov et al. 2010). This includes the marginal zone, primary B cell follicles and follicular dendritic cell network (FDC). The latter two are major components of the germinal center (GC). The GC provides a niche for B cell : T cell cross-talk, clonal expansion of activated B cells, plasma cell differentiation, antibody isotype switching and affinity maturation.

Antigens are presented in the FDC for positive and negative selection of maturing B cells and stimulation of naïve B cells, GC B cells and memory B cells (Su and Rawlings 2002, Allen and Cyster 2008). Lymphoid organogenesis and humoral responses are severely disrupted in mice with specific ablation of TNFα in B cells (Tumanov, Grivennikov et al. 2010).

It is also highly probable that B2-derived TNFα regulates ectopic lymphangiogenesis. This can directly influence atherosclerosis with the formation of aortic tertiary lymphoid organs (ATLO). ATLOs containing GC B cells, T cells, plasma cells and DCs are located in the adventitia immediately adjacent to atherosclerotic lesions (Houtkamp, de Boer et al. 2001, Maiellaro and Taylor 2007, Galkina and Ley 2009, Campbell, Lipinski et al. 2012). Their importance is reflected by the immune cell clusters becoming increasingly organized as atherosclerosis progresses. A significant proportion of adventitial $CD45^+$ leukocytes are IFNγ-expressing Th1 cells (Butcher, Herre et al. 2011). It is worthwhile to point out that B2-derived TNFα potentiates the production of IFNγ by T cells during Toxoplasma gondii infection in mice (Menard, Minns et al. 2007). A similar affair may occur in the ATLO since the B cell and T cell nodules are in close contact with each other. IgG antibodies specific for oxLDL have been shown in rabbit and human atherosclerotic lesions, a clear sign of interaction between B2 cells and CD4 T cells (Yla-Herttuala, Palinski et al. 1994).

Immune cells may commute between the intimal lesion and ATLO through the vasa vasorum (Campbell, Lipinski et al. 2012). A mosaic of chemotactic factors, lectins and selectins, has been implicated in directing the cellular traffic. There is also

speculation that the vasa vasorum provides a passage for cytokines, chemokines and antibodies to pass from either side (Campbell, Lipinski et al. 2012).

For future advances in this area, three hurdles need to be overcome. The first is to determine whether ATLOs modulate atherosclerosis independently of the peripheral lymphoid organs (e.g. spleen). This may aid in designing therapies specifically against immune responses only within ATLOs. The second concerns the role of B2-derived TNFα in the ATLO. This can be addressed with the recent finding that CCR6 expression is required for B cells to home to the aorta (Doran, Lipinski et al. 2012). $CCR6^{-/-}$ mice have markedly reduced aortic B cell numbers. This creates the opportunity to reconstitute the aorta of B cell-restricted CCR6-deficient chimeric mice with TNFα-deficient or TNFα-sufficient B2 cells by adoptive transfer. The adoptively transferred B2 cells are expected to localise and accumulate in the aortic adventitia (Galkina, Kadl et al. 2006). Third, the identification of other cytokines/chemokines that are co-expressed in TNFα-expressing B2 cells may provide more options to block the inflammatory effects of these cells. A recent report of local B2 cells expressing TNFα, IL-6 and GM-CSF in the aortic adventitia and atherosclerotic plaque of humans corroborates further characterization of cytokine-producing B2 cells in these regions (Hamze, Desmetz et al. 2013).

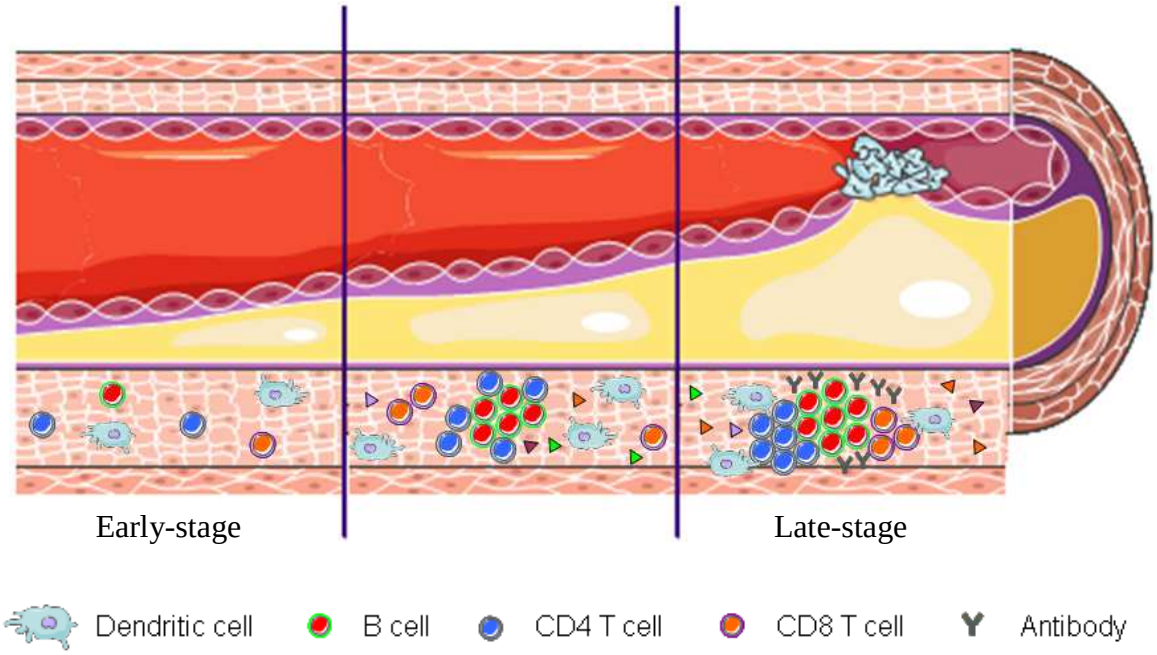

Figure 4 : As atherosclerosis develops, immune cells in the adventitial layer adjacent to the lesion become increasingly organized and form clusters that resemble germinal centres in lymphoid organs. Germinal centre responses in the adventitia are typified by clonal expansion of activated B and T cells, plasma cell differentiation and affinity maturation. These result in the production of oxLDL-specific IgG antibodies, oxLDL-specific T cells and the secretion of cytokines and chemokines.

1.12.2 Role of TNFα-producing B2 cells in atherosclerotic lesions

Within the atherosclerotic lesion, B2 cells may foster a cycle of inflammation by releasing TNFα. This feature could endow B2 cells with the ability to promote atherosclerosis T-independently as shown in lymphocyte-deficient $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice following adoptive B2 cell transfer (Kyaw, Tay et al. 2010). TNFα is a pleiotropic cytokine that promotes inflammation, cell proliferation, migration, differentiation, survival and death. It is linked to an extensive list of pathophysiological disorders. Treatment with thalidomide, an inhibitor of TNFα expression, or soluble TNFα decoy receptor reduces atherosclerosis in $ApoE^{-/-}$ mice (Elhage, Maret et al. 1998, Chew, Zhou et al. 2003). $ApoE^{-/-}$ mice genetically deficient in TNFα were shown to have significantly less atherosclerosis compared to

control ApoE$^{-/-}$ mice after high fat feeding (Ohta, Wada et al. 2005, Kober, Canault et al. 2007). Similarly, less advanced atherosclerotic lesions were present in TNFα$^{-/-}$ ApoE*3-leiden mice (Boesten, Zadelaar et al. 2005). Moreover, bone marrow transplantation from ApoE$^{-/-}$TNFα$^{-/-}$ mice to ApoE$^{-/-}$ mice was carried out to selectively ablate TNFα in leukocytes. As expected, atherosclerosis was reduced by nearly 80% in recipients of ApoE$^{-/-}$TNFα$^{-/-}$ bone marrow cells compared to control recipients (Branen, Hovgaard et al. 2004). To differentiate the atherogenic effects of membrane-bound TNFα and soluble TNFα, ApoE$^{-/-}$ mice transgenic for a mutated form of TNFα that could only be expressed on but not released from the cell membrane was evaluated. Although atherosclerosis was reduced in these mice compared to ApoE$^{-/-}$ mice expressing wild type TNFα, the degree of reduction was markedly less than that in ApoE$^{-/-}$ mice with total TNFα deficiency (Canault, Peiretti et al. 2008). This indicates that TNFα is more atherogenic when secreted (Canault, Peiretti et al. 2004, Canault, Peiretti et al. 2008).

There are two receptors for TNFα - TNFR1 and TNFR2. While TNFR1 is more reactive to soluble TNFα and is constitutively produced in all cell types, TNFR2 has a preference for membrane-bound TNFα and is mainly expressed in immune cells (Wajant, Pfizenmaier et al. 2003). Both receptors have distinct responses when activated by TNFα. The majority of TNFα-mediated inflammatory processes (e.g. activation of vascular adhesion molecules), including TNFα-induced cell death by apoptosis, are transduced through TNFR1 (Wajant, Pfizenmaier et al. 2003, Zhang, Peppel et al. 2007). Quantification of apoptotic cells in atherosclerotic plaques indicated that TNFα-deficient mice had a lower frequency of plaque apoptotic cells compared to TNFα-sufficient mice (Canault, Peiretti et al. 2008). A second reason

that can explain the decrease in plaque apoptosis in TNFα-deficient mice relates to TNFα-mediated inhibition of apoptotic cell clearance by macrophages (McPhillips, Janssen et al. 2007). This was shown to be especially prevalent in the presence of oxidants and inflammatory factors – a classic environment in atherosclerotic plaques. The efferocytic function of mature macrophages can be impaired by TNFα through TNFR1 and TNFR2 (McPhillips, Janssen et al. 2007).

Besides, other studies have claimed that TNFα stimulates the production of MCP-1 in endothelial cells (Murao, Ohyama et al. 2000, Park, Yang et al. 2004). MCP-1 and its receptor, CCR2, are crucial for monocyte infiltration into atherosclerotic plaques. Mice that are deficient in MCP-1 and CCR2 have not only smaller but more stable atherosclerotic plaques (Gu, Okada et al. 1998, Guo, Van Eck et al. 2003). TNFα may also promote the formation of foam cells by upregulating scavenger receptors in macrophages for LDL uptake (Hsu and Twu 2000). The diverse range of TNFα producers (e.g. macrophages and T cells) poses a conundrum for deciphering the role of TNFα-producing B2 cells in atherosclerotic lesions. However, studies have presented distinct inflammatory effects of TNFα derived from discrete cellular sources (de Boer, van der Wal et al. 1999, Grivennikov, Tumanov et al. 2005). This firmly supports the impetus to define the specific role of B2-derived TNFα in atherosclerosis.

1.12.3 Blocking the effects of B2-derived TNFα

Anti-TNFα agents are currently employed to treat patients with inflammatory diseases. Insufficient remission and recurrence of disease are amongst the outstanding problems (Engel, Gomez-Puerta et al. 2011). Until recently, the principle function of TNFα blockers had been assumed to antagonise the effects of TNFα. Remarkably,

there is new evidence of anti-TNFα agents reducing GC B2 cells in lymphoid organs as well as circulating memory B2 cells in the blood (Anolik, Ravikumar et al. 2008). It is suspected that the occasional relapse of disease is due to resurgence of pathogenic B2 cells post anti-TNFα treatment. Lately, one countermeasure has taken shape to overcome this shortfall of anti-TNFα agents. The scheme engages the therapeutic depletion of B cells and has proven to be effective in patients who do not respond adequately to anti-TNFα therapies (Engel, Gomez-Puerta et al. 2011). Three key questions need to be answered before a similar strategy can be applied to treat atherosclerosis. The first is to resolve the roles of GC B2 cells and memory B2 cells in atherosclerosis. The second requires the assessment of whether TNFα-producing B2 cells are neutralised by anti-TNFα agents. The third entails comparing the efficacies of anti-TNFα agents and B2 cell depletion therapies in modulating atherosclerosis.

1.12.4 Role of IFNγ-producing B2 cells in inflammation

B2 cells can also augment inflammation by producing IFNγ. In vivo expression of IFNγ was evident in wild type B2 cells that were adoptively transferred into IFNγ$^{-/-}$ recipient mice treated with IL-12 and IL-18 (Yoshimoto, Okamura et al. 1997). The same was observed in B2 cells that were activated with Th1 cells or a combination of anti-CD40 antibody, IL-12 and IL-18 in vitro (Yoshimoto, Okamura et al. 1997, Harris, Haynes et al. 2000). IFNγ-producing B2 cells were also found to induce resting CD4 T-cells to differentiate into Th1 cells (Harris, Haynes et al. 2000, Lund 2008). They were predominant in Toxoplasma gondii-infected mice as well as Borrelia burgdorferi-infected mice (Harris, Haynes et al. 2000, Ganapamo, Dennis et al. 2001). B2 cells that were isolated from such mice secreted IFNγ at levels similar to those of non-B cells ex vivo. Furthermore, the production of IFNγ by B2 cells gave rise to a Th1-skewed setting as shown by decreased IgG1 and increased IgG2a

production (Yoshimoto, Okamura et al. 1997). IgG1 and IgG2a are signature antibody isotypes of Th2 and Th1 responses in mice respectively. Naïve B2 cells have low basal expression of IFNγ. When activated by Th1 cells, a chain of signals is initiated by IFNγ-receptor (IFNγR) to upregulate IFNγ expression in B2 cells. The increase in IFNγ mRNA transcription can be as high as 100-fold (Harris, Goodrich et al. 2005). One characteristic that has enticed much interest is the specific molecular factors that distinguish IFNγ-producing Be-1 cells from other cytokine-producing Be-1 cells (e.g. TNFα and IL-2). In the case of Th1-mediated activation, the pathway is believed to begin with Th1 cells recognizing MHCII-restricted antigens on B2 cells. The cognate Th1 cells release IFNγ that in turn binds to IFNγR on B2 cells. This results in the downregulation of GATA-3 and upregulation of T-bet in B2 cells (Harris, Goodrich et al. 2005). Similar to Th1 cells, T-bet is indispensable for B2 cells to produce IFNγ. T-bet$^{-/-}$ B2 cells, however, can differentiate into TNFα-producing and IL2-producing Be-1 cells. Beyond this stage, Th1-derived IFNγ is not required to maintain IFNγ-producing Be-1 cells. Instead, an autocrine feedback system is formed to sustain IFNγ production in Be-1 cells (Harris, Goodrich et al. 2005). The same occurs when B2 cells are stimulated by IL-12, IL-18 and other antigens/ligands. IFNγ production is first turned on by the IL-12 receptor and subsequently taken over by the IFNγ : IFNγR : T-bet axis (Harris, Goodrich et al. 2005). This commits IFNγ-producing Be-1 cells to chronic Type 1 inflammation.

1.12.5 Role of IFNγ in atherosclerosis

IFNγ imposes a spectrum of effects on atherosclerosis. Human and mouse atherosclerotic lesions contain considerable amounts of IFNγ (Hansson, Holm et al. 1989, George, Shoenfeld et al. 2000, Huber, Sakkinen et al. 2001). Injection of

recombinant IFNγ produced larger atherosclerotic lesions in spite of a reduction in plasma cholesterol compared to injection of saline in ApoE$^{-/-}$ mice (Whitman, Ravisankar et al. 2000). This damaging action prevailed even in a lymphocyte free environment where vascular smooth muscle cells proliferated in response to IFNγ-mediated expression of platelet derived growth factor resulting in intimal expansion (Tellides, Tereb et al. 2000). In addition, ApoE$^{-/-}$ mice deficient in IFNγ or IFNγR had significantly less atherosclerosis (Gupta, Pablo et al. 1997, Whitman, Ravisankar et al. 2002). This lends credence to IFNγ as an atherogenic cytokine.

1.12.6 Atherogenic effects of IFNγ

The atherogenic role of IFNγ is mainly due to its inflammatory properties. One of the most potent manners which IFNγ promotes inflammation is through antigen presentation. Professional APCs (B2 cells, macrophages and DCs) respond rapidly to IFNγ by upregulating MHCII and regulatory proteins (e.g. cathepsins B, H, L) that prepare and load antigens onto MHCII complexes for surveillance by CD4 T cells (Wong, Clark-Lewis et al. 1983, Stemme, Fager et al. 1990, McLaren and Ramji 2009). MHCII antigen presentation is a major component of the adaptive immune system behind atherosclerosis. This was validated in LDLR$^{-/-}$ mice deficient in CD74 (Sun, Hartvigsen et al. 2010). CD74 is known as the invariant chain of MHCII. It chaperones the folding and assembly of the α and β chains of MHCII in the endoplasmic reticulum and ushers the assembled MHCII molecule into transport organelles for antigen loading. Without CD74, the MHCII α and β chains remain as single entities and the total CD4 T cell pool in the thymus and periphery is reduced (Pieters 2000). LDLR$^{-/-}$CD74$^{-/-}$ mice showed a marked decrease in atherosclerosis compared to control LDLR$^{-/-}$ mice. Lesion numbers of CD4 T cells and CD25^{+} activated T cells were also lower in LDLR$^{-/-}$CD74$^{-/-}$ mice (Sun, Hartvigsen et al.

2010). Furthermore, HSP65-immunization only exacerbated atherosclerosis in LDLR$^{-/-}$ mice but not in LDLR$^{-/-}$CD74$^{-/-}$ mice (Sun, Hartvigsen et al. 2010). The latter was likely due to a dysfunction in APC activity. In accord with the capacity of IFNγ in enhancing MHCII expression, increased MHCII^{+} cells and CD4 T cells were found in the lesions of ApoE$^{-/-}$ mice administered with IFNγ (Tellides, Tereb et al. 2000, Whitman, Ravisankar et al. 2000). These were reversed in ApoE$^{-/-}$IFNγ$^{-/-}$ mice (Whitman, Ravisankar et al. 2002). The relationship between IFNγ and MHCII antigen presentation clearly speaks volumes of the atherogenic potential of IFNγ.

Apart from CD4 T cells, IFNγ can mobilise cytotoxic CD8 T cells by inducing the formation of antigen : MHCI complex (Schroder, Hertzog et al. 2004). MHCI antigen presentation operates in professional and non-professional APCs. It can be active in the late phases of atherosclerosis when cytotoxic CD8 T cells have been shown to target smooth muscle cells, endothelial cells and macrophages for apoptosis (Kyaw, Winship et al. 2013). The premature death of these cells predisposes atherosclerotic plaques to necrotic inflammation and increased vulnerability to rupture. Given that IFNγ-producing Be-1 cells autoregulate their production of IFNγ, they are conditioned to present antigens to T cells. This assigns B2 cells with a site-specific atherogenic role that may only come about locally within inflamed tissues where an abundance of antigen-specific T cells congregate.

A more direct atherogenic function of IFNγ is the support that it provides for the formation of foam cells. IFNγ promotes the uptake of oxLDL by enhancing the expression of SR-A and scavenger receptor for phosphatidylserine and oxLDL in macrophages and VSMCs (Greaves, Gough et al. 1998, Shimaoka, Kume et al. 2000,

McLaren and Ramji 2009). IFNγ also mediates the retention of cholesterol esters within cells as it upregulates Acyl coenzyme A acylcholesterol transferase (ACAT) (Panousis and Zuckerman 2000). ACAT catalyses the esterification of cholesterol. Cholesterol esters are highly lipophobic and cannot be integrated in the cell membrane lipid bilayer. They are packaged and stored in lipid droplets in the cytosol (Li and Glass 2002). Finally, IFNγ inhibits the expression of ABCA1, a member of the ATP-binding cassette transporter family, and mitochondrial P450 enzyme cholesterol 27-hydroxylase in macrophages (Panousis and Zuckerman 2000, Wang, Panousis et al. 2002). These two molecules aid in expelling cholesterol from cells (Oram and Heinecke 2005). From a T-independent perspective, Be-1 derived IFNγ may act directly on plaque macrophages to mediate their transformation into foam cells. Notwithstanding its contribution to the accumulation of foam cells, IFNγ is known to increase the expression of pro-apoptotic factors such as TNFα receptor 1, FAS and caspase 8 in monocytes, macrophages and VSMCs (Tamura, Ueda et al. 1996, Inagaki, Yamagishi et al. 2002). This can inevitably lead to the growth of necrotic cores in advanced lesions. Two other atherogenic effects of IFNγ are the induction of adhesion molecules (e.g. ICAM-1 and VCAM-1) and chemokines (e.g. MCP-1) that recruit immune cells into lesions (Li, Cybulsky et al. 1993, Boisvert 2004).

1.13 Concluding remarks

The contrasting roles of B1a and B2 cells in atherosclerosis have renewed interest in the search of novel therapies to prevent CVD. Whereas B1a cells protect against atherosclerosis, B2 cells aggravate atherosclerosis. The production of natural IgM antibodies is key to the atheroprotective function of B1a cells. Although there are

several features of natural IgM that are asserted to modulate inflammation, their impact on atherosclerosis is not known. The proposed atheroprotective role of B1a-derived natural IgM in facilitating efferocytosis in atherosclerotic lesions needs to be more robustly tested. Studies have shown that the defect in efferocytosis occurs not at the fatty streak stage but during the development of atherosclerotic plaques. By increasing B1a-derived natural IgM in developing plaques, efferocytosis is expected to be restored and the plaques rescued from necrosis-induced inflammation. This has been partially addressed in B1a-reconstituted splenectomised $ApoE^{-/-}$ mice. However, the site of atheroprotective action of natural IgM-secreting B1a cells has still not been determined. For instance, the need for B1a cells to traffick to atherosclerotic lesions to secrete natural IgM remains unresolved. Subsequent experiments should consider selectively depleting and expanding B1a cells in uncompromised mice to assess whether the resulting differences in natural IgM deposition affect efferocytosis and inflammation in developing lesions.

As for B2 cells, a potential strategy is to delineate their atherogenic role into T-dependent and T-independent effector functions. B2 cells can promote atherosclerosis without help from other lymphocytes. Resident B2 cells are present in the aortic wall even before the initiation of atherosclerosis. They gradually increase in number as atherosclerosis develops and are also found within developing atherosclerotic lesions. Thus, it is possible that B2 cells exert atherogenic effects directly at the site of lesions. They may respond to atherogenic signals by releasing inflammatory cytokines such as TNFα and IFNγ as they do in other inflammatory settings. These cytokines can promote atherosclerosis in a variety of ways through leukocyte recruitment, macrophage activation and apoptosis and VSMC proliferation. The T-dependent

effect of B2 cells is likely drawn from GC response in secondary lymphoid organs and possibly ATLOs. GC is a point of contact for B2 cells, CD4 T cells and DCs. The activities that take place in the GC are related to adaptive immunity. The clearest indications of GC involvement in the development of atherosclerosis are the increased levels of oxLDL-IgG and oxLDL-specific CD4 T cells. Antigen presentation is the main event that is responsible for these responses. Work is needed to measure the redundancy shared between B2 cells and DCs in presenting oxLDL antigens to CD4 T cells and compare the atherogenicity of oxLDL-specific CD4 T cells pulsed by either of them.

There is certainly a long road ahead to completely understand the immune processes that beget atherosclerosis. Advancements in real time in vivo imaging techniques would add a new dimension to examine the cellular activities that take place in atherosclerosis.

1.14 Aims of thesis

Atherosclerosis is a chronic inflammatory disorder. There is substantial evidence of natural IgM antibodies protecting against the development of atherosclerosis. Given that B1a B cells are the primary producers of natural IgM antibodies, it is highly possible that B1a cells play a major atheroprotective role. On the other hand, conventional B2 B cells have been ascribed pathogenic roles in autoimmune diseases. They are known to drive inflammation by producing pathogenic IgG antibodies, activating T cells and producing inflammatory cytokines. It is likely that B2 cells contribute to the development of atherosclerosis. The focus of this thesis is to resolve the differential roles and functions of B1a cells and B2 cells in atherosclerosis.

- To determine the effect of depleting B cells on the development and progression of atherosclerosis
- To determine the role and mechanistic action of B1a cells in atherosclerosis
- To determine the role and mechanistic action of B2 cells in atherosclerosis
- To determine the effect of specifically depleting B2 cells on atherosclerosis

1.15 References

Ait-Oufella, H., O. Herbin, J. D. Bouaziz, C. J. Binder, C. Uyttenhove, L. Laurans, S. Taleb, E. Van Vre, B. Esposito, J. Vilar, J. Sirvent, J. Van Snick, A. Tedgui, T. F. Tedder and Z. Mallat (2010). "B cell depletion reduces the development of atherosclerosis in mice." J Exp Med **207**(8): 1579-1587.

Allen, C. D. and J. G. Cyster (2008). "Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function." Semin Immunol **20**(1): 14-25.

Amano, M., N. Baumgarth, M. D. Dick, L. Brossay, M. Kronenberg, L. A. Herzenberg and S. Strober (1998). "CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms." J Immunol **161**(4): 1710-1717.

Ameli, S., A. Hultgardh-Nilsson, J. Regnstrom, F. Calara, J. Yano, B. Cercek, P. K. Shah and J. Nilsson (1996). "Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits." Arterioscler Thromb Vasc Biol **16**(8): 1074-1079.

Anolik, J. H., R. Ravikumar, J. Barnard, T. Owen, A. Almudevar, E. C. Milner, C. H. Miller, P. O. Dutcher, J. A. Hadley and I. Sanz (2008). "Cutting edge: anti-tumor necrosis factor therapy in rheumatoid arthritis inhibits memory B lymphocytes via effects on lymphoid germinal centers and follicular dendritic cell networks." J Immunol **180**(2): 688-692.

Arnon, T. I., R. M. Horton, I. L. Grigorova and J. G. Cyster (2013). "Visualization of splenic marginal zone B-cell shuttling and follicular B-cell egress." Nature **493**(7434): 684-688.

Baumgarth, N., J. W. Tung and L. A. Herzenberg (2005). "Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion." Springer Semin Immunopathol **26**(4): 347-362.

Berland, R. and H. H. Wortis (2002). "Origins and functions of B-1 cells with notes on the role of CD5." Annu Rev Immunol **20**: 253-300.

Berliner, J. A., M. C. Territo, A. Sevanian, S. Ramin, J. A. Kim, B. Bamshad, M. Esterson and A. M. Fogelman (1990). "Minimally modified low density lipoprotein stimulates monocyte endothelial interactions." J Clin Invest **85**(4): 1260-1266.

Binder, C. J., M. K. Chang, P. X. Shaw, Y. I. Miller, K. Hartvigsen, A. Dewan and J. L. Witztum (2002). "Innate and acquired immunity in atherogenesis." Nat Med **8**(11): 1218-1226.

Binder, C. J., K. Hartvigsen, M. K. Chang, M. Miller, D. Broide, W. Palinski, L. K. Curtiss, M. Corr and J. L. Witztum (2004). "IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis." J Clin Invest **114**(3): 427-437.

Binder, C. J., P. X. Shaw, M. K. Chang, A. Boullier, K. Hartvigsen, S. Horkko, Y. I. Miller, D. A. Woelkers, M. Corr and J. L. Witztum (2005). "The role of natural antibodies in atherogenesis." J Lipid Res **46**(7): 1353-1363.

Boesten, L. S., A. S. Zadelaar, A. van Nieuwkoop, M. J. Gijbels, M. P. de Winther, L. M. Havekes and B. J. van Vlijmen (2005). "Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice." Cardiovasc Res **66**(1): 179-185.

Boisvert, W. A. (2004). "Modulation of atherogenesis by chemokines." Trends Cardiovasc Med **14**(4): 161-165.

Bourchier M, B. K., Chau T, McIntosh N, Moon L, Woodall J (2011). "Cardiovascular disease: Australian facts 2011. Cardiovascular disease series. Cat. no. CVD 53. Canberra: AIHW.".

Branen, L., L. Hovgaard, M. Nitulescu, E. Bengtsson, J. Nilsson and S. Jovinge (2004). "Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice." Arterioscler Thromb Vasc Biol **24**(11): 2137-2142.

Buono, C., C. J. Binder, G. Stavrakis, J. L. Witztum, L. H. Glimcher and A. H. Lichtman (2005). "T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses." Proc Natl Acad Sci U S A **102**(5): 1596-1601.

Butcher, M. J., M. Herre, K. Ley and E. Galkina (2011). "Flow cytometry analysis of immune cells within murine aortas." J Vis Exp(53).

Caligiuri, G., A. Nicoletti, B. Poirier and G. K. Hansson (2002). "Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice." J Clin Invest **109**(6): 745-753.

Campbell, K. A., M. J. Lipinski, A. C. Doran, M. D. Skaflen, V. Fuster and C. A. McNamara (2012). "Lymphocytes and the adventitial immune response in atherosclerosis." Circ Res **110**(6): 889-900.

Canault, M., F. Peiretti, C. Mueller, F. Kopp, P. Morange, S. Rihs, H. Portugal, I. Juhan-Vague and G. Nalbone (2004). "Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus." Atherosclerosis **172**(2): 211-218.

Canault, M., F. Peiretti, M. Poggi, C. Mueller, F. Kopp, B. Bonardo, D. Bastelica, A. Nicolay, M. C. Alessi and G. Nalbone (2008). "Progression of atherosclerosis in ApoE-deficient mice that express distinct molecular forms of TNF-alpha." J Pathol **214**(5): 574-583.

Carbone, F. R., G. T. Belz and W. R. Heath (2004). "Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity." Trends Immunol **25**(12): 655-658.

Cariappa, A., C. Chase, H. Liu, P. Russell and S. Pillai (2007). "Naive recirculating B cells mature simultaneously in the spleen and bone marrow." Blood **109**(6): 2339-2345.

Casola, S. (2007). "Control of peripheral B-cell development." Curr Opin Immunol **19**(2): 143-149.

Casola, S., K. L. Otipoby, M. Alimzhanov, S. Humme, N. Uyttersprot, J. L. Kutok, M. C. Carroll and K. Rajewsky (2004). "B cell receptor signal strength determines B cell fate." Nat Immunol **5**(3): 317-327.

Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner and J. L. Witztum (2004). "Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory." J Exp Med **200**(11): 1359-1370.

Chen, Y., S. Khanna, C. S. Goodyear, Y. B. Park, E. Raz, S. Thiel, C. Gronwall, J. Vas, D. L. Boyle, M. Corr, D. H. Kono and G. J. Silverman (2009). "Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis." J Immunol **183**(2): 1346-1359.

Chew, M., J. Zhou, A. Daugherty, T. Eriksson, S. Ellermann-Eriksen, P. R. Hansen and E. Falk (2003). "Thalidomide inhibits early atherogenesis in apoE-deficient mice." APMIS Suppl(109): 113-116.

Chou, M. Y., L. Fogelstrand, K. Hartvigsen, L. F. Hansen, D. Woelkers, P. X. Shaw, J. Choi, T. Perkmann, F. Backhed, Y. I. Miller, S. Horkko, M. Corr, J. L. Witztum and C. J. Binder (2009). "Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans." J Clin Invest **119**(5): 1335-1349.

Chung, J. B., R. A. Sater, M. L. Fields, J. Erikson and J. G. Monroe (2002). "CD23 defines two distinct subsets of immature B cells which differ in their responses to T cell help signals." Int Immunol **14**(2): 157-166.

Chung, J. B., M. Silverman and J. G. Monroe (2003). "Transitional B cells: step by step towards immune competence." Trends Immunol **24**(6): 343-349.

Cinamon, G., M. A. Zachariah, O. M. Lam, F. W. Foss, Jr. and J. G. Cyster (2008). "Follicular shuttling of marginal zone B cells facilitates antigen transport." Nat Immunol **9**(1): 54-62.

Coutinho, A., M. D. Kazatchkine and S. Avrameas (1995). "Natural autoantibodies." Curr Opin Immunol **7**(6): 812-818.

Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz and A. M. Fogelman (1990). "Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells." Proc Natl Acad Sci U S A **87**(13): 5134-5138.

Davenport, P. and P. G. Tipping (2003). "The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice." Am J Pathol **163**(3): 1117-1125.

de Boer, O. J., A. C. van der Wal, C. E. Verhagen and A. E. Becker (1999). "Cytokine secretion profiles of cloned T cells from human aortic atherosclerotic plaques." J Pathol **188**(2): 174-179.

Doran, A. C., M. J. Lipinski, S. N. Oldham, J. C. Garmey, K. A. Campbell, M. D. Skaflen, A. Cutchins, D. J. Lee, D. K. Glover, K. A. Kelly, E. V. Galkina, K. Ley, J. L. Witztum, S. Tsimikas, T. P. Bender and C. A. McNamara (2012). "B-cell aortic homing and atheroprotection depend on Id3." Circ Res **110**(1): e1-12.

Dorshkind, K. and E. Montecino-Rodriguez (2007). "Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential." Nat Rev Immunol **7**(3): 213-219.

Ehrenstein, M. R. and C. A. Notley (2010). "The importance of natural IgM: scavenger, protector and regulator." Nat Rev Immunol **10**(11): 778-786.

Elhage, R., A. Maret, M. T. Pieraggi, J. C. Thiers, J. F. Arnal and F. Bayard (1998). "Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice." Circulation **97**(3): 242-244.

Endres, R., M. B. Alimzhanov, T. Plitz, A. Futterer, M. H. Kosco-Vilbois, S. A. Nedospasov, K. Rajewsky and K. Pfeffer (1999). "Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells." J Exp Med **189**(1): 159-168.

Engel, P., J. A. Gomez-Puerta, M. Ramos-Casals, F. Lozano and X. Bosch (2011). "Therapeutic targeting of B cells for rheumatic autoimmune diseases." Pharmacol Rev **63**(1): 127-156.

Fagarasan, S., N. Watanabe and T. Honjo (2000). "Generation, expansion, migration and activation of mouse B1 cells." Immunol Rev **176**: 205-215.

Ferguson, A. R., M. E. Youd and R. B. Corley (2004). "Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells." Int Immunol **16**(10): 1411-1422.

Fredrikson, G. N., B. Hedblad, G. Berglund, R. Alm, M. Ares, B. Cercek, K. Y. Chyu, P. K. Shah and J. Nilsson (2003). "Identification of immune responses against aldehyde-modified peptide sequences in apoB associated with cardiovascular disease." Arterioscler Thromb Vasc Biol **23**(5): 872-878.

Fredrikson, G. N., I. Soderberg, M. Lindholm, P. Dimayuga, K. Y. Chyu, P. K. Shah and J. Nilsson (2003). "Inhibition of atherosclerosis in apoE-null mice by immunization with apoB-100 peptide sequences." Arterioscler Thromb Vasc Biol **23**(5): 879-884.

Freigang, S., S. Horkko, E. Miller, J. L. Witztum and W. Palinski (1998). "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**(12): 1972-1982.

Friedman, P., S. Horkko, D. Steinberg, J. L. Witztum and E. A. Dennis (2002). "Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids. Importance of Schiff base formation and aldol condensation." J Biol Chem **277**(9): 7010-7020.

Gagro, A., D. Servis, A. M. Cepika, K. M. Toellner, G. Grafton, D. R. Taylor, S. Branica and J. Gordon (2006). "Type I cytokine profiles of human naive and memory B lymphocytes: a potential for memory cells to impact polarization." Immunology **118**(1): 66-77.

Galkina, E., A. Kadl, J. Sanders, D. Varughese, I. J. Sarembock and K. Ley (2006). "Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent." J Exp Med **203**(5): 1273-1282.

Galkina, E. and K. Ley (2009). "Immune and inflammatory mechanisms of atherosclerosis (*)." Annu Rev Immunol **27**: 165-197.

Ganapamo, F., V. A. Dennis and M. T. Philipp (2001). "CD19(+) cells produce IFN-gamma in mice infected with Borrelia burgdorferi." Eur J Immunol **31**(12): 3460-3468.

Garaud, S., C. Le Dantec, A. R. de Mendoza, R. A. Mageed, P. Youinou and Y. Renaudineau (2009). "IL-10 production by B cells expressing CD5 with the alternative exon 1B." Ann N Y Acad Sci **1173**: 280-285.

George, J., Y. Shoenfeld, B. Gilburd, A. Afek, A. Shaish and D. Harats (2000). "Requisite role for interleukin-4 in the acceleration of fatty streaks induced by heat shock protein 65 or Mycobacterium tuberculosis." Circ Res **86**(12): 1203-1210.

Glass, C. K. and J. L. Witztum (2001). "Atherosclerosis. the road ahead." Cell **104**(4): 503-516.

Gonzalez, M., F. Mackay, J. L. Browning, M. H. Kosco-Vilbois and R. J. Noelle (1998). "The sequential role of lymphotoxin and B cells in the development of splenic follicles." J Exp Med **187**(7): 997-1007.

Greaves, D. R., P. J. Gough and S. Gordon (1998). "Recent progress in defining the role of scavenger receptors in lipid transport, atherosclerosis and host defence." Curr Opin Lipidol **9**(5): 425-432.

Griffin, D. O., N. E. Holodick and T. L. Rothstein (2011). "Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70." J Exp Med **208**(1): 67-80.

Grivennikov, S. I., A. V. Tumanov, D. J. Liepinsh, A. A. Kruglov, B. I. Marakusha, A. N. Shakhov, T. Murakami, L. N. Drutskaya, I. Forster, B. E. Clausen, L. Tessarollo, B. Ryffel, D. V. Kuprash and S. A. Nedospasov (2005). "Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects." Immunity **22**(1): 93-104.

Gronwall, C., Y. Chen, J. Vas, S. Khanna, S. Thiel, M. Corr, D. H. Kono and G. J. Silverman (2012). "MAPK phosphatase-1 is required for regulatory natural

autoantibody-mediated inhibition of TLR responses." Proc Natl Acad Sci U S A **109**(48): 19745-19750.

Gronwall, C., J. Vas and G. J. Silverman (2012). "Protective Roles of Natural IgM Antibodies." Front Immunol **3**: 66.

Gu, L., Y. Okada, S. K. Clinton, C. Gerard, G. K. Sukhova, P. Libby and B. J. Rollins (1998). "Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice." Mol Cell **2**(2): 275-281.

Guo, J., M. Van Eck, J. Twisk, N. Maeda, G. M. Benson, P. H. Groot and T. J. Van Berkel (2003). "Transplantation of monocyte CC-chemokine receptor 2-deficient bone marrow into ApoE3-Leiden mice inhibits atherogenesis." Arterioscler Thromb Vasc Biol **23**(3): 447-453.

Gupta, S., A. M. Pablo, X. Jiang, N. Wang, A. R. Tall and C. Schindler (1997). "IFN-gamma potentiates atherosclerosis in ApoE knock-out mice." J Clin Invest **99**(11): 2752-2761.

Hamaguchi, Y., J. Uchida, D. W. Cain, G. M. Venturi, J. C. Poe, K. M. Haas and T. F. Tedder (2005). "The peritoneal cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in mice." J Immunol **174**(7): 4389-4399.

Hamze, M., C. Desmetz, M. L. Berthe, P. Roger, N. Boulle, P. Brancherau, E. Picard, C. Guzman, C. Tolza and P. Guglielmi (2013). "Characterization of resident B cells of vascular walls in human atherosclerotic patients." J Immunol **191**(6): 3006-3016.

Hansson, G. K. (2005). "Inflammation, atherosclerosis, and coronary artery disease." N Engl J Med **352**(16): 1685-1695.

Hansson, G. K. (2012). "How to chew up cells: lessons for the atherosclerotic plaque." Circ Res **111**(6): 669-671.

Hansson, G. K. and A. Hermansson (2011). "The immune system in atherosclerosis." Nat Immunol **12**(3): 204-212.

Hansson, G. K., J. Holm and L. Jonasson (1989). "Detection of activated T lymphocytes in the human atherosclerotic plaque." Am J Pathol **135**(1): 169-175.

Hansson, G. K., A. K. Robertson and C. Soderberg-Naucler (2006). "Inflammation and atherosclerosis." Annu Rev Pathol **1**: 297-329.

Hardy, R. R. (2006). "B-1 B cell development." J Immunol **177**(5): 2749-2754.

Hardy, R. R. (2006). "B-1 B cells: development, selection, natural autoantibody and leukemia." Curr Opin Immunol **18**(5): 547-555.

Hardy, R. R., K. Hayakawa, D. R. Parks and L. A. Herzenberg (1983). "Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence." Nature **306**(5940): 270-272.

Harris, D. P., S. Goodrich, A. J. Gerth, S. L. Peng and F. E. Lund (2005). "Regulation of IFN-gamma production by B effector 1 cells: essential roles for T-bet and the IFN-gamma receptor." J Immunol **174**(11): 6781-6790.

Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain and F. E. Lund (2000). "Reciprocal regulation of polarized cytokine production by effector B and T cells." Nat Immunol **1**(6): 475-482.

Harwood, N. E. and F. D. Batista (2009). "The antigen expressway: follicular conduits carry antigen to B cells." Immunity **30**(2): 177-179.

Hastings, W. D., S. M. Gurdak, J. R. Tumang and T. L. Rothstein (2006). "CD5+/Mac-1- peritoneal B cells: a novel B cell subset that exhibits characteristics of B-1 cells." Immunol Lett **105**(1): 90-96.

Hayakawa, K. and R. R. Hardy (1988). "Normal, autoimmune, and malignant CD5+ B cells: the Ly-1 B lineage?" Annu Rev Immunol **6**: 197-218.

Hernandez-Vargas, P., G. Ortiz-Munoz, O. Lopez-Franco, Y. Suzuki, J. Gallego-Delgado, G. Sanjuan, A. Lazaro, V. Lopez-Parra, L. Ortega, J. Egido and C. Gomez-Guerrero (2006). "Fcgamma receptor deficiency confers protection against atherosclerosis in apolipoprotein E knockout mice." Circ Res **99**(11): 1188-1196.

Herzenberg, L. A., N. Baumgarth and J. A. Wilshire (2000). "B-1 cell origins and VH repertoire determination." Curr Top Microbiol Immunol **252**: 3-13.

Ho, I. C. and L. H. Glimcher (2002). "Transcription: tantalizing times for T cells." Cell **109 Suppl**: S109-120.

Houtkamp, M. A., O. J. de Boer, C. M. van der Loos, A. C. van der Wal and A. E. Becker (2001). "Adventitial infiltrates associated with advanced atherosclerotic plaques: structural organization suggests generation of local humoral immune responses." J Pathol **193**(2): 263-269.

Hsu, H. Y. and Y. C. Twu (2000). "Tumor necrosis factor-alpha -mediated protein kinases in regulation of scavenger receptor and foam cell formation on macrophage." J Biol Chem **275**(52): 41035-41048.

Huber, S. A., P. Sakkinen, C. David, M. K. Newell and R. P. Tracy (2001). "T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia." Circulation **103**(21): 2610-2616.

Inagaki, Y., S. Yamagishi, S. Amano, T. Okamoto, K. Koga and Z. Makita (2002). "Interferon-gamma-induced apoptosis and activation of THP-1 macrophages." Life Sci **71**(21): 2499-2508.

Ishida, H., R. Hastings, J. Kearney and M. Howard (1992). "Continuous anti-interleukin 10 antibody administration depletes mice of Ly-1 B cells but not conventional B cells." J Exp Med **175**(5): 1213-1220.

Itabe, H., T. Obama and R. Kato (2011). "The Dynamics of Oxidized LDL during Atherogenesis." J Lipids **2011**: 418313.

Jongstra-Bilen, J., M. Haidari, S. N. Zhu, M. Chen, D. Guha and M. I. Cybulsky (2006). "Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis." J Exp Med **203**(9): 2073-2083.

Kanellakis, P., A. Agrotis, T. S. Kyaw, C. Koulis, I. Ahrens, S. Mori, H. K. Takahashi, K. Liu, K. Peter, M. Nishibori and A. Bobik (2011). "High-mobility group

box protein 1 neutralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice." Arterioscler Thromb Vasc Biol **31**(2): 313-319.

Kantor, A. B., C. E. Merrill, L. A. Herzenberg and J. L. Hillson (1997). "An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells." J Immunol **158**(3): 1175-1186.

Karras, J. G., Z. Wang, L. Huo, R. G. Howard, D. A. Frank and T. L. Rothstein (1997). "Signal transducer and activator of transcription-3 (STAT3) is constitutively activated in normal, self-renewing B-1 cells but only inducibly expressed in conventional B lymphocytes." J Exp Med **185**(6): 1035-1042.

Katoh, S., M. M. Bendig, Y. Kanai, L. D. Shultz, Y. Hitoshi, K. Takatsu and A. Tominaga (1993). "Maintenance of CD5+ B cells at an early developmental stage by interleukin-5: evidence from immunoglobulin gene usage in interleukin-5 transgenic mice." DNA Cell Biol **12**(6): 481-491.

Kaveri, S. V., G. J. Silverman and J. Bayry (2012). "Natural IgM in immune equilibrium and harnessing their therapeutic potential." J Immunol **188**(3): 939-945.

Kelly, J. A., M. E. Griffin, R. A. Fava, S. G. Wood, K. A. Bessette, E. R. Miller, S. A. Huber, C. J. Binder, J. L. Witztum and P. M. Morganelli (2010). "Inhibition of arterial lesion progression in CD16-deficient mice: evidence for altered immunity and the role of IL-10." Cardiovasc Res **85**(1): 224-231.

Kober, F., M. Canault, F. Peiretti, C. Mueller, F. Kopp, M. C. Alessi, P. J. Cozzone, G. Nalbone and M. Bernard (2007). "MRI follow-up of TNF-dependent differential progression of atherosclerotic wall-thickening in mouse aortic arch from early to advanced stages." Atherosclerosis **195**(2): e93-99.

Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young and K. I. Matthaei (1996). "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**(1): 15-24.

Krieger, M. (1997). "The other side of scavenger receptors: pattern recognition for host defense." Curr Opin Lipidol **8**(5): 275-280.

Kroese, F. G., W. A. Ammerlaan and G. J. Deenen (1992). "Location and function of B-cell lineages." Ann N Y Acad Sci **651**: 44-58.

Kyaw, T., C. Tay, H. Hosseini, P. Kanellakis, T. Gadowski, F. MacKay, P. Tipping, A. Bobik and B. H. Toh (2012). "Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation." PLoS One **7**(1): e29371.

Kyaw, T., C. Tay, A. Khan, V. Dumouchel, A. Cao, K. To, M. Kehry, R. Dunn, A. Agrotis, P. Tipping, A. Bobik and B. H. Toh (2010). "Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis." J Immunol **185**(7): 4410-4419.

Kyaw, T., C. Tay, S. Krishnamurthi, P. Kanellakis, A. Agrotis, P. Tipping, A. Bobik and B. H. Toh (2011). "B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions." Circ Res **109**(8): 830-840.

Kyaw, T., A. Winship, C. Tay, P. Kanellakis, H. Hosseini, A. Cao, P. Li, P. Tipping, A. Bobik and B. H. Toh (2013). "Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice." Circulation **127**(9): 1028-1039.

Lewis, M. J., T. H. Malik, M. R. Ehrenstein, J. J. Boyle, M. Botto and D. O. Haskard (2009). "Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice." Circulation **120**(5): 417-426.

Li, A. C. and C. K. Glass (2002). "The macrophage foam cell as a target for therapeutic intervention." Nat Med **8**(11): 1235-1242.

Li, H., M. I. Cybulsky, M. A. Gimbrone, Jr. and P. Libby (1993). "Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma." Am J Pathol **143**(6): 1551-1559.

Li, S., Y. Sun, C. P. Liang, E. B. Thorp, S. Han, A. W. Jehle, V. Saraswathi, B. Pridgen, J. E. Kanter, R. Li, C. L. Welch, A. H. Hasty, K. E. Bornfeldt, J. L. Breslow, I. Tabas and A. R. Tall (2009). "Defective phagocytosis of apoptotic cells by

macrophages in atherosclerotic lesions of ob/ob mice and reversal by a fish oil diet." Circ Res **105**(11): 1072-1082.

Liao, F., J. A. Berliner, M. Mehrabian, M. Navab, L. L. Demer, A. J. Lusis and A. M. Fogelman (1991). "Minimally modified low density lipoprotein is biologically active in vivo in mice." J Clin Invest **87**(6): 2253-2257.

Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers and R. Carsetti (1999). "B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals." J Exp Med **190**(1): 75-89.

Lu, T. T. and J. G. Cyster (2002). "Integrin-mediated long-term B cell retention in the splenic marginal zone." Science **297**(5580): 409-412.

Lund, F. E. (2008). "Cytokine-producing B lymphocytes-key regulators of immunity." Curr Opin Immunol **20**(3): 332-338.

Maiellaro, K. and W. R. Taylor (2007). "The role of the adventitia in vascular inflammation." Cardiovasc Res **75**(4): 640-648.

Malek, A. M., S. L. Alper and S. Izumo (1999). "Hemodynamic shear stress and its role in atherosclerosis." JAMA **282**(21): 2035-2042.

Mallavia, B., A. Oguiza, O. Lopez-Franco, C. Recio, G. Ortiz-Munoz, I. Lazaro, V. Lopez-Parra, J. Egido and C. Gomez-Guerrero (2013). "Gene Deficiency in Activating Fcgamma Receptors Influences the Macrophage Phenotypic Balance and Reduces Atherosclerosis in Mice." PLoS One **8**(6): e66754.

Marcos, M. A., F. Huetz, P. Pereira, J. L. Andreu, A. C. Martinez and A. Coutinho (1989). "Further evidence for coelomic-associated B lymphocytes." Eur J Immunol **19**(11): 2031-2035.

Martin, F. and J. F. Kearney (2000). "B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory"." Immunol Rev **175**: 70-79.

Martin, F., A. M. Oliver and J. F. Kearney (2001). "Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens." Immunity **14**(5): 617-629.

Matsuura, E., G. R. Hughes and M. A. Khamashta (2008). "Oxidation of LDL and its clinical implication." Autoimmun Rev **7**(7): 558-566.

Matsuura, E., K. Kobayashi, M. Tabuchi and L. R. Lopez (2006). "Oxidative modification of low-density lipoprotein and immune regulation of atherosclerosis." Prog Lipid Res **45**(6): 466-486.

Maynard, C. L., L. E. Harrington, K. M. Janowski, J. R. Oliver, C. L. Zindl, A. Y. Rudensky and C. T. Weaver (2007). "Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10." Nat Immunol **8**(9): 931-941.

McLaren, J. E. and D. P. Ramji (2009). "Interferon gamma: a master regulator of atherosclerosis." Cytokine Growth Factor Rev **20**(2): 125-135.

McPhillips, K., W. J. Janssen, M. Ghosh, A. Byrne, S. Gardai, L. Remigio, D. L. Bratton, J. L. Kang and P. Henson (2007). "TNF-alpha inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms." J Immunol **178**(12): 8117-8126.

Menard, L. C., L. A. Minns, S. Darche, D. W. Mielcarz, D. M. Foureau, D. Roos, F. Dzierszinski, L. H. Kasper and D. Buzoni-Gatel (2007). "B cells amplify IFN-gamma production by T cells via a TNF-alpha-mediated mechanism." J Immunol **179**(7): 4857-4866.

Mendez-Fernandez, Y. V., B. G. Stevenson, C. J. Diehl, N. A. Braun, N. S. Wade, R. Covarrubias, S. van Leuven, J. L. Witztum and A. S. Major (2011). "The inhibitory FcgammaRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice." Atherosclerosis **214**(1): 73-80.

Miller, A. M., D. Xu, D. L. Asquith, L. Denby, Y. Li, N. Sattar, A. H. Baker, I. B. McInnes and F. Y. Liew (2008). "IL-33 reduces the development of atherosclerosis." J Exp Med **205**(2): 339-346.

Miller, Y. I., S. Viriyakosol, C. J. Binder, J. R. Feramisco, T. N. Kirkland and J. L. Witztum (2003). "Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells." J Biol Chem **278**(3): 1561-1568.

Millonig, G., H. Niederegger, W. Rabl, B. W. Hochleitner, D. Hoefer, N. Romani and G. Wick (2001). "Network of vascular-associated dendritic cells in intima of healthy young individuals." Arterioscler Thromb Vasc Biol **21**(4): 503-508.

Montecino-Rodriguez, E. and K. Dorshkind (2006). "New perspectives in B-1 B cell development and function." Trends Immunol **27**(9): 428-433.

Moon, B. G., S. Takaki, K. Miyake and K. Takatsu (2004). "The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production." J Immunol **172**(10): 6020-6029.

Moore, K. J., F. J. Sheedy and E. A. Fisher (2013). "Macrophages in atherosclerosis: a dynamic balance." Nat Rev Immunol **13**(10): 709-721.

Moore, K. J. and I. Tabas (2011). "Macrophages in the pathogenesis of atherosclerosis." Cell **145**(3): 341-355.

Murao, K., T. Ohyama, H. Imachi, T. Ishida, W. M. Cao, H. Namihira, M. Sato, N. C. Wong and J. Takahara (2000). "TNF-alpha stimulation of MCP-1 expression is mediated by the Akt/PKB signal transduction pathway in vascular endothelial cells." Biochem Biophys Res Commun **276**(2): 791-796.

Nagarajan, S. (2007). "Anti-OxLDL IgG blocks OxLDL interaction with CD36, but promotes FcgammaR, CD32A-dependent inflammatory cell adhesion." Immunol Lett **108**(1): 52-61.

Nauta, A. J., N. Raaschou-Jensen, A. Roos, M. R. Daha, H. O. Madsen, M. C. Borrias-Essers, L. P. Ryder, C. Koch and P. Garred (2003). "Mannose-binding lectin engagement with late apoptotic and necrotic cells." Eur J Immunol **33**(10): 2853-2863.

Navab, M., G. M. Ananthramaiah, S. T. Reddy, B. J. Van Lenten, B. J. Ansell, G. C. Fonarow, K. Vahabzadeh, S. Hama, G. Hough, N. Kamranpour, J. A. Berliner, A. J. Lusis and A. M. Fogelman (2004). "The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL." J Lipid Res **45**(6): 993-1007.

Ngo, V. N., R. J. Cornall and J. G. Cyster (2001). "Splenic T zone development is B cell dependent." J Exp Med **194**(11): 1649-1660.

Nicoletti, A., S. Kaveri, G. Caligiuri, J. Bariety and G. K. Hansson (1998). "Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice." J Clin Invest **102**(5): 910-918.

Nimmerjahn, F. and J. V. Ravetch (2006). "Fcgamma receptors: old friends and new family members." Immunity **24**(1): 19-28.

Nisitani, S., T. Tsubata, M. Murakami and T. Honjo (1995). "Administration of interleukin-5 or -10 activates peritoneal B-1 cells and induces autoimmune hemolytic anemia in anti-erythrocyte autoantibody-transgenic mice." Eur J Immunol **25**(11): 3047-3052.

O'Garra, A., R. Chang, N. Go, R. Hastings, G. Haughton and M. Howard (1992). "Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10." Eur J Immunol **22**(3): 711-717.

O'Garra, A. and M. Howard (1992). "IL-10 production by CD5 B cells." Ann N Y Acad Sci **651**: 182-199.

Ogden, C. A., R. Kowalewski, Y. Peng, V. Montenegro and K. B. Elkon (2005). "IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo." Autoimmunity **38**(4): 259-264.

Ohta, H., H. Wada, T. Niwa, H. Kirii, N. Iwamoto, H. Fujii, K. Saito, K. Sekikawa and M. Seishima (2005). "Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice." Atherosclerosis **180**(1): 11-17.

Oram, J. F. and J. W. Heinecke (2005). "ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease." Physiol Rev **85**(4): 1343-1372.

Palinski, W., E. Miller and J. L. Witztum (1995). "Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis." Proc Natl Acad Sci U S A **92**(3): 821-825.

Palinski, W., M. E. Rosenfeld, S. Yla-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg and J. L. Witztum (1989). "Low density lipoprotein undergoes oxidative modification in vivo." Proc Natl Acad Sci U S A **86**(4): 1372-1376.

Palinski, W., R. K. Tangirala, E. Miller, S. G. Young and J. L. Witztum (1995). "Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis." Arterioscler Thromb Vasc Biol **15**(10): 1569-1576.

Panousis, C. G. and S. H. Zuckerman (2000). "Interferon-gamma induces downregulation of Tangier disease gene (ATP-binding-cassette transporter 1) in macrophage-derived foam cells." Arterioscler Thromb Vasc Biol **20**(6): 1565-1571.

Parhami, F., Z. T. Fang, A. M. Fogelman, A. Andalibi, M. C. Territo and J. A. Berliner (1993). "Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate." J Clin Invest **92**(1): 471-478.

Park, S. K., W. S. Yang, N. J. Han, S. K. Lee, H. Ahn, I. K. Lee, J. Y. Park, K. U. Lee and J. D. Lee (2004). "Dexamethasone regulates AP-1 to repress TNF-alpha induced MCP-1 production in human glomerular endothelial cells." Nephrol Dial Transplant **19**(2): 312-319.

Peng, Y., R. Kowalewski, S. Kim and K. B. Elkon (2005). "The role of IgM antibodies in the recognition and clearance of apoptotic cells." Mol Immunol **42**(7): 781-787.

Pieters, J. (2000). "MHC class II-restricted antigen processing and presentation." Adv Immunol **75**: 159-208.

Pricop, L., P. Redecha, J. L. Teillaud, J. Frey, W. H. Fridman, C. Sautes-Fridman and J. E. Salmon (2001). "Differential modulation of stimulatory and inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines." J Immunol **166**(1): 531-537.

Rajavashisth, T. B., A. Andalibi, M. C. Territo, J. A. Berliner, M. Navab, A. M. Fogelman and A. J. Lusis (1990). "Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins." Nature **344**(6263): 254-257.

Ramos-Casals, M., M. J. Soto, M. J. Cuadrado and M. A. Khamashta (2009). "Rituximab in systemic lupus erythematosus: A systematic review of off-label use in 188 cases." Lupus **18**(9): 767-776.

Rapaka, R. R., D. M. Ricks, J. F. Alcorn, K. Chen, S. A. Khader, M. Zheng, S. Plevy, E. Bengten and J. K. Kolls (2010). "Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina." J Exp Med **207**(13): 2907-2919.

Ratcliffe, N. R., S. M. Kennedy and P. M. Morganelli (2001). "Immunocytochemical detection of Fcgamma receptors in human atherosclerotic lesions." Immunol Lett **77**(3): 169-174.

Rauch, M., R. Tussiwand, N. Bosco and A. G. Rolink (2009). "Crucial role for BAFF-BAFF-R signaling in the survival and maintenance of mature B cells." PLoS One **4**(5): e5456.

Ravetch, J. V. and S. Bolland (2001). "IgG Fc receptors." Annu Rev Immunol **19**: 275-290.

Rezende, A. B., N. N. Neto, L. R. Fernandes, A. C. Ribeiro, J. I. Alvarez-Leite and H. C. Teixeira (2011). "Splenectomy increases atherosclerotic lesions in apolipoprotein E deficient mice." J Surg Res **171**(2): e231-236.

Roger, V. L., A. S. Go, D. M. Lloyd-Jones, E. J. Benjamin, J. D. Berry, W. B. Borden, D. M. Bravata, S. Dai, E. S. Ford, C. S. Fox, H. J. Fullerton, C. Gillespie, S. M. Hailpern, J. A. Heit, V. J. Howard, B. M. Kissela, S. J. Kittner, D. T. Lackland, J. H. Lichtman, L. D. Lisabeth, D. M. Makuc, G. M. Marcus, A. Marelli, D. B. Matchar, C. S. Moy, D. Mozaffarian, M. E. Mussolino, G. Nichol, N. P. Paynter, E. Z. Soliman, P. D. Sorlie, N. Sotoodehnia, T. N. Turan, S. S. Virani, N. D. Wong, D. Woo and M. B. Turner (2012). "Heart disease and stroke statistics--2012 update: a report from the American Heart Association." Circulation **125**(1): e2-e220.

Rose, N. and M. Afanasyeva (2003). "Autoimmunity: busting the atherosclerotic plaque." Nat Med **9**(6): 641-642.

Sage, A. P., D. Tsiantoulas, L. Baker, J. Harrison, L. Masters, D. Murphy, C. Loinard, C. J. Binder and Z. Mallat (2012). "BAFF receptor deficiency reduces the development of atherosclerosis in mice--brief report." Arterioscler Thromb Vasc Biol **32**(7): 1573-1576.

Salonen, J. T., S. Yla-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, K. Nyyssonen, W. Palinski and J. L. Witztum (1992). "Autoantibody against oxidised LDL and progression of carotid atherosclerosis." Lancet **339**(8798): 883-887.

Sampi, M., O. Ukkola, M. Paivansalo, Y. A. Kesaniemi, C. J. Binder and S. Horkko (2008). "Plasma interleukin-5 levels are related to antibodies binding to oxidized low-density lipoprotein and to decreased subclinical atherosclerosis." J Am Coll Cardiol **52**(17): 1370-1378.

Scapini, P., C. Lamagna, Y. Hu, K. Lee, Q. Tang, A. L. DeFranco and C. A. Lowell (2011). "B cell-derived IL-10 suppresses inflammatory disease in Lyn-deficient mice." Proc Natl Acad Sci U S A **108**(41): E823-832.

Schiopu, A., J. Bengtsson, I. Soderberg, S. Janciauskiene, S. Lindgren, M. P. Ares, P. K. Shah, R. Carlsson, J. Nilsson and G. N. Fredrikson (2004). "Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis." Circulation **110**(14): 2047-2052.

Schiopu, A., B. Frendeus, B. Jansson, I. Soderberg, I. Ljungcrantz, Z. Araya, P. K. Shah, R. Carlsson, J. Nilsson and G. N. Fredrikson (2007). "Recombinant antibodies to an oxidized low-density lipoprotein epitope induce rapid regression of atherosclerosis in apobec-1(-/-)/low-density lipoprotein receptor(-/-) mice." J Am Coll Cardiol **50**(24): 2313-2318.

Schrijvers, D. M., G. R. De Meyer, A. G. Herman and W. Martinet (2007). "Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability." Cardiovasc Res **73**(3): 470-480.

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." J Leukoc Biol **75**(2): 163-189.

Schultze, J. L., S. Michalak, J. Lowne, A. Wong, M. H. Gilleece, J. G. Gribben and L. M. Nadler (1999). "Human non-germinal center B cell interleukin (IL)-12 production is primarily regulated by T cell signals CD40 ligand, interferon gamma, and IL-10: role of B cells in the maintenance of T cell responses." J Exp Med **189**(1): 1-12.

Seimon, T. and I. Tabas (2009). "Mechanisms and consequences of macrophage apoptosis in atherosclerosis." J Lipid Res **50 Suppl**: S382-387.

Sfikakis, P. P., J. N. Boletis and G. C. Tsokos (2005). "Rituximab anti-B-cell therapy in systemic lupus erythematosus: pointing to the future." Curr Opin Rheumatol **17**(5): 550-557.

Shaw, P. X., C. S. Goodyear, M. K. Chang, J. L. Witztum and G. J. Silverman (2003). "The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells." J Immunol **170**(12): 6151-6157.

Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman and J. L. Witztum (2000). "Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity." J Clin Invest **105**(12): 1731-1740.

Shimaoka, T., N. Kume, M. Minami, K. Hayashida, H. Kataoka, T. Kita and S. Yonehara (2000). "Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages." J Biol Chem **275**(52): 40663-40666.

Shimizu, K., M. Shichiri, P. Libby, R. T. Lee and R. N. Mitchell (2004). "Th2-predominant inflammation and blockade of IFN-gamma signaling induce aneurysms in allografted aortas." J Clin Invest **114**(2): 300-308.

Shirota, H., K. Sano, N. Hirasawa, T. Terui, K. Ohuchi, T. Hattori and G. Tamura (2002). "B cells capturing antigen conjugated with CpG oligodeoxynucleotides induce Th1 cells by elaborating IL-12." J Immunol **169**(2): 787-794.

Shoenfeld, Y., R. Wu, L. D. Dearing and E. Matsuura (2004). "Are anti-oxidized low-density lipoprotein antibodies pathogenic or protective?" Circulation **110**(17): 2552-2558.

Sjogren, P., G. N. Fredrikson, A. Samnegard, C. G. Ericsson, J. Ohrvik, R. M. Fisher, J. Nilsson and A. Hamsten (2008). "High plasma concentrations of autoantibodies against native peptide 210 of apoB-100 are related to less coronary atherosclerosis and lower risk of myocardial infarction." Eur Heart J **29**(18): 2218-2226.

Skalen, K., M. Gustafsson, E. K. Rydberg, L. M. Hulten, O. Wiklund, T. L. Innerarity and J. Boren (2002). "Subendothelial retention of atherogenic lipoproteins in early atherosclerosis." Nature **417**(6890): 750-754.

Smith, S. C., Jr., A. Collins, R. Ferrari, D. R. Holmes, Jr., S. Logstrup, D. V. McGhie, J. Ralston, R. L. Sacco, H. Stam, K. Taubert, D. A. Wood and W. A. Zoghbi (2012). "Our time: a call to save preventable death from cardiovascular disease (heart disease and stroke)." Circulation **126**(23): 2769-2775.

Stemme, S., B. Faber, J. Holm, O. Wiklund, J. L. Witztum and G. K. Hansson (1995). "T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein." Proc Natl Acad Sci U S A **92**(9): 3893-3897.

Stemme, S., G. Fager and G. K. Hansson (1990). "MHC class II antigen expression in human vascular smooth muscle cells is induced by interferon-gamma and modulated by tumour necrosis factor and lymphotoxin." Immunology **69**(2): 243-249.

Stocker, R. and J. F. Keaney, Jr. (2004). "Role of oxidative modifications in atherosclerosis." Physiol Rev **84**(4): 1381-1478.

Su, T. T. and D. J. Rawlings (2002). "Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development." J Immunol **168**(5): 2101-2110.

Sun, J., K. Hartvigsen, M. Y. Chou, Y. Zhang, G. K. Sukhova, J. Zhang, M. Lopez-Ilasaca, C. J. Diehl, N. Yakov, D. Harats, J. George, J. L. Witztum, P. Libby, H. Ploegh and G. P. Shi (2010). "Deficiency of antigen-presenting cell invariant chain reduces atherosclerosis in mice." Circulation **122**(8): 808-820.

Tamura, T., S. Ueda, M. Yoshida, M. Matsuzaki, H. Mohri and T. Okubo (1996). "Interferon-gamma induces Ice gene expression and enhances cellular susceptibility to apoptosis in the U937 leukemia cell line." Biochem Biophys Res Commun **229**(1): 21-26.

Tellides, G., D. A. Tereb, N. C. Kirkiles-Smith, R. W. Kim, J. H. Wilson, J. S. Schechner, M. I. Lorber and J. S. Pober (2000). "Interferon-gamma elicits arteriosclerosis in the absence of leukocytes." Nature **403**(6766): 207-211.

Thorp, E., D. Cui, D. M. Schrijvers, G. Kuriakose and I. Tabas (2008). "Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice." Arterioscler Thromb Vasc Biol **28**(8): 1421-1428.

Thurnheer, M. C., A. W. Zuercher, J. J. Cebra and N. A. Bos (2003). "B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice." J Immunol **170**(9): 4564-4571.

Tominaga, A., S. Takaki, N. Koyama, S. Katoh, R. Matsumoto, M. Migita, Y. Hitoshi, Y. Hosoya, S. Yamauchi, Y. Kanai and et al. (1991). "Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production." J Exp Med **173**(2): 429-437.

Tsimikas, S., W. Palinski and J. L. Witztum (2001). "Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized LDL in LDL receptor-deficient mice." Arterioscler Thromb Vasc Biol **21**(1): 95-100.

Tsokos, G. C. (2004). "B cells, be gone--B-cell depletion in the treatment of rheumatoid arthritis." N Engl J Med **350**(25): 2546-2548.

Tumanov, A. V., S. I. Grivennikov, A. A. Kruglov, Y. V. Shebzukhov, E. P. Koroleva, Y. Piao, C. Y. Cui, D. V. Kuprash and S. A. Nedospasov (2010). "Cellular source and molecular form of TNF specify its distinct functions in organization of secondary lymphoid organs." Blood **116**(18): 3456-3464.

VanderLaan, P. A., C. A. Reardon and G. S. Getz (2004). "Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators." Arterioscler Thromb Vasc Biol **24**(1): 12-22.

Wagner, M., H. Poeck, B. Jahrsdoerfer, S. Rothenfusser, D. Prell, B. Bohle, E. Tuma, T. Giese, J. W. Ellwart, S. Endres and G. Hartmann (2004). "IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA." J Immunol **172**(2): 954-963.

Wajant, H., K. Pfizenmaier and P. Scheurich (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.

Wang, X. Q., C. G. Panousis, M. L. Alfaro, G. F. Evans and S. H. Zuckerman (2002). "Interferon-gamma-mediated downregulation of cholesterol efflux and ABC1 expression is by the Stat1 pathway." Arterioscler Thromb Vasc Biol **22**(5): e5-9.

Wardemann, H., T. Boehm, N. Dear and R. Carsetti (2002). "B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen." J Exp Med **195**(6): 771-780.

Wen, L., J. Brill-Dashoff, S. A. Shinton, M. Asano, R. R. Hardy and K. Hayakawa (2005). "Evidence of marginal-zone B cell-positive selection in spleen." Immunity **23**(3): 297-308.

Whitman, S. C., P. Ravisankar and A. Daugherty (2002). "IFN-gamma deficiency exerts gender-specific effects on atherogenesis in apolipoprotein E-/- mice." J Interferon Cytokine Res **22**(6): 661-670.

Whitman, S. C., P. Ravisankar, H. Elam and A. Daugherty (2000). "Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice." Am J Pathol **157**(6): 1819-1824.

Witztum, J. L. and A. H. Lichtman (2013). "The Influence of Innate and Adaptive Immune Responses on Atherosclerosis." Annu Rev Pathol.

Wong, G. H., I. Clark-Lewis, L. McKimm-Breschkin, A. W. Harris and J. W. Schrader (1983). "Interferon-gamma induces enhanced expression of Ia and H-2 antigens on B lymphoid, macrophage, and myeloid cell lines." J Immunol **131**(2): 788-793.

Xiu, Y., C. P. Wong, J. D. Bouaziz, Y. Hamaguchi, Y. Wang, S. M. Pop, R. M. Tisch and T. F. Tedder (2008). "B lymphocyte depletion by CD20 monoclonal antibody prevents diabetes in nonobese diabetic mice despite isotype-specific differences in Fc gamma R effector functions." J Immunol **180**(5): 2863-2875.

Yanaba, K., Y. Hamaguchi, G. M. Venturi, D. A. Steeber, E. W. St Clair and T. F. Tedder (2007). "B cell depletion delays collagen-induced arthritis in mice: arthritis induction requires synergy between humoral and cell-mediated immunity." J Immunol **179**(2): 1369-1380.

Yla-Herttuala, S., W. Palinski, S. W. Butler, S. Picard, D. Steinberg and J. L. Witztum (1994). "Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL." Arterioscler Thromb **14**(1): 32-40.

Yoshida, T., K. Ikuta, H. Sugaya, K. Maki, M. Takagi, H. Kanazawa, S. Sunaga, T. Kinashi, K. Yoshimura, J. Miyazaki, S. Takaki and K. Takatsu (1996). "Defective B-1 cell development and impaired immunity against Angiostrongylus cantonensis in IL-5R alpha-deficient mice." Immunity **4**(5): 483-494.

Yoshimoto, T., H. Okamura, Y. I. Tagawa, Y. Iwakura and K. Nakanishi (1997). "Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells." Proc Natl Acad Sci U S A **94**(8): 3948-3953.

Yvan-Charvet, L., T. A. Pagler, T. A. Seimon, E. Thorp, C. L. Welch, J. L. Witztum, I. Tabas and A. R. Tall (2010). "ABCA1 and ABCG1 protect against oxidative stress-induced macrophage apoptosis during efferocytosis." Circ Res **106**(12): 1861-1869.

Zhang, L., K. Peppel, P. Sivashanmugam, E. S. Orman, L. Brian, S. T. Exum and N. J. Freedman (2007). "Expression of tumor necrosis factor receptor-1 in arterial wall cells promotes atherosclerosis." Arterioscler Thromb Vasc Biol **27**(5): 1087-1094.

Zhao, M., M. Wigren, P. Duner, D. Kolbus, K. E. Olofsson, H. Bjorkbacka, J. Nilsson and G. N. Fredrikson (2010). "FcgammaRIIB inhibits the development of atherosclerosis in low-density lipoprotein receptor-deficient mice." J Immunol **184**(5): 2253-2260.

Zhou, X., G. Caligiuri, A. Hamsten, A. K. Lefvert and G. K. Hansson (2001). "LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis." Arterioscler Thromb Vasc Biol **21**(1): 108-114.

Zhou, X. and G. K. Hansson (1999). "Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice." Scand J Immunol **50**(1): 25-30.

Zhou, X., G. Paulsson, S. Stemme and G. K. Hansson (1998). "Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice." J Clin Invest **101**(8): 1717-1725.

Chapter 2: Conventional B2 B Cell Depletion Ameliorates whereas Its Adoptive Transfer Aggravates Atherosclerosis

2.1 Short Introduction

B cell depletion mediated by anti-CD20 monoclonal antibody has been shown to improve a wide variety of inflammatory disorders in human and mice. Here, I examined the effects of anti-CD20 mediated B cell depletion on atherosclerosis in hypercholesterolemic ApoE$^{-/-}$ mice. Depletion of B cells by anti-CD20 resulted in not only reduced development but also reduced progression of atherosclerosis in ApoE$^{-/-}$ mice fed a high fat diet. Body weight and plasma lipid levels were not affected by anti-CD20 treatment. Moreover, atherosclerosis was significantly increased in lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice as well as B cell-deficient ApoE$^{-/-}$μMT mice that received adoptive transfers of the B2 subset of B cells compared to control mice that did not receive any cells after 8 weeks of high fat diet. Conversely, adoptive transfer of the B1a subset of B cells did not bring about any significant impact on atherosclerosis. These findings suggest that B2 B cells but not B1a B cells promote atherosclerosis. *J Immunol.* 2010; 185(7):4410-9.

PART B: Suggested Declaration for Thesis Chapter

[This declaration to be completed for each conjointly authored publication and to be placed at the start of the thesis chapter in which the publication appears.]

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter [2], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	45

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Tin Kyaw	Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	
Abdul Khan	Experimentation	
Vanessa Dumouchel	Experimentation	
Anh Cao	Experimentation	
Kelly To	Experimentation	
Merilyn Kehry	Experimentation	
Robert Dunn	Experimentation	
Alex Agrotis	Experimental analysis	
Peter Tipping	Study concept, Study design, Result Interpretation	
Alex Bobik	Study concept, Study design, Result Interpretation, Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Result Interpretation, Preparation of manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 7/2/2014
Main Supervisor's Signature	[redacted]	**Date** 7/2/2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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Conventional B2 B Cell Depletion Ameliorates whereas Its Adoptive Transfer Aggravates Atherosclerosis

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Atherosclerosis is a chronic inflammatory arterial disease characterized by focal accumulation of lipid and inflammatory cells. It is the number one cause of deaths in the Western world because of its complications of heart attacks and strokes. Statins are effective in only approximately one third of patients, underscoring the urgent need for additional therapies. B cells that accumulate in atherosclerotic lesions and the aortic adventitia of humans and mice are considered to protect against atherosclerosis development. Unexpectedly, we found that selective B cell depletion in apolipoprotein E-deficient ($ApoE^{-/-}$) mice using a well-characterized mAb to mouse CD20 reduced atherosclerosis development and progression without affecting the hyperlipidemia imposed by a high-fat diet. Adoptive transfer of 5×10^6 or 5×10^7 conventional B2 B cells but not 5×10^6 B1 B cells to a lymphocyte-deficient $ApoE^{-/-}$ $Rag\text{-}2^{-/-}$ common cytokine receptor γ-chain–deficient mouse that was fed a high-fat diet augmented atherosclerosis by 72%. Transfer of 5×10^6 B2 B cells to an $ApoE^{-/-}$ mouse deficient only in B cells aggravated atherosclerosis by >300%. Our findings provide compelling evidence for the hitherto unrecognized proatherogenic role of conventional B2 cells. The data indicate that B2 cells can potently promote atherosclerosis development entirely on their own in the total absence of all other lymphocyte populations. Additionally, these B2 cells can also significantly augment atherosclerosis development in the presence of T cells and all other lymphocyte populations. Our findings raise the prospect of B cell depletion as a therapeutic approach to inhibit atherosclerosis development and progression in humans. *The Journal of Immunology*, **2010, 185: 4410–4419.**

Today, atherosclerosis is considered a chronic inflammatory disease of major arteries that leads to heart attacks and strokes caused by rupture of atherosclerotic plaques and thrombotic arterial occlusion. Current therapies for atherosclerosis are mainly based on drugs that lower plasma cholesterol concentration and blood pressure. The potent cholesterol-lowering agents (statins) significantly reduce cardiovascular events, not only as a consequence of their cholesterol lowering properties but also through their more recently described anti-inflammatory and immunomodulatory effects. However, atherosclerosis remains the main cause of heart attacks and strokes that are responsible for the majority of deaths in Western countries. The identification and development of promising new anti-inflammatory and immunomodulatory therapies is therefore of great interest in the management of atherosclerosis.

In early human plaques, B cells are minor populations found near lipid cores, whereas advanced human lesions contain adventitial nodular B cell aggregates (1, 2). In apolipoprotein E-deficient ($ApoE^{-/-}$) mice, B cells are found in early fatty-streak lesions and accumulate in basal regions of advanced plaques containing high concentrations of proinflammatory cytokines TNF-α and IL-6 and abundant IgG and IgM deposits (3).

It is widely held that B cells protect against atherosclerosis development by producing IgM Abs to oxidized low-density lipoprotein (ox-LDL) (4, 5). This view is supported by the following observations: first, $Ldlr^{-/-}$ mice rendered B cell deficient by transplantation of bone marrow from μ-chain–deficient mice showed increased aortic atherosclerotic lesions accompanied by reduced anti–ox-LDL Abs (6). Secondly, $Ldlr^{-/-}$ mice, deficient in serum IgM, displayed substantially larger and more complex atherosclerotic lesions (7). The protective role of B cells in atherosclerosis has stimulated interest in developing a protective vaccine for atherosclerosis, targeted toward harnessing the protective B cell-derived IgM response to oxidized LDL (8).

B cell depletion mediated by mAb to CD20 remits a wide variety of inflammatory diseases in humans (9–12) and mice (13–16). In humans, rituximab, a chimeric Ab to human CD20, has been approved for treating intractable rheumatoid arthritis, although its value for other inflammatory diseases, such as systemic lupus erythematosus (10, 11) and multiple sclerosis (12), is currently being assessed. These observations highlight a key role for B lymphocytes in inflammatory diseases.

Based on the observations with B cell-depletion therapy, we revisited the role of B cells in atherosclerosis by depleting B lymphocytes using a mAb to mouse CD20 that has ameliorated murine thyroidits (13), lupus (14), and arthritis (15). We found that B cell depletion prevented the development and progression of atherosclerosis. Moreover, transfer of conventional B2 B cells into a lymphocyte-deficient $ApoE^{-/-}$ $Rag\text{-}2^{-/-}$ common cytokine receptor γ-chain (γc)-deficient mouse or to B cell-deficient atherogenic mice lacking B lymphocytes due to deleted μ-chain of Ig

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Abbreviations used in this paper: $ApoE^{-/-}$, apolipoprotein E-deficient; $ApoE^{-/-}$ $\mu MT^{-/-}$, atherogenic mice lacking B lymphocytes due to deleted μ-chain of Ig; Blood, peripheral blood; γc, common cytokine receptor γ-chain; HFD, high-fat diet; IA, innominate artery; LCCA, left common carotid artery; LN, peripheral lymph node; LS, left subclavian artery; ox-LDL, oxidized low-density lipoprotein; PC, peritoneal cavity; TKO, $ApoE^{-/-}$ $Rag\text{-}2^{-/-}$ $\gamma c^{-/-}$ atherogenic mice lacking all lymphocyte populations; T-reg, regulatory T cell.

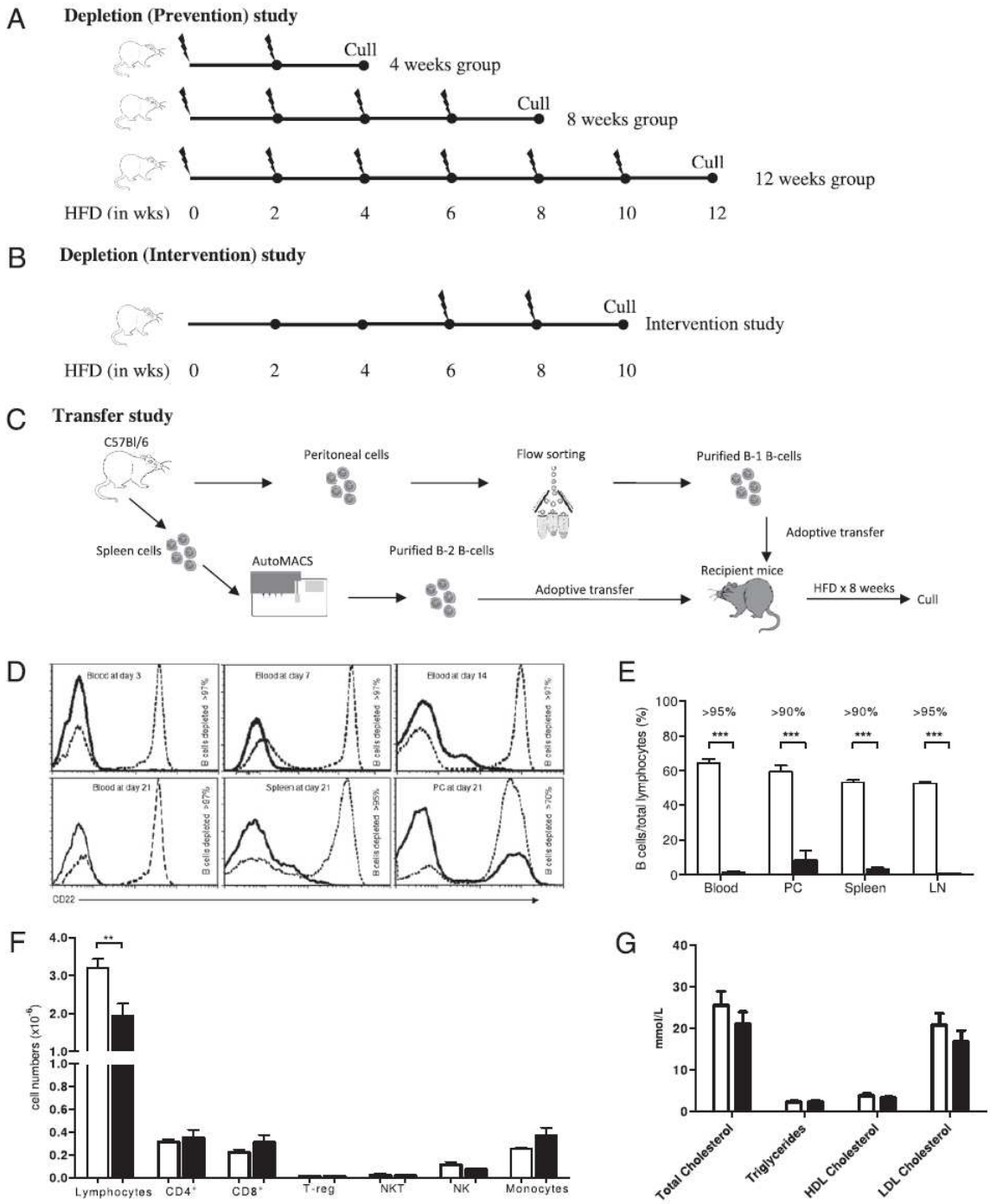

FIGURE 1. Experimental design and specific B cell depletion by anti-CD20 Ab. *A–C*, Schema of experimental design of B cell depletion and adoptive transfer of B2 B cells (n = 8–10/group). *A*, Depletion (prevention) study: ApoE$^{-/-}$ mice were fed a HFD for 4, 8, or 12 wk while maintaining B cell depletion by anti-CD20 Ab. *B*, Depletion (intervention study): ApoE$^{-/-}$ mice were fed a HFD for 10 wk during which two injections of anti-CD20 Ab were given. *C*, Adoptive transfer study: Ab administered. *D*, Single injection of anti-CD20 Ab depletes B cells up to 3 wk. ApoE$^{-/-}$ mice were given a single i.v. injection of anti-CD20 Ab and culled at days 3, 7, 14, and 21 postinjection. Shown are data for peripheral blood, spleen, and PC. Continuous and dotted lines, respectively, represent mice injected with anti-CD20 18B12 Ab (test) and 2B8 (control) Ab (n = 2 in each group). *E*, Repeated injections of CD20 Ab deplete >90% of B cells during atherosclerosis development. ApoE$^{-/-}$ mice were fed a HFD for 12 wk. Anti-CD20 Ab was given i.v. at the start of HFD and every 2 wk thereafter. Mice were culled after 4, 8, or 12 wk. Data show >90% depletion of CD22$^+$ B cells in mice fed a HFD for the 12 wk (n = 9 in each group). Similar B cell depletion efficiency was seen in both the 4 and 8 wk groups. *F*, Non-B lymphocytes and monocytes are unaffected by anti-CD20 Ab treatment. Shown are data for blood lymphocytes (CD4$^+$ T cells, CD8$^+$ T cells, CD4$^+$ CD25$^+$ Foxp3$^+$ T-reg cells, NK1.1$^+$ NK cells, and NK1.1$^+$ TCR-β$^+$ NKT cells) and monocytes (CD11b$^+$ monocytes) in the 12 wk groups (n = 9 in each group). Similar findings were observed in spleen, PC, and lymph nodes in the 12-wk groups. *G*, Lipid profiles are unaffected by anti-CD20 Ab treatment. Shown are data from the depletion (prevention) 12-wk groups (n = 9 in each group). Similar data were seen in both depletion (prevention) 4- and 8-wk groups. White bars, mice injected with control (2B8) Ab. Black bars, mice injected with test (18B12) Ab. Data are mean values ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$. Blood, peripheral blood; LN, peripheral lymph nodes; PC, peritoneal cavity; T-reg, regulatory T cell.

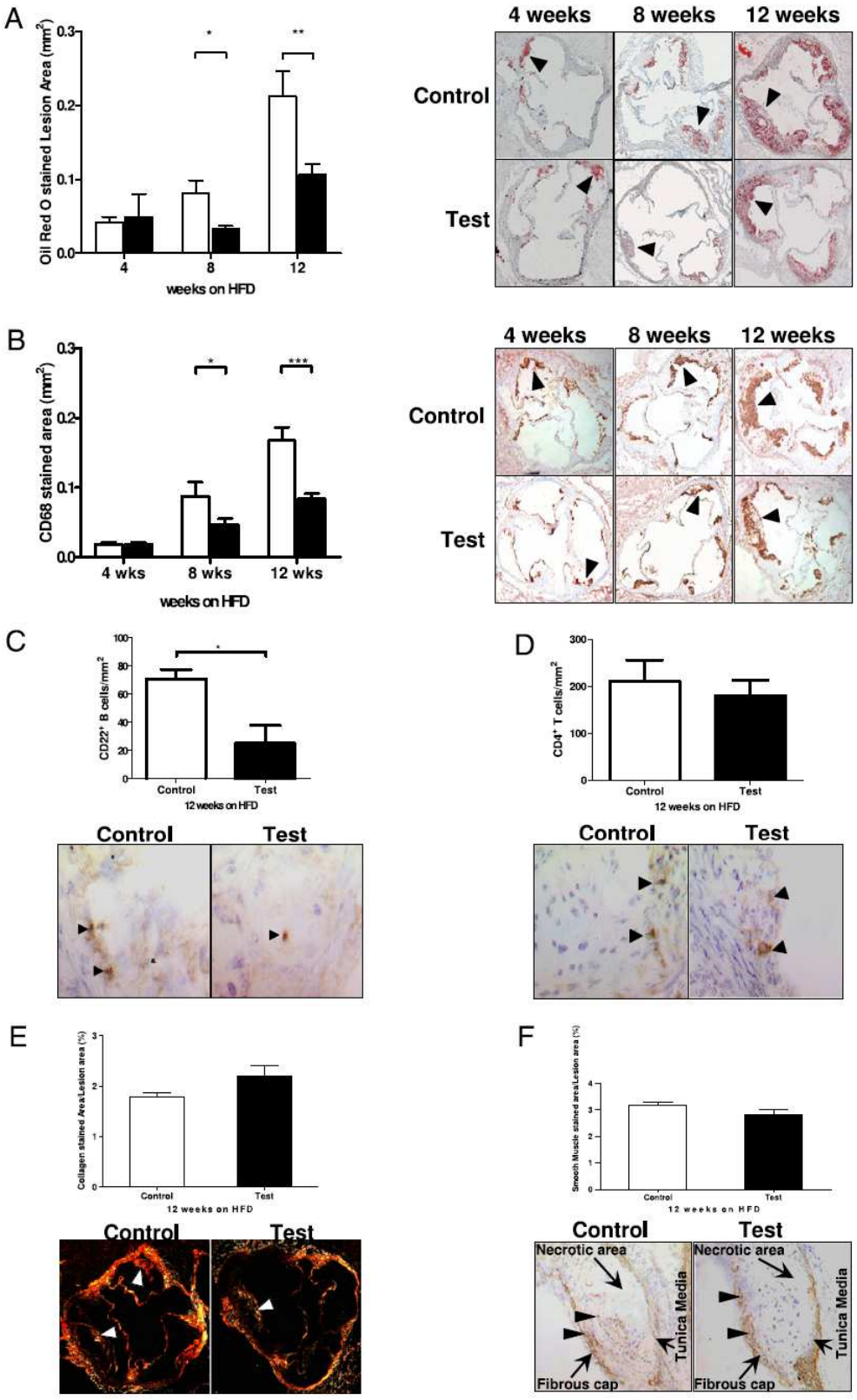

FIGURE 2. B cell depletion attenuates atherosclerosis development. *A* and *B*, Reduced atherosclerotic lesion development and macrophage accumulation. ApoE$^{-/-}$ mice were treated with anti-CD20 Ab fortnightly while being fed a HFD for 4, 8, or 12 wk ($n = 9$ in each group). Aortic sinus lesions were stained by Oil Red O (*A*, *right panel*) for lipid content or by anti-CD68 Ab (*B*, *right panel*) for macrophages. Arrows indicate positively stained areas. *C* and *D*, Reduction of lesion B cells. Shown are CD22^{+} B cells (*C*) in aortic sinus lesions of mice treated with anti-CD20 Ab fortnightly while being fed a HFD for 12 wk compared with that in control group; lesion CD4^{+} T cells (*D*) were unaffected ($n = 9$ in each group). Similar results were observed in the 8-wk group. Arrows indicate positively stained cells. *E* and *F*, Collagen and smooth muscle cell contents are unaffected by anti-CD20 Ab. Shown are collagen (*E*) and smooth muscle cell (*F*) contents in the 12-wk group ($n = 9$ in each group). Similar results were observed in all experiments of both prevention and intervention studies. Arrows indicate positively stained areas. Original magnification ×60 (*A*, *B*, *E*) and ×160 (*C*, *D*, *F*). White bars, mice injected with control (2B8) Ab. Black bars, mice injected with test (18B12) Ab. Data are mean values ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

(ApoE$^{-/-}$ μMT$^{-/-}$) potently aggravated atherosclerosis. In contrast, transfer of B1 B cells failed to aggravate atherosclerosis. Taken together, our studies clearly identify the B2 B cell subset as a proatherogenic B cell population.

Materials and Methods

Depleting Abs

A unique mouse anti-mouse CD20 IgG2a (18B12) used to deplete murine B cells and an isotype-matched control Ab (mouse anti-human CD20 Ab; 2B8) were supplied by Biogen Idec (San Diego, CA). The dosage used in depletion study was 10 mg/kg body weight for both Abs.

Animals and experimental design

In the depletion (prevention) study, 6–8-wk-old ApoE$^{-/-}$ mice were fed a high-fat diet (HFD; Specialty Feeds, Glen Forrest, Western Australia) for 4, 8, and 12 wk, during which either control Ab (2B8) or test Ab (18B12) was given once every 2 wk i.v. via tail vein (Fig. 1*A*). To induce atherosclerosis in the depletion (intervention) study, 6–8-wk-old ApoE$^{-/-}$ mice were fed an HFD for 6 wk and then given Ab treatment once every 2 wk for a further 4 wk on an HFD (Fig. 1*B*).

We generated a lymphocyte-deficient atherogenic ApoE$^{-/-}$ Rag-2$^{-/-}$ $\gamma c^{-/-}$ (TKO) mouse model by crossing an ApoE$^{-/-}$ Rag-2$^{-/-}$ mouse and an ApoE$^{-/-}$ $\gamma c^{-/-}$ mouse on a C57BL/6J background. We also generated an atherosclerosis-prone mouse deficient only in B cells by crossing an ApoE$^{-/-}$ mouse and a μMT$^{-/-}$ mouse lacking the μ-chain of Ig on C57BL/6J background. Genotype analysis confirmed the deleted gene status (data not shown). Phenotype analysis confirmed absence of all lymphocyte populations in the TKO mouse and absence of only B cells in the ApoE$^{-/-}$$\mu$MT$^{-/-}$ mouse (data not shown).

In the transfer study (Fig. 1*C*), we adoptively transferred 5×10^6 or 5×10^7 spleen B2 B cells to 6–8-wk-old lymphocyte-deficient TKO mice at the start of an 8-wk HFD. The experiments were controlled by transfer of PBS and with the transfer of 5×10^6 peritoneal B1 B cells. In a separate study, we adoptively transferred 5×10^6 spleen B2 B cells to B cell-deficient ApoE$^{-/-}$$\mu$MT$^{-/-}$ mice in a similar approach.

All animal experiments were conducted at Precinct Animal Centre, Alfred Medical Research and Education Precinct, Praharn, Victoria, Australia. All animal procedures were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee.

Isolation of spleen conventional B2 B cells and peritoneal B1 B cells

Conventional B2 B cells were purified from C57BL/6J spleens by using a B cell isolation kit containing a mixture of Abs against CD4, CD43, and Ter-113 (Miltenyi Biotec, Auburn, CA) according to the manufacturer's recommendation. Cell purity (>99%) was confirmed by FACS analysis using fluorochrome-labeled CD22 and CD5 Abs (BD Pharmingen, San Diego, CA). Cell viability (>98%) was assessed by trypan blue exclusion method (data not shown).

Peritoneal cells from C57BL/6J mice were obtained by flushing the peritoneum with FACS buffer and labeled with fluorochrome-conjugated CD22 and CD5 Abs (BD Pharmingen). To obtain 5×10^6 B1 B cells, a pool of peritoneal cells from 10 donor mice, each of which the peritoneum was flushed with 10 ml FACS buffer, was used. CD22$^+$ CD5$^+$ B1 B cells were isolated by FACSAria flow sorter (BD Biosciences, San Jose, CA) to a purity of >99% (data not shown). Cell viability assessed by trypan blue exclusion method was >98% (data not shown).

FACS analysis

B cell and non-B cell populations in peripheral blood, peritoneal fluid, peripheral lymph nodes, and spleen were analyzed using fluorochrome-conjugated Abs (BD Pharmingen) on a BD FACSCanto II (BD Biosciences). For B cells and monocytes, PE-conjugated CD22, allophycocyanin-conjugated CD5, and allophycocyanin-Cy7–conjugated CD11b Abs were used. For non-B lymphocyte populations, Pacific Blue-conjugated CD4, PerCP-conjugated CD8a, allophycocyanin-Cy7–conjugated CD25, PE-conjugated Foxp3, allophycocyanin-conjugated TCR-β, and PE-Cy7–conjugated NK1.1 Abs were used. Data analysis was performed using BD FACSDiva software (BD Biosciences).

Lipid analysis

Plasma lipid profile was measured by a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyzer, with reagents and calibrators supplied by Beckman Coulter Diagnostics, New South Wales, Australia.

Atherosclerotic lesion size analysis

The heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-tek, Sakura Finetek, Torrance, CA) and frozen at −80°C. Frozen sections (6 μm) were cut from the aortic sinus, defined as the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off (17). The aortic sinus was evaluated because this region of the aorta is particularly susceptible to development of atherosclerosis in mice fed a HFD (17). Sections were stained with Oil Red O and examined using light microscopy and cross-sectional area of lipid deposition quantified using image analysis software (Optimas 6.2 Video Pro-32, Bedford Park, South Australia, Australia). For each mouse, lesion size was measured in 6 cross-sectional areas at 30μm intervals and averaged. Collagen stained with Picosirus Red was analyzed using Polarizing microscope and Optimas software (Bedford Park).

Immunohistochemical analysis

Abs against CD68, CD22, CD4, and α smooth muscle actin were used in immunohistochemical analyses of macrophages, B cells, CD4$^+$ T cells, and

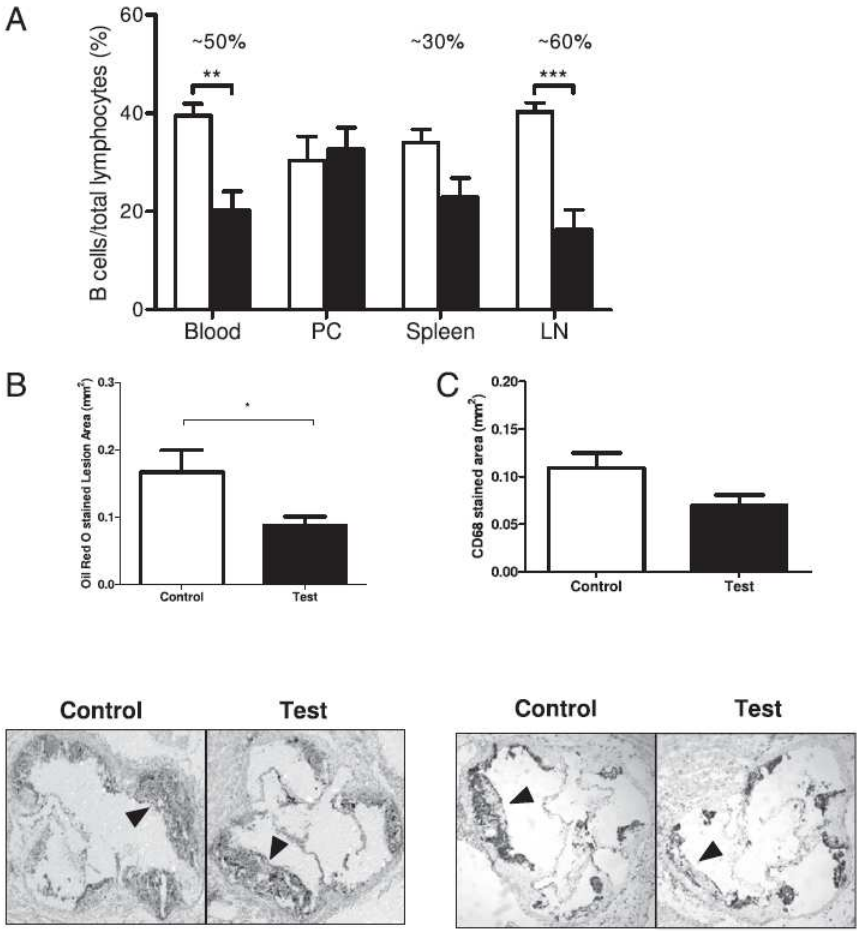

FIGURE 3. Partial B cell depletion retards established atherosclerosis. *A*, Anti-CD20 Ab partially depletes B cells during atherosclerosis progression. Anti-CD20 Ab depleted CD22$^+$ B cells in the blood by 50% and in the lymph node by 60% in the depletion (intervention) study ($n = 9$ in each group). *B* and *C*, Anti-CD20 Ab retards lipid but not macrophage accumulation in atherosclerotic lesions. Anti-CD20 Ab-treated mice only showed significant reduction in lipid content. Aortic sinus lesions were stained with Oil Red O for lipid content (*B*) and by anti-CD68 Ab for macrophages (*C*). Arrows indicate positively stained areas. Original magnification ×60. White bar, control mice injected with control (2B8) Ab; black bar, test mice injected with test (18B12) depleting Ab. Data are mean values ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

smooth muscle cells in frozen sections of the aortic roots, respectively. CD68- and α smooth muscle actin positively stained areas were quantified by Optimas software (Bedford Park). We microscopically counted CD22-stained B cells and CD4-stained T cells within atherosclerotic lesions in the intima of the aortic root. Mean values for positively stained areas and cell counts were calculated from three sections in the same way as described for the assessment of lesion size by Oil Red O stain.

Measurement of ox-LDL–specific IgG and IgM Abs

The ox-LDL–specific Abs were measured using ELISA. Briefly, copper-oxidized human LDL and native human LDL (Calbiochem, Darmstadt, Germany) were used to coat 96-well ELISA plates at 50 μl of 10 μg/ml overnight at 4°C. Duplicate samples of 50 μl mouse plasma diluted 1:100 were added into the ELISA plates for 2 h at room temperature after blocking with 1% BSA, followed by addition of anti-mouse IgG or IgM Abs conjugated with HRP. Color development was done by addition of TMB solution, and plates were read at 450 nm wavelength. ox-LDL–specific Ab was determined by subtracting the native LDL OD from the ox-LDL OD.

Measurement of plasma Igs

To determine plasma Igs titers using ELISA, 50 μl anti-mouse Ig (1 μg/ml) was used to coat 96-well ELISA plates overnight at 4°C. After blocking with 1% BSA, duplicate samples of 50 μl plasma (diluted $1:10^5$ for total Ig and IgG and $1:10^4$ for IgM) was added into ELISA plates for 2 h at room temperature. Respective secondary anti-mouse Abs conjugated with HRP were added into the wells, followed by addition of TMB substrate for color development. The OD at 450 nm was read by ELISA reader.

Analysis of gene expression

Total RNA was extracted from aortic arches using TRIzol reagent (Invitrogen, Carlsbad, CA) and quantitated by measuring absorbance at 260nm wavelength. cDNA was generated from 1 μg total RNA using TaqMan reverse transcription (Applied Biosystems, Foster City, CA) reagents according to the manufacturer's recommendation. Quantitative gene expression analysis to determine the mRNA levels of TNF-α, IFN-γ, IL-2, IL-4, IL-12, and 18S was performed on an ABI PRISM 7500 real-time PCR system (Applied Biosystems) using SYBR Green technology (Applied Biosystems). Each reaction was analyzed in triplicate, and the changes in target gene expression levels were quantitated using the comparative cycle threshold method with 18S rRNA primers (Applied Biosystems) as an endogenous control (18, 19). Sequences of primers used were as follows: TNF-α forward, 5′-TCT TCT GTC TAC TGA ACT TCG-3′; TNF-α reverse, 5′-GAA GAT GAT CTG AGT GTG AGG-3′; IFN-γ forward, 5′-CTG GAC CTG TGG GTT GTT GAC-3′; IFN-γ reverse, 5′-CAA CAG CAA GGC GAA AAA GG-3′; IL-2 forward, 5′-CGC AGA GGT CCA AGT TCA TCT T-3′; IL-2 reverse, 5′-CAG GAT GCT CAC CTT CAA ATT TTA C-3′; IL-4 forward, 5′-CGA GCT CAC TCT CTG TGG TGT T-3′; IL4 reverse, 5′-GGA GAT GGA TGT GCC AAA CG-3′; IL-12 forward, 5′-GGT GTA ACC AGA AAG GTG CG-3′; and IL-12 reverse, 5′-GAG GAA TTG TAA TAG CGA TCC TGA G-3′.

Statistical analysis

GraphPad Prism 4 (GraphPad, San Diego, CA) was used for statistical analyses. Results are presented as mean ± SEM. Two-tailed unpaired Student *t* tests (for comparisons between two groups) or one-way ANOVA (for comparisons of ≥3 groups) were used for statistical analyses. The *p* values were considered significant at <0.05.

Results

Mouse anti-mouse CD20 Ab (18B12) selectively depletes B cells in ApoE$^{-/-}$ mice

To test the efficiency of anti-CD20–mediated B cell depletion in C57BL/6J ApoE$^{-/-}$ mice, we administered a single i.v. injection of mouse IgG2a anti-mouse CD20 Ab (18B12) and performed flow cytometric (FACS) analysis of B cells in peripheral blood and lymphoid tissues at days 3, 7, 14, and 21 postinjection. We observed sustained depletion of circulating B cells (>97%) through to the third week. Spleen and peritoneal B cells were depleted by >95 and >70%, respectively (Fig. 1*D*). Relative resistance of peritoneal B cells to anti-CD20–mediated depletion has been reported (20). We injected anti-CD20 Ab once every 2 wk to maintain B cell depletion.

To determine whether B cell depletion can prevent atherosclerosis development, we fed 6–8-wk-old C57BL/6J ApoE$^{-/-}$ mice

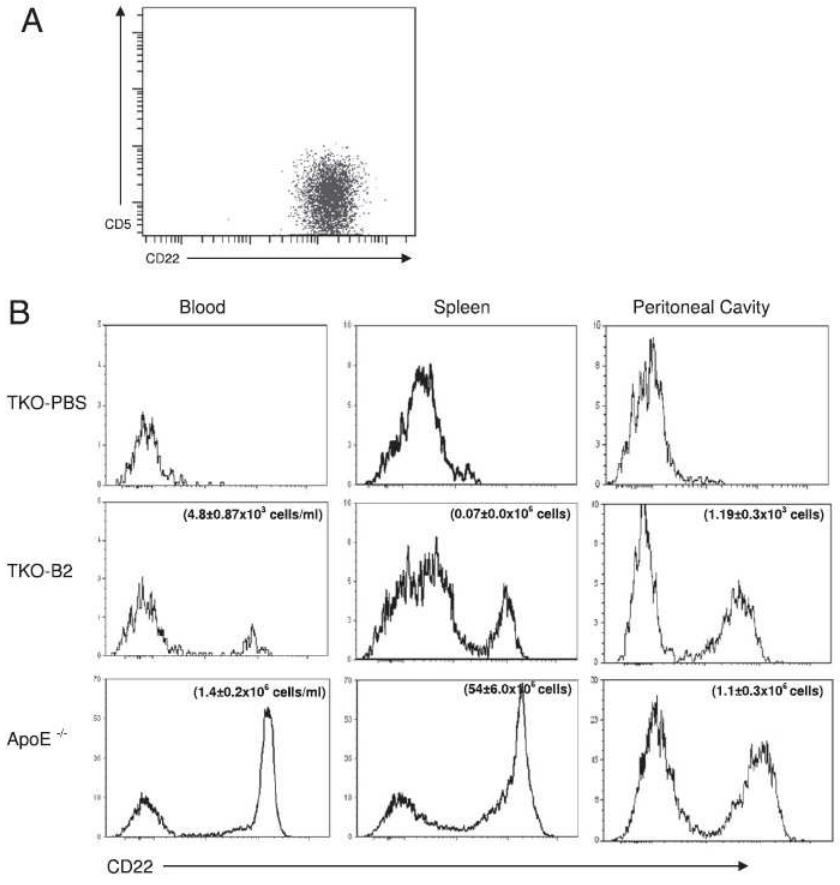

FIGURE 4. Transferred B2 B cells are found in blood, spleen, and peritoneal cavity. *A*, Isolation of purified B2 B cells. FACS analysis of magnetically sorted spleen B2 B cells using anti-CD22 and anti-CD5 Abs showed purity (>99%) of CD22$^+$CD5$^-$ B2 B cells. *B*, Identification of transferred B cells in peripheral blood, spleen, and peritoneal cavity. Shown are representative FACS data of CD22$^+$ B cells in blood, spleen, and peritoneal cavity 8 wk posttransfer of 5×10^6 magnetically sorted spleen B2 B cells into TKO mice ($n = 8$ in each group). Compared to ApoE$^{-/-}$ mice and TKO with PBS transfer (TKO-PBS), B cells transferred to TKO mice (TKO-B2) represented 0.1–0.3% of B cells in blood, spleen, and peritoneal cavity. Note difference in scale between ApoE$^{-/-}$ and TKO mice.

an HFD for 4, 8, or 12 wk, during which we maintained B cell depletion by injecting anti-CD20 Ab (Fig. 1*A*). At the end of experiments, B cells remained depleted by 90–95% in peripheral blood, spleen, lymph nodes, and peritoneal cavity in anti-CD20–treated mice compared with control mice (Fig. 1*E*). Non-B lymphocytes and monocytes (Fig. 1*F*), total plasma cholesterol, LDL cholesterol, HDL cholesterol, triglyceride levels (Fig. 1*G*), and body weights (data not shown) were unaffected.

B cell depletion by anti-CD20 Ab ameliorates atherosclerosis development in ApoE$^{-/-}$ mice

To investigate the effect of B cell depletion on atherosclerosis development, we measured lipid content and macrophage accumulation within the intima of the aortic sinus. We observed marked reductions in B cell-depleted mice fed an HFD for 8 wk and 12 wk, but not for 4 wk. Lipid content (Fig. 2*A*) and macrophage accumulation (Fig. 2*B*) were reduced by 59 and 47%, respectively, in the 8-wk group and by 50% in the 12-wk group. CD22-stained B cells (Fig. 2*C*), but not CD4-stained T cells (Fig. 2*D*), were reduced in atherosclerotic lesions in aortic roots of anti-CD20–treated mice. Plaque stability assessed by collagen (Fig. 2*E*) and smooth muscle cell content (Fig. 2*F*) in relation to lesion size were unaffected in anti-CD20–treated mice.

B cell depletion by anti-CD20 Ab retards established atherosclerosis in ApoE$^{-/-}$ mice

Next, we asked whether CD20-targeted B cell depletion can reduce established atherosclerotic lesions. We employed an intervention approach in which 6–8-wk-old C57BL/6J ApoE$^{-/-}$ mice were initially fed an HFD for 6 wk to allow establishment of atherosclerosis before commencement of anti-CD20 Ab treatment (Fig. 1*B*). In this study, anti-CD20 Ab reduced B cells by 50% in the peripheral blood and 60% in the lymph nodes (Fig. 3*A*), whereas other lymphocyte and monocyte populations, body weight, and plasma lipids were unaffected (data not shown). Atherosclerotic lesion size was reduced by 46% (Fig. 3*B*) and macrophage accumulation by 33% (Fig. 3*C*) in the test group. Plaque stability assessed by smooth muscle and collagen contents was unaffected (data not shown). Lesion CD22^{+} B cell numbers and lesion CD4^{+} T cell numbers remained unchanged (data not shown), suggesting that activated B cells in established atherosclerosis may be more resistant to anti-CD20–mediated depletion.

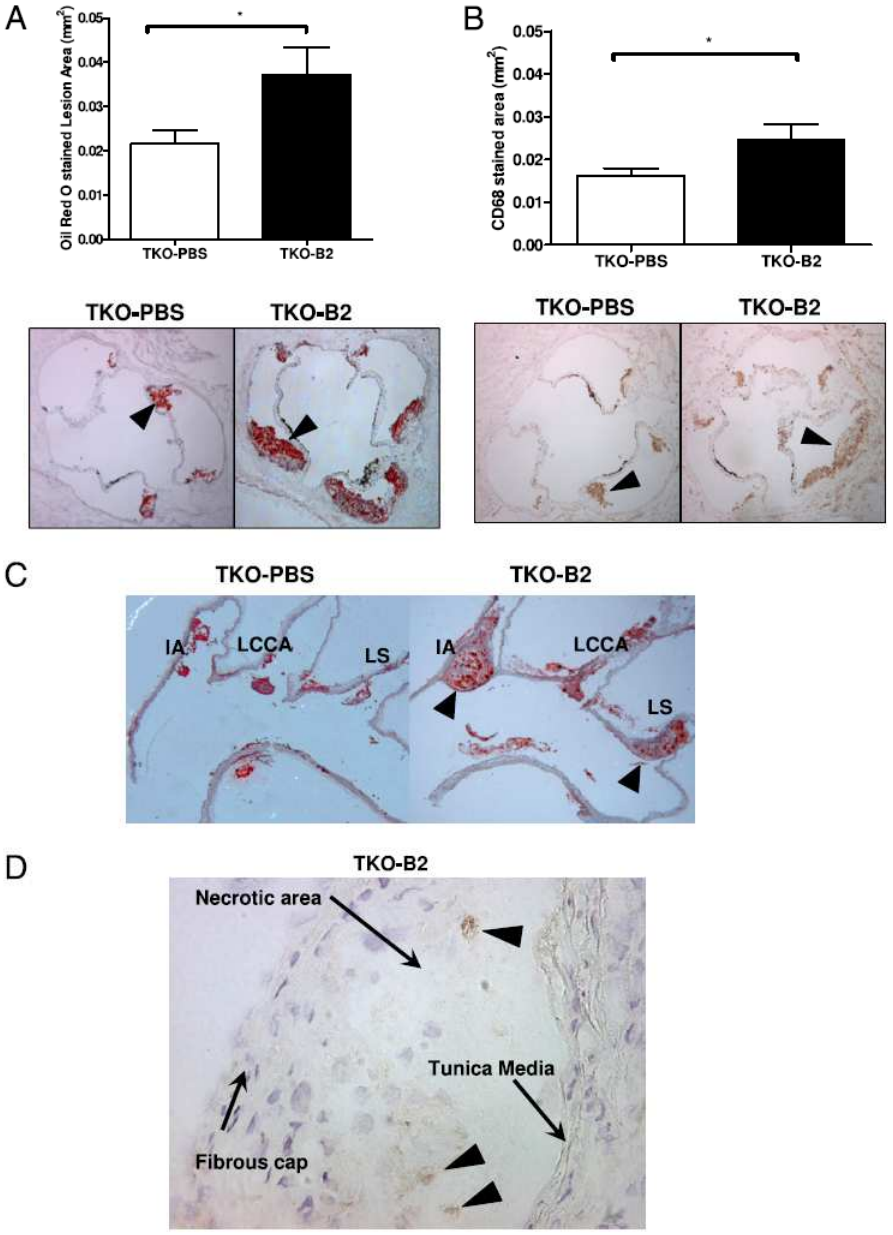

FIGURE 5. B2 B cell transfer increases atherosclerotic lesions in lymphocyte-deficient ApoE$^{-/-}$ mice. *A* and *B*, Dramatic increase in atherosclerotic lipid content and macrophage accumulation. Shown are aortic sinus lesions in TKO mice after spleen B2 B cell transfer (TKO-B2, black bars) compared with PBS transfer (TKO-PBS, white bars) (n = 8 in each group). Atherosclerotic lesions stained with Oil Red O (*A*) for lipid content and with anti-CD68 Ab (*B*) for macrophages. Arrows indicate positively stained areas. Original magnification ×60. *C*, Exaggerated atherosclerotic lesions in aortic arches. Shown are representative Oil Red O-stained aortic arches of TKO-B2 transfer group compared with TKO-PBS transfer group (n = 8 in each group). Arrows indicate Oil Red O-stained areas. Original magnification ×40. *D*, Lesion B cells identified in atherosclerotic lesions of TKO-B2 mice. Shown is aortic sinus lesion stained with anti-CD22 Ab for B cells in TKO-B2 transfer. Arrows indicate CD22^{+} B cells. Original magnification ×160. Data are mean values ± SEM. *$p < 0.05$. IA, innominate artery; LCCA, left common carotid artery; LS, left subclavian artery.

Transferred conventional B2 B lymphocytes are found in lymphocyte-deficient ApoE$^{-/-}$ mice

One potential mechanism by which anti-CD20 Ab attenuates atherosclerosis is by depleting conventional B2 B cells. To test this hypothesis, we reintroduced B2 B cells into a lymphocyte-deficient TKO mouse that is devoid of Rag-2–dependent lymphocytes (including B, T, and NKT cells) and γc-dependent NK cells. We used a B cell isolation kit (Miltenyi Biotec) to negatively select spleen B2 B cells from non-B cells and CD43-expressing B-1 B cells (21). Purity of the isolated B2 B cell preparation assessed by FACS analysis was >99% (Fig. 4*A*). We transferred 5×10^6 B2 B cells into lymphocyte-deficient TKO mice and fed the mice an HFD for 8 wk. At the end of the experiment, B2 B cells were found in peripheral blood, spleen, and peritoneal cavity, albeit in very small numbers of 0.1–0.3% compared with B cells in ApoE$^{-/-}$ mice (Fig. 4*B*).

Conventional B2 B lymphocytes potently augment atherosclerosis development

Despite the very small numbers of transferred B2 B cells in the peripheral blood, spleen, and peritoneal cavity of the lymphocyte-deficient TKO mice, atherosclerotic lesion size and macrophage accumulation in the aortic sinus were dramatically increased by 72 and 53%, respectively (Fig. 5*A*, 5*B*), effects not observed following transfer of vehicle (PBS) only. Larger atherosclerotic lesions were also seen in the aortic arch (Fig. 5*C*). Transferred B2 B cells were present in these enlarged atherosclerotic lesions (Fig. 5*D*). Hyperlipidemia in the lymphocyte-deficient TKO mice was unaffected (data not shown).

In a separate experiment, we transferred 5×10^7 conventional B2 B cells into lymphocyte-deficient TKO mice. Atherosclerotic lesion size and macrophage accumulation were again increased, but they were not significantly different from those that followed 5×10^6 B2 B cell transfer (Fig. 6*A*). This result shows that 5×10^6 adoptively transferred B2 B cells is sufficient to accelerate atherosclerosis in lymphocyte-deficient TKO mice and supports the proatherogenic role of B2 B cells.

In another experiment, we examined the effect on atherosclerosis following the adoptive transfer of 5×10^6 conventional B2 B cells compared with the adoptive transfer of 5×10^6 B1 B cells into lymphocyte-deficient TKO mice. We found that only the transfer of B2 B cells, but not the transfer of B1 B cells, augmented atherosclerosis development (Fig. 6*B*).

Conventional B2 B lymphocytes also increase atherosclerosis development in ApoE$^{-/-}$ mice deficient only in B lymphocytes

Because TKO mice are deficient not only in B cells but also in non-B lymphocytes, we asked whether conventional B2 B cells are

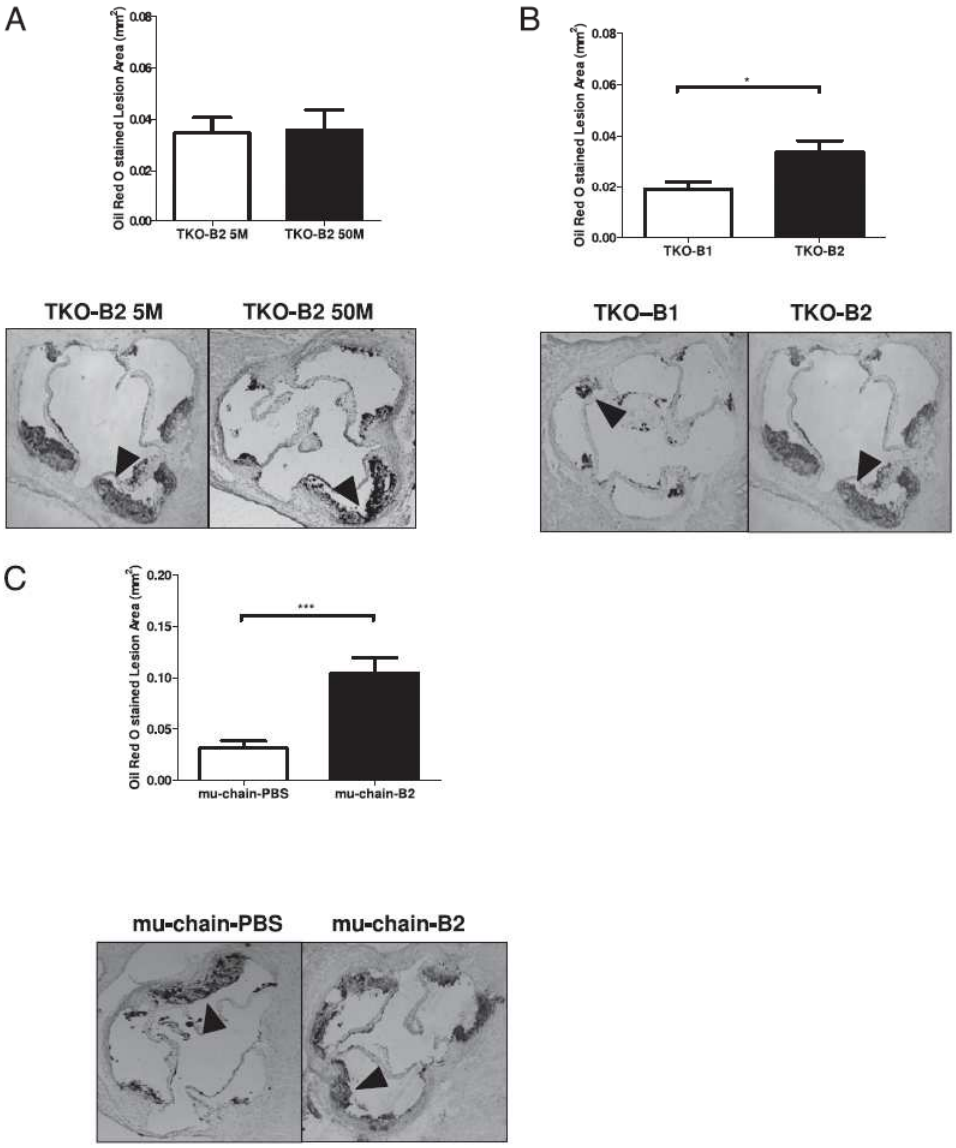

FIGURE 6. Comparison of atherosclerotic lesions after B2 B cell transfer into lymphocyte deficient and into B cell-deficient ApoE$^{-/-}$ mice. *A*, Comparison of atherosclerotic lesions between TKO mice transferred with 5×10^6 (TKO-B2 5M) and 5×10^7 (TKO-B2 50M) B2 B cells. Shown are mean atherosclerotic lesion areas stained with Oil Red O for lipid content. White bar represents 5×10^6 B2 B cell transfer group ($n = 8$), and black bar represents 5×10^7 B2 B cell transfer group ($n = 4$). Arrows indicate Oil Red O-stained areas. Original magnification ×60. *B*, Conventional B2 B cells, not B1 B cells, increase atherosclerosis in lymphocyte-deficient TKO mice. Shown are mean atherosclerotic lesion areas stained with Oil Red O for lipid content. Either 5×10^6 peritoneal B1 B cells (TKO-B1) or conventional spleen B2 B cells (TKO-B2) were transferred into TKO mice. White bar represents B1 B cells transfer group ($n = 4$), and black bar represents B2 B cells transfer group ($n = 8$). Arrows indicate Oil Red O-stained areas. Original magnification ×60. *C*, Conventional B2 B cells increase atherosclerotic lesions in atherogenic mice deficient only in B cells (ApoE$^{-/-}$ μMT$^{-/-}$ mice). Shown are mean atherosclerotic lesion areas stained with Oil Red O for lipid content. ApoE$^{-/-}$ μMT$^{-/-}$ mice were transferred with either PBS (μ-chain–PBS) or 5×10^6 B2 B cells (μ-chain–B2). White bar represents μ-chain–PBS group ($n = 7$), and black bar represents μ-chain–B2 group ($n = 8$). Arrows indicate Oil Red O stained areas. Original magnification ×60. Data are mean values ± SEM. $*p < 0.05$; $***p < 0.001$.

proatherogenic in ApoE$^{-/-}$ mice selectively deficient only in B cells. We generated ApoE$^{-/-}$ μMT$^{-/-}$ mice deficient in the μ-chain of Ig and lacking only in B cells (data not shown). We then transferred 5×10^6 conventional B2 B cells into these B cell-deficient ApoE$^{-/-}$ mice. Upon completion of 8 wk HFD, we found that the atherosclerotic lesions in ApoE$^{-/-}$ μMT$^{-/-}$ mice were increased >3-fold as compared with those from a control group that had not received B2 B cells (Fig. 6*C*).

Plasma Ig and ox-LDL–specific Ab levels

As conventional B cells are best known for Ab production, we examined plasma Igs as well as IgG and IgM Abs to ox-LDL. We used human LDL (Calbiochem) as a coating Ag in ELISA to compare the levels of ox-LDL–specific Abs in our studies because comparable ox-LDL–specific Abs against oxidized human LDL as coating Ag in ELISA have been reported in mice immunized with murine LDL (22). In our depletion (prevention) study, plasma Igs and ox-LDL–specific Abs remained unchanged with anti-CD20–mediated B cell depletion and 4 wk of HFD. With B cell depletion and 8 or 12 wk of HFD, total Igs and IgG but not IgM decreased (Fig. 7*A*), and only ox-LDL–specific IgG Ab decreased in the 12 wk group (Fig. 7*B*). In our depletion (intervention) study, total Ig, IgG, and IgM levels and IgG and IgM Abs to ox-LDL remained unchanged (Fig. 7*C, 7D*). The data suggest that atherosclerosis was ameliorated by B cell depletion irrespective of Ab levels. The observations are consistent with the resistance of long-lived plasma cells that do not express CD20 (23) to B cell depletion with anti-CD20 Ab (24). After B2 B cell transfer into TKO mice, Igs and ox-LDL–specific Abs, absent in TKO mice, were detectable at low levels of 3–16% of immunocompetent ApoE$^{-/-}$ mice (Fig. 8*A, 8B*). Ig production by B cells in the absence of T cell help has been reported in T cell-deficient (25) and immunodeficient (26) mice.

TNF-α expression is increased in atherosclerotic lesions in TKO mice transferred with conventional B2 B lymphocytes

As B cells produce a broad range of pro- and anti-inflammatory cytokines (27, 28), we next examined atherosclerotic lesions, augmented by B2 B cell transfer into lymphocyte-deficient TKO mice, for cytokines by RT-PCR. We found elevated levels of TNF-α but not of IFN-γ, IL-2, IL-4, and IL-12 (Fig. 8*C*). To account for differences in lesion sizes, we expressed TNF-α levels relative to F4/80, a macrophage marker (Fig. 8*D*). TNF-α, expressed as a proportion of macrophages, was increased but was not statistically significant (Fig. 8*E*).

Discussion

In the current study, we have shown that B cell depletion by anti-CD20 Ab not only prevents the development of atherosclerosis but also ameliorates established atherosclerosis. Contrary to the widely held view that B cells are protective (4, 5), our observations demonstrate that B cells can have a damaging role in atherosclerosis. We identified conventional B2 B cells as a proatherogenic B cell population because transfer of these B2 B cells into lymphocyte-depleted ApoE$^{-/-}$ mice or to B cell-deficient ApoE$^{-/-}$ mice potently augmented atherosclerosis development. These B2 B cells comprise the majority of circulating B cells and reside in lymphoid follicles in lymph nodes and in the spleen, where they are also known as follicular B cells. Augmented atherosclerosis development following transfer of B2 B cells into TKO mice totally lacking in Rag2-dependent T cells, B cells, NKT cells, and γc-dependent NK cells demonstrates that these conventional B2 B cells can directly promote atherosclerosis development entirely on their own in the complete absence of all other lymphocyte populations. Augmented atherosclerosis development following

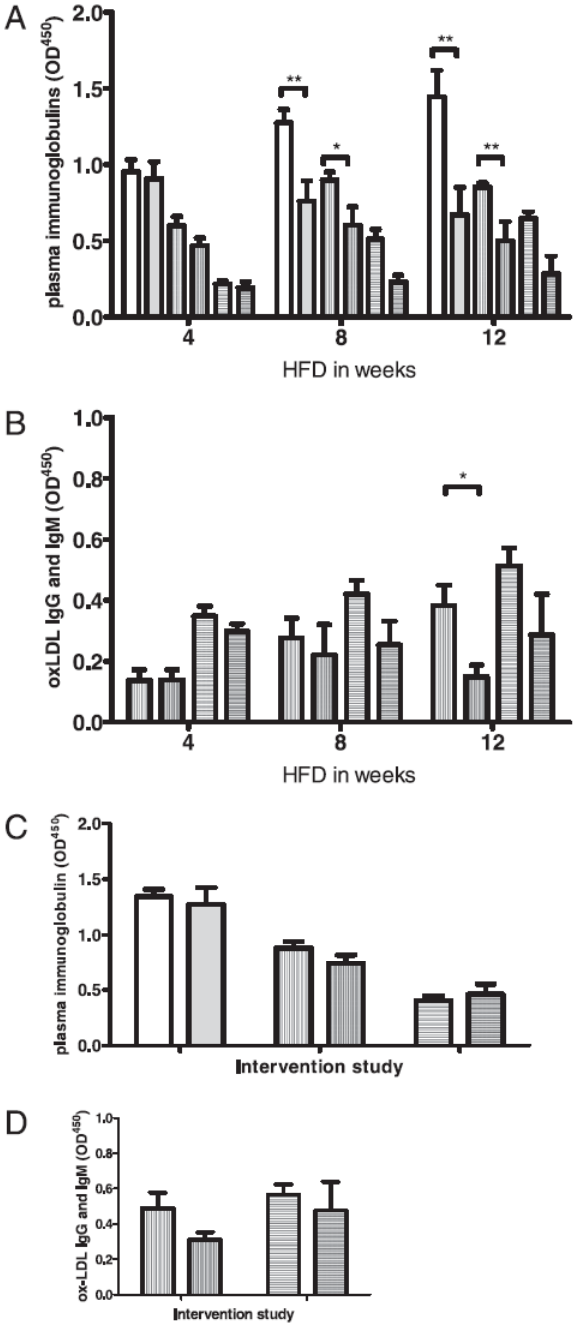

FIGURE 7. Igs and ox-LDL–specific Abs in B cell depletion studies. *A* and *B*, Igs and ox-LDL–specific Abs levels in 12-wk depletion (prevention) study. ApoE$^{-/-}$ mice were treated with anti-CD20 Ab and fed a HFD for 12 wk. *A*, Total Ig, IgG, and IgM. White bars, control mice injected with control (2B8) Ab; gray bars, test mice injected with test (18B12) Ab; open bar, total Ig; bar with vertical lines, IgG; bar with horizontal line, IgM. *B*, ox-LDL–specific IgG and IgM Abs. White bars, control mice injected with control (2B8) Ab; gray bars, test mice injected with test (18B12) Ab; bar with vertical lines, ox-LDL–specific IgG; bar with horizontal line, ox-LDL–specific IgM ($n = 9$ in each group). Data are mean values ± SEM. *C* and *D*, Igs and ox-LDL–specific Abs levels in depletion (intervention) study. ApoE$^{-/-}$ mice were fed a HFD for 6 wk, after which they were treated with anti-CD20 Ab while maintained on a HFD for a further 4 wk. *C*, Total Ig, IgG, and IgM; legend as in *A*. *D*, ox-LDL–specific IgG and IgM Abs; legend as in *B* ($n = 9$ in each group). Data are mean values ± SEM. $*p < 0.05$; $**p < 0.01$.

transfer of B2 B cells into ApoE$^{-/-}$ μMT$^{-/-}$ mice deficient only in B cells indicates that B2 B cells can also augment atherosclerosis development in the presence of all other lymphocyte populations. B2 B cells transfer into ApoE$^{-/-}$ deficient only in B cells

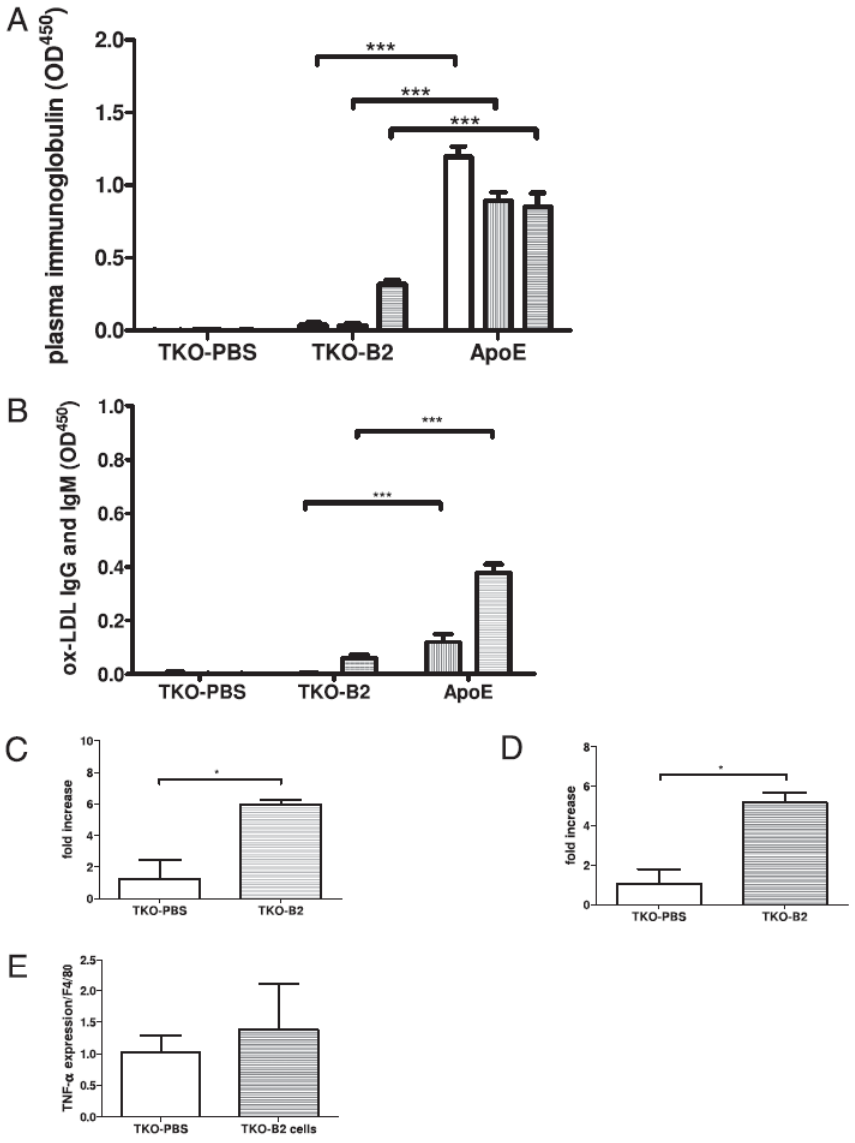

FIGURE 8. Abs and lesion TNF-α after B2 B cell transfer in TKO mice. *A* and *B*, Igs and ox-LDL–specific Abs levels. Data shown are Ab levels in TKO-PBS, TKO-B2, and ApoE$^{-/-}$ mice being fed a HFD for 8 wk. *A*, Open bar, total Ig; bar with vertical lines, IgG; bar with horizontal line, IgM. *B*, Bar with vertical lines, ox-LDL–specific IgG; bar with horizontal line, ox-LDL–specific IgM. *C–E*, TNF-α expression in atherosclerotic lesions of TKO-B2 and TKO-PBS mice. RT-PCR was carried out using cDNA derived from aortic arches as described in *Materials and Methods*. *C*, TNF-α expression. *D*, Macrophage marker F4/80 expression. *E*, TNF-α expression normalized by F4/80 expression. Data are mean values ± SEM. $*p < 0.05$; $***p < 0.001$.

increased lesion size by >300%, whereas transfer into ApoE$^{-/-}$ mice deficient in all lymphocyte populations increased lesion size by 72%. The findings suggest that proatherogenic B2 B cells can act in concert with other proatherogenic lymphocyte populations to augment atherosclerosis development.

B cells are considered to have important roles in immunity by producing Abs. However, emerging evidence shows that B cells can influence immune responses without Ab involvement. For instance, in MRL/lpr mice, whereas B cell deficiency generated by Jh mutation prevented lupus nephritis (29), mice expressing a mutant transgene that prevented IgG secretion developed disease (30). With initial anti-CD20 treatment, plasma Igs were unaffected at 4 wk of HFD, but declined at 8 and 12 wk of HFD in subclass IgG but not IgM levels. These findings may reflect the relative resistance of IgM-producing B1 B cells to anti-CD20 Ab depletion (20). In contrast, plasma Igs and ox-LDL–specific Abs were largely unaffected by anti-CD20 treatment in established atherosclerosis. This may reflect the failure of anti-CD20 Ab treatment to deplete Ab-producing plasma cells (23, 24). The data suggest that atherosclerosis was ameliorated by B cell depletion irrespective of Ab levels. TKO mice did not show any detectable plasma Igs and ox-LDL–specific Abs, but those that received conventional B2 B cells were found to have very low plasma Igs and IgM Ab to ox-LDL compared with immunocompetent ApoE$^{-/-}$ mice. The presence of circulating Igs in the absence of T cell help is consistent with reports of Ig production by B cells from T cell-deficient (25) and immunodeficient mouse models (26). The significance, if any, for atherosclerosis of these low-level Abs generated without T cell help remains unknown.

In addition to Ab production, conventional B2 B cells produce a broad range of pro- and anti-inflammatory cytokines (27, 28). Significant increase in expression of TNF-α was detected in atherosclerotic lesions of TKO mice transferred with conventional B2 B cells. This finding was not significant when TNF-α expression levels were expressed relative to a macrophage marker, F4/80. Although a macrophage origin for TNF-α cannot be excluded, TNF-α is also produced by B lymphocytes (31, 32). It enhances B cell locomotion and migration (32) and is found in lesions in the vicinity of B lymphocytes (3). This finding suggests that B2 B cells may have augmented inflammation in a cytokine-dependent manner to promote atherosclerosis.

Although Ag presentation to proatherogenic CD4$^+$ T cells by conventional B2 B cells can conceivably activate these T cells and further augment atherosclerosis, the notable finding in our study is that B2 B cells can promote atherosclerosis in the complete absence of T cells in the lymphocyte-deficient TKO mice. Our findings in these lymphocyte-deficient mice indicate that B cells can promote atherosclerosis in the absence of Ag presentation to

T cells. These findings are consistent with the suggestion that B cells may be important in the rate-limiting step in the genesis of autoimmune reactions (33). We suggest that our findings are likely to have relevance for other inflammatory diseases in which B cells have a role. Our identification of a proatherogenic role for B2 B cells provides a therapeutic target for attenuating lesion development/progression in hyperlipidemic subjects and other subjects susceptible to atherosclerosis-related cardiovascular complications.

Disclosures

The authors have no financial conflicts of interest.

References

1. Houtkamp, M. A., O. J. de Boer, C. M. van der Loos, A. C. van der Wal, and A. E. Becker. 2001. Adventitial infiltrates associated with advanced atherosclerotic plaques: structural organization suggests generation of local humoral immune responses. *J. Pathol.* 193: 263–269.
2. Ylä-Herttuala, S., W. Palinski, S. W. Butler, S. Picard, D. Steinberg, and J. L. Witztum. 1994. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler. Thromb.* 14: 32–40.
3. Zhou, X., and G. K. Hansson. 1999. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of apolipoprotein E knockout mice. *Scand. J. Immunol.* 50: 25–30.
4. Weber, C., A. Zernecke, and P. Libby. 2008. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat. Rev. Immunol.* 8: 802–815.
5. Galkina, E., and K. Ley. 2009. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu. Rev. Immunol.* 27: 165–197.
6. Major, A. S., S. Fazio, and M. F. Linton. 2002. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler. Thromb. Vasc. Biol.* 22: 1892–1898.
7. Lewis, M. J., T. H. Malik, M. R. Ehrenstein, J. J. Boyle, M. Botto, and D. O. Haskard. 2009. Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* 120: 417–426.
8. Hansson, G. K., and J. Nilsson. 2009. Vaccination against atherosclerosis? Induction of atheroprotective immunity. *Semin. Immunopathol.* 31: 95–101.
9. Dörner, T., A. Radbruch, and G. R. Burmester. 2009. B-cell-directed therapies for autoimmune disease. *Nat. Rev. Rheumatol.* 5: 433–441.
10. Looney, R. J., J. H. Anolik, D. Campbell, R. E. Felgar, F. Young, L. J. Arend, J. A. Sloand, J. Rosenblatt, and I. Sanz. 2004. B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab. *Arthritis Rheum.* 50: 2580–2589.
11. Ramos-Casals, M., C. Díaz-Lagares, and M. A. Khamashta. 2009. Rituximab and lupus: good in real life, bad in controlled trials. Comment on the article by Lu et al. *Arthritis Rheum.* 61: 1281–1282.
12. Hauser, S. L., E. Waubant, D. L. Arnold, T. Vollmer, J. Antel, R. J. Fox, A. Bar-Or, M. Panzara, N. Sarkar, S. Agarwal, et al; HERMES Trial Group. 2008. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N. Engl. J. Med.* 358: 676–688.
13. Yu, S., R. Dunn, M. R. Kehry, and H. Braley-Mullen. 2008. B cell depletion inhibits spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. *J. Immunol.* 180: 7706–7713.
14. Ahuja, A., J. Shupe, R. Dunn, M. Kashgarian, M. R. Kehry, and M. J. Shlomchik. 2007. Depletion of B cells in murine lupus: efficacy and resistance. *J. Immunol.* 179: 3351–3361.
15. Hamel, K., P. Doodes, Y. Cao, Y. Wang, J. Martinson, R. Dunn, M. R. Kehry, B. Farkas, and A. Finnegan. 2008. Suppression of proteoglycan-induced arthritis by anti-CD20 B Cell depletion therapy is mediated by reduction in autoantibodies and CD4+ T cell reactivity. *J. Immunol.* 180: 4994–5003.
16. Hu, C. Y., D. Rodriguez-Pinto, W. Du, A. Ahuja, O. Henegariu, F. S. Wong, M. J. Shlomchik, and L. Wen. 2007. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J. Clin. Invest.* 117: 3857–3867.
17. Paigen, B., A. Morrow, P. A. Holmes, D. Mitchell, and R. A. Williams. 1987. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 68: 231–240.
18. Bas, A., G. Forsberg, S. Hammarström, and M. L. Hammarström. 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand. J. Immunol.* 59: 566–573.
19. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ Method. *Methods* 25: 402–408.
20. Hamaguchi, Y., J. Uchida, D. W. Cain, G. M. Venturi, J. C. Poe, K. M. Haas, and T. F. Tedder. 2005. The peritoneal cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in mice. *J. Immunol.* 174: 4389–4399.
21. Wells, S. M., A. B. Kantor, and A. M. Stall. 1994. CD43 (S7) expression identifies peripheral B cell subsets. *J. Immunol.* 153: 5503–5515.
22. Freigang, S., S. Hörkkö, E. Miller, J. L. Witztum, and W. Palinski. 1998. 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.
23. Edwards, J. C., and G. Cambridge. 2006. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nat. Rev. Immunol.* 6: 394–403.
24. Hoyer, B. F., R. A. Manz, A. Radbruch, and F. Hiepe. 2005. Long-lived plasma cells and their contribution to autoimmunity. *Ann. N. Y. Acad. Sci.* 1050: 124–133.
25. Wen, L., S. J. Roberts, J. L. Viney, F. S. Wong, C. Mallick, R. C. Findly, Q. Peng, J. E. Craft, M. J. Owen, and A. C. Hayday. 1994. Immunoglobulin synthesis and generalized autoimmunity in mice congenitally deficient in alpha beta(+) T cells. *Nature* 369: 654–658.
26. Reininger, L., T. Radaszkiewicz, M. Kosco, F. Melchers, and A. G. Rolink. 1992. Development of autoimmune disease in SCID mice populated with long-term "in vitro" proliferating (NZB x NZW)F1 pre-B cells. *J. Exp. Med.* 176: 1343–1353.
27. Lund, F. E. 2008. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr. Opin. Immunol.* 20: 332–338.
28. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat. Immunol.* 1: 475–482.
29. Chan, O. T., M. P. Madaio, and M. J. Shlomchik. 1999. B cells are required for lupus nephritis in the polygenic, Fas-intact MRL model of systemic autoimmunity. *J. Immunol.* 163: 3592–3596.
30. Chan, O. T., L. G. Hannum, A. M. Haberman, M. P. Madaio, and M. J. Shlomchik. 1999. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J. Exp. Med.* 189: 1639–1648.
31. Sung, S. S., L. K. Jung, J. A. Walters, W. Chen, C. Y. Wang, and S. M. Fu. 1988. Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. *J. Exp. Med.* 168: 1539–1551.
32. Corcione, A., L. Ottonello, G. Tortolina, P. Tasso, F. Ghiotto, I. Airoldi, G. Taborelli, F. Malavasi, F. Dallegri, and V. Pistoia. 1997. Recombinant tumor necrosis factor enhances the locomotion of memory and naive B lymphocytes from human tonsils through the selective engagement of the type II receptor. *Blood* 90: 4493–4501.
33. Shlomchik, M. J. 2008. Sites and stages of autoreactive B cell activation and regulation. *Immunity* 28: 18–28.

Chapter 3: B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM Deposits and Reduces Necrotic Cores in Atherosclerotic Lesions

3.1 Short Introduction

The role of B1a B cells in atherosclerosis has not been defined. Splenectomy has been shown to aggravate atherosclerosis and other studies indicate that splenectomy abolishes the B1a B cell population. Here, I have used splenectomised ApoE$^{-/-}$ mice to define the role of B1a B cells in atherosclerosis. I have also used sIgM$^{-/-}$ mice to determine the mechanism by which B1a cells affect atherosclerosis. Splenectomy was first performed to deplete peritoneal B1a cells in ApoE$^{-/-}$ mice. In comparison to sham-operated ApoE$^{-/-}$ mice, ApoE$^{-/-}$ mice that underwent splenectomy developed significantly larger atherosclerotic lesions. Remarkably, the reconstitution of B1a B cells by adoptive transfer in splenectomised ApoE$^{-/-}$ mice not only ameliorated the aggravation of atherosclerosis caused by splenectomy but also markedly suppressed the development of atherosclerosis. However, the adoptive transfer of B1a cells deficient in secretory IgM (sIgM) did not exhibit any modulatory effect. Further studies indicated that the atheroprotective function of B1a cells may be associated with increased IgM deposition in the atherosclerotic plaque with concomitant decrease in plaque apoptotic cells and regions of necrosis. As such, it is reasonable to deduce that B1a cells restrict the development of atherosclerosis by producing IgM antibodies that facilitate the clearance of apoptotic cells, thereby curtailing necrotic inflammation in atherosclerotic plaques. Circ Res. 2011; 109(8):830-40.

PART B: Suggested Declaration for Thesis Chapter

[This declaration to be completed for each conjointly authored publication and to be placed at the start of the thesis chapter in which the publication appears.]

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter [3], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	45

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Tin Kyaw	Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	
Surendran Krishnamurthi	Study design, Experimentation, Data analysis, Result Interpretation	
Peter Kanellakis	Experimentation, Data analysis	
Alex Agrotis	Data analysis	
Peter Tipping	Study concept, Study design, Result Interpretation	
Alex Bobik	Study concept, Study design, Result Interpretation, Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Result Interpretation, Preparation of manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 7/2/2014
Main Supervisor's Signature	[redacted]	**Date** 7/2/2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM Deposits and Reduces Necrotic Cores in Atherosclerotic Lesions

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B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM Deposits and Reduces Necrotic Cores in Atherosclerotic Lesions

Tin Kyaw,* Christopher Tay,* Surendran Krishnamurthi,* Peter Kanellakis, Alex Agrotis, Peter Tipping,† Alex Bobik,† Ban-Hock Toh†

Rationale: **Aggravated atherosclerosis in B lymphocyte-deficient chimeric mice and reduced atherosclerosis after transfer of unfractionated spleen B lymphocytes into splenectomized mice have led to the widely held notion that B lymphocytes are atheroprotective. However, B lymphocytes can be pathogenic, because their depletion by anti-CD20 antibody ameliorated atherosclerosis, and transfer of B2 lymphocytes aggravated atherosclerosis. These observations raise the question of the identity of the atheroprotective B-lymphocyte population.**

Objective: **The purpose of the study was to identify an atheroprotective B-lymphocyte subset and mechanisms by which they confer atheroprotection.**

Methods and Results: **Splenectomy of apolipoprotein E–deficient mice selectively reduced peritoneal B1a lymphocytes, plasma IgM, and oxidized low-density lipoprotein IgM levels and lesion IgM deposits. These reductions were accompanied by increased oil red O–stained atherosclerotic lesions and increased necrotic cores, oxidized low-density lipoproteins, and apoptotic cells in lesions. Plasma lipids, body weight, collagen, and smooth muscle content were unaffected. Transfer of B1a lymphocytes into splenectomized mice increased peritoneal B1a lymphocytes; restored plasma IgM, oxidized low-density lipoprotein IgM levels, and lesion IgM deposits; and potently attenuated atherosclerotic lesions, with reduced lesion necrotic cores, oxidized low-density lipoprotein, and apoptotic cells. In contrast, transfer of B1a lymphocytes that cannot secrete IgM failed to protect against atherosclerosis development in splenectomized mice despite reconstitution in the peritoneum.**

Conclusions: **B1a lymphocytes are an atheroprotective B-lymphocyte population. Our data suggest that natural IgM secreted by these lymphocytes offers protection by depositing IgM in atherosclerotic lesions, which reduces the necrotic cores of lesions. (*Circ Res*. 2011;109:830-840.)**

Key Words: B-lymphocytes ■ apoptosis ■ inflammation ■ immunoglobulin M ■ splenectomy

Atherosclerosis is an inflammatory disease of arteries initiated by lipid entry into the arterial intima. Despite introduction of lipid-lowering statins, it remains the major cause of cardiovascular deaths related to heart attacks and strokes. There is therefore a pressing need to precisely understand the role of leukocytes in the inflammatory infiltrate in atherosclerosis that can be exploited to develop novel antiinflammatory therapies.

The presence of B lymphocytes in the inflammatory infiltrate in humans[1,2] and in mice[3] has led to a search for their role in atherosclerosis. Earlier studies led to a widely held notion that B lymphocytes are protective.[4] This conclusion is based primarily on 2 principal reports. The first was that adoptive transfer of large numbers of unfractionated spleen B lymphocytes reduced splenectomy-aggravated atherosclerosis.[5] The next was the report of aggravated atherosclerosis in low-density lipoprotein receptor–deficient (LDL-$R^{-/-}$) mice rendered B lymphocyte deficient by bone marrow transplantation from mice deficient in the μ-chain of immunoglobulin into irradiated recipients.[6] However, our recent observation[7] and that of Ait-Oufella et al[8] that depletion of B lymphocytes by anti-CD20 antibody ameliorated atherosclerosis indicate that B lymphocytes can be proatherogenic. Furthermore, we identified conventional B2 lymphocytes as a proatherogenic B-lymphocyte population, because transfer of this population to lymphocyte-deficient or B lymphocyte–

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Non-standard Abbreviations and Acronyms	
MDA	malondialdehyde
sIgM$^{-/-}$	secretory IgM-deficient
SX	splenectomy

deficient apolipoprotein E–deficient (ApoE$^{-/-}$) mice aggravated atherosclerosis.[7] These observations raise the question of the identity of the protective B-lymphocyte population in atherosclerosis.

In a search for an atheroprotective B-lymphocyte subset, we were guided by reports that splenectomy is associated with augmented atherosclerosis in humans and in experimental animals. In a 19-year follow-up of World War II veterans with trauma-induced splenectomy, a high incidence of acute myocardial infarction was reported compared with those without splenectomy.[9] In animal studies, splenectomized mice, rats, and rabbits showed significantly increased atherosclerotic lesions compared with sham-operated controls.[5,10,11]

The report that B1a lymphocytes are depleted in splenectomized or asplenic mice[12] suggests that the aggravated atherosclerosis in splenectomized mice[5] may be the consequence of the loss of peritoneal B1a lymphocytes. Because B1a lymphocytes produce the majority of natural IgM,[13] and IgM has been shown to protect against atherosclerosis,[14] diminished B1a lymphocytes and B1a-produced natural IgM antibodies are rational explanations for aggravated atherosclerosis in splenectomized mice. Therefore, we hypothesized that B1a lymphocytes, unlike B2 lymphocytes, are an atheroprotective B-lymphocyte subset.

To test this hypothesis, we revisited the splenectomy-aggravated atherosclerosis experiments. We confirmed that splenectomy reduced peritoneal B1a lymphocytes and at the same time aggravated atherosclerosis in ApoE$^{-/-}$ mice. Furthermore, we found that adoptive transfer of B1a lymphocytes repopulated B1a lymphocytes in the peritoneum and potently attenuated atherosclerosis aggravated by splenectomy. We have also demonstrated that IgM produced by B1a lymphocytes is required for their atheroprotective action.

Methods

Animals

All animal procedures and protocols were approved by the Animal Ethics Committee of the Alfred Medical, Research, and Education Precinct, Prahran, Melbourne, Australia. All male mice were bred on a C57BL/6 background. ApoE$^{-/-}$ and C57BL/6 mice were from the Precinct Animal Centre, Alfred Medical, Research, and Education Precinct. Ly5.1 mice were from the Walter and Eliza Hall Institute, Melbourne, Australia. Secretory IgM-deficient (sIgM$^{-/-}$) mice generated by Jianzhu Chen, Massachusetts Institute of Technology, were supplied by Troy Randall, University of Rochester.

ApoE$^{-/-}$ mice were subjected to either splenectomy or sham operation and fed a high-fat diet with 21% fat and 0.15% cholesterol (Specialty Feeds, Glen Forrest, Western Australia) for 8 weeks. At the end of experiments, mice were killed and peritoneal fluids collected for differential cell analysis by flow cytometry; aortic roots were frozen in OCT embedding medium, and plasma was kept in a −80°C freezer.

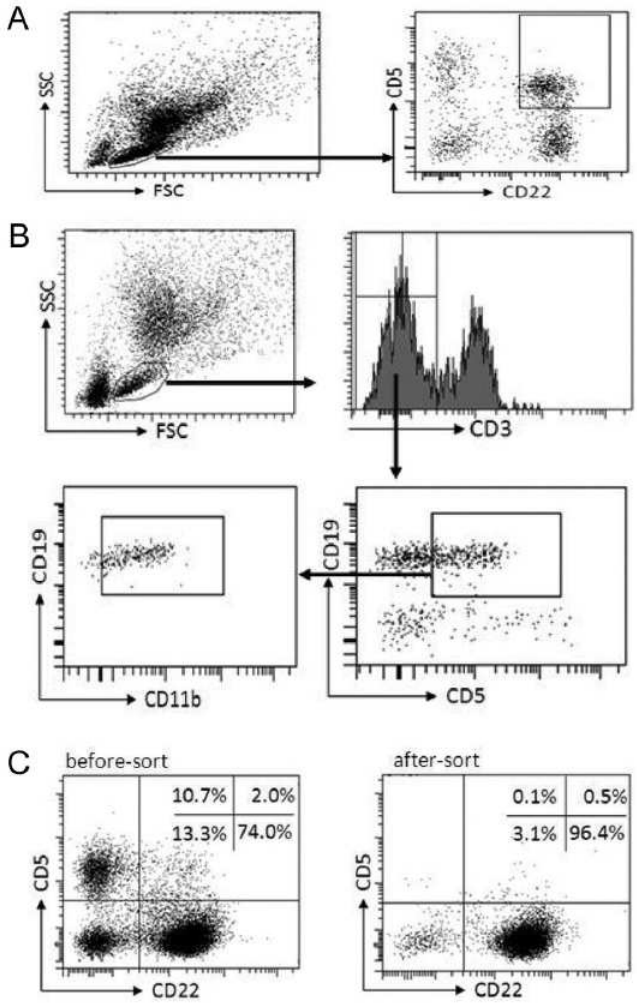

Figure 1. Schematic of peritoneal B1a and spleen B2 lymphocyte isolation and assessment by flow cytometry. A, Peritoneal cells from donor mice were fluorochrome-labeled with anti-CD22 and anti-CD5 antibodies and sorted by BD FACSAria. Lymphocytes gated on forward scatter (FSC) and side scatter (SSC; **left**) were examined for CD22^{+} CD5^{+} coexpression (right). **B**, In some experiments, CD3^{-} CD19^{+} CD5^{+} CD11b^{+} peritoneal B1a lymphocytes were sorted by BD FACSAria. Peritoneal lymphocytes were gated (**upper left panel**), and CD3^{-} B1a lymphocytes were isolated (**upper right panel**) and sequentially sorted for CD19^{+} CD5^{+} coexpression (**lower left panel**) and CD19+CD11b^{+} coexpression (**lower right panel**). **C**, Using AutoMACS and a B lymphocyte isolation kit (Miltenyi Biotech) that contained a cocktail of anti-CD43, anti-CD4, and anti-Ter119 antibodies, activated B2 and B1 lymphocytes and non-B lymphocytes were positively removed and naïve B2 lymphocytes negatively sorted. Purity of naïve CD22^{+} and CD5^{-} B2 lymphocytes was confirmed. All experimental groups that included transfer experiments with WT and sIgM$^{-/-}$ B1a lymphocytes were performed in 1 experiment.

Cell Isolation

Peritoneal fluid and/or spleens were collected from donor mice.[7] CD19^{+} CD5^{+} or CD19^{+} CD5^{+} peritoneal B1a B lymphocytes were isolated by FACSAria (BD Biosciences, San Jose, CA; Figures 1A and 1B), and splenic B2 B lymphocytes were magnetically purified by AutoMACS (Miltenyi Biotec, Auburn, CA; Figure 1C). Cell viability by the Trypan blue exclusion method was >95%.

Flow Cytometry

Peritoneal B- and non–B-lymphocyte populations were analyzed with fluorochrome-conjugated antibodies (BD Pharmingen, San Diego, CA) on a BD FACSCanto II (BD Biosciences) as described previously.[7]

Histological Lesion Analysis at Aortic Roots

Atherosclerotic lesion sizes were assessed with oil red O and collagen content with picrosirius red as described previously.[7] To analyze necrotic core areas, aortic root atherosclerotic lesions were stained with hematoxylin and eosin.[15]

Immunohistochemical Analysis at Aortic Roots

Macrophages, smooth muscles, and oxidized low-density lipoprotein (oxLDL) at aortic root atherosclerotic lesions were assessed by immunohistochemical analyses as described previously.[7] Apoptotic cells were identified by terminal dUTP nick end-labeling (TUNEL).

Enzyme-Linked Immunosorbent Assay

Plasma IgG and IgM levels were assessed at 4 and 8 weeks after splenectomy as described previously.[7] A modified ELISA protocol adapted from Caliguiri et al[5] was used to measure anti-malondialdehyde-oxidized (MDA)-LDL IgM antibody with human LDL modified and assessed by spectrophotometry as described previously.[16]

Lipid Profiles

Plasma lipids (total cholesterol, high-density lipoprotein cholesterol, very-low-density lipoprotein/LDL cholesterol, and triglycerides) were measured by a standard commercial enzymatic assay as described previously.[7]

Statistical Analysis

GraphPad Prism 4 software was used for statistical analyses. Results are presented as mean±SEM. One-way ANOVA with Newman-Keuls post hoc test was used for comparisons of multiple groups of ≥3. A 2-tailed unpaired Student *t* test was used for comparison of 2 groups. A 1-tailed unpaired Student *t* test was used for confirmatory transfer experiments, with B1a lymphocytes selected for $CD19^+CD5^+$ expression and for Ly5.1 expression. $P<0.05$ was considered significant.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Splenectomy Selectively Decreases Peritoneal B1a Lymphocytes Without Affecting Other Lymphocyte Populations, Body Weight, or Plasma Lipids

Because splenectomy has been reported to reduce peritoneal B1a lymphocytes in the C57BL/6 mouse,[12] we investigated whether splenectomy causes a similar B1a lymphocyte reduction in the $ApoE^{-/-}$ mouse. Splenectomy depleted $CD22^+$ $CD5^+$ B1a lymphocytes in the peritoneal cavity by 68% compared with sham-operated mice (Figures 2A and 2B). B2 and other lymphocytes in the peritoneal cavity (Figures 2B and 2C) were unaffected.

Increased plasma lipids are important initiating factors in the early stage of atherosclerosis. We questioned whether splenectomy has an effect on these lipids in the $ApoE^{-/-}$ mouse. Both body weight (Figure 2D) and lipid profile (Figure 2E) imposed by an 8-week high-fat diet were unaffected by splenectomy, because they were similar to the body weight (mean±SEM 26.81±1.11 g) and lipid profile (mean±SEM: total cholesterol 21.8±1.73 mmol/L, high-density lipoprotein cholesterol 4.12±0.26 mmol/L, very-low-density lipoprotein/LDL cholesterol 17.34±1.42 mmol/L, triglycerides 3.02±0.25 mmol/L) of $ApoE^{-/-}$ mice fed a high-fat diet for 8 weeks.

Splenectomy Increases Atherosclerotic Lesions but Does Not Increase Lesion Smooth Muscle and Collagen Content

In agreement with previous reports,[5,10,11,17] splenectomized mice exhibited a 35% increase in oil red O–stained atherosclerotic lesions (Figure 3A) and a 49% increase in $CD68^+$ macrophage accumulation (Figure 3B) compared with sham-operated mice; however, macrophage accumulation expressed as a percentage of total atherosclerotic lesion area was unaffected by splenectomy (Figure 3C). We next examined smooth muscle and collagen content in atherosclerotic lesion to determine whether splenectomy alters plaque stability. Smooth muscle and collagen content stained by α-actin smooth muscle antibody and picrosirius red, respectively, were unaffected (Figures 3C and 3D).

Adoptive Transfer of B1a Lymphocytes Reconstitutes the Peritoneal Compartment

Given that splenectomy reduced peritoneal B1a lymphocytes, we first set out to ascertain whether adoptive transfer of B1a lymphocytes to splenectomized mice would reconstitute the peritoneal compartment. We isolated $CD22^+$ $CD5^+$ B1a lymphocytes (purity >96%; Figure 1A) from the peritoneum of C57BL/6 mice. Because peritoneal B1a lymphocytes require the spleen for survival, and splenectomy reduced their numbers after 1 week and sustained this reduction up to 3 weeks,[12] we transferred 10^5 peritoneal B1a lymphocytes at weeks 1, 4, and 7 after splenectomy to maintain a pool of replenished peritoneal B1a lymphocytes. We controlled the experiment with transfers of the same numbers of spleen B2 lymphocytes (purity >98%; Figure 1C) and PBS in a similar manner. Transfer of B1a lymphocytes, but not B2 lymphocytes or PBS, reconstituted peritoneal B1a lymphocytes by approximately 67% compared with sham-operated mice and increased this population by approximately 90% compared with splenectomized mice (Figure 2A).

Transfer of B1a but Not B2 Lymphocytes Potently Reduces Atherosclerosis Aggravated by Splenectomy

Next, we examined atherosclerotic lesions at the aortic root to determine whether adoptively transferred B1a lymphocytes can influence the augmented atherosclerosis in splenectomized mice. After assessment of atherosclerotic lesion size by lipid accumulation with oil red O stain, transfer of B1a lymphocytes was shown to potently decrease atherosclerotic lesions by ≈70% compared with sham-operated mice and by ≈80% compared with splenectomized mice (Figure 3A). Similarly, $CD68^+$ macrophage accumulation was also markedly decreased by ≈60% compared with sham-operated mice and by ≈75% compared with splenectomized mice (Figure 3B), although $CD68^+$ macrophage accumulation expressed as a percentage of total atherosclerotic lesion area was unaffected by splenectomy (Figure 3C). There were no differences in body weights and plasma lipids among the experimental groups (Figures 2D and 2E).

The Vast Majority (>98%) of $CD22^+$ $CD5^+$ Peritoneal B1a Lymphocytes Are $CD19^+CD5^+$

We used anti-CD22 and anti-CD5 antibodies to isolate peritoneal B1a lymphocytes. Because CD22 has been reported to be expressed on murine T cells,[18] we compared the phenotype of peritoneal B1a lymphocytes isolated with $CD22^+$ $CD5^+$ antibodies to those isolated with $CD19^+$ $CD5^+$ antibodies (Figure 4A). The vast majority (>98%) of $CD22^+$ $CD5^+$ peritoneal B lymphocytes expressed CD19 antigen but not CD3, whereas 95% of $CD19^+$ $CD5^+$ peritoneal B lymphocytes expressed CD22. Both populations expressed CD11b but not CD23. Furthermore, adoptive transfer to splenectomized mice of peri-

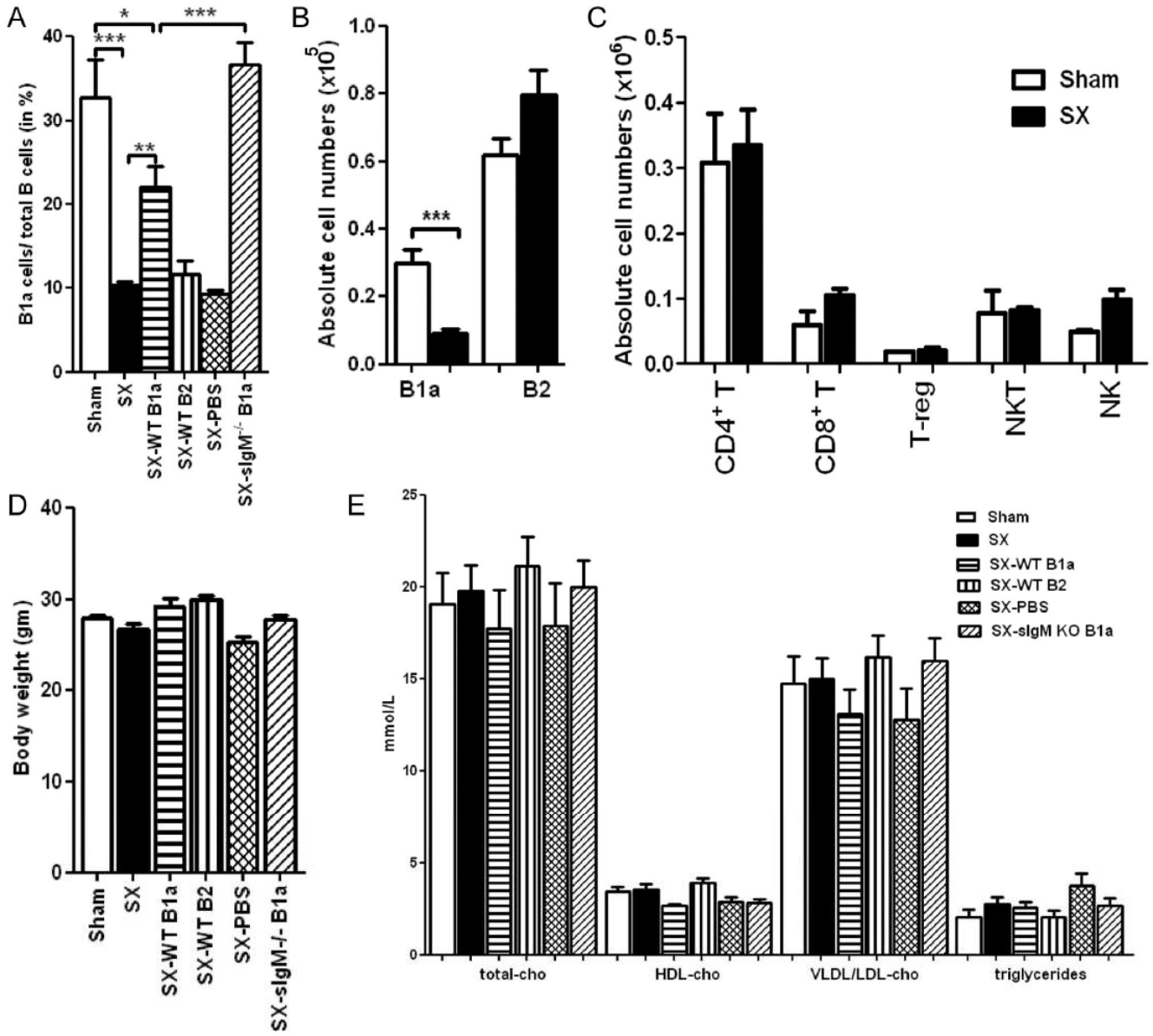

Figure 2. Splenectomy selectively reduces peritoneal B1a lymphocytes without affecting other peritoneal lymphocytes, body weight, or plasma lipids. Splenectomized (SX) mice reduced peritoneal B1a lymphocytes (**A**, **B**) without affecting peritoneal B2 lymphocytes (**B**) or other non-B lymphocytes (**C**). Transfer of WT Bla and sIgM-deficient Bla lymphocytes, but not B2 lymphocytes or saline, increased peritoneal B1a lymphocytes (**A**). **D** and **E**, Splenectomy and adoptive transfers did not affect body weight or lipid profiles. Graphs present mean±SEM values (n=7–8). ***$P<0.001$, **$P<0.01$, *$P<0.05$, 1-way ANOVA with Newman-Keuls post hoc test (**A**, **C–E**) and 2-tailed unpaired *t* test (**B**). SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM$^{-/-}$B1a, splenectomy with sIgM-deficient Bla transfer; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; KO, knockout; and cho, cholesterol.

toneal B1a lymphocytes isolated by anti-CD19, anti-CD3, anti-CD5, and anti-CD11b antibodies gave very similar results (Figure 4B) to those observed with transfer of CD22$^+$ CD5$^+$ Bla lymphocytes (Figure 3A). To assess the contribution of donor Bla lymphocytes to reconstitution of this population, we isolated peritoneal CD3$^-$ CD19$^+$ CD5$^+$ CD11b$^+$ Bla lymphocytes from allelic Ly5.1 mice for transfer to splenectomized mice (n=3). Assessment of donor Ly5.1$^+$ cells in the B1a lymphocyte population at the end of an 8-week high-fat diet showed that they constituted 47.0±8.0% (mean±SEM) of this population (Figure 4C). These findings are consistent with the percentage increase in this population after transfer of Bla cells to splenectomized mice compared with their percentage in splenectomized mice (Figure 2A).

IgM in Plasma and Lesions Is Reduced by Splenectomy and Markedly Increased by Transfer of B1a Lymphocytes

Because the majority of circulating natural IgM antibodies are constitutively produced by B1a lymphocytes throughout life,[13] and these antibodies react to oxLDL, we examined whether IgM and oxLDL-specific IgM antibody produced by B1a lymphocytes are selectively affected by splenectomy. Plasma levels of IgM assessed at 4 and 8 weeks after splenectomy were decreased by 45% in splenectomized mice compared with sham-operated mice (Figures 5B and 5D). Plasma IgM levels of ApoE mice fed a high-fat diet for 8 weeks and normal C57Bl/6 mice (mean±SEM 0.82±0.14 OD^{450}) were almost identical to those of sham-operated mice.

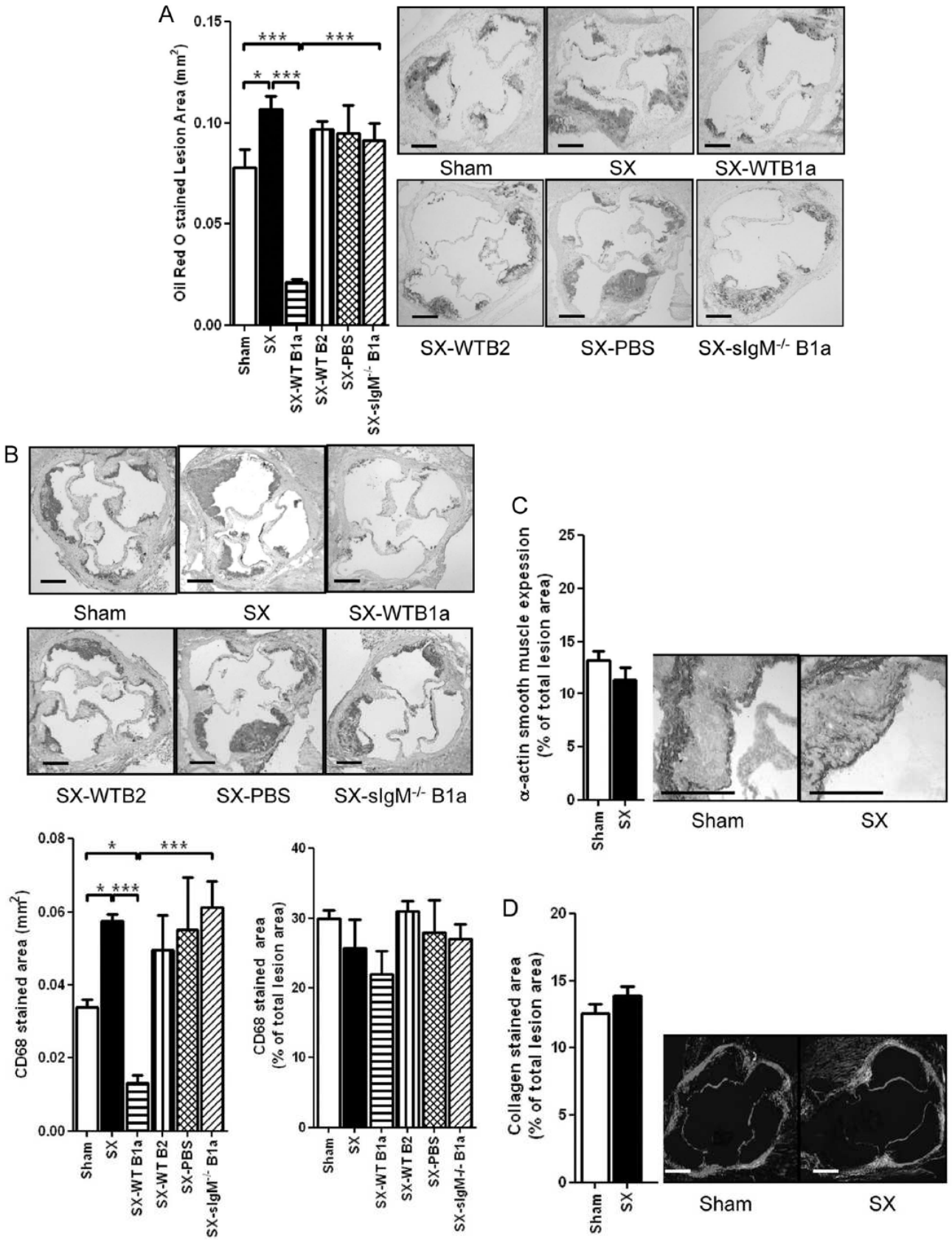

Figure 3. Splenectomy increases atherosclerosis development in ApoE$^{-/-}$ mice. At the end of an 8-week high-fat diet, splenectomized (SX) mice showed (**A**) increased oil red O–stained atherosclerotic lesions and (**B**) CD68-stained macrophage areas. However, macrophage (**B**), lesion smooth muscle (**C**), and collagen content (**D**) expressed by lesion size were unaffected by splenectomy. Transfer of WT B1a lymphocytes but not sIgM$^{-/-}$ B1a lymphocytes, B2 lymphocytes, or saline decreased postsplenectomy aggravated atherosclerotic lesions and macrophage content (**A**, **B**). Graphs present mean±SEM values (n=7–8). ***$P<0.001$, **$P<0.01$, *$P<0.05$, 1-way ANOVA with Newman-Keuls post hoc test (**A**, **B**) and 2-tailed unpaired *t* test (**C**, **D**). **Bars**, 100 μm in **A**, **B**, and **D** and 200 μm in **C**. SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM B1a, splenectomy with sIgM-deficient B1a transfer.

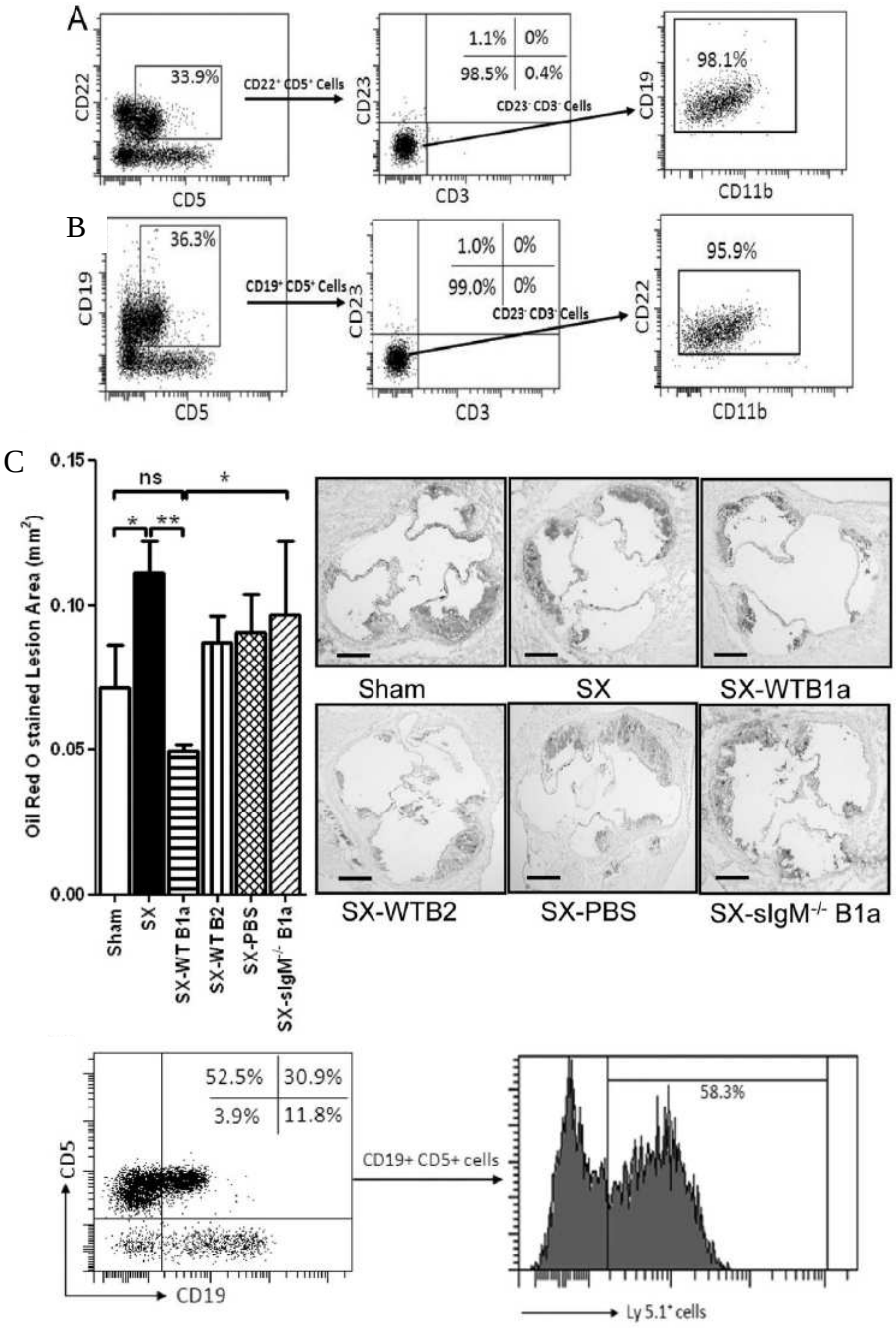

Figure 4. CD22$^+$ CD5$^+$ peritoneal lymphocytes are CD3$^-$ CD23$^-$ CD19$^+$ CD5$^+$ CD11b$^+$ B1a lymphocytes. A, Peritoneal cells were fluorochrome-stained with anti-CD3, anti-CD22, anti-CD19, anti-CD23, anti-CD5, and anti-CD11b antibodies. Lymphocyte-gated CD22$^+$ CD5$^+$ peritoneal cells were CD3$^-$ CD23$^-$ CD19$^+$ CD11b$^+$. **B**, Similarly, lymphocyte-gated CD19$^+$ CD5$^+$ peritoneal cells were CD3$^-$ CD23$^-$ CD22$^+$CD11b$^+$. To confirm the atheroprotective role of peritoneal B1a lymphocytes, CD3$^-$ CD19$^+$ CD5$^+$ CD11b$^+$ peritoneal B1a lymphocytes were transferred to splenectomized mice. **C**, Atherosclerotic lesion assessment by oil red O staining showed that CD3$^-$ CD19$^+$ CD5$^+$ CD11b$^+$ peritoneal B1a lymphocytes from WT but not from sIgM$^{-/-}$ donor mice decreased atherosclerosis. To assess the contribution of donor Bla lymphocytes to reconstitution of this population, peritoneal CD3$^-$ CD19$^+$ CD5$^+$ CD11b$^+$ Bla lymphocytes were isolated from Ly5.1 mice and transferred to splenectomized mice. Donor Ly5.1$^+$ cells assessed at the end of an 8-week high-fat diet comprised 58.3% of the CD19$^+$CD5$^+$ Bla population. Graphs present mean±SEM values (n=3–4). $^{**}P<0.01$, $^{*}P<0.05$, 1-tailed unpaired *t* test. **Bars**, 100 μm in panel **C**. SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM/B1a, splenectomy with sIgM-deficient B1a transfer.

In contrast, plasma levels of IgG were unaffected (Figure 5A). Splenectomized mice also showed decreased IgM plasma level against MDA-LDL compared with sham-operated mice (Figure 5C).

Next, we determined whether adoptively transferred B1a lymphocytes can restore IgM and oxLDL-specific IgM levels decreased by splenectomy. Plasma levels of IgM assessed at 4 and 8 weeks after splenectomy increased by 68% in splenectomized mice into which B1a lymphocytes had been transferred, to levels that were almost the same as in sham-operated mice (Figures 5B and 5d). Plasma IgM levels from splenectomized mice into which B2 lymphocytes or PBS had been transferred did not show any difference compared with splenectomized mice (Figure 5B). Transfer of B1a lymphocytes also restored plasma MDA-LDL–specific IgM levels decreased by splenectomy (Figure 5C). Plasma levels of IgG were unaffected in all mice in the transfer experiments (Figure 5A).

Natural IgM antibodies, abundant in the circulation, can also be found in the subintimal space of atherosclerotic lesions.[19] Our demonstration of a direct correlation between plasma IgM level and B1a lymphocytes led us to examine atherosclerotic lesions for IgM deposits. IgM deposits in atherosclerotic lesions were decreased by 58% after splenectomy (Figure 6). After transfer of B1a lymphocytes to splenectomized mice, lesion IgM deposits were increased by >100% compared with splenectomized mice, to a level similar to that in sham-operated mice. In contrast, splenectomized mice into which B2 lymphocytes or PBS had been transferred exhibited similar percentages of areas of lesion IgM deposits as splenectomized mice (Figure 6).

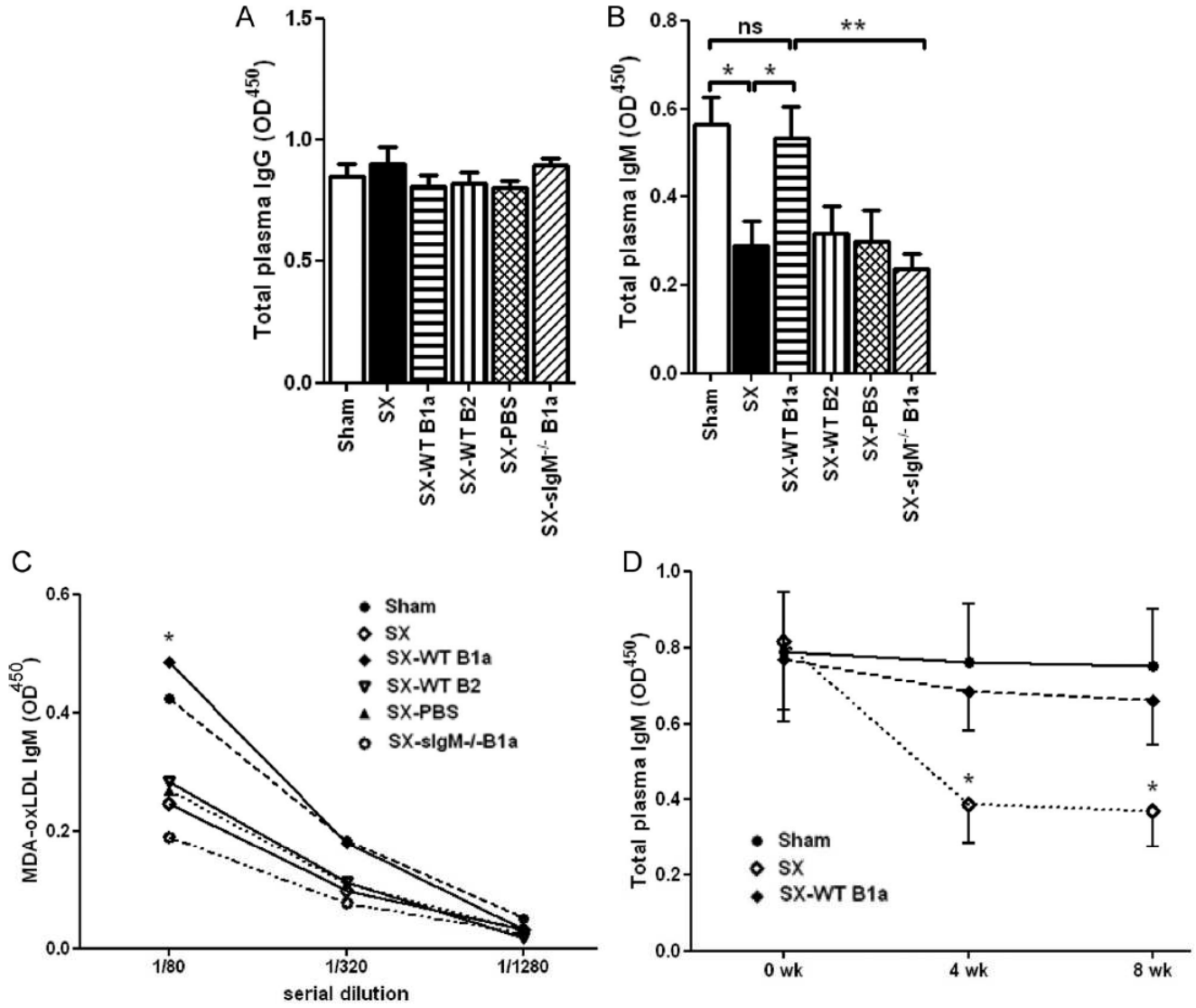

Figure 5. Plasma IgM and MDA-oxLDL-IgM antibodies are reduced by splenectomy and reversed by transfer of WT but not sIgM-deficient peritoneal B1a lymphocytes. Plasma IgM (**A**) but not plasma IgG (**B**) and MDL-oxLDL IgM (**C**) antibodies were reduced by splenectomy, whereas transfer of WT but not sIgM-deficient B1a lymphocytes increased plasma IgM and MDA-oxLDL IgM almost to levels in sham-operated mice. Transfer of WT-B2 lymphocyte and PBS did not affect plasma IgM levels or MDA-oxLDL-IgM antibodies. Plasma IgM levels were reduced at 4 weeks after splenectomy and remained at this decreased level at 8 weeks. **D**, WT B1a lymphocytes restored plasma IgM levels. Graphs present mean±SEM values (n=7–8 in panels **A–C** and n=3–4 in panel **D**). $^{**}P<0.01$, $^{*}P<0.05$, 1-way ANOVA with Newman-Keuls post hoc test (**A–C**) and 1-tailed unpaired *t* test (**D**). SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM/B1a, splenectomy with sIgM-deficient B1a transfer.

B1a Lymphocytes That Cannot Secrete IgM Fail to Reduce Atherosclerosis Augmented by Splenectomy and Do Not Increase Plasma or Lesion IgM Despite Reconstitution of the Peritoneal Compartment

The observation that splenectomy-aggravated atherosclerosis is accompanied by decreased plasma and lesion IgM whereas reduction of atherosclerosis by transfer of B1a lymphocytes is accompanied by elevated plasma and lesion IgM suggests that B1a-derived IgM is atheroprotective. To test this hypothesis, we isolated peritoneal B1a lymphocytes from $sIgM^{-/-}$ donor mice, which cannot secrete IgM,[20] and transferred these lymphocytes into splenectomized mice. Despite reconstitution of the peritoneal compartment (Figure 2A), atherosclerotic lesions of splenectomized mice given $sIgM^{-/-}$ B1a lymphocytes did not change compared with lesions of splenectomized mice assessed by lipid and macrophage accumulation (Figures 3A and 3B).

To further assess the role of IgM in atherosclerosis, we determined the levels of IgM in the circulation and in atherosclerotic lesions of splenectomized mice into which $sIgM^{-/-}$ B1a lymphocytes had been transferred. As expected, the levels of plasma IgM and oxLDL-specific IgM antibody in these mice were decreased to levels similar to splenectomized mice (Figures 5B and 5c) without affecting plasma IgG (Figure 5A). Furthermore, the transferred $sIgM^{-/-}$ B1a lymphocytes also failed to increase IgM deposits in lesions, which contrasted with the effects of transferred wild-type (WT) B1a lymphocytes (Figure 6).

Necrotic Cores in Atherosclerotic Lesion Are Increased by Splenectomy and Reduced by Transfer of B1a Lymphocytes

We next examined atherosclerotic lesions for necrotic cores, because it has been suggested that a major function of natural

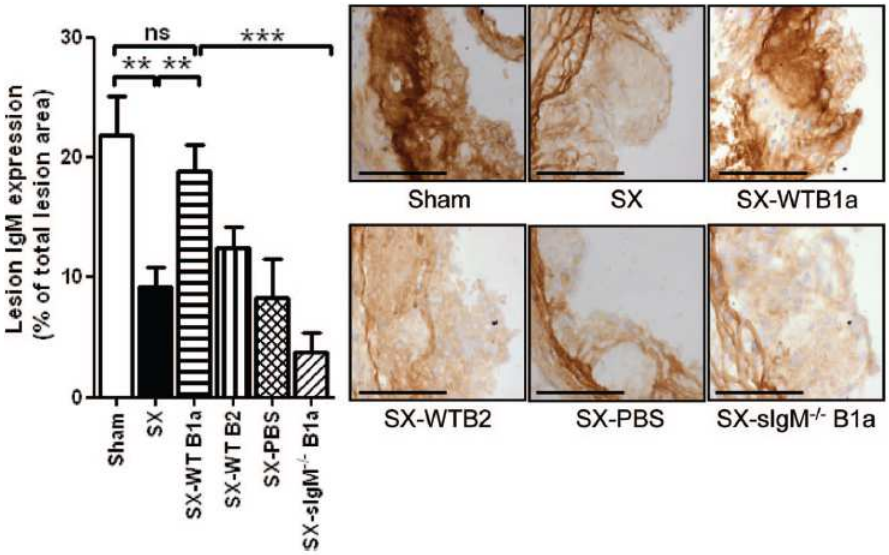

Figure 6. Lesion IgM deposits are decreased by splenectomy and restored by transfer of WT but not sIgM-deficient B1a lymphocytes. Immunohistochemical analysis showed that IgM deposits decreased by splenectomy were increased by transfer of WT but not sIgM-deficient B1a lymphocytes. Transfer of B2 lymphocytes or PBS did not affect lesion IgM deposits. Graphs present mean±SEM values (n=7–8). ***$P<0.001$, **$P<0.01$, 1-way ANOVA with Newman-Keuls post hoc test. **Bar**, 200 μm. SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM/B1a, splenectomy with sIgM-deficient Bla transfer.

IgM is to clear apoptotic cells[21] by binding to oxidation-specific epitopes.[19] We assessed the size of the necrotic core identified as acellular areas after hematoxylin-and-eosin staining of atherosclerotic lesions.[15] Necrotic core areas in atherosclerotic lesions increased by 19% after splenectomy and decreased by 14% with the transfer of B1a lymphocytes. In contrast, sIgM$^{-/-}$ B1a lymphocytes failed to decrease the necrotic core area augmented by splenectomy (Figure 7A).

Given that approximately 30% of natural IgM antibodies bind to oxidation-specific epitopes,[22] we next examined atherosclerotic lesions for oxLDL using an antibody to MDA-LDL. OxLDL in lesions was increased by splenec-

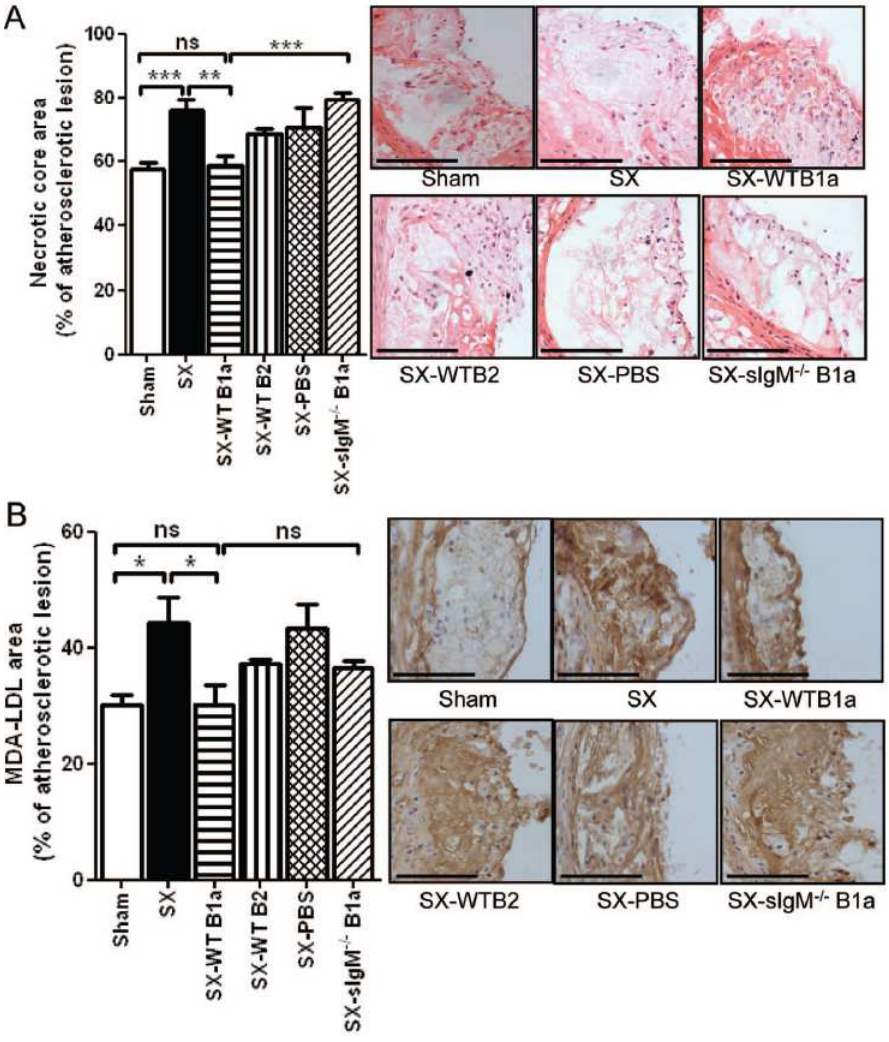

Figure 7. Atherosclerotic necrotic core areas and OxLDL in lesions are increased by splenectomy and reduced by transfer of B1a lymphocytes. A, Necrotic cores of atherosclerotic lesions, identified as acellular areas in atherosclerotic lesions, were increased by splenectomy and decreased by transfer of WT-B1a lymphocytes but not sIgM$^{-/-}$ B1a lymphocytes (**B**). OxLDL in lesions assessed by antibody to MDA-LDL was increased after splenectomy and decreased by transfer of WT B1a lymphocytes. Graphs present mean±SEM (n=7–8). ***$P<0.001$; **$P<0.01$; *$P<0.05$, 1-way ANOVA with Newman-Keuls post hoc test. **Bars**, 200 μm. SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM/B1a, splenectomy with sIgM-deficient B1a transfer.

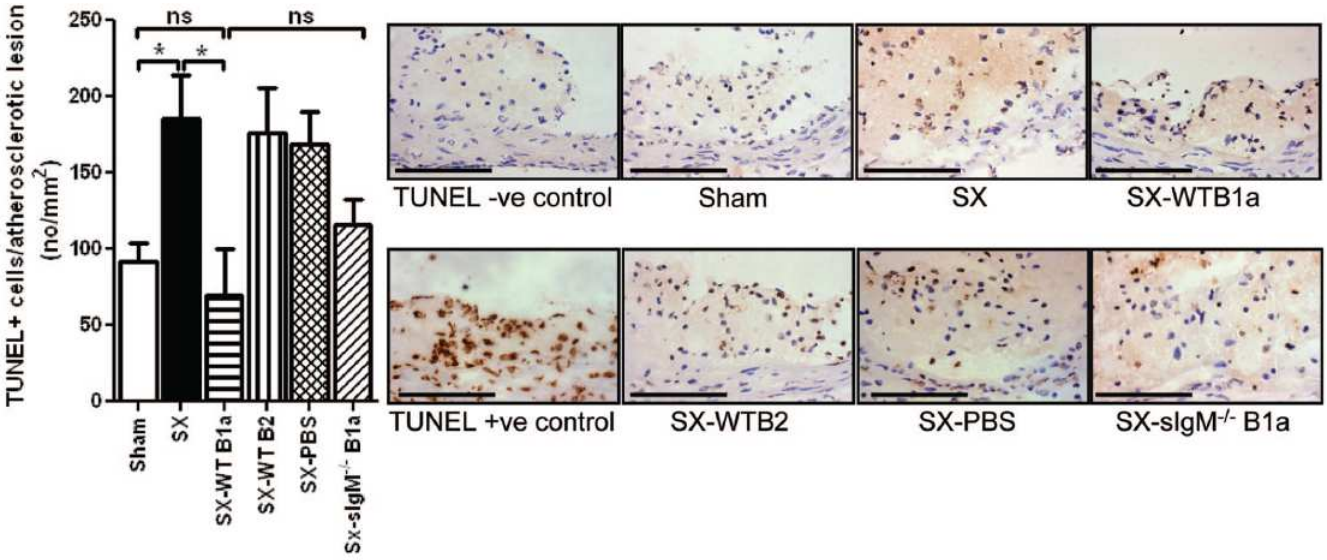

Figure 8. Apoptotic cells are increased by splenectomy and reduced by transfer of B1a lymphocytes. TUNEL-positive cells, expressed relative to atherosclerotic lesion size assessed by oil red O, were increased in splenectomized mice and decreased by transfer of WT B1a lymphocytes but not B2 lymphocytes or PBS. Graphs present mean±SEM (n=10). *$P<0.05$, **$P<0.01$, 1-way ANOVA with Newman-Keuls post hoc test. **Bar**, 200 μm. SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM/B1a, splenectomy with sIgM-deficient B1a transfer.

tomy and decreased by transfer of WT B1a lymphocytes (Figure 7B).

Next, we assessed TUNEL-positive apoptotic cells in atherosclerotic lesions, because natural IgM antibodies are responsible for apoptotic cell clearance in chronic inflammation.[14] Apoptotic cells were increased in splenectomized mice and decreased by transfer of WT B1a lymphocytes (Figure 8).

Discussion

B1 lymphocytes differ from B2 lymphocytes in origin, distribution, surface markers, and function. Whereas conventional B2 lymphocytes have important roles in adaptive immunity, B1 lymphocytes have key roles in innate immunity. B1 lymphocytes are self-replenishing, long-lived B lymphocytes that reside mainly in serosal cavities, such as in the peritoneum and the pleura. They are a minor B-lymphocyte population in the circulation and in the spleen[23,24] and comprise B1a ($CD11b^+$, $CD5^+$) and B1b ($CD11b^+$, $CD5^-$) subsets. B1a lymphocytes are implicated in first-line defense against bacterial and viral infections.[24] They respond rapidly and strongly to pathogen-derived products such as phosphorylcholine but poorly to receptor-mediated activation.[13,25,26] B1a lymphocytes rarely enter germinal centers to undergo affinity maturation and are thus highly restricted in their ability to produce high-affinity antibodies. Instead, in humans and mice, they produce low-affinity, polyreactive IgM antibodies throughout life. These are called "natural antibodies" because their levels in germ-free mice are virtually identical to those of mice housed in conventional facilities.[27] Thus, natural antibodies are generated in the absence of external antigenic stimulation and are selected presumably by endogenous ligands.[28] These antibodies are produced mainly, if not exclusively, by B1a lymphocytes and constitute most, if not all, circulating IgM.[13]

Our observation that splenectomy depleted peritoneal B1a lymphocytes and augmented atherosclerosis, assessed by oil red O–stained lipid accumulation, without affecting body weight and hypercholesterolemia provides a direct relationship between peritoneal B1a lymphocytes and atherosclerosis. Macrophage accumulation assessed by CD68-stained areas was also increased by splenectomy and decreased by WT but not by sIgM-deficient peritoneal B1a lymphocytes. However, the percentage of $CD68^+$ macrophage content expressed in relation to total lesion area remained unchanged, which suggests little if any effect on lesion composition. The observation that splenectomy did not affect collagen or smooth muscle content of atherosclerotic lesions further supports the notion that splenectomy-induced depletion of B1a lymphocytes did not affect lesion composition or plaque stability. Atherosclerosis aggravated by splenectomy-induced B1a lymphocyte depletion coupled with reduction of the aggravated atherosclerosis by repletion of the B1a lymphocyte population by adoptive transfer provides compelling evidence for an atheroprotective role for B1a lymphocytes. Reconstitution of peritoneal B1a lymphocytes should have reduced atherosclerosis to the level in sham-operated mice. Instead, the transferred B1a lymphocytes reduced atherosclerotic lesions to a level far below that in sham-operated mice. These results highlight the potency of the B1a lymphocytes in suppressing atherosclerosis development.

The present results implicate natural IgM antibodies produced by B1a lymphocytes and deposited in atherosclerotic lesions in mediating protection against lesion development. Thus, we showed that plasma IgM and lesion IgM deposits were decreased on depletion of peritoneal B1a lymphocytes after splenectomy, without affecting plasma IgG. Only the adoptive transfer of B1a lymphocytes, and not B2 lymphocytes, reversed plasma and lesion IgM, with 67% reconstitution in the peritoneal cavity compared with sham-operated mice. The observation that transfer of B1a lymphocytes that cannot secrete IgM failed to increase plasma and lesion IgM and to attenuate atherosclerosis despite reconstitution of the peritoneal compartment confirms the key role played by natural IgM produced by B1a lymphocytes in protection against atherosclerosis development. Our

findings are consistent with B1a lymphocytes being responsible for generating the majority of natural IgM[13] and with the report that genetically manipulated asplenic mice and splenectomized mice did not produce plasma IgM.[12] Transfer of fetal liver cells, not bone marrow cells, from both Hox11$^{-/-}$ or C57BL/6 donors regenerated peritoneal B1a lymphocytes.[12] These regenerated B1a lymphocytes produced IgM antibodies against streptococcal polysaccharides regardless of the immunization route, in contrast to splenectomized, asplenic mice and irradiated Rag-2$^{-/-}$ mice transferred with bone marrow that failed to produce these antibodies.[12]

Circulating IgM antibodies comprise B1a-produced natural IgM and B2-produced antigen-specific IgM. The former is generated in the absence of antigen stimulation, whereas the latter requires antigen stimulation. Both natural and antigen-specific IgM antibodies are required for optimal protection against microbial antigens. Natural IgMs play an important role in initial defense, whereas antigen-specific IgMs act together with natural IgMs to enhance IgG responses in late immune responses.[29] Our finding that splenectomy decreased both natural IgM antibodies and IgM antibodies specific to oxLDL is consistent with selective depletion of Bla lymphocytes by splenectomy.[12] The observation that transfer of WT B1a but not sIgM-deficient B1a lymphocytes rescued postsplenectomy aggravated atherosclerosis indicates that natural IgM produced by B1a lymphocytes protects against atherosclerosis development.

The present data suggest possible mechanisms by which natural IgMs produced by B1a lymphocytes and deposited in atherosclerotic lesions mediate atheroprotection. The finding that atherosclerotic lesions aggravated by splenectomy-induced B1a lymphocyte depletion are accompanied by reduced IgM deposits in lesions and increased necrotic core, oxLDL, and apoptotic cells in lesions suggests that IgM deposited in lesions plays a role in clearing apoptotic cells and proinflammatory oxLDL. This suggestion is further supported by the finding that transfer of WT B1a lymphocytes to splenectomized mice is accompanied by increased IgM deposits in lesions and decreased necrotic core, oxLDL, and apoptotic cells. Failure of transferred sIgM$^{-/-}$ B1a lymphocytes to increase lesion IgM and reduce necrotic cores is consistent with this suggestion. Together, the present data support a scavenger role for natural IgM in clearing apoptotic cell debris and oxLDL.[30–32] IgM has been suggested to bind to apoptotic cells[21] through oxidation-specific epitopes,[19,32] resulting in clearance of apoptotic cells and cellular debris from atherosclerotic lesions. Approximately 30% of these IgM bind to model oxidation-specific epitopes and to atherosclerotic lesions.[20] Natural IgM also blocks oxLDL uptake by macrophages, preventing foam cell formation.[22,33,34]

IgM antibodies assessed at 4 and 8 weeks after splenectomy showed that the WT B1a-transferred group had approximately the same IgM level as the sham-operated group. The significant and persistently lower plasma IgM level as a result of splenectomy-induced depletion of natural IgM-antibody–producing Bla lymphocytes is consistent with the short half-life of IgM (2 days).[35] Transfer of WT B1a lymphocytes reconstituted the peritoneal compartment and restored plasma IgM level to almost the same level as in sham-operated mice. In contrast, transfer of IgM-deficient B1a lymphocytes failed to increase plasma IgM level despite reconstitution of peritoneal B1a lymphocytes. Enhanced reconstitution by peritoneal sIgM$^{-/-}$ B1a lymphocytes may reflect homeostatic expansion in an sIgM-deficient environment.

Identification of IgM-secreting B1a lymphocytes as an atheroprotective B-lymphocyte subset coupled with our previous identification of B2 lymphocytes as an atherogenic population highlights the opposing roles played by these B-lymphocyte subsets in atherosclerosis. We previously reported that transfer of B2 lymphocytes to lymphocyte-deficient mice aggravated atherosclerosis; however, transfer of B2 lymphocytes did not aggravate atherosclerosis in splenectomized mice. This outcome is probably because the transferred naïve B2 lymphocytes (3×10^5 cells) comprise only a small fraction of the host's total reservoir of B2 lymphocytes.

Our findings have therapeutic implications. They indicate that a rational approach in B lymphocyte–depletion therapy for inflammatory diseases[36] including atherosclerosis should be directed toward selective depletion of damaging B lymphocytes while sparing protective B lymphocytes such as Bla lymphocytes. The findings also suggest that therapeutic strategies directed toward expansion of atheroprotective B1a lymphocytes can be exploited to attenuate atherosclerosis development and progression. Our findings lend credence to the search for a protective vaccine in atherosclerosis.[37]

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Disclosures

None.

References

1. Houtkamp MA, de Boer OJ, van der Loos CM, van der Wal AC, Becker AE. Adventitial infiltrates associated with advanced atherosclerotic plaques: structural organization suggests generation of local humoral immune responses. *J Pathol*. 2001;193:263–269.
2. Yla-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler Thromb*. 1994;14:32–40.
3. Zhou X, Hansson GK. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. *Scand J Immunol*. 1999;50:25–30.
4. Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol*. 2009;27:165–197.
5. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest*. 2002;109:745–753.
6. Major AS, Fazio S, Linton MF. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol*. 2002; 22:1892–1898.
7. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol*. 2010;185:4410–4419.
8. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vre E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF, Mallat Z. B cell depletion reduces the development of atherosclerosis in mice. *J Exp Med*. 2010;207:1579-1587.
9. Robinette CD, Fraumeni JF Jr. Splenectomy and subsequent mortality in veterans of the 1939–45 war. *Lancet*. 1977;2:127–129.
10. Asai K, Kuzuya M, Naito M, Funaki C, Kuzuya F. Effects of splenectomy on serum lipids and experimental atherosclerosis. *Angiology*. 1988;39:497–504.
11. Shapiro S. The influence of thyroidectomy, splenectomy, gonadectomy, and suprarenalectomy upon the development of experimental atherosclerosis in rabbits. *J Exp Med*. 1927;45:595–607.

12. Wardemann H, Boehm T, Dear N, Carsetti R. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J Exp Med*. 2002;195:771–780.
13. Baumgarth N, Tung JW, Herzenberg LA. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin Immunopathol*. 2005;26:347–362.
14. Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO. Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation*. 2009;120:417–426.
15. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe−/− mice. *Arterioscler Thromb Vasc Biol*. 2008;28:1421–1428.
16. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest*. 1998;101:1717–1725.
17. Akan AA, Sengul N, Simsek S, Demirer S. The effects of splenectomy and splenic autotransplantation on plasma lipid levels. *J Invest Surg*. 2008;21:369–372.
18. Sathish JG, Walters J, Luo JC, Johnson KG, Leroy FG, Brennan P, Kim KP, Gygi SP, Neel BG, Matthews RJ. CD22 is a functional ligand for SH2 domain-containing protein-tyrosine phosphatase-1 in primary T cells. *J Biol Chem*. 2004;279:47783–47791.
19. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, Witztum JL. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest*. 2000;105:1731–1740.
20. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol*. 1998;160:4776–4787.
21. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol*. 2010;10:778–786.
22. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Backhed F, Miller YI, Horkko S, Corr M, Witztum JL, Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest*. 2009;119:1335–1349.
23. Hayakawa K, Hardy RR, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J Exp Med*. 1985;161:1554–1568.
24. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. *Curr Opin Immunol*. 2001;13:195–201.
25. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med*. 2008;205:3053–3064.
26. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity*. 2001;14:617–629.
27. Coutinho A, Kazatchkine MD, Avrameas S. Natural autoantibodies. *Curr Opin Immunol*. 1995;7:812–818.
28. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *Eur J Immunol*. 1997;27:1557–1563.
29. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med*. 2000;192:271–280.
30. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med*. 2003;9:736–743.
31. Binder CJ, Shaw PX, Chang MK, Boullier A, Hartvigsen K, Horkko S, Miller YI, Woelkers DA, Corr M, Witztum JL. The role of natural antibodies in atherogenesis. *J Lipid Res*. 2005;46:1353–1363.
32. Hartvigsen K, Chou MY, Hansen LF, Shaw PX, Tsimikas S, Binder CJ, Witztum JL. The role of innate immunity in atherogenesis. *J Lipid Res*. 2009;50(suppl):S388–S393.
33. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Friedman P, Dennis EA, Curtiss LK, Palinski W, Witztum JL. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest*. 1999;103:117–128.
34. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP, Witztum JL. Human-derived anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions in vivo. *Arterioscler Thromb Vasc Biol*. 2001;21:1333–1339.
35. Vieira P, Rajewsky K. The half-lives of serum immunoglobulins in adult mice. *Eur J Immunol*. 1988;18:313–316.
36. Townsend MJ, Monroe JG, Chan AC. B-cell targeted therapies in human autoimmune diseases: an updated perspective. *Immunol Rev*. 2010;237:264–283.
37. Hansson GK, Nilsson J. Vaccination against atherosclerosis? Induction of atheroprotective immunity. *Semin Immunopathol*. 2009;31:95–101.

Novelty and Significance

What Is Known?

- B lymphocytes have been reported to reduce atherosclerosis, but the specific subset that exerts this effect has not been identified previously.
- Depletion of B lymphocytes by anti-CD20 antibody decreased atherosclerosis, whereas adoptive transfer studies identified B2 lymphocytes as a damaging B-lymphocyte subset.
- Splenectomy aggravated atherosclerosis and depleted B1a B lymphocytes, whereas IgM was atheroprotective.

What New Information Does This Article Contribute?

- Peritoneal Bla lymphocytes are an atheroprotective B-cell subset.
- B1a lymphocytes protect against atherosclerosis development by producing natural IgM that circulates in the blood and is deposited in atherosclerotic lesions.
- IgM deposits in atherosclerotic lesions remove dead cells from lesions.

B lymphocytes have been reported to be atheroprotective. However, B lymphocytes are heterogeneous, and although the B2 subset has been identified as atherogenic, the identity of the atheroprotective B-lymphocyte subset remains unknown. Here, we have identified IgM-producing B1a lymphocytes as an atheroprotective B-lymphocyte subset based on the following salient observations: (1) B1a depletion by splenectomy aggravated atherosclerosis, accompanied by reduced plasma and lesion IgM and increased dead cells in lesions; (2) B1a repletion by their transfer to splenectomized mice reduced atherosclerosis, increased plasma and lesion IgM, and reduced dead cells in lesions; (3) transfer of Bla lymphocytes, which cannot produce IgM, failed to protect against atherosclerosis. Our data provide the novel concept that B1a lymphocytes are atheroprotective by producing natural IgM antibodies deposited in atherosclerotic lesions that remove dead cells from lesions. This process explains the exaggerated atherosclerosis after splenectomy and an atheroprotective role of IgM. The significance of this new knowledge lies not only in providing a better insight into the role of B-lymphocyte subsets in atherosclerosis, but also in its potential clinical translation. Our findings suggest that therapeutic strategies directed toward amelioration of atherosclerosis should be directed toward deletion of damaging B2 lymphocytes and/or expansion of protective Bla lymphocytes.

Supplemental Materials

Splenectomy

In splenectomy experiments, spleens of 6-8 week-old ApoE$^{-/-}$ mice were removed surgically under aseptic conditions. Briefly, under anesthesia with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg), a 10-mm left flank incision was made to expose the spleen and the whole spleen was removed using diathermy. The peritoneum and skin were closed separately using 2-0 monofilament suture after checking for any hemorrhage in the abdominal cavity. Upon subcutaneous injection of Atipamezole HCl (antisedan 100mg/kg), mice were placed in 37˚C recovery chambers before they were returned to their cages. Sham-operation was performed according to the splenectomy procedure but without removing the spleen. For transfer experiments, purified 10^5 B1a lymphocytes were intravenously transferred via the tail vein.

Cell isolation

Peritoneal fluid and/or spleens were collected from donor mice[1] . After lysing red blood cells and preparing single cell suspensions, peritoneal B1a lymphocytes were isolated by FACS Aria (BD Biosciences) using anti-CD22 and anti-CD5 antibodies (BD Pharmingen) (Fig. 1 a). In some experiments, anti-CD3, anti-CD19, anti-CD5 and anti-CD11b antibodies (BD Pharmigen) were used to isolate peritoneal B1a lymphocytes (Fig. 1 b). Spleen B2 lymphocytes were sorted magnetically by negative selection using a B lymphocyte isolation kit (Miltenyi Biotec) (Fig. 1 c). Purity of cells was checked by anti-CD22/anti-CD19 and anti-CD5 antibodies (BD Pharmingen) on FACS Aria (BD Biosciences) or FACS Cantos II (BD Biosciences). Cell viability checked by trypan blue exclusion method was >95%. To assess the contribution of donor Bla lymphocytes to the reconstitution of this population, peritoneal CD3$^-$ CD19$^+$ CD5$^+$ CD11b$^+$ Bla lymphocytes were also isolated from allelic Ly5.1 mice, and transferred to splenectomised mice. Bla lymphocytes isolated from these mice at the end of the 8 week experiment were then assessed for Ly5.1$^+$ donor cells in this population.

Flow cytometry

B and non-B lymphocyte populations in peritoneal fluid were analyzed using fluorochrome-conjugated antibodies (BD Pharmingen) on BD FACSCanto II (BD Biosciences) as described[1].

Histological lesion analysis at aortic roots

The heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-teck) and frozen at -80˚C. Frozen sections (6µm) were cut from the aortic sinus, defined as the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off[2]. The aortic sinus was evaluated because this region of the aorta is particularly susceptible to development of atherosclerosis in mice fed a HFD[2]. Atherosclerotic lesion sizes were assessed using oil red-O and collagen content using picrosirius red as previously described[1]. To analyse necrotic core areas, aortic root atherosclerotic lesions were stained with hematoxylin and eosin (H&E) stain. Acellular (non-stained) areas were measured by Optimas software and expressed as a percentage of lesion areas[3].

Immunohistochemical analysis

Immunohistochemical analyses were performed at aortic root atherosclerotic lesions as described[1]. Rat anti-mouse CD68 antibody (Serotec, Raleigh, NC), rabbit anti-α-actin smooth muscle antibody (Abcam, Cambridge, UK) and mouse anti-MDA- oxidized low-density lipoproteins (oxLDL) antibody (Abcam, Cambridge, UK) were used to detect macrophages, smooth muscles, and oxLDL in atherosclerotic lesions.

Some sections were used to identify apoptotic cells using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) system and alkaline phosphatase [in-situ cell death detection kit-AP assay (Roche)] according to the manufacturer's instruction.

Detection of plasma IgG and IgM

Plasma levels of IgG and IgM were measured by enzyme linked immunosorbent assay (ELISA) as described[1]. In time-course analysis, IgM levels were assessed at 4 and 8 weeks after splenectomy.

Dilution factors used for sample preparation were 10^5 for plasma IgG and 10^4 for IgM. To determine plasma immunoglobulins titers using ELISA, 50 µl of anti-mouse immunoglobulin (1µg/ml) was used to coat 96-well ELISA plates overnight at 4°C. After blocking with 1% bovine serum albumin, duplicate samples of 50 µl of plasma (diluted 1:105 for total Ig and IgG and 1:104 for IgM) was added into ELISA plates for 2 hours at room temperature. Respective secondary anti-mouse antibodies conjugated with horseradish peroxidase were added into the wells, followed by addition of TMB substrate for colour development. The OD at 450 nm was read by ELISA reader.

Measurement of anti–malondialdehyde(MDA)-LDL IgM antibody

Modification of human LDL (Calbiochem, Darmstadt, Germany) was carried out and assessed by spectrophotometer as described[4]. A modified ELISA protocol adapted from Caliguiri et al.[5] was used to measure anti-MDA-LDL IgM antibody. Briefly, MDA-LDL and native LDL were used to coat 96-well ELISA plates at 50µl of 10 µg/ml overnight at 4˚C. Duplicate samples of 50 µl mouse plasma diluted 1:80, 1:320 and 1:1280 were added into the ELISA plates for 1 hour at 37˚C after blocking with 1% BSA, followed by addition of 1:10,000 diluted anti-mouse IgM (BD Pharmingen) antibody conjugated with HRP. Color was developed by addition of TMB solution. Plates were read at 450 nm wavelength. MDA-LDL–specific IgM antibody was determined by subtracting the native LDL OD from the oxLDL OD.

Lipid profiles

Plasma lipids (total cholesterol, HDL-cholesterol, VLDL/LDL-cholesterol and triglycerides) were measured by a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyser, with reagents and calibrators supplied by Beckman Coulter Diagnostics Australia as described[1].

Statistical analysis

GraphPad Prism 4 software was used for statistical analyses. Results are presented as mean ± SEM. One-way ANOVA with Newman-Keul post-hoc test was used for comparisons of multiple groups of ≥3. Two-tailed unpaired student t-test was used for comparison of 2 groups. One-tailed unpaired student t-test was used for confirmatory transfer experiments with Bla lymphocytes selected for $CD19^+CD5^+$ expression and for congenic Ly5.1 expression. P values were considered significant at $P<0.05$.

1. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol.* 2010;185(7):4410-4419.

2. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 1987;68(3):231-240.

3. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice. *Arterioscler Thromb Vasc Biol.* 2008;28(8):1421-1428.

4. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest.* 1998;101(8):1717-1725.

5. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest.* 2002;109(6):745-753.

Chapter 4: Depletion of B2 but Not B1a B Cells in BAFF Receptor-Deficient ApoE$^{-/-}$ Mice Attenuates Atherosclerosis by Potently Ameliorating Arterial Inflammation

4.1 Short Introduction

In earlier studies, I demonstrated that B2 B cells are atherogenic but their contribution to atherosclerosis in immune intact mice was not evaluated. Here, I used BAFF-R-deficient ApoE$^{-/-}$ mice to specifically assess the role of B2 B cells in the development of atherosclerosis. ApoE$^{-/-}$ mice that were genetically deficient in the receptor for B-cell activating factor (BAFF-R) provided additional evidence of the differential roles of B1a and B2 B cells in atherosclerosis. BAFF is a ligand of the tumor necrosis factor superfamily. The association of BAFF and BAFF-R is crucial for the survival and maintenance of B2 cells but not B1a B cells. ApoE$^{-/-}$BAFF-R$^{-/-}$ mice showed diminished B2 but normal B1a B cell populations. Indeed, the concept of anti-atherogenic B1a B cells and pro-atherogenic B2 B cells was reinforced by ApoE$^{-/-}$ BAFF-R$^{-/-}$ mice displaying smaller atherosclerotic plaques compared to ApoE$^{-/-}$ mice after eight weeks of high fat diet. Furthermore, the reduction in atherosclerosis was accompanied by lower expression of the proinflammatory markers - TNFα, IL-1β and MCP-1 in the aorta. These results indicate that B2 cells may accentuate atherosclerosis by enhancing local plaque inflammation. *PLoS One.* 2012; 7(1):e29371.

PART B: Suggested Declaration for Thesis Chapter

[This declaration to be completed for each conjointly authored publication and to be placed at the start of the thesis chapter in which the publication appears.]

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter [4], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	45

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Tin Kyaw	Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	
Hamid Hosseini	Experimentation	5%
Peter Kanellakis	Experimentation	
Tahlia Gadowski	Experimentation	
Fabeinne Mackay	Study concept, Study design	
Peter Tipping	Study concept, Study design, Result Interpretation	
Alex Bobik	Study concept, Study design, Result Interpretation, Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Result Interpretation, Preparation of manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 7/2/2014
Main Supervisor's Signature	[redacted]	**Date** 7/2/2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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Depletion of B2 but Not B1a B Cells in BAFF Receptor-Deficient ApoE$^{-/-}$ Mice Attenuates Atherosclerosis by Potently Ameliorating Arterial Inflammation

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Abstract

We have recently identified conventional B2 cells as atherogenic and B1a cells as atheroprotective in hypercholesterolemic ApoE$^{-/-}$ mice. Here, we examined the development of atherosclerosis in BAFF-R deficient ApoE$^{-/-}$ mice because B2 cells but not B1a cells are selectively depleted in BAFF-R deficient mice. We fed BAFF-R$^{-/-}$ ApoE$^{-/-}$ (*BaffR.ApoE* DKO) and BAFF-R$^{+/+}$ApoE$^{-/-}$ (*ApoE* KO) mice a high fat diet (HFD) for 8-weeks. B2 cells were significantly reduced by 82%, 81%, 94%, 72% in blood, peritoneal fluid, spleen and peripheral lymph nodes respectively; while B1a cells and non-B lymphocytes were unaffected. Aortic atherosclerotic lesions assessed by oil red-O stained-lipid accumulation and CD68+ macrophage accumulation were decreased by 44% and 50% respectively. B cells were absent in atherosclerotic lesions of *BaffR.ApoE* DKO mice as were IgG1 and IgG2a immunoglobulins produced by B2 cells, despite low but measurable numbers of B2 cells and IgG1 and IgG2a immunoglobulin concentrations in plasma. Plasma IgM and IgM deposits in atherosclerotic lesions were also reduced. BAFF-R deficiency in ApoE$^{-/-}$ mice was also associated with a reduced expression of VCAM-1 and fewer macrophages, dendritic cells, CD4+ and CD8+ T cell infiltrates and PCNA+ cells in lesions. The expression of proinflammatory cytokines, TNF-α, IL1-β and proinflammatory chemokine MCP-1 was also reduced. Body weight and plasma cholesterols were unaffected in *BaffR.ApoE* DKO mice. Our data indicate that B2 cells are important contributors to the development of atherosclerosis and that targeting the BAFF-R to specifically reduce atherogenic B2 cell numbers while preserving atheroprotective B1a cell numbers may be a potential therapeutic strategy to reduce atherosclerosis by potently reducing arterial inflammation.

Citation: Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, et al. (2012) Depletion of B2 but Not B1a B Cells in BAFF Receptor-Deficient ApoE$^{-/-}$ Mice Attenuates Atherosclerosis by Potently Ameliorating Arterial Inflammation. PLoS ONE 7(1): e29371. doi:10.1371/journal.pone.0029371

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☯ These authors contributed equally to this work.

Introduction

Atherosclerosis is a chronic inflammatory disease of large arteries initiated by lipid entry. Despite the therapeutic application of lipid-lowering statins; atherosclerosis-related vascular disease remains the major cause of mortality from heart attacks and strokes. New therapies to attenuate the chronic inflammation in atherosclerosis are therefore urgently sought that can be combined with current lipid-control medications and healthy life-style adaptation [1,2]. B cells together with other immune cells are implicated in the pathogenesis and progression of atherosclerosis. Previous studies have suggested that these B cells are atheroprotective [3,4]. However, in a major paradigm shift, we and Ait-Oufella et al have reported that these B cells can be pathogenic because their depletion by anti-CD20 monoclonal antibody ameliorated atherosclerosis in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice [5,6]. In adoptive transfer experiments we have identified conventional B2 B cells as an atherogenic B cell subset and peritoneal B1a B cells as an atheroprotective B cell subset in atherosclerosis [5,7]. Consequentially, we have proposed a potential therapeutic strategy for atherosclerosis based on selective depletion of atherogenic B2 B cells without depleting atheroprotective peritoneal B1a B cells [8].

B-cell activating factor (BAFF), also known as BlyS, TALL-1, zTNF4 and THANK, is a member of the TNF superfamily (TNFSF13B) that is produced by myeloid cells, non-lymphoid cells and epithelial cells [9]. BAFF is required for maturation and survival of B2 cells [10]. Its biological activities are mediated by three receptors, BAFF-receptor (BAFF-R; TNFRSF13C), transmembrane activator-calcium modulator and cyclophilin ligand interactor (TACI; TNFRSF13B) and B-cell maturation antigen (BCMA; TNFRSF17) [11]. While TACI and BCMA can also interact with the BAFF homologue, a proliferation-inducing ligand (APRIL; TNFSF13), BAFF-R is expressed by all mature B cells

and only binds BAFF to initiate signaling that is crucial for B cell development and survival [12].

Mice with genetically disrupted BAFF-R gene and spontaneous mutation in the BAFF-R gene show a significant reduction in mature B2 cells without affecting B1a B cells [13,14]. Therefore BAFF-R has properties that are suitable for therapeutic targeting in atherosclerosis. Here, we have examined the role of BAFF-R in atherosclerosis using ApoE$^{-/-}$ mice deficient in BAFF-R. We report that atherosclerosis and arterial inflammation is markedly reduced in hypercholesterolemic BAFF-R deficient ApoE$^{-/-}$ mice.

Results

Generation and characteristics of BAFF-R-deficient ApoE$^{-/-}$ mouse

We generated BAFF-R$^{-/-}$ ApoE$^{-/-}$ (*BaffR.ApoE* DKO) mice by crossing C57Bl/6 BAFF-R$^{-/-}$ mice with atherosclerosis-prone C57Bl/6 ApoE$^{-/-}$ (*ApoE* KO) mice. Genotypes of *BaffR.ApoE* DKO and *ApoE* KO mice were verified by PCR (**Figure 1 A**). *BaffR.ApoE* DKO and *ApoE* KO mice were fed a high fat diet (HFD) containing 21% fat and 0.15% cholesterol (Specialty Feed, Western Australia) for eight weeks to study the role of BAFF-R in atherosclerosis.

Genes encoding BAFF-R disrupted by spontaneous mutation [13] or gene-targeted depletion [14] showed a decrease in mature B cells. To confirm similar effects in the *BaffR.ApoE* DKO mice, CD22+ B cell population was analyzed in different tissues at the end of 8-week HFD period. We found that B cells were significantly reduced in peripheral blood, peritoneal cavity, spleen and lymph node by 82% ($p<0.01$), 81% ($p<0.001$), 94% ($p<0.001$) and 72% ($p<0.01$) respectively in BAFF-R-deficient ApoE$^{-/-}$ mice (**Figure 1 B**).

Although BAFF-R is necessary for maturation of B cells from transitional B cells to follicular B cells and Marginal Zone B cells, BAFF-R deletion has either no or minimal effects on the B1 B cell linage [13,14]. In accordance with the literature, peritoneal CD22+ CD5+ B1a cell population was not affected by depletion of BAFF-R gene in ApoE$^{-/-}$ mice ($p>0.05$; **Figure 1 C and D**). FACS analysis of splenic cells showed that non-B cell populations, including CD4+CD25+ Foxp3 regulatory T cells were unaffected in BAFF-R-deficient ApoE$^{-/-}$ mice ($p>0.05$; **Table 1**), consistent with a previous report [14].

Hypercholesterolemia is an initiating factor in the pathogenesis of atherosclerosis. Plasma lipid analysis showed that there was no difference in plasma total cholesterol, HDL-cholesterol, VLDL/LDL-cholesterol and triglycerides between BAFF-R-deficient and –competent ApoE$^{-/-}$ mice fed a HFD for eight weeks ($p>0.05$; **Table 2**). Similarly, BAFF-R depletion did not affect the body weight in ApoE$^{-/-}$ mice ($p>0.05$; **Table 2**).

BaffR.ApoE DKO mice exhibit reduced atherosclerosis

Next, we asked if the deficiency in BAFF-R influences the development of atherosclerosis in hypercholesterolemic ApoE$^{-/-}$ mice. Assessment of atherosclerotic lesions after feeding mice a HFD by oil red-O stained lipid accumulation showed that *BaffR.ApoE* DKO mice had a significant ~44% reduction in aortic atherosclerosis compared to *ApoE* KO mice ($p<0.01$; **Figure 2 A**). We found that a similar ~50% reduction in atherosclerosis as assessed by CD68-stained macrophage accumulation in *BaffR.ApoE* DKO mice ($p<0.05$; **Figure 2 B**).

Lack of B cells and immunoglobulins G in atherosclerotic lesions of *BaffR.ApoE* DKO mice

We next determined whether B cell numbers were reduced in lesions of *BaffR.ApoE* DKO mice using anti-CD22 immunohistochemistry. In marked contrast to *ApoE* KO mice, there was no accumulation of B cells in atherosclerotic lesions in *BaffR.ApoE* DKO mice ($p<0.001$; **Figure 3 A**). As IgG1 and IgG2a are produced by B2 cells, we also assessed their accumulation in lesions. Again, in contrast to lesions of *ApoE* KO mice, those of *BaffR.ApoE* DKO mice did not contain detectable quantities of these two immunoglobulins ($p<0.001$ and $p<0.05$ respectively; **Figure 3 B and C**), confirming the marked differences in B cell accumulation between *ApoE* KO and *BaffR.ApoE* DKO mice. To ensure that the reductions in lesion IgG1 and IgG2a were the consequence of markedly different B cell numbers in lesions of the two groups of mice, we also assessed circulating levels of IgG1 and IgG2a. As expected, ELISA results revealed that the plasma levels of IgG1 and IgG2a were decreased by 65% and 82% as the result of BAFF-R gene disruption (both $p<0.01$; **Figure 3 E**).

BAFF-R deficiency reduced plasma IgM and IgM deposits in lesions

ELISA results revealed that levels of plasma IgM in *BaffR.ApoE* DKO mice were significantly decreased to about ~80% of plasma IgM levels of ApoE$^{-/-}$ mice ($p<0.001$; **Figure 3 E**). Significant reductions of plasma IgM prompted us to investigate whether IgM deposits were also altered in atherosclerotic lesions of BAFF-R$^{-/-}$ ApoE$^{-/-}$ mice. Indeed, we found that IgM deposits in atherosclerotic lesion were also decreased to 60% in *BaffR.ApoE* DKO mice compared to ApoE$^{-/-}$ mice ($p<0.01$; **Figure 3 D**).

Decreased T cells and PCNA-positive cells in lesions of *BaffR.ApoE* DKO mice

Next we asked if there were any reductions in T cells and PCNA-positive cells in lesions of *BaffR.ApoE* DKO mice that could account for the reductions in lesion size. CD4+ and CD 8+ T cells as well as PCNA+ cells are cellular markers of inflammatory responses and active proliferative activity in atherosclerosis [15–17]. Using CD4, CD8 and PCNA-specific antibodies, we found that CD4+ T cells and proliferating PCNA+ cells were reduced by 69% ($p<0.05$) and 76% ($p<0.05$) respectively in lesions of the *BaffR.ApoE* DKO mice compared to *ApoE* KO mice. In addition CD8+ T cell numbers were reduced by 49% ($p<0.05$; **Figure 4**).

Reductions in VCAM-1 expression and dendritic cells in lesions of BAFF-R$^{-/-}$ ApoE$^{-/-}$ mice

We carried out immunohistochemical assays to determine whether expression of VCAM-1 in atherosclerotic lesions was affected by BAFF-R gene disruption. VCAM-1 regulates the migration of leukocytes, including monocyte infiltration into developing lesions [18] and promotes the differentiation of monocytes into macrophages and dendritic cells to sites of inflammation [19]. Data analysis indicated that expression of lesional VCAM-1 in BAFF-R-deficient mice showed a ~79% decrease compared to BAFF-R-competent mice ($p<0.001$; **Figure 5 A**). We also found that CD11c+ immature dendritic cells and CD83+ mature dendritic cells were reduced by 54% ($p<0.05$) and 68% ($p<0.001$) in *BaffR.ApoE* DKO mice compared to *ApoE* KO mice (**Figure 5 B and C**).

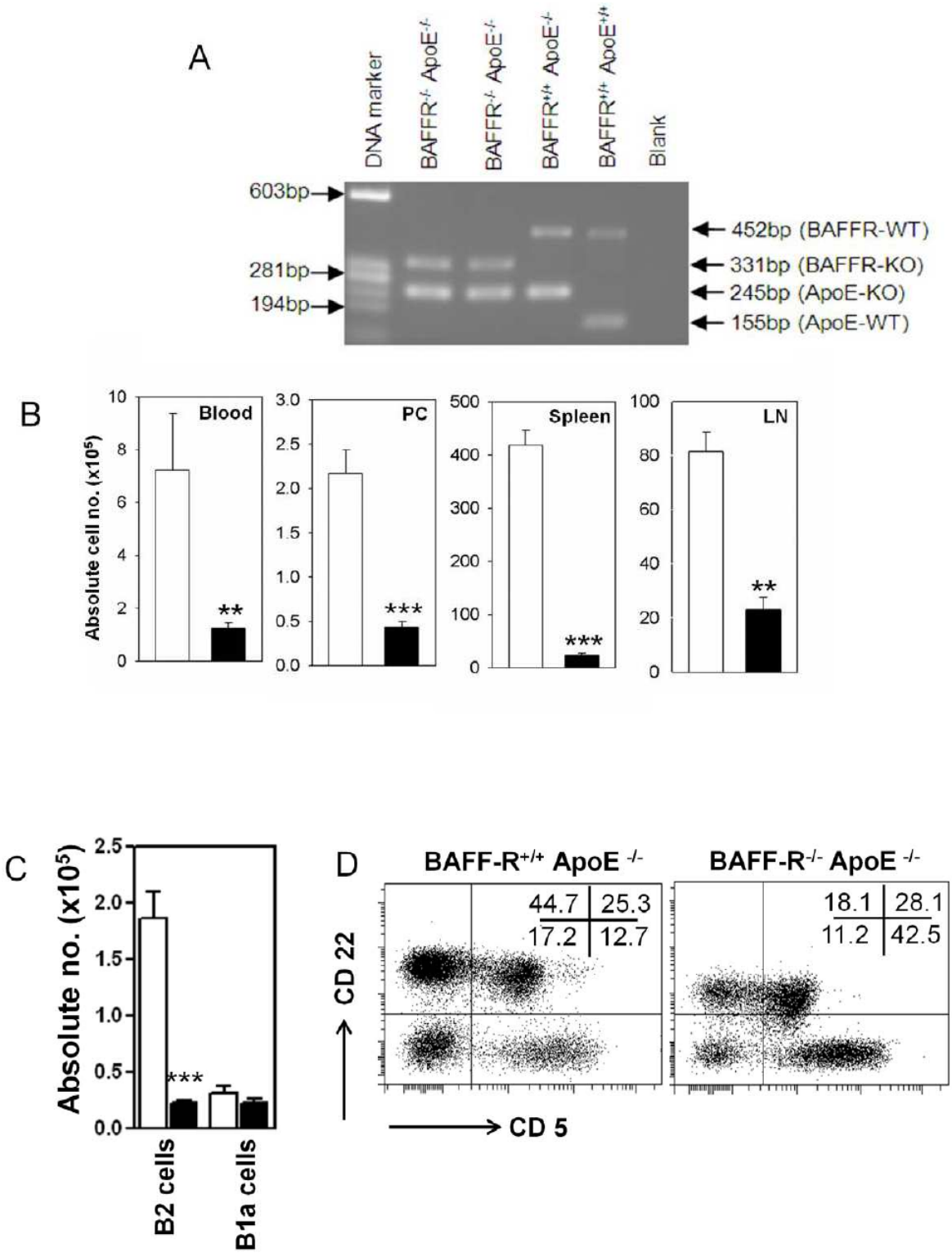

Figure 1. BAFF-R deficiency attenuates selectively conventional B2 cells, not peritoneal B1a cells. (**A**) PCR analysis of BAFF-R gene disruption was performed using DNA extracted from tails of BAFF-$R^{-/-}$ $ApoE^{-/-}$, BAFF-$R^{+/+}$ $ApoE^{-/-}$ and BAFF-$R^{+/+}$ $ApoE^{+/+}$ mice. (**B**) *BaffR.ApoE* DKO mice showed that $CD22^{+}$ B cells were lowered in peripheral blood, peritoneal cavity, spleen and peripheral lymph nodes. (**C**) FACS analysis on peritoneal cavity revealed that depleted B cells were conventional CD22+ CD5- B2 cells, not CD22+ CD5+ B1a cells comparing to *ApoE* KO mice. (**D**) A representative FACS analysis of peritoneal cavity showed that only conventional CD22+ CD5- B2 cell population (left upper quadrant) was decreased in *BaffR.ApoE* DKO and peritoneal CD22+ CD5+ B1a cell (right upper quadrant) was unaffected. Open bar = *ApoE* KO; Black bar = *BaffR.ApoE* DKO; n = 9–11 mice; **: $p<0.01$, ***: $p<0.001$.

Table 1. Non-B lymphocyte populations in spleen ($\times 10^6$).

	Non-B cells	*ApoE* KO	*BaffR.ApoE* DKO
Spleen	CD4+ T cells	17.13±2.86	18.97±3.15
	CD8+ T cells	13.91±2.33	19.42±3.29
	NK cells	1.44±0.09	1.02±0.11
	NKT cells	1.00±0.04	0.8±0.13
	Regulatory T cells	2.67±0.42	2.31±0.04

At the end of 8 week HFD, spleen cells are analyzed for non-B lymphocyte populations (n = 9–11 mice).
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Decreased inflammatory cytokines and MCP-1 expression in lesions of *BaffR.ApoE* DKO mice

IFN-γ expression by CD4+ T cells is reduced by anti-CD20 B cell depletion in atherosclerotic mice [6]. To determine whether IFN-γ and other proinflammatory cytokines and chemokines were also reduced in BAFF-receptor deficient mice, we performed real-time PCR analysis using mRNA extracted from aorta containing atherosclerotic aortic arches. We found that expression of mRNA encoding the proinflammatory cytokines TNF-α and IL1-β as well as monocyte attractant MCP-1 were decreased to 57%, 77% and 51% respectively in *BaffR.ApoE* DKO mice (All $p<0.05$; **Figure 6**). The expression level of IFN-γ was reduced by 24%, but it was not statistically significant (**Figure 6**).

Discussion

In this study, we have provided evidence that the attenuation of atherosclerosis and decreased inflammatory responses in atherosclerotic lesions in *BaffR.ApoE* DKO mice is linked to a reduced B2 cell population. In accordance with our previous reports [5,7], the development of atherosclerosis is potently ameliorated in an environment where atherogenic B2 cells but not atheroprotective B1a cells are reduced, as occurs in BAFF-R-deficient ApoE$^{-/-}$ mice.

The vast majority of conventional B2 B cells express BAFF-R which is essential for BAFF mediated maturation as well as their survival. Our data demonstrate that B2 cells are major regulators of atherosclerosis development. These findings are consistent with our previous observation that the adoptive transfer of B2 but not B1a cells to either lymphocyte deficient or B cell deficient mice aggravates atherosclerosis [5]. We suggest that targeting B2 cells via the BAFF receptor that spares B1a cells may be more therapeutically efficacious than B cell depletion strategies using monoclonal antibody to CD20 that depletes both B2 as well as B1a cells [5,6].

Table 2. Factors contributing in atherosclerosis pathogenesis.

	ApoE KO	*BaffR.ApoE* DKO
Bodyweight	30.92±0.59	28.97±1.06
Total cholesterol	19.85±2.03	18.56±1.74
HDL cholesterol	3.54±0.33	2.95±0.27
VLDL/LDL cholesterol	15.35±1.77	13.42±1.25
Triglycerides	2.67±0.58	4.21±1.00

At the end of 8 week HFD, body weight and plasma lipids were analyzed (see text) (n = 9–11 mice).
doi:10.1371/journal.pone.0029371.t002

BAFF-R gene defect is associated with a strong, but not complete, reduction of mature B cells, sparing the B1a cell in both spontaneous mutation and knockout mice [13,14]. B cells contribute to the production of immunoglobulins, particularly subclass IgG type. IgG constitutes about 75% of immunoglobulins in the circulation and is the major Ig class generated in adaptive immune responses. Our finding of reduced circulating immunoglobulin IgG1 and IgG2a in mature B cell-depleted *BaffR.ApoE* DKO mice is consistent with the inability of immature transitional B cells to efficiently differentiate into mature B cells [13,14].

IgG deposits are reported in both human and animal atherosclerotic lesions [20–22]. The origin of IgG is yet to be determined. It could arise from B cells and plasma cells in the intimal or adventitial layer and/or the circulating IgG pool [21,22]. In our study, both IgG1 and IgG2a detected in both intimal and medial layers in BAFF-R-competent ApoE$^{-/-}$ mice were almost undetectable in BAFF-R-deficient ApoE$^{-/-}$ mice, despite reduced but still easily detectable, levels of these IgGs in the circulation. Concurrently CD22+ B cells in atherosclerotic lesions were reduced to almost none in BAFF-R deficient ApoE$^{-/-}$ mice. Taken together, it is likely that lesion IgG deposits found in BAFF-R-competent ApoE$^{-/-}$ mice are mostly derived from B cell and/or plasma cells located in the intimal or adventitial layers. Plasma IgM levels at the end of the 8 week-HFD were also decreased in *BaffR.ApoE* DKO mice consistent with a previous report [14] and with the suggestion that BAFF-R also has a role in germinal centre responses and in Ig class-switch DNA recombination [14,23,24]. The reduction in IgM deposits that we observed in atherosclerotic lesions may be a consequence of the reduced levels of circulating IgM. The residual levels of IgM deposits in atherosclerotic lesions may represent natural IgM produced by peritoneal B1a cells as we have previously reported [7].

Accelerated local inflammation responsible for progression of atherosclerosis and its complications is mediated by atherogenic cells as well as proinflammatory proteins in atherosclerotic lesions [25]. Immune cells such as monocytes, macrophages, dendritic cells and lymphocytes migrate into atherosclerotic lesions, to contribute to inflammation by interacting with other proinflammatory immune cells [16–19]. Activated immune cells secrete a range of pro-inflammatory cytokines to intensify the inflammatory process locally. These cytokines promote both their own production and the production of other cytokines and can also target the cells that produce them as well as neighboring cells to promote inflammation [26]. Thus, proinflammatory cytokines produced by macrophages, dendritic cells, CD4+ and CD8+ T cells promote the development and progression of atherosclerosis as positive regulators.

VCAM-1 is an adhesion molecule required for recruitment of lymphocytes and monocytes from the circulation into atherosclerotic lesions [18,27–31]. Monocytes recruited into atherosclerotic lesions further differentiate into macrophages and dendritic cells [32]. Our results suggest that the recruitment and retention of proinflammatory macrophages, dendritic cells and CD4 and CD8 T cells in atherosclerotic lesions are decreased due to low expression of VCAM-1 in *BaffR.ApoE* DKO mice.

Macrophages are major proinflammatory cells in the pathogenesis of atherosclerosis. They appear early in the atherosclerotic lesion to drive the inflammatory process in atherosclerotic lesions. We suggest that the reduced accumulation of macrophages contributes to the attenuated inflammatory responses that result in decreased atherosclerosis. It is also likely that the decreased accumulation of T cells, contributes to the reduced arterial

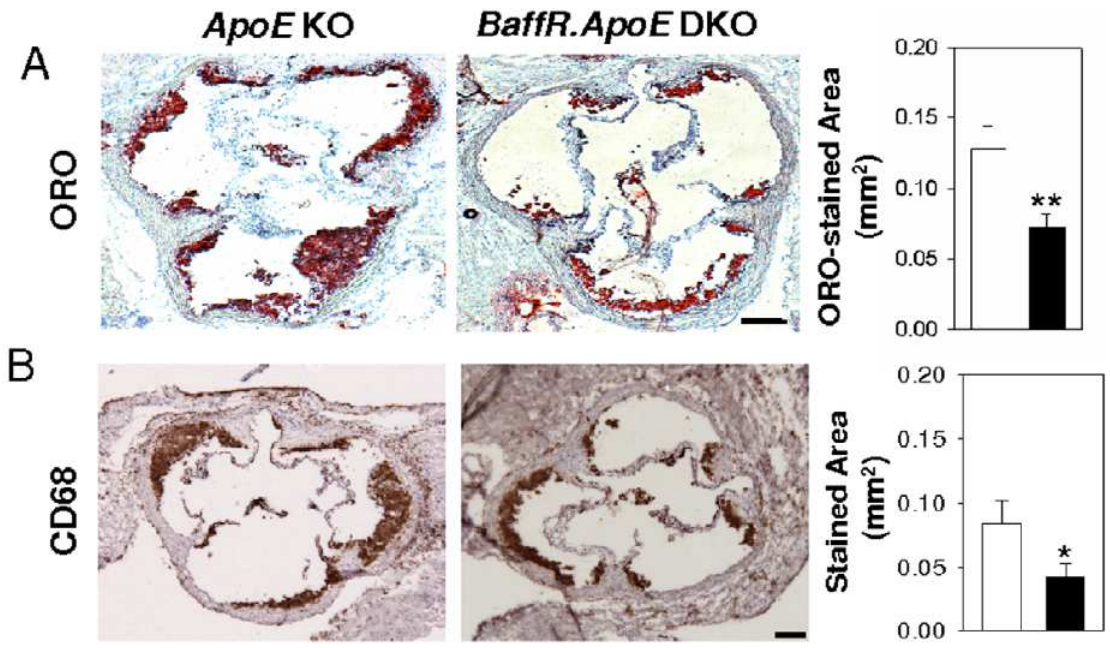

Figure 2. Deficiency of BAFF-R attenuates atherosclerosis. After feeding a HFD for eight weeks, *BaffR.ApoE* DKO showed decreased atherosclerosis at aortic sinus as assessed by (**A**) oil-red O stained lipid accumulation and (**B**) CD68+ macrophage accumulation compared to *ApoE* KO. Open bar = *ApoE* KO; Black bar = *BaffR.ApoE* DKO; n = 9–11 mice; scale bar = 100 μm; *: $p<0.05$, **: $p<0.01$.
doi:10.1371/journal.pone.0029371.g002

inflammation. CD8+ T cells are important not only in recruitment, differentiation and activation of macrophages in the early stage of inflammatory responses, but also in maintenance of subsequent inflammatory responses [33]. The reduction of CD4 T cells in atherosclerotic lesions may be attributed at least in part to BAFF-R providing co-stimulatory signals [34] that augment Th1 inflammatory responses [35]. CD4+ T cells can activate macrophages to produce pro-inflammatory cytokines such as TNF-α, IL1-β, MCP-1 [36].

Our findings also clearly show that selective deficiency of B2 but not B1a cells is accompanied by reduction in the lesions of, the proinflammatory cytokines TNF-α, IL1-β and MCP-1 [16–18,25–31,33,36]. We have also previously reported reduced TNF-α in atherosclerotic lesions that accompany the reduced atherosclerosis following B cell depletion by anti-CD20 monoclonal antibody treatment [5]. The reduction in TNF-α appears to be the consequence of depletion of B2 cells as these cells are known to be significant producers of TNF-α [34,37]. Expression of IFN-γ showed a trend in reduction in *BaffR-ApoE* DKO mice, but the result was not statistically significant. As TNF-α has been reported to upregulate VCAM-1 expression [38], the reduced VCAM-1 expression is likely the result of reduced TNF-α expression. In turn, the reduced VCAM-1 and MCP-1 expression in lesions may likely contribute, at least in part, to the reduced numbers of pro-inflammatory macrophages, dendritic cells, CD4 T cells and CD8 T cells in the lesions directly and to the reduced proinflammatory cytokines produced by these cells indirectly. Given that B2 cells have been ascribed antigen-presenting functions, it is likely that the depletion in B2 cells directly contributes to the reduced entry of CD4 and CD8 T cells into the lesions.

In addition to pro-inflammatory cytokines, actively proliferating cells are an indicator for progression of advanced atherosclerosis. In early and progressing atherosclerotic plaques, intimal macrophages are principal proliferative cells constituting about 50% of cell proliferation as assessed by the proliferative cell nuclear antigen antibody [15] whilst three dominant PCNA+ cell types found in advanced plaques are macrophages, vascular endothelial cells and smooth muscle cells. The reduced PCNA+ cells in intimal atherosclerotic lesion in *BaffR.ApoE* DKO mice suggest less proliferative activity in atherosclerotic lesions.

Control of inflammation comes to the forefront in the future management of atherosclerosis. In a meta-analysis of studies on the relationship between methotrexate and cardiovascular disease, methotrexate was associated with a lower risk for CVD patients with chronic inflammatory diseases, suggesting that direct treatment of inflammation will reduce cardiovascular events [39]. Currently the use of BAFF antagonists to target B cells to modulate immune responses in autoimmune diseases is being trialled and shows promising results in animal and human studies [9,40–43].

Given that we have provided compelling evidence that BAFF-R deficiency is associated with potent reduction in inflammation in atherosclerotic lesions, we suggest that targeting the BAFF-R has potential for therapeutic application in the management of patients with atherosclerotic vascular disease.

Materials and Methods

Animals

C57Bl/6 mice deficient in both BAFF-R and ApoE genes were generated from crossing BAFF-R$^{-/-}$ mice (from Klaus Rajewsky of the Harvard Medical School) and ApoE$^{-/-}$ mice (from the Jackson Laboratory). Experimental mice (6–8 week- old male mice) were fed a high fat diet containing 21% fat and 0.15% cholesterol (Specialty Feeds, Western Australia) for eight weeks.

Ethics statement

All animal experiments approved by the Alfred Medical Research and Education Precinct (AMREP) animal ethic committee (E/0708/2008/B) were carried out at the Precinct Animal Centre, AMREP.

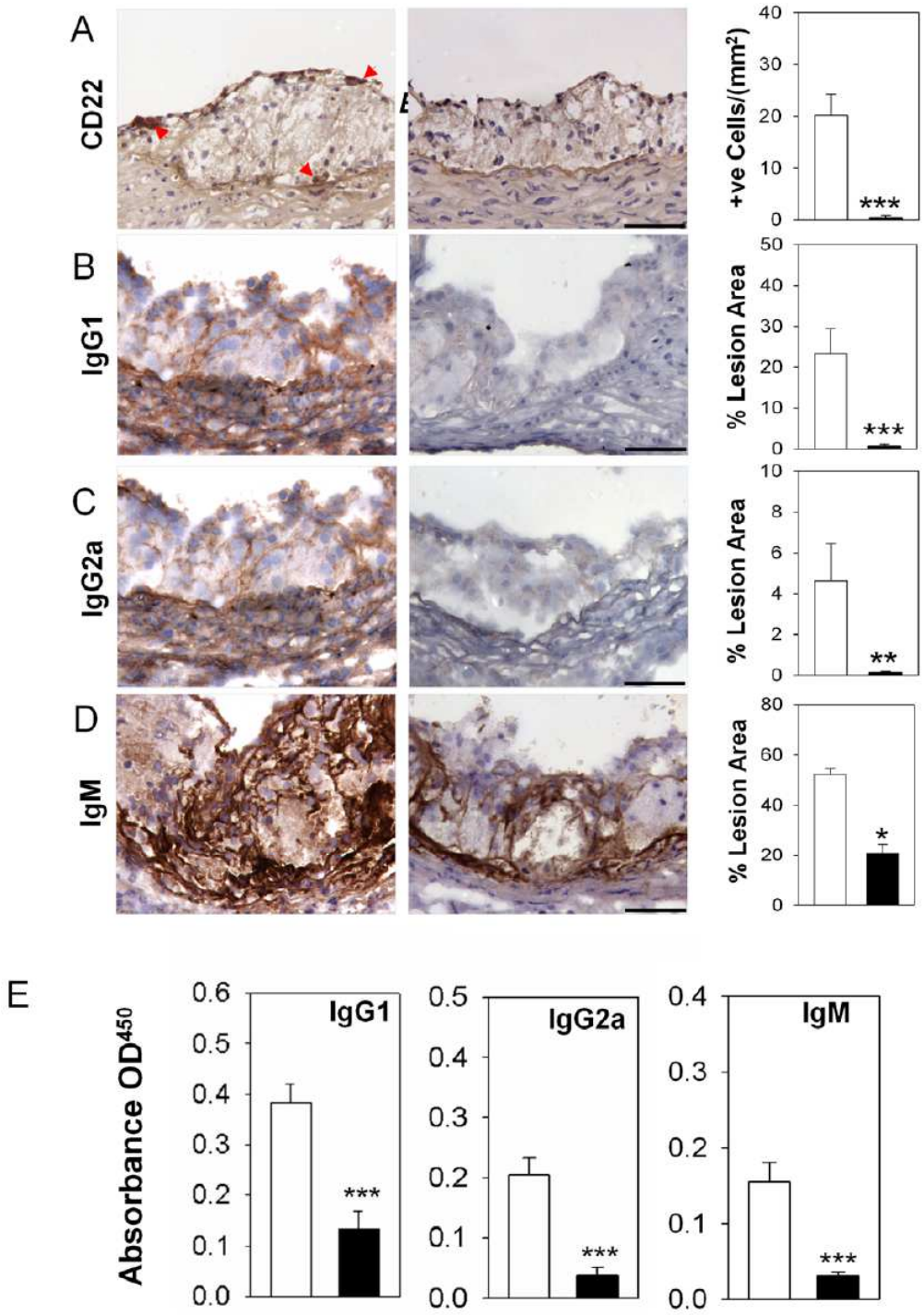

Figure 3. BAFF-R deficiency responsible for loss of B2 cells affects loss of B cells and IgG deposits in atherosclerotic lesion and attenuates plasma IgG levels. (**A**) CD22+ B cells and (**B–D**) immunoglobulins deposits (IgG1, IgG2a and IgM) found in the atherosclerotic lesion of *ApoE* KO mice were not detected in *BaffR.ApoE* DKO mice. (**E**)The plasma IgG1, IgG2a and IgM levels are also significantly decreased in *BaffR.ApoE* DKO but to a lesser extent than in lesions. Open bar = *ApoE* KO; Black bar = *BaffR.ApoE* DKO; n = 9–11 mice; scale bar = 100 μm; **: $p<0.01$, ***: $p<0.001$.
doi:10.1371/journal.pone.0029371.g003

PCR-genotyping of BAFF-R and ApoE genes

Genomic DNAs were extracted from tail samples using DNeasy blood and tissue kit (Qiagen, Germany). PCR application was carried out with 50 ng of genomic DNA in a single reaction vessel containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 uM of each primer and .2 unit of Taq DNA polymerase (Invitrogen). PCR condition was as follow: initial denaturation step of 5 minutes at 95°C, 35 cycles of 10 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C and final amplification step of 5 minutes of 72°C. Primers used were ApoE-Com 5′-GCC TAG CCG AGG GAG AGC CG-3′; ApoE-WT 5′- TGT GAC TTG GGA GCT CTG CAG C-3′; ApoE-KO 5′- GCC GCC CCG ACT GCA TCT-3′; BAFF-R-Com 5′-TTC TTT GAG CGG AGG CCA GG-3′; BAFF-R-WT

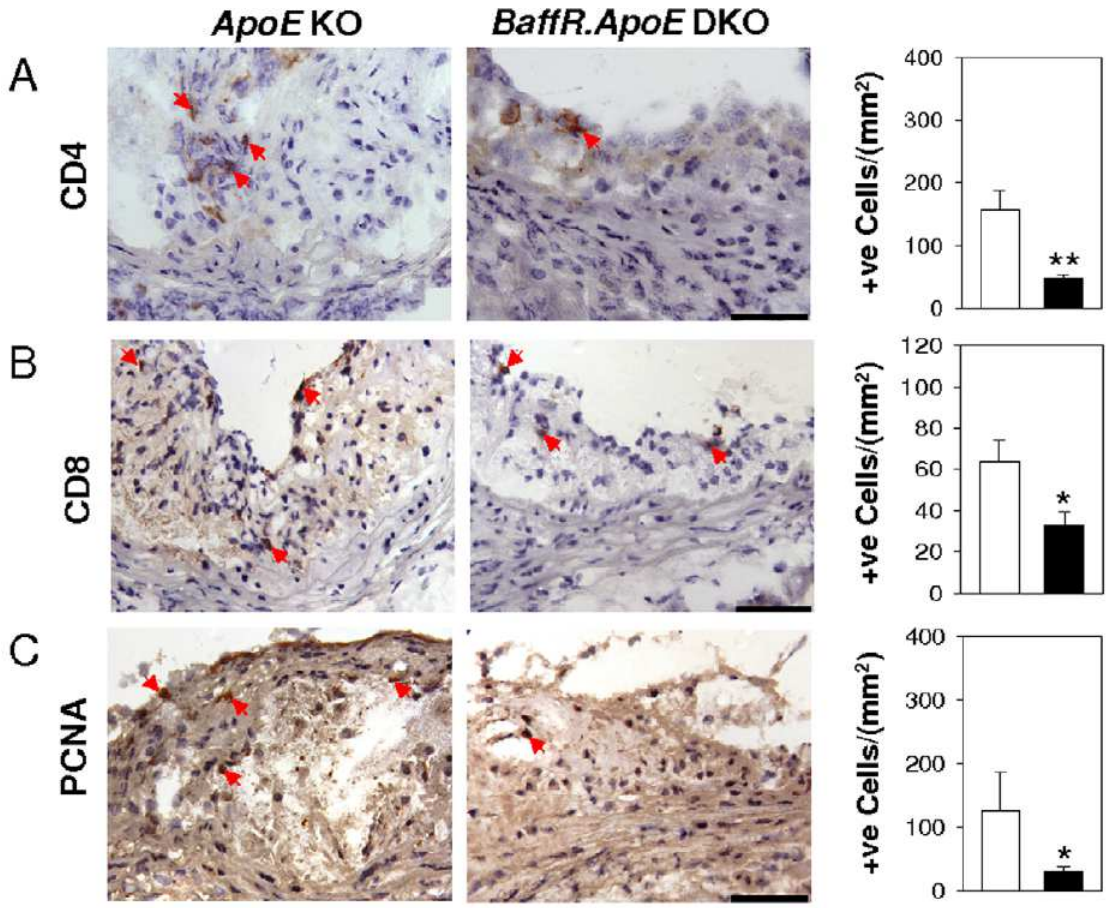

Figure 4. T cell infiltrates and cellular proliferative activity are decreased in atherosclerotic lesions of *BaffR.ApoE* DKO. (**A and B**) T cell infiltrates as assessed by anti-CD4 and anti-CD8 antibodies and (**C**) cellular proliferative activity as assessed by PCNA antibody were reduced by deficiency of BAFF-R. Open bar = *ApoE* KO; Black bar = *BaffR.ApoE* DKO; n = 9–11 mice; scale bar = 100 μm; *: $p<0.05$, **: $p<0.01$.
doi:10.1371/journal.pone.0029371.g004

5′-CTG AGG GAG ACC TGG AGT TG-3′ and BAFF-R-KO 5′-ATG GGC CAC TCA AGA TGA TCT G-3′ (Genework, Australia). PCR products were visualized on ethidium-stained 1.5% TAE agarose gel electrophoresis and digitally recorded.

Lymphocyte analysis

Lymphocytes were analyzed using flow cytometry as described [5]. Flurochrome-labeled antibodies (all from BD Biosciences unless otherwise stated) used were anti-CD22 (PE-Cat# 553384), anti-CD5 (APC-Cat# 550035), anti-CD25 (APC-Cy7-Cat# 557658), anti-CD4 (Pacific Blue-Cat# MCD0428, Caltag Laboratories), anti-CD8 (PerCP-Cat# 553036), anti-TCR-β (FITC-Cat# 11-596185, eBioscience), anti-NK1.1 (PE-Cy7-Cat# 552878) and anti-foxp3 (PE-Cat# 12-5773-82, eBioscicence). For surface markers, single cell suspension was stained with multiple antibodies at 4°C for 30 minutes, washed away unbounded antibodies and suspended in PBS with 1% FCS. For regulatory T cells, anti-CD4 and anti-CD25 stained cells were fixed, permeabilized and further stained with anti-foxp3 antibody. FACS CantoII (BD Biosciences) was used to collect data from different flurochrome-labeled cells. FACSDiva software (BD Biosciences) was used to analyze the data.

Assessment of atherosclerosis

To assess the atherosclerosis, the aortic roots embedded in OCT media were sectioned at 6 μ thickness and stained with Oil Red O (Sigma) to visualize lipid accumulation in aortic intima as previously described by us [5]. Immunohistological staining was also carried with anti-CD68 antibody (Cat# MAC1957, Serotec) to assess macrophage accumulation in the atherosclerotic lesions [5]. The lesion assessments were carried out by quantifying the Oil Red O stained lipid accumulation and CD68+ macrophage accumulation by Optimas software [5].

Lesion cellular content analysis

Immunohistochemical staining was performed on aortic root sections. Anti-mouse antibodies targeting CD4 (Cat# 550280, BD Pharmigen), CD8 (Cat# 550281 BD Pharmigen), CD22 (Cat# 553382, BD Pharmigen), CD83 (Cat# 14-0831, eBioscience), CD11c (Cat# 14-0114, eBioscience), VCAM-1 (Cat# 550547, BD Pharmigen), PCNA (Cat# ab2426, Abcam), IgM (Cat# 550588, BD Pharmigen), IgG1 (Cat# 559626, BD Pharmigen) and IgG2a (553391, BD Pharmigen) were used in primary antibody incubation, followed by horse radish peroxidase-conjugated secondary antibody for DAB substrate. All slides were counter stained with H&E stain. Areas staining by CD11c, VCAM, IgM, IgG1 and IgG2a antibodies were quantified by Optimas software whilst cells stained with CD22, CD4 and CD8, CD83 and PCNA were counted under light microscopy [5,44]. Both positive areas and positive cells were corrected to total lesion areas as quantified by Optima software.

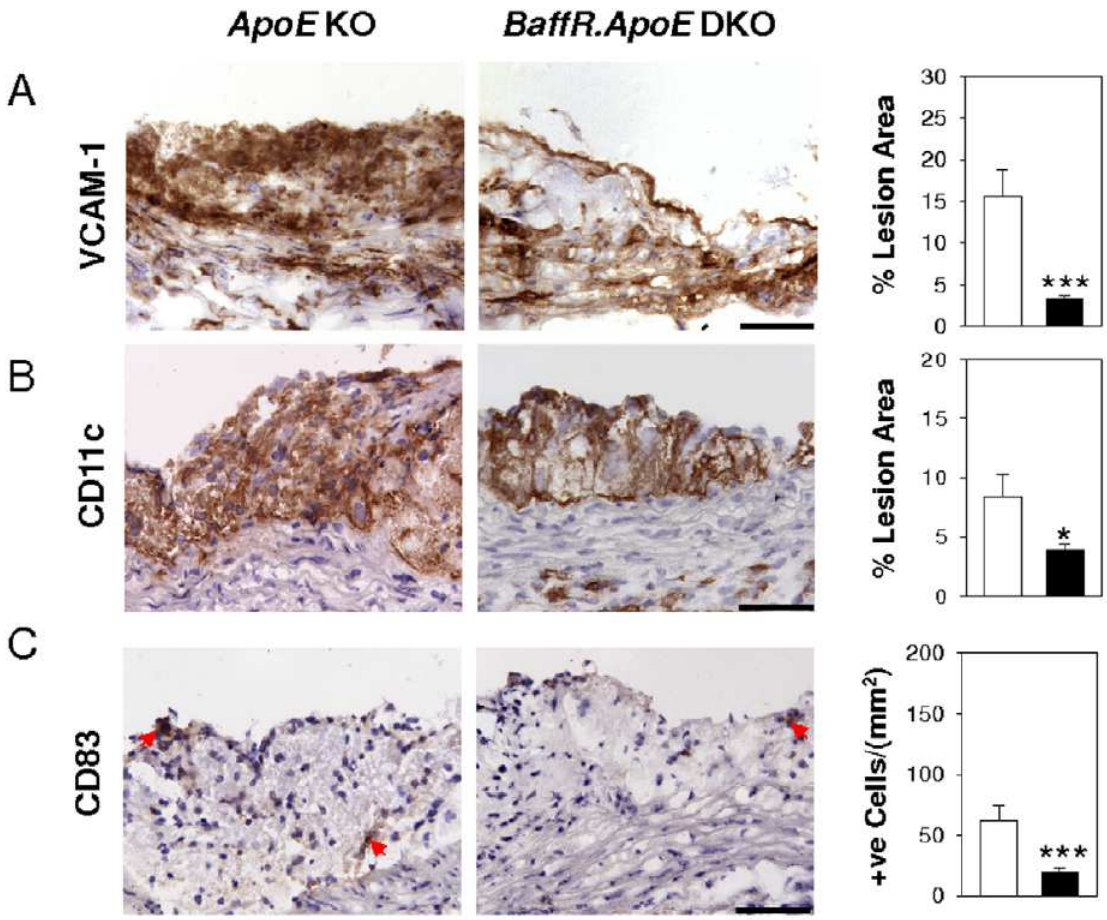

Figure 5. Low expression of adhesion molecule, VCAM-1, is associated with reduction in immature and mature dendritic cells in atherosclerotic lesions of BAFF-R$^{-/-}$ ApoE$^{-/-}$ mice. (**A**) VCAM-1 expression decreased in atherosclerotic lesions by disruption of BAFF-R gene was accompanied by (**B**) decreased immature dendritic cells as assessed by anti-CD11c antibody and (**C**) mature dendritic cells as assessed by anti-CD83 antibody in *BaffR.ApoE* DKO mice. Open bar = *ApoE* KO; Black bar = *BaffR.ApoE* DKO; n = 9–11 mice; scale bar = 100 μm; *: $p<0.05$, ***: $p<0.001$.
doi:10.1371/journal.pone.0029371.g005

Plasma lipid analysis

Determination of plasma cholesterol and triglycerides was done using Beckman Coulter LX20PRO analyzer according to manufacturer's instructions.

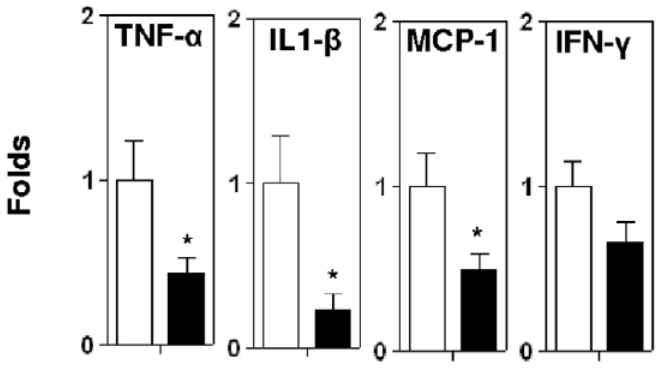

Figure 6. Real-time PCR analysis of proinflammatory cytokines. RNAs extracted from aortic arches were analysed for proinflammatory cytokines, TNF-α, IL1-β, MCP-1 and IFN-γ. *BaffR.ApoE* DKO showed reduced expression of proinflammatory cytokines in aortic arches compared to *ApoE* KO. Open bar = *ApoE* KO; Black bar = *BaffR.ApoE* DKO; n = 9–11 mice; *: $p<0.05$.
doi:10.1371/journal.pone.0029371.g006

Immunoglobulin analysis

Plasma immunoglobulin IgG1 and IgG2a were measured by enzyme linked immnosorbent assay (ELISA). Nunc Maxisorp 96-well ELISA plates were coated with 50 μl of 2 mg/ml goat anti-mouse Ig antibody (Cat# 1010-01, Southern Biotech) overnight at 4°C. After addition of 50 μl of plasma diluted at 10^5, the plates were incubated for 1 hour at room temperature. Secondary antibody incubation was done for 1 hour at room temperature with HRP-conjugated goat anti-mouse IgG1 (Cat# 1070-05, Southern Biotech) and IgG2a (Cat# 1080-05, Southern Biotech) antibodies for respective antibody measurement. TMB substrate was used for color development. After stopping the reaction, optic density was measured at 450 nm wave length using ELISA reader. Plasma IgM was measured using rabbit polyclonal anti-mouse Ig antibody (Cat Z0259, Dako) as coating antigen and HRP-conjugated goat anti-mouse IgM (Cat GM-90P, ICL) as detection antibody as previously described [7].

Real-time analysis of different mRNA

Aortas containing aortic arch and thoracic aorta were immediately frozen in liquid nitrogen. According to manufacturer's instructions, total RNA were extracted from aortas using RNeasy fibrous tissue mini kit (Qiagen, Germany). RNA integrity and quantity were determined using MultiNA electrophoresis system (Shimadzu, Japan). One-step real-time PCR was carried

out with QuantiFast SYBR Green RT-PCR kit (Qiagen, Germany) on 7500 Fast Real-Time PCR system (Applied Biosystem). The target gene expression levels were analyzed using comparative cycle threshold method [45] with 18S rRNA primers (Applied Biosystems). The primers used were as follows: IFN-γ sense (S) 5′-AAG TTT GAG GTC AAC AAC CCA C-3′, IFN-γ antisense (AS) 5′-GCT GGC AGA ATT ATT CTT ATT GGG-3′; TNF-α (S) 5′- TCT CAG CCT CTT CTC ATT CCT-3′, TNF-α (AS) 5′- ACT TGG TGG TTT GCT ACG AC-3′; MCP-1 (S) 5′-CTC AGC CAG ATG CAG TTA ACG-3′, MCP-1 (AS) 5′-GGG TCA ACT TCA CAT TCA AAG G-3′; IL1-β (S) 5′-CCA CCT CAA TGG ACA GAA TCT CAA-3′, IL1-β (S) 5′-GTC GTT GCT TGG TTC TCC TTG T-3′.

Statistical analysis

SigmaPlot 10.0 was used for statistical analyses. Results are presented as mean ± SEM. Two-tailed unpaired student t tests with Welch's correction were used for statistical analyses. *P* values were considered significant at $P<0.05$.

Author Contributions

Conceived and designed the experiments: TK FM PT AB BT. Performed the experiments: TK CT HH PK TG. Analyzed the data: TK CT HH PK TG. Contributed reagents/materials/analysis tools: TK PK AB BT. Wrote the paper: TK FM PT AB BT.

References

1. Ridker PM (2009) Testing the inflammatory hypothesis of atherothrombosis: scientific rationale for the cardiovascular inflammation reduction trial (CIRT). J Thromb Haemost 7 Suppl 1: 332–339.
2. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., et al. (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 359: 2195–2207.
3. Caligiuri G, Nicoletti A, Poirier B, Hansson GK (2002) Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. J Clin Invest 109: 745–753.
4. Major AS, Fazio S, Linton MF (2002) B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler Thromb Vasc Biol 22: 1892–1898.
5. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, et al. (2010) Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. J Immunol 185: 4410–4419.
6. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, et al. (2010) B cell depletion reduces the development of atherosclerosis in mice. J Exp Med 207: 1579–1587.
7. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, et al. (2011) B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM Deposits and Reduces Necrotic Cores in Atherosclerotic Lesions. Circ Res.
8. Kyaw T, Tipping P, Toh BH, Bobik A (2011) Current understanding of the role of B cell subsets and intimal and adventitial B cells in atherosclerosis. Curr Opin Lipidol 22: 373–379.
9. Lied GA, Berstad A (2011) Functional and clinical aspects of the B-cell-activating factor (BAFF): a narrative review. Scand J Immunol 73: 1–7.
10. Mackay F, Browning JL (2002) BAFF: a fundamental survival factor for B cells. Nat Rev Immunol 2: 465–475.
11. Brink R (2006) Regulation of B cell self-tolerance by BAFF. Semin Immunol 18: 276–283.
12. Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, et al. (2001) BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 293: 2108–2111.
13. Miller DJ, Hayes CE (1991) Phenotypic and genetic characterization of a unique B lymphocyte deficiency in strain A/WySnJ mice. Eur J Immunol 21: 1123–1130.
14. Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M (2004) TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. J Immunol 173: 2245–2252.
15. Rekhter MD, Gordon D (1995) Active proliferation of different cell types, including lymphocytes, in human atherosclerotic plaques. Am J Pathol 147: 668–677.
16. Mallat Z, Taleb S, Ait-Oufella H, Tedgui A (2009) The role of adaptive T cell immunity in atherosclerosis. J Lipid Res 50 Suppl: S364–369.
17. Kolbus D, Ramos OH, Berg KE, Persson J, Wigren M, et al. (2010) CD8+ T cell activation predominate early immune responses to hypercholesterolemia in Apoe(/) mice. BMC Immunol 11: 58.
18. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, et al. (2001) A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 107: 1255–1262.
19. Ou R, Zhang M, Huang L, Flavell RA, Koni PA, et al. (2008) Regulation of immune response and inflammatory reactions against viral infection by VCAM-1. J Virol 82: 2952–2965.
20. Hansson GK, Holm J, Kral JG (1984) Accumulation of IgG and complement factor C3 in human arterial endothelium and atherosclerotic lesions. Acta Pathol Microbiol Immunol Scand A 92: 429–435.
21. Yla-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, et al. (1994) Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. Arterioscler Thromb 14: 32–40.
22. Parums D, Mitchinson MJ (1981) Demonstration of immunoglobulin in the neighbourhood of advanced atherosclerotic plaques. Atherosclerosis 38: 211–216.
23. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, et al. (2002) DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol 3: 822–829.
24. Rahman ZS, Rao SP, Kalled SL, Manser T (2003) Normal induction but attenuated progression of germinal center responses in BAFF and BAFF-R signaling-deficient mice. J Exp Med 198: 1157–1169.
25. Galkina E, Ley K (2009) Immune and inflammatory mechanisms of atherosclerosis (*). Annu Rev Immunol 27: 165–197.
26. Tedgui A, Mallat Z (2006) Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 86: 515–581.
27. Ramos CL, Huo Y, Jung U, Ghosh S, Manka DR, et al. (1999) Direct demonstration of P-selectin- and VCAM-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein E-deficient mice. Circ Res 84: 1237–1244.
28. Steiner O, Coisne C, Cecchelli R, Boscacci R, Deutsch U, et al. (2010) Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium. J Immunol 185: 4846–4855.
29. de Chaisemartin L, Goc J, Damotte D, Validire P, Magdeleinat P, et al. (2011) Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer. Cancer Res.
30. Hortelano S, Lopez-Fontal R, Traves PG, Villa N, Grashoff C, et al. (2010) ILK mediates LPS-induced vascular adhesion receptor expression and subsequent leucocyte trans-endothelial migration. Cardiovasc Res 86: 283–292.
31. Hyun YM, Chung HL, McGrath JL, Waugh RE, Kim M (2009) Activated integrin VLA-4 localizes to the lamellipodia and mediates T cell migration on VCAM-1. J Immunol 183: 359–369.
32. Galkina E, Ley K (2007) Vascular adhesion molecules in atherosclerosis. Arterioscler Thromb Vasc Biol 27: 2292–2301.
33. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, et al. (2009) CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med 15: 914–920.
34. Ng LG, Sutherland AP, Newton R, Qian F, Cachero TG, et al. (2004) B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol 173: 807–817.
35. Sutherland AP, Ng LG, Fletcher CA, Shum B, Newton RA, et al. (2005) BAFF augments certain Th1-associated inflammatory responses. J Immunol 174: 5537–5544.
36. Chan T, Pek EA, Huth K, Ashkar AA (2011) CD4(+) T-cells are important in regulating macrophage polarization in C57BL/6 wild-type mice. Cell Immunol 266: 180–186.
37. Sung B, Na HS, Kim YI, Yoon YW, Han HC, et al. (1998) Supraspinal involvement in the production of mechanical allodynia by spinal nerve injury in rats. Neurosci Lett 246: 117–119.
38. Cid MC, Kleinman HK, Grant DS, Schnaper HW, Fauci AS, et al. (1994) Estradiol enhances leukocyte binding to tumor necrosis factor (TNF)-stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1. J Clin Invest 93: 17–25.
39. Micha R, Imamura F, Wyler von Ballmoos M, Solomon DH, Hernan MA, et al. (2011) Systematic Review and Meta-Analysis of Methotrexate Use and Risk of Cardiovascular Disease. Am J Cardiol.
40. Townsend MJ, Monroe JG, Chan AC (2010) B-cell targeted therapies in human autoimmune diseases: an updated perspective. Immunol Rev 237: 264–283.
41. Sanz I, Lee FE (2010) B cells as therapeutic targets in SLE. Nat Rev Rheumatol 6: 326–337.
42. Davidson A (2010) Targeting BAFF in autoimmunity. Curr Opin Immunol 22: 732–739.
43. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, et al. (2011) Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. Lancet 377: 721–731.

44. Kanellakis P, Agrotis A, Kyaw TS, Koulis C, Ahrens I, et al. (2011) High-mobility group box protein 1 neutralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. Arterioscler Thromb Vasc Biol 31: 313–319.

45. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.

Chapter 5: Tumor necrosis factor α produced by conventional B2 lymphocytes promotes atherosclerosis development in hyperlipidaemic ApoE-/- mice

5.1 Short Introduction

As mentioned before, the adoptive transfer of B2 B cells into lymphocyte-deficient $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice resulted in increased atherosclerosis. This suggests that B2 cells can promote atherosclerosis independently of T cell help and IgG antibody production. The present study examined whether B2 cells promote atherosclerosis by producing inflammatory cytokines such as TNFα and IFNγ. The assessment of spleen B2 cells in $ApoE^{-/-}$ mice fed a high fat diet showed that 18.4% expressed TNFα and 2.7% expressed IFNγ. A similar result was obtained in the atherosclerotic aorta. Next, adoptive transfers of B2 cells from wildtype, $TNF\alpha^{-/-}$ or $IFN\gamma^{-/-}$ mice into $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice were carried out. At the end of 8 weeks high fat diet, significant increases in atherosclerosis were recorded for the wild type B2 and $IFN\gamma^{-/-}$ B2 groups but not for the $TNF\alpha^{-/-}$ B2 group in comparison to the control group that did not receive any cells. This was affirmed by the adoptive transfer of wild type B2, $IFN\gamma^{-/-}$ B2 and $TNF\alpha^{-/-}$ B2 cells into B cell-deficient $ApoE^{-/-}\mu MT$ mice. As such, B2 cells can promote atherosclerosis in a TNFα-dependent manner. The data also suggest that TNFα-expressing B2 cells may promote atherosclerosis by enhancing plaque inflammation, increasing the recruitment of macrophages into atherosclerotic plaques and inducing apoptosis in plaque cells. This study is currently under revision for re-submission to the journal *Circulation.*

PART B: Suggested Declaration for Thesis Chapter

[This declaration to be completed for each conjointly authored publication and to be placed at the start of the thesis chapter in which the publication appears.]

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter [5], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	85

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Tin Kyaw	Study design, Experimentation, Data analysis, Result Interpretation, Preparation of manuscript	
Hamid Hosseini	Experimentation	5%
Peter Kanellakis	Experimentation	
Edgar Liu	Experimentation	
Anh Cao	Experimentation	
Peter Tipping	Study concept, Study design, Result Interpretation	
Alex Bobik	Study concept, Study design, Result Interpretation, Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Result Interpretation, Preparation of manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 7/2/2014
Main Supervisor's Signature	[redacted]	**Date** 7/2/2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Tumor necrosis factor *α* produced by conventional B2 lymphocytes promotes atherosclerosis development in hyperlipidaemic ApoE$^{-/-}$ mice

Tay: B2-derived TNFα promotes atherosclerosis

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Abstract

B2 cells are atherogenic but their mechanisms of action are not known. While there is increasing recognition of cytokine production by B2 cells as key regulators of immunity, the role of cytokines produced by B cells in atherosclerosis is also not known. To determine the role of TNFα and IFNγ derived from B2 cells in atherosclerosis development, we compared the adoptive transfer of B2 cells isolated from wildtype (WT) mice, with the transfer of B2 cells from TNFα- or IFNγ-deficient mice into lymphocyte-deficient ($Rag2^{-/-}\gamma c^{-/-}ApoE^{-/-}$) mice. After 8 weeks high fat diet, mice that had received TNFα-deficient B2 cells failed to augment atherosclerosis compared to those that had received TNFα-sufficient (WT or IFNγ-deficient) B2 cells. All transfer groups had comparable body weight and lipid levels. Transferred B2 cells were detected in the spleen as well as in atherosclerotic lesions of the recipient mice. Within atherosclerotic lesions, mice that received TNFα-deficient B2 cells exhibited reduced TNFα expression, reduced macrophage accumulation, reduced MCP1 and IL1β expression, fewer apoptotic cells including apoptotic macrophages and smaller necrotic cores. Transfer of B2 cells from WT or IFNγ-deficient donors, but not B2 cells from TNFα-deficient donors, into B cell-deficient μMT-$ApoE^{-/-}$ mice also showed increased lipid and macrophage accumulation and increased apoptosis and necrotic cores in lesions. Our data indicate that TNFα derived from B2 cells increases arterial TNFα expression to promote inflammation, apoptosis and development of necrotic cores that are characteristic features of unstable rupture-prone atherosclerotic plaques.

Key words: atherosclerosis, inflammation, apoptosis, B2 cells, TNFα

Introduction

Atherosclerosis is a chronic inflammatory disease of large and medium arteries. It begins with a net influx of LDL-cholesterol into the vascular wall. Overtime, oxidation of LDL occurs and immune responses are triggered against oxidized LDL within the intimal layer, resulting in the growth of atherosclerotic lesions[1]. Advanced unstable atherosclerotic lesions are characterized by enhanced inflammation, abundant apoptotic cells and large necrotic cores[2]. In the event that a lesion ruptures, thrombotic occlusion of coronary and cerebral arteries can occur. These may culminate in heart attack and stroke which are the leading causes of global mortality.

Early studies on the role of B cells suggested that they are atheroprotective because the transfer of unfractionated spleen B cells ameliorated splenectomy-aggravated atherosclerosis[3]. However we and Ait-Oufella et al reported that B cell depletion by monoclonal antibody to CD20 ameliorated atherosclerosis in ApoE$^{-/-}$ mice and LDLR$^{-/-}$ mice fed a high fat diet (HFD)[4, 5]. These findings are consistent with the amelioration of human and experimental autoimmune diseases by CD20-targeted B cell depletion and strongly suggest that B cells can be atherogenic[6].

We previously identified conventional B2 cells as the atherogenic B cell subset because the transfer of B2, not B1a cells, into B cell- and lymphocyte-deficient ApoE$^{-/-}$ mice augmented atherosclerosis[4]. We confirmed the atherogenicity of B2 cells by further demonstrating reduced atherosclerosis in ApoE$^{-/-}$ mice that were genetically deficient in the B-cell activating factor receptor (BAFF-R)[7]. ApoE$^{-/-}$BAFF-R$^{-/-}$ mice are selectively deficient in B2 cells[7, 8] but not B1a cells. BAFF is a member of the

tumor necrosis factor superfamily and its association with BAFF-R is crucial for B2 cell development[8]. Similar findings were reported in chimeric BAFF-R-deficient LDLR$^{-/-}$ mice[9]. Furthermore, we recently reported that treatment of ApoE$^{-/-}$ mice with monoclonal antibody to BAFF-R also reduced atherosclerosis[10]. The conundrum of an atheroprotective B cell population reported by Caliguiri et al[3] was resolved by our findings of an atheroprotective B cell subset termed natural IgM-secreting B1a cells[11, 12]. Since then, we have reviewed the opposing roles of atherogenic B2 cells and atheroprotective B1a cells[13].

The aforementioned studies firmly establish B2 cells as an atherogenic B cell subset. However, their mechanisms of action are not known. Cytokines produced by activated B cells have been ascribed important roles in enhancing inflammation[14, 15]. Of note, pivotal roles have been assigned to B2-derived TNFα and IFNγ[14-16]. Moreover, we previously reported that the expression of TNFα was increased in atherosclerotic lesions following B2 cell transfer into lymphocyte-deficient mice[4] and reduced in lesions of BAFF-R-deficient[7] mice and BAFF-R antibody treated mice[10]. CD22^{+} B cells and TNFα have been reported in atherosclerotic lesions of hypercholesterolemic ApoE$^{-/-}$ mice[17]. In humans, TNFα gene transcription is highly and rapidly induced in B cells that are activated through their antigen receptor[18]. A later study found that TNFα expression occurred in memory but not naïve B cells in autoimmune diseases[19]. To date, our knowledge on the roles of TNFα and IFNγ in atherosclerosis is restricted to studies on the effects of global genetic deficiency and blockade of TNFα[20, 21] and IFNγ[22-24]. The cellular sources of these atherogenic cytokines have not hitherto been identified.

Here we show that TNFα but not IFNγ produced by B2 cells is a key cytokine that promotes atherosclerosis development and that it can do so in the absence of all other lymphocyte populations. Our findings indicate that TNFα-producing B2 cells promote atherosclerosis by increasing macrophage recruitment, increasing MCP1 and IL1β expression and increasing apoptosis and necrotic cores in atherosclerotic lesions. Hence, it is likely that B2-derived TNFα enhances the development of unstable and rupture-prone atherosclerotic lesions[2].

Materials and Methods

Animals and B2 cell transfer protocol

Lymphocyte-deficient ApoE$^{-/-}$ mice (ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$), B cell-deficient ApoE$^{-/-}$ mice (μMT-ApoE$^{-/-}$), TNFα$^{-/-}$ and IFNγ$^{-/-}$ mice were maintained at Animal Facilities, Monash Medical Centre, Australia. ApoE$^{-/-}$ mice were maintained at Precinct Animal Centre, Alfred Medical, Research and Education Precinct, Australia. All mice were on C57Bl/6 background. Mice were allowed ad libitum access to water and HFD comprising of 21% fat and 0.15% cholesterol (Specialty Feeds, Glen Forrest, Western Australia). B2 cells (5x10^6 cells) isolated from donor spleens were transferred into 6-8 week old male mice via tail vein injection at the beginning of 8 week HFD. Recipient mice were killed at the end of experiments. Animal procedures were approved by institutional Animal Ethics Committee.

B2 cell isolation

B2 cells were isolated using magnetic B cell isolation kit (Miltenyi Biotech) as described[4].

Flow Cytometry

The following fluorochrome-conjugated anti-mouse antibodies were used: anti-CD19 (V450; Clone 1D3 BD Bioscience), anti-CD22 (PE; clone Cy 34.1 BD Biosciences) and anti-CD5 (APC; Clone 53-7.3 BD Biosciences). Aortic cells were isolated and prepared as described[25, 26]. Briefly, aortas were perfused with PBS containing 20mM EDTA and separated from adipose tissue. Dissected aortas were digested in a cocktail of enzymes namely 125U/ml collagenase XI, 60U/ml hyaluronidase I-s, 60U/ml

DNase I and 450U/ml Collagenase I. After 1 hour incubation at 37^0C, aortas were mashed through a 70μm cell-strainer to prepare single cells suspensions. Aortic cells were pelleted by centrifugation and resuspended in FACS buffer (PBS with 0.5% FCS and 20mM EDTA). FACS analysis of lymphocyte populations in the aorta and spleen was performed as described[7].

Quantification of Atherosclerosis

Atherosclerosis was assessed by total lesion areas and oil red-O (ORO)-stained lipid accumulation as described[7].

Quantification of necrotic core area in atherosclerotic lesions

Necrotic core areas defined as acellular areas in hematoxylin and eosin stained atherosclerotic lesions were analyzed as described[11].

Immunohistochemical staining

Anti-CD68 (Clone FA-11, AbD Serotec), anti-MCP1 (Rabbit polyclonal, Abcam) and anti-IL1β (Rabbit polyclonal, Abcam) antibodies were used to immunostain lesions in aortic roots as described[11]. Apoptotic cells were identified by terminal dUTP nick end-labeling (TUNEL assay kit, Roche) as described[11].

Immunofluorescence staining

To investigate the presence of transferred B2 cells, frozen aortic roots were stained with anti-CD19 (FITC; Clone 1D3 BD Bioscience). Sections counterstained with 4', 6-diamidino-2-phenylindole (DAPI) were observed under Olympus BX61 fluorescence microscope and images captured using FVII Olympus camera.

In co-localization experiments, frozen aortic root sections were stained by TUNEL assay kit (Roche) for apoptotic cells and by anti-CD68 (Clone FA-11, AbD Serotec) for macrophages. Secondary goat anti-rat antibody conjugated with Alexa-Flour 546 (Molecular Probes) was used to detect $CD68^+$ cells in red. TUNEL-positive nuclei were detected by green fluorescence. Nuclei were counterstained with DAPI (blue). Images were scanned using Carl Zeiss Laser Scanning System LSM 510 and analyzed using Zeiss LSM imaging software.

Plasma lipids

Plasma lipids were determined as described before[4].

Real-time PCR analysis

Total RNA from aortic arch and thoracic aorta was extracted and one-step real-time PCR carried out as described[4]. Primers were

IL1β (S) 5'-CCACCTCAATGGACAGAATCTCAA-3'

IL1β (AS) 5'-GTCGTTGCTTGGTTCTCCTTGT-3'

VCAM1 (S) 5'-AGAACCCAGACAGACAGTCC-3'

VCAM1 (AS) 5'-GGATCTTCAGGGAATGAGTAGAC-3'

TNFα (S) 5'-TCTCAGCCTCTTCTCATTCCT-3'

TNFα (AS) 5'-ACTTGGTGGTTTGCTACGAC-3'

IFNγ (S) 5'-AAGTTTGAGGTCAACAACCCAC-3'

IFNγ (AS) 5'-GCTGGCAGAATTATTCTTATTGGG-3'.

Statistical analysis

Comparisons between groups expressed as mean ±SEM were performed by Student-t test or Mann-Whitney U test, depending on whether data were normally distributed, as assessed by Kolmogorov-Smirnov test. For multiple comparisons, results were analyzed using one way analysis of variance (ANOVA) with Dunnett's post-test for data with normal distribution or Kruskal Wallis test with Dunns post-test for data that is not normal. $P < 0.05$ was considered significant.

Results

High fat diet-fed ApoE$^{-/-}$ mice have increased expression of TNFα and IFNγ in B2 cells

TNFα and IFNγ are highly potent inflammatory cytokines. Several studies have shown that TNFα and IFNγ produced by B2 cells are involved in the stimulation of immune responses against infections[27, 28]. Hence, we measured the expression of TNFα and IFNγ in B2 cells during the development of atherosclerosis in ApoE$^{-/-}$ mice. We found that 18.4% of B2 cells expressed TNFα in ApoE$^{-/-}$ mice fed a HFD compared to 16.5% in ApoE$^{-/-}$ mice and 13.7% in wild type (WT) mice on chow diet (Figure 1A). The degree of TNFα expression in B2 cells was significantly higher in ApoE$^{-/-}$ (HFD) mice compared to ApoE$^{-/-}$ and WT mice, as measured by mean fluorescence intensity (Figure 1B).

The expression of IFNγ in B2 cells was considerably lower. We found that only 2.67% of B2 cells expressed IFNγ in ApoE$^{-/-}$ (HFD) mice, 0.67% in ApoE$^{-/-}$ mice and 1.07% in WT mice (Figure 1C). The mean expression of IFNγ in B2 cells was

markedly higher in ApoE$^{-/-}$ (HFD) mice compared to ApoE$^{-/-}$ and WT mice (Figure 1D).

Besides, we carried out FACS analysis of aortic cells from ApoE$^{-/-}$ (HFD) mice and found that 16.7% of aortic B2 cells expressed TNFα while only 0.8% expressed IFNγ (Figure 1E). This is consistent with a recent finding of human aortic B cells expressing TNFα but not IFNγ in the atherosclerotic aorta[29].

B2 cells can promote atherosclerosis in a T-independent but TNFα-dependent manner

To determine the roles of TNFα and IFNγ produced by B2 cells in the development of atherosclerosis, we adoptively transferred WT B2 cells or B2 cells deficient in TNFα or IFNγ into lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice. At the end of 8 weeks HFD, WT B2 and IFNγ$^{-/-}$ B2 cells had markedly increased atherosclerosis. In comparison to the PBS control group, total intimal lesion area and ORO-stained lipid area of lesions were increased by 134% (p=0.002) and 91% (p=0.017) in the WT B2 group and by 169% (p=0.002) and 133% (p=0.006) in the IFNγ$^{-/-}$ B2 group respectively (Figure 2A). In striking contrast, TNFα$^{-/-}$ B2 cells failed to promote atherosclerosis (Figure 2A).

To determine the atherogenic effect of B2-derived TNFα on the entire aorta, we performed en face staining of aorta and found that atherosclerosis was increased by 197% (p=0.008) in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice that received WT B2 cells compared to control mice. As expected, TNFα$^{-/-}$ B2 cells failed to augment atherosclerosis in the

aorta (Figure 2B). There was no significant difference in body weight (Figure 2D) and plasma lipid levels (Figure 2E) among all groups.

B2-derived TNFα promotes arterial inflammation

TNFα is known to be a strong inducer of inflammation. For TNFa to potently promote atherosclerosis in a lymphocyte-free environment, we believe that TNFa may synergize with other inflammatory cytokines. One such cytokine that closely associates with TNFa is IL-1b[30, 31]. Many studies have shown that TNFα acts in concert with IL1β to promote inflammation in diseases including atherosclerosis and post-myocardial infarction[32, 33].

We first measured the expression of TNFa in the lesion by immunohistochemical staining. Eight weeks after WT B2 or IFNγ$^{-/-}$ B2 cells were transferred into ApoE$^{-/-}$ Rag2$^{-/-}$γc$^{-/-}$ mice, the level of TNFα in atherosclerotic lesions was about 531% (p=0.009) and 427% (p=0.019) higher compared to control respectively. However, there was no significant increase following the transfer of TNFα$^{-/-}$ B2 cells (Figure 3A). A similar result was obtained for the mRNA expression of TNFα in the aorta. Using total RNA extracted from the aorta, our assessment showed that aortic TNFα mRNA expression was increased by 104% (p=0.003) and 71% (p=0.019) respectively in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice that received WT B2 cells and IFNγ$^{-/-}$ B2 cells, but not in mice that received TNFα$^{-/-}$ B2 cells, compared to control mice that received PBS (Figure 3B).

We next measured the expression of IL1β in the lesion and found that it was significantly increased by 148% (p<0.001) and 123% (p=0.001) in the WT B2 and

IFNγ$^{-/-}$ B2 transfer groups respectively, but not in the TNFα$^{-/-}$ B2 transfer group, compared to the control group (Figure 3C). Again, this was reflected in our measurement of aortic IL1β mRNA expression which was increased by 109% (p=0.045) and 170% (p=0.007) respectively in mice that received WT B2 cells and IFNγ$^{-/-}$ B2 cells, but not in mice that received TNFα$^{-/-}$ B2 cells, compared to control mice (Figure 3D).

There was no significant difference in arterial mRNA expression of IFNγ in all groups. VCAM-1 expression was also unaffected in all groups (data not shown).

B2-derived TNFα promotes intimal accumulation of macrophages

Given that the degree of inflammation in atherosclerotic lesions is related to macrophage content in lesions, we measured the accumulation of CD68^{+} macrophages in lesions by immunohistochemical staining. We found that the transfer of WT B2 cells and IFNγ$^{-/-}$ B2 cells, but not TNFα$^{-/-}$ B2 cells, into ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice markedly increased the build up of macrophages in the intima by 139% (p=0.022) and 153% (p=0.037) respectively compared to PBS transfer (Figure 4A).

This prompted us to assess another inflammatory cytokine, MCP1, which is mainly responsible for the recruitment of macrophages into lesions[34]. We found that the expression of MCP1 relative to lesion area was significantly higher in mice with WT B2 cells or IFNγ$^{-/-}$ B2 cells (100%; p=0.015 and 130%; p=0.014 respectively), but not in mice with TNFα$^{-/-}$ B2 cells, compared to control mice (Figure 4B). This may explain the reduction in intimal macrophage accumulation in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice that received TNFα $^{-/-}$ B2 cells compared to ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice that received WT

B2 cells and is consistent with studies that reported a role for TNFa in stimulating MCP1 expression in vascular cells[35].

TNFα produced by B2 cells increases apoptotic cell numbers and necrotic areas in atherosclerotic plaques

Besides acting as a proinflammatory cytokine, TNFα can induce apoptotic cell death[36, 37] and inhibit phagocytosis of apoptotic cells by macrophages, a process known as efferocytosis[38, 39]. To determine whether B2-derived TNFα affects the number of apoptotic cells in atherosclerotic lesions, we carried out TUNEL assay to identify apoptotic cells in the atherosclerotic plaque. Our results indicated that the percentage of TUNEL-positive plaque cells relative to total plaque cells was 63% ($p<0.001$) and 53% ($p=0.002$) lower in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice that received $TNF\alpha^{-/-}$ B2 cells compared to those that received WT B2 cells and $IFN\gamma^{-/-}$ B2 cells respectively (Figure 5A). In addition, necrotic plaque areas, measured as acellular regions in H&E stained lesions, were also lower by 21% ($p=0.008$) and 20% ($p=0.008$) in the $TNF\alpha^{-/-}$ B2 group compared to the WT B2 and $IFN\gamma^{-/-}$ B2 groups respectively (Figure 5B). Collectively, the data suggests that B2-derived TNFα increases the number of apoptotic cells and size of necrotic cores in developing lesions. Our findings are consistent with the report that TNFα-deficiency not only results in less advanced plaques but also less plaque apoptosis and necrosis in $ApoE^{-/-}$ mice[20].

B2-derived TNFα may target macrophages in lesions for apoptosis

We examined whether macrophages are apoptotic targets of B2-derived TNFα in atherosclerotic lesions. We carried out double immunofluorescence staining to detect

CD68-postive macrophages and TUNEL-positive apoptotic cells. We found that atherosclerotic plaques of $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice that received WT B2 cells contained $CD68^{+}Tunel^{+}$ apoptotic macrophages (Figure 6A). However, there were less $CD68^{+}Tunel^{+}$ cells observed in mice with $TNF\alpha^{-/-}$ B2 cells (Figure 6B). Apoptotic macrophages may not only contribute to the total accumulation of apoptotic cells but also result in inefficient efferocytosis. In time, secondary necrosis is likely to become prevalent and larger necrotic cores develop in atherosclerotic lesions[40].

TNFα-deficient B2 cells do not promote atherosclerosis in B cell-deficient μMT-ApoE-/- mice

To determine whether TNFα produced by B2 cells also augment atherosclerosis in mice deficient only in B cells, we adoptively transferred WT B2 cells or B2 cells deficient in TNFα or IFNγ into B cell-deficient $ApoE^{-/-}$μMT mice. After 8 weeks of HFD, WT B2 cells and $IFN\gamma^{-/-}$ B2 cells were found to have markedly increased atherosclerosis in $ApoE^{-/-}$μMT mice. The assessment of lesions in the aortic sinus showed that the transfers of WT B2 cells and $IFN\gamma^{-/-}$ B2 cells augmented atherosclerosis by 87% ($p=0.003$) and 119% ($p<0.001$) respectively, as measured by total lesion area, and by 85% ($p=0.008$) and 144% ($p<0.001$) respectively, as measured by ORO-stained lipid area of lesions, compared to PBS control transfer (Figure 7A). In contrast, the transfer of $TNF\alpha^{-/-}$ B2 cells failed to augment atherosclerosis (Figure 7A). A similar result was obtained for $CD68^{+}$ macrophage accumulation (Figure 7B). All B2 recipient groups had comparable reconstitution of B2 cells in the spleen (Figure 7C). Body weight (Figure 7D) and plasma lipid levels (Figure 7E) did not differ significantly in all groups. Furthermore, the number of apoptotic cells (Figure 7F) and size of necrotic cores (Figure 7G) were markedly

increased by TNFα-sufficient B2 cells (WT and IFNγ$^{-/-}$) but not TNFα-deficient B2 cells.

Adoptively transferred B2 cells traffick to the atherosclerotic lesions of ApoE-/-Rag2-/-γc-/- mice and ApoE-/-μMT mice

To determine whether adoptively transferred B2 cells home to the atherosclerotic lesions, we carried out immunofluorescence staining to detect CD19^{+} cells in lesions. Adoptively transferred WT B2 cells, TNFα-/- B2 cells and IFNγ-/- B2 cells were found in atherosclerotic lesions of the respective recipient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice (Figure 8A). We further confirmed the specificity by adoptively transferring B2 cells from Ly5.1 congenic mice into B cell-deficient ApoE$^{-/-}$μMT mice. Indeed, CD19^{+}Ly5.1^{+} B2 cells were localized in the atherosclerotic lesion of ApoE$^{-/-}$μMT mice (Figure 8B). Similarly, CD19^{+} WT B2 cells, TNFα-/- B2 cells and IFNγ-/- B2 cells were present in the lesions of the respective recipient ApoE$^{-/-}$μMT mice (Figure 8C). Our observation is consistent with reports of B cells in human and mouse atherosclerotic lesions[17, 41] and other reports of adoptively transferred B2 cells trafficking to the site of lesions in mice[4, 26]. Hence, it is likely that TNFa-producing B2 cells elicit atherogenic responses directly within lesions.

Discussion

In the present study, wild type B2 cells markedly increased atherosclerosis in lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice and in B cell-deficient ApoE$^{-/-}$μMT mice. These findings are consistent with our previous report[4]. In striking contrast, TNFα-deficient but not IFNγ- deficient B2 cells failed to augment atherosclerosis in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ as well as in ApoE$^{-/-}$μMT mice. Our findings indicate that TNFα but not IFNγ produced by B2 cells is essential for B2 cells to promote atherosclerosis. The results from lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice suggest that the TNFα-mediated atherogenic response of B2 cells can occur independently of T-dependent antibody production and of all other lymphocyte populations. Our findings are consistent with the notion that cytokine-producing B cells are key regulators of immunity[14-16, 42]. Previous studies have shown a role for TNFα in atherogenesis given that their inhibition or the absence of their receptors reduces atherosclerosis[20, 43-47]. Our findings identify TNFα-producing B2 cells as key atherogenic cells.

Following the adoptive transfer of B2 cells into ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice, we found that the transferred B2 cells not only trafficked to the spleen but also to atherosclerotic lesions at the end of 8 weeks HFD. These findings are consistent with previous reports of detecting adoptively transferred B cells in the lymphoid organs and aorta of recipient mice[4, 26, 48]. Moreover, our observations suggest that B2 cells persist for at least eight weeks in lymphocyte-deficient and B cell-deficient ApoE$^{-/-}$ mice.

Despite the low proportion of B2 cells identified in recipient mice at the end of our experiments, the transfer of WT but not TNFα-deficient B2 cells significantly

augmented arterial TNFα mRNA and protein in lesions. As TNFα is predominantly produced by macrophages[49], we propose that B2-derived TNFα may contribute to total TNFα production by macrophages, the largest cell constituent in atherosclerotic lesions. This is supported by reports of TNFα inducing macrophages to produce even more TNFα through a positive feedback loop[49-51]. Our findings are in agreement with the report in humans that significant amounts of TNFα is produced by activated memory B2 cells and, as a result, enhances pathological immune responses in autoimmunity[19]. In addition, TNFα gene transcription is one of the earliest events activated in human B cells that are stimulated through their antigen receptors or CD40 and interleukin-4[18]. Hence, our data is in line with B2 cells as a key cellular source of TNFα that augments TNFα expression in atherosclerotic lesions[4, 7, 10].

The site-specific inflammatory role of B2-derived TNFα in atherosclerotic lesions is supported by the reduction in lesion macrophage accumulation and the reduction in IL1β and MCP1 expression in lesions after adoptive transfer of $TNF\alpha^{-/-}$ B2 cells compared to transfer of WT B2 cells into $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice. TNFα is known to stimulate MCP1 production in a variety of cell types including macrophages and endothelial cells in the vasculature[35, 52]. MCP1 is a key chemotactic factor for macrophage recruitment into atherosclerotic lesions[34]. The increase in MCP1 expression likely brought about increased macrophage accumulation in lesions of mice with WT B2 cells. Conversely, mice with $TNF\alpha^{-/-}$ B2 cells did not exhibit increased MCP1 expression and macrophage accumulation in lesions.

The increased arterial and lesion IL1β expression in mice with WT B2 cells but not in mice with $TNF\alpha^{-/-}$ B2 cells may be explained by the role of TNFα in upregulating

IL1β expression in macrophages[53-55]. Consequently, IL1β can induce macrophages to produce more IL1β and even TNFα[55-57]. IL1β has also been shown to synergize with TNFα to promote inflammation by elevating TNF-receptor 2 expression. This can result in enhanced cell sensitivity to the pathologic effects of TNFα[58]. A more direct atherogenic response of IL1β and TNFα pertains to their involvement in enhancing foam cell formation through the inhibition of lipid catabolism in macrophages[59]. In addition to promoting inflammation, IL1β and TNFα are alleged to combine in rendering cells vulnerable to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[60]. This could be a reason behind the increase in lesion apoptotic cells in mice with WT B2 cells but not $TNF\alpha^{-/-}$ B2 cells.

There is substantial evidence of TNFα promoting apoptosis in atherosclerotic lesions[36,37] . The accumulation of apoptotic cells is a hallmark of advanced unstable, rupture-prone atherosclerotic lesions in humans[61, 62] and mice[63]. Due to the impairment of efferocytosis in advanced lesions, increased secondary necrosis can transpire and become dominant[64]. The resultant expansion of necrotic cores is also a characteristic feature of unstable plaques[2]. The lower number of lesion apoptotic cells in mice that were given $TNF\alpha^{-/-}$ B2 cells compared to those that were given WT B2 cells indicates a role for TNFα produced by B2 cells in exerting cell death by apoptosis in atherosclerotic lesions. Several studies have claimed that TNFα is cytotoxic to a variety of cell lines in vitro[65] and induces macrophage apoptosis by inhibiting NF-κB and through caspase-dependent and independent pathways[66, 67]. These may be the reasons behind the increased presence of $TUNEL^{+}CD68^{+}$ macrophages in lesions of mice transferred with WT B2 cells compared to mice with $TNF\alpha^{-/-}$ B2 cells. TNFα mainly induces apoptosis by binding to TNF receptor 1[36, 68].

Efferocytosis is the process whereby professional and non-professional phagocytes engulf and dispose apoptotic cells[39]. Macrophages are the predominant phagocytes within atherosclerotic lesions. Hence, it is paramount that they remain functional and conduct efferocytosis efficiently to contain apoptosis and necrosis in lesions. However, this may not be the case when macrophages, themselves, are targeted for apoptosis as suggested above. Furthermore, a recent report showed that TNFα could inhibit the efferocytic function of macrophages[39]. This was particularly pervasive when the macrophages were exposed to oxidants and inflammatory factors – a classic environment in advanced atherosclerotic plaques.

Together, our data suggest that B2-derived TNFα promotes the development of vulnerable, rupture-prone atherosclerotic plaques. It is conceivable that TNFα-producing B2 cells share a similar role with cytotoxic CD8 T cells to form unstable atherosclerotic lesions, given that we have also recently reported CD8 T cells enhancing apoptosis in lesions[69].

Accelerated atherosclerosis is associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. Inflammatory cytokines are deemed to be the culprits behind the enhancement of atherosclerosis in autoimmune patients[70]. Our results corroborate studies that claim TNFα-targeted and B cell-depletion therapies reduce cardiovascular risks in patients with rheumatoid arthritis[71, 72].

In summary, our study demonstrates that TNFα-producing B2 cells augment the development of atherosclerosis. They may do so by eliciting multiple effects which

result in increased recruitment of macrophages into lesions, increased expression of other inflammatory cytokines (IL1β and MCP1) in lesions and accumulation of apoptotic cells and expansion of necrotic cores in mature lesions. These findings provide novel insights into the atherogenic behavior of TNFα-producing B2 cells that can facilitate future therapeutic design.

References

1. Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nature immunology*. 2011;12:204-212
2. Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:1282-1292
3. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by b cells of hypercholesterolemic mice. *The Journal of clinical investigation*. 2002;109:745-753
4. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional b2 b cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *Journal of immunology*. 2010;185:4410-4419
5. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vre E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF, Mallat Z. B cell depletion reduces the development of atherosclerosis in mice. *The Journal of experimental medicine*. 2010;207:1579-1587
6. Townsend MJ, Monroe JG, Chan AC. B-cell targeted therapies in human autoimmune diseases: An updated perspective. *Immunological reviews*. 2010;237:264-283
7. Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, Tipping P, Bobik A, Toh BH. Depletion of b2 but not b1a b cells in baff receptor-deficient apoe mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PloS one*. 2012;7:e29371
8. Mackay F, Schneider P. Cracking the baff code. *Nature reviews. Immunology*. 2009;9:491-502
9. Sage AP, Tsiantoulas D, Baker L, Harrison J, Masters L, Murphy D, Loinard C, Binder CJ, Mallat Z. Baff receptor deficiency reduces the development of atherosclerosis in mice--brief report. *Arteriosclerosis, thrombosis, and vascular biology*. 2012;32:1573-1576
10. Kyaw T, Cui P, Tay C, Kanellakis P, Hosseini H, Liu E, Rolink AG, Tipping P, Bobik A, Toh BH. Baff receptor mab treatment ameliorates development and progression of atherosclerosis in hyperlipidemic apoe(-/-) mice. *PloS one*. 2013;8:e60430
11. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, Bobik A, Toh BH. B1a b lymphocytes are atheroprotective by secreting natural igm that increases

igm deposits and reduces necrotic cores in atherosclerotic lesions. *Circulation research*. 2011;109:830-840
12. Kyaw T, Tipping P, Bobik A, Toh BH. Protective role of natural igm-producing b1a cells in atherosclerosis. *Trends in cardiovascular medicine*. 2012;22:48-53
13. Kyaw T, Tipping P, Toh BH, Bobik A. Current understanding of the role of b cell subsets and intimal and adventitial b cells in atherosclerosis. *Current opinion in lipidology*. 2011;22:373-379
14. Lund FE. Cytokine-producing b lymphocytes-key regulators of immunity. *Curr Opin Immunol*. 2008;20:332-338
15. Pistoia V. Production of cytokines by human b cells in health and disease. *Immunol Today*. 1997;18:343-350
16. Vaughan AT, Roghanian A, Cragg MS. B cells--masters of the immunoverse. *Int J Biochem Cell Biol*. 2011;43:280-285
17. Zhou X, Hansson GK. Detection of b cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein e knockout mice. *Scandinavian journal of immunology*. 1999;50:25-30
18. Boussiotis VA, Nadler LM, Strominger JL, Goldfeld AE. Tumor necrosis factor alpha is an autocrine growth factor for normal human b cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;91:7007-7011
19. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, Kim HJ, Bar-Or A. Distinct effector cytokine profiles of memory and naive human b cell subsets and implication in multiple sclerosis. *Journal of immunology*. 2007;178:6092-6099
20. Canault M, Peiretti F, Poggi M, Mueller C, Kopp F, Bonardo B, Bastelica D, Nicolay A, Alessi MC, Nalbone G. Progression of atherosclerosis in apoe-deficient mice that express distinct molecular forms of tnf-alpha. *The Journal of pathology*. 2008;214:574-583
21. Wollin M, Abele S, Bruns H, Weyand M, Kalden JR, Ensminger SM, Spriewald BM. Inhibition of tnf-alpha reduces transplant arteriosclerosis in a murine aortic transplant model. *Transpl Int*. 2009;22:342-349
22. Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. Ifn-gamma potentiates atherosclerosis in apoe knock-out mice. *The Journal of clinical investigation*. 1997;99:2752-2761
23. Whitman SC, Ravisankar P, Elam H, Daugherty A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein e-/- mice. *The American journal of pathology*. 2000;157:1819-1824
24. Koga M, Kai H, Yasukawa H, Yamamoto T, Kawai Y, Kato S, Kusaba K, Kai M, Egashira K, Kataoka Y, Imaizumi T. Inhibition of progression and stabilization of plaques by postnatal interferon-gamma function blocking in apoe-knockout mice. *Circulation research*. 2007;101:348-356
25. Butcher MJ, Herre M, Ley K, Galkina E. Flow cytometry analysis of immune cells within murine aortas. *J Vis Exp*. 2011
26. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially l-selectin dependent. *J Exp Med*. 2006;203:1273-1282
27. Menard LC, Minns LA, Darche S, Mielcarz DW, Foureau DM, Roos D, Dzierszinski F, Kasper LH, Buzoni-Gatel D. B cells amplify ifn-gamma production by t cells via a tnf-alpha-mediated mechanism. *J Immunol*. 2007;179:4857-4866
28. Ganapamo F, Dennis VA, Philipp MT. Cd19(+) cells produce ifn-gamma in mice infected with borrelia burgdorferi. *Eur J Immunol*. 2001;31:3460-3468
29. Hamze M, Desmetz C, Berthe ML, Roger P, Boulle N, Brancherau P, Picard E, Guzman C, Tolza C, Guglielmi P. Characterization of resident b cells of vascular walls in human atherosclerotic patients. *J Immunol*. 2013;191:3006-3016
30. Moore PE, Lahiri T, Laporte JD, Church T, Panettieri RA, Jr., Shore SA. Selected contribution: Synergism between tnf-alpha and il-1 beta in airway smooth muscle

cells: Implications for beta-adrenergic responsiveness. *J Appl Physiol (1985)*. 2001;91:1467-1474
31. Wankowicz Z, Megyeri P, Issekutz A. Synergy between tumour necrosis factor alpha and interleukin-1 in the induction of polymorphonuclear leukocyte migration during inflammation. *J Leukoc Biol*. 1988;43:349-356
32. Zhou C, Pridgen B, King N, Xu J, Breslow JL. Hyperglycemic ins2akitaldlr(-)/(-) mice show severely elevated lipid levels and increased atherosclerosis: A model of type 1 diabetic macrovascular disease. *J Lipid Res*. 2011;52:1483-1493
33. Turner NA, Mughal RS, Warburton P, O'Regan DJ, Ball SG, Porter KE. Mechanism of tnfalpha-induced il-1alpha, il-1beta and il-6 expression in human cardiac fibroblasts: Effects of statins and thiazolidinediones. *Cardiovasc Res*. 2007;76:81-90
34. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell*. 1998;2:275-281
35. Murao K, Ohyama T, Imachi H, Ishida T, Cao WM, Namihira H, Sato M, Wong NC, Takahara J. Tnf-alpha stimulation of mcp-1 expression is mediated by the akt/pkb signal transduction pathway in vascular endothelial cells. *Biochem Biophys Res Commun*. 2000;276:791-796
36. Rath PC, Aggarwal BB. Tnf-induced signaling in apoptosis. *J Clin Immunol*. 1999;19:350-364
37. Micheau O, Tschopp J. Induction of tnf receptor i-mediated apoptosis via two sequential signaling complexes. *Cell*. 2003;114:181-190
38. Thorp E, Subramanian M, Tabas I. The role of macrophages and dendritic cells in the clearance of apoptotic cells in advanced atherosclerosis. *European journal of immunology*. 2011;41:2515-2518
39. McPhillips K, Janssen WJ, Ghosh M, Byrne A, Gardai S, Remigio L, Bratton DL, Kang JL, Henson P. Tnf-alpha inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase a2 and oxidant-dependent mechanisms. *Journal of immunology*. 2007;178:8117-8126
40. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: The importance of lesion stage and phagocytic efficiency. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2255-2264
41. Burioni R, Canducci F, Saita D, Perotti M, Mancini N, De Marco D, Clementi N, Chieffo A, Denaro M, Cianflone D, Manfredi AA, Colombo A, Maseri A, Clementi M. Antigen-driven evolution of b lymphocytes in coronary atherosclerotic plaques. *J Immunol*. 2009;183:2537-2544
42. Leon B, Ballesteros-Tato A, Misra RS, Wojciechowski W, Lund FE. Unraveling effector functions of b cells during infection: The hidden world beyond antibody production. *Infectious disorders drug targets*. 2012;12:213-221
43. Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein e knockout mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:2137-2142
44. Xanthoulea S, Thelen M, Pottgens C, Gijbels MJ, Lutgens E, de Winther MP. Absence of p55 tnf receptor reduces atherosclerosis, but has no major effect on angiotensin ii induced aneurysms in ldl receptor deficient mice. *PloS one*. 2009;4:e6113
45. Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in apoe*3-leiden transgenic mice. *Cardiovasc Res*. 2005;66:179-185
46. Ohta H, Wada H, Niwa T, Kirii H, Iwamoto N, Fujii H, Saito K, Sekikawa K, Seishima M. Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in apoe-deficient mice. *Atherosclerosis*. 2005;180:11-17
47. Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G. Exclusive expression of transmembrane tnf-alpha in mice

reduces the inflammatory response in early lipid lesions of aortic sinus. *Atherosclerosis*. 2004;172:211-218
48. Roth R, Mamula MJ. Trafficking of adoptively transferred b lymphocytes in b-lymphocyte-deficient mice. *J Exp Biol*. 1997;200:2057-2062
49. Trinchieri G. Regulation of tumor necrosis factor production by monocyte-macrophages and lymphocytes. *Immunol Res*. 1991;10:89-103
50. Mace KF, Ehrke MJ, Hori K, Maccubbin DL, Mihich E. Role of tumor necrosis factor in macrophage activation and tumoricidal activity. *Cancer Res*. 1988;48:5427-5432
51. Heidenreich S, Weyers M, Gong JH, Sprenger H, Nain M, Gemsa D. Potentiation of lymphokine-induced macrophage activation by tumor necrosis factor-alpha. *Journal of immunology*. 1988;140:1511-1518
52. Ciesielski CJ, Andreakos E, Foxwell BM, Feldmann M. Tnfalpha-induced macrophage chemokine secretion is more dependent on nf-kappab expression than lipopolysaccharides-induced macrophage chemokine secretion. *European journal of immunology*. 2002;32:2037-2045
53. Danis VA, Kulesz AJ, Nelson DS, Brooks PM. Cytokine regulation of human monocyte interleukin-1 (il-1) production in vitro. Enhancement of il-1 production by interferon (ifn) gamma, tumour necrosis factor-alpha, il-2 and il-1, and inhibition by ifn-alpha. *Clinical and experimental immunology*. 1990;80:435-443
54. Shingu M, Nobunaga M, Ezaki I, Yoshioka K. Recombinant human il-1 beta and tnf-alpha stimulate production of il-1 alpha and il-1 beta by vascular smooth muscle cells and il-1 alpha by vascular endothelial cells. *Life sciences*. 1991;49:241-246
55. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA, Jr., O'Connor JV. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *The Journal of experimental medicine*. 1986;163:1433-1450
56. Ledesma E, Martinez I, Cordova Y, Rodriguez-Sosa M, Monroy A, Mora L, Soto I, Ramos G, Weiss B, Santiago Osorio E. Interleukin-1 beta (il-1beta) induces tumor necrosis factor alpha (tnf-alpha) expression on mouse myeloid multipotent cell line 32d cl3 and inhibits their proliferation. *Cytokine*. 2004;26:66-72
57. Ikejima T, Okusawa S, Ghezzi P, van der Meer JW, Dinarello CA. Interleukin-1 induces tumor necrosis factor (tnf) in human peripheral blood mononuclear cells in vitro and a circulating tnf-like activity in rabbits. *The Journal of infectious diseases*. 1990;162:215-223
58. Saperstein S, Chen L, Oakes D, Pryhuber G, Finkelstein J. Il-1beta augments tnf-alpha-mediated inflammatory responses from lung epithelial cells. *J Interferon Cytokine Res*. 2009;29:273-284
59. Persson J, Nilsson J, Lindholm MW. Interleukin-1beta and tumour necrosis factor-alpha impede neutral lipid turnover in macrophage-derived foam cells. *BMC Immunol*. 2008;9:70
60. Mezosi E, Wang SH, Utsugi S, Bajnok L, Bretz JD, Gauger PG, Thompson NW, Baker JR, Jr. Interleukin-1beta and tumor necrosis factor (tnf)-alpha sensitize human thyroid epithelial cells to tnf-related apoptosis-inducing ligand-induced apoptosis through increases in procaspase-7 and bid, and the down-regulation of p44/42 mitogen-activated protein kinase activity. *J Clin Endocrinol Metab*. 2004;89:250-257
61. Bjorkerud S, Bjorkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and t cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol*. 1996;149:367-380
62. Geng YJ, Libby P. Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme. *Am J Pathol*. 1995;147:251-266
63. Tabas I. Apoptosis and efferocytosis in mouse models of atherosclerosis. *Current drug targets*. 2007;8:1288-1296
64. Silva MT. Secondary necrosis: The natural outcome of the complete apoptotic program. *FEBS letters*. 2010;584:4491-4499

65. Williamson BD, Carswell EA, Rubin BY, Prendergast JS, Old LJ. Human tumor necrosis factor produced by human b-cell lines: Synergistic cytotoxic interaction with human interferon. *Proceedings of the National Academy of Sciences of the United States of America*. 1983;80:5397-5401
66. Liu H, Ma Y, Pagliari LJ, Perlman H, Yu C, Lin A, Pope RM. Tnf-alpha-induced apoptosis of macrophages following inhibition of nf-kappa b: A central role for disruption of mitochondria. *Journal of immunology*. 2004;172:1907-1915
67. Tran TM, Temkin V, Shi B, Pagliari L, Daniel S, Ferran C, Pope RM. Tnfalpha-induced macrophage death via caspase-dependent and independent pathways. *Apoptosis*. 2009;14:320-332
68. Kleemann R, Zadelaar S, Kooistra T. Cytokines and atherosclerosis: A comprehensive review of studies in mice. *Cardiovasc Res*. 2008;79:360-376
69. Kyaw T, Winship A, Tay C, Kanellakis P, Hosseini H, Cao A, Li P, Tipping P, Bobik A, Toh BH. Cytotoxic and proinflammatory cd8+ t lymphocytes promote development of vulnerable atherosclerotic plaques in apoe-deficient mice. *Circulation*. 2013;127:1028-1039
70. Shoenfeld Y, Gerli R, Doria A, Matsuura E, Cerinic MM, Ronda N, Jara LJ, Abu-Shakra M, Meroni PL, Sherer Y. Accelerated atherosclerosis in autoimmune rheumatic diseases. *Circulation*. 2005;112:3337-3347
71. Greenberg JD, Furer V, Farkouh ME. Cardiovascular safety of biologic therapies for the treatment of ra. *Nat Rev Rheumatol*. 2012;8:13-21
72. Novikova DS, Popkova TV, Nasonov EL. The effect of anti-b-cell therapy on the development of atherosclerosis in patients with rheumatoid arthritis. *Curr Pharm Des*. 2012;18:1512-1518

Figure 1

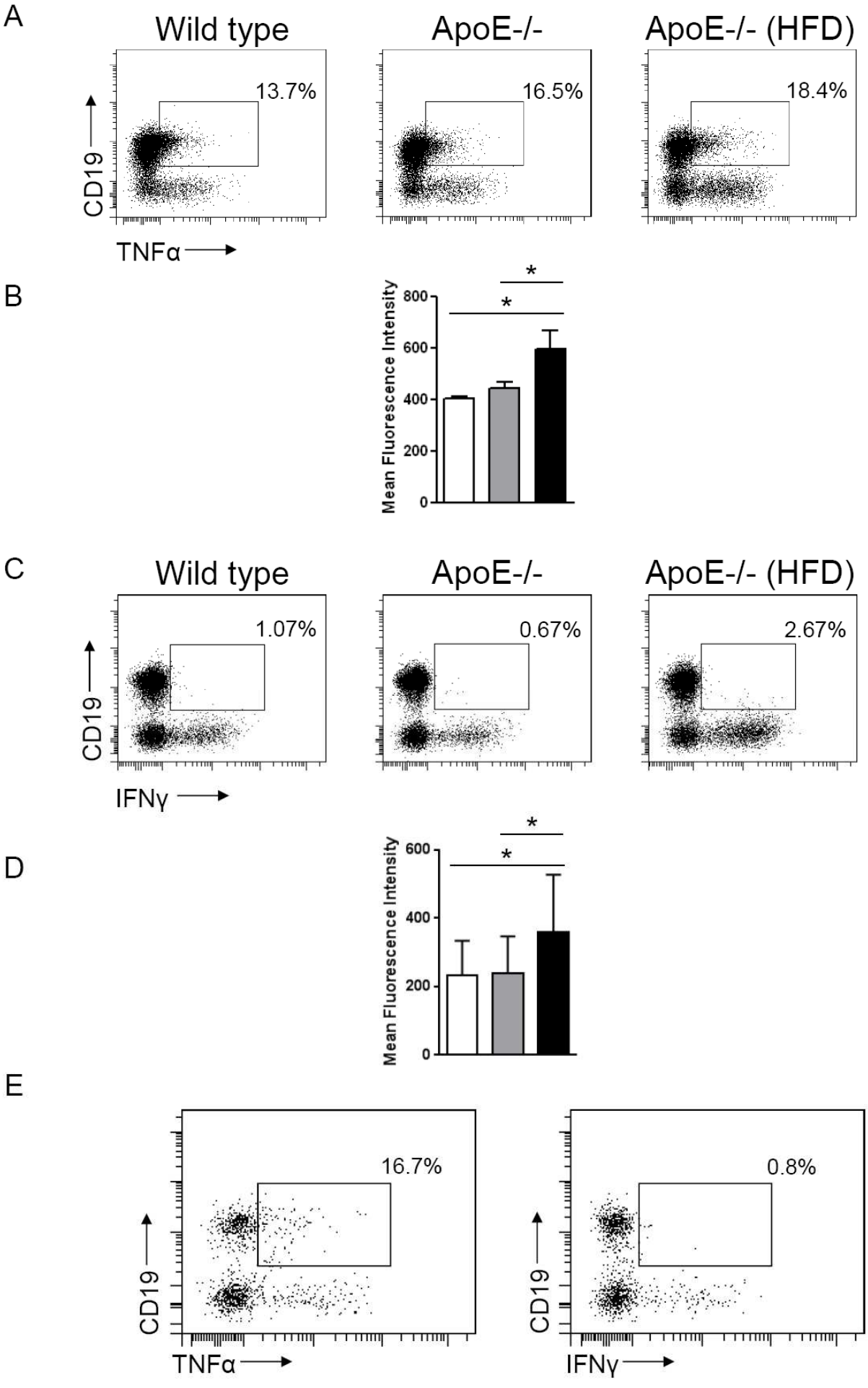

A
Wild type
ApoE-/-
ApoE-/- (HFD)
13.7%
16.5%
18.4%
CD19
TNFα
B
Mean Fluorescence Intensity
800
600
400
200
0
*
*
C
Wild type
ApoE-/-
ApoE-/- (HFD)
1.07%
0.67%
2.67%
CD19
IFNγ
D
Mean Fluorescence Intensity
600
400
200
0
*
*
E
16.7%
0.8%
CD19
TNFα
CD19
IFNγ

Figure 1 High fat diet fed ApoE$^{-/-}$mice have increased expression of TNFα and IFNγ in B2 cells.

Spleen cells were obtained from wild type mice, ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice fed a high fat diet (HFD). Spleen cells were stimulated with a cocktail of PMA, ionomycin, brefeldin A and monensin for 5-6 hours. Cells were subsequently stained for surface CD19 and for intracellular TNFα and IFNγ. **(A)** Representative FACS plots show gated CD19^{+} TNFα^{+} cells. Figures indicate the percentage of CD19^{+} cells that express TNFα. **(B)** Mean fluorescence intensity of TNFα expression in CD19^{+} cells was significantly higher in ApoE$^{-/-}$ mice after HFD. **(C)** Representative FACS plots show gated CD19^{+} IFNγ $^{+}$ cells. Figures indicate the percentage of CD19^{+} cells that express IFNγ. **(D)** Mean fluorescence intensity of IFNγ expression in CD19^{+} cells was significantly higher in ApoE$^{-/-}$ mice after HFD. Data represent (mean±SEM) and n=8-9 per group. * P<0.05. **(E)** Aortic cells were obtained from ApoE$^{-/-}$ mice after HFD. Cells were stimulated and stained for CD19, TNFα and IFNγ as described above. Representative FACS plots show gated CD19^{+} TNFα^{+} cells and CD19^{+} IFNγ $^{+}$ cells. Figures indicate the percentage of CD19^{+} cells that express TNFα and IFNγ respectively.

Figure 2

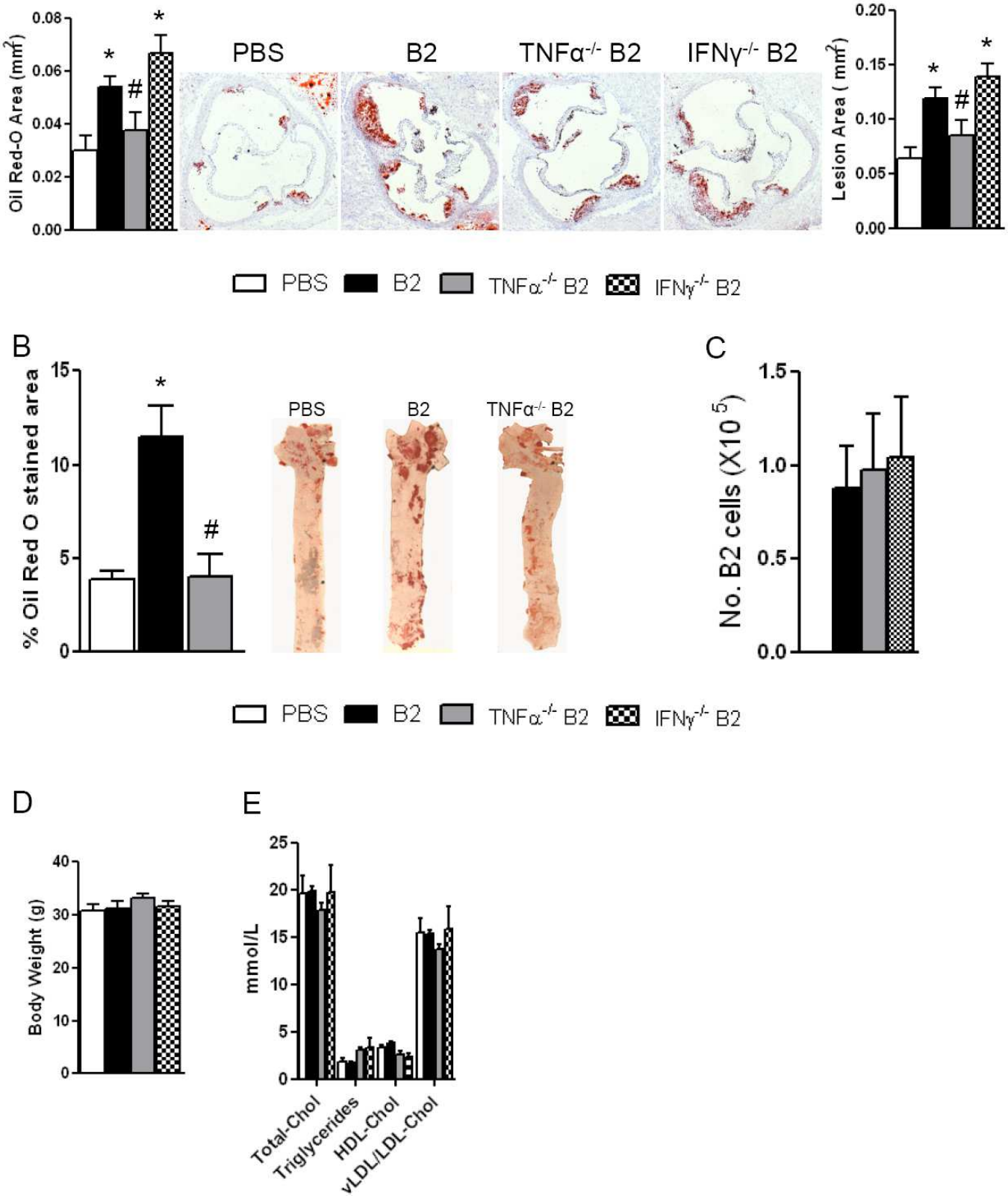
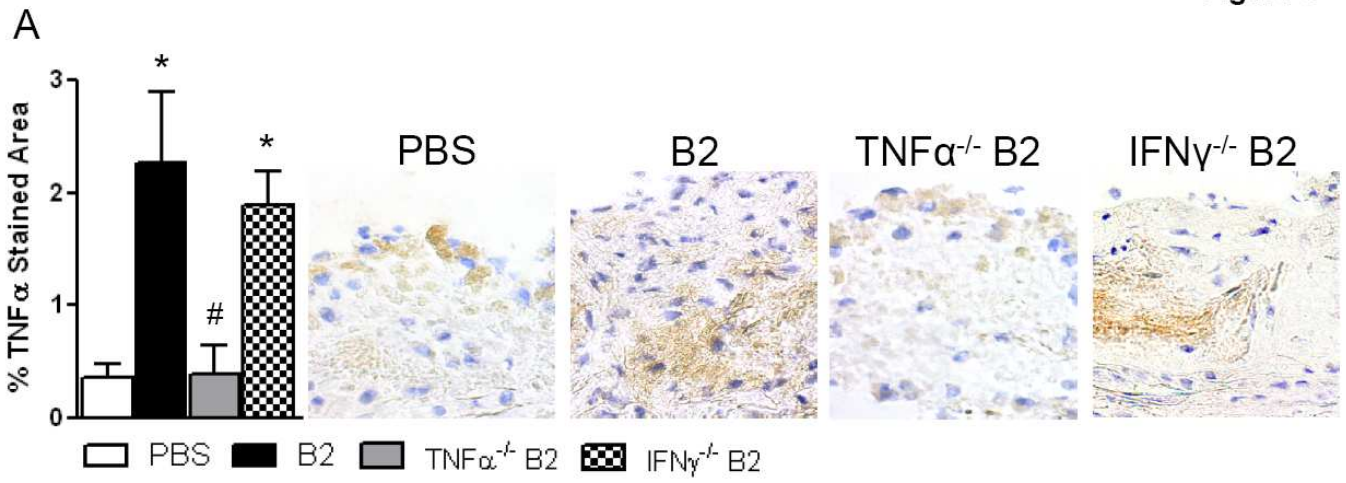
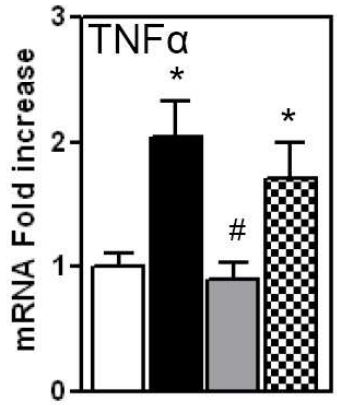
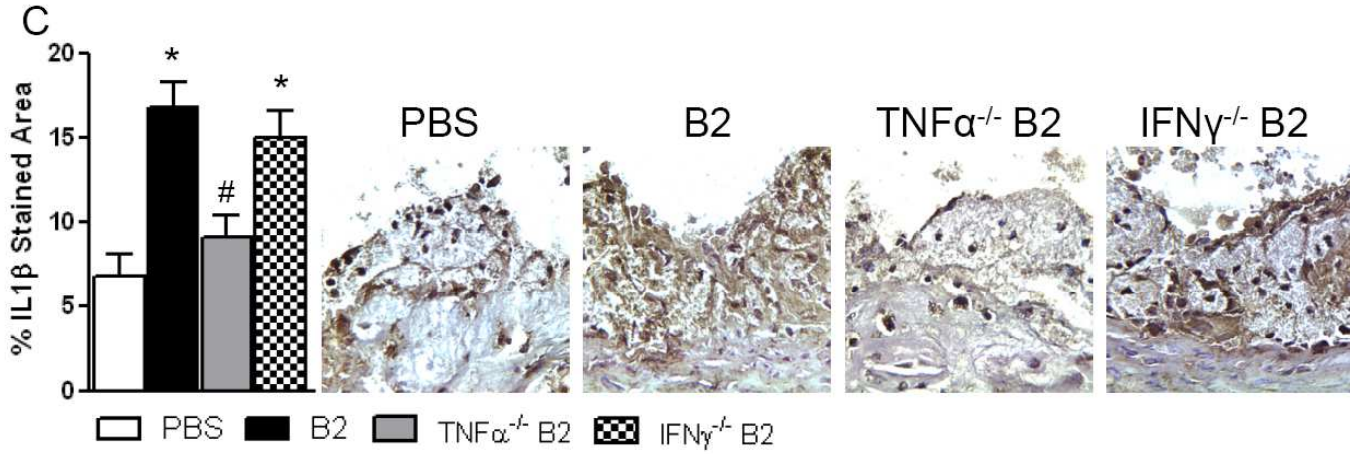

A
Oil Red-O Area (mm²)
0.08
0.06
0.04
0.02
0.00
*
#
*
PBS
B2
TNFα-/- B2
IFNγ-/- B2
Lesion Area (mm²)
0.20
0.15
0.10
0.05
0.00
*
#
*
PBS
B2
TNFα-/- B2
IFNγ-/- B2
B
% Oil Red O stained area
15
10
5
0
*
#
PBS
B2
TNFα-/- B2
C
No. B2 cells (X10 5)
1.5
1.0
0.5
0.0
PBS
B2
TNFα-/- B2
IFNγ-/- B2
D
Body Weight (g)
40
30
20
10
0
E
mmol/L
25
20
15
10
5
0
Total-Chol
Triglycerides
HDL-Chol
vLDL/LDL-Chol

Figure 2 Wild type B2 and IFNγ$^{-/-}$ B2, but not TNFα$^{-/-}$ B2 cells, augment atherosclerosis in lymphocyte-deficient Rag2$^{-/-}$γc$^{-/-}$ApoE$^{-/-}$mice. B2 cells from WT, TNFα$^{-/-}$ and IFNγ$^{-/-}$ mice were adoptively transferred into lymphocyte-deficient ApoE$^{-/-}$ mice. Control mice were given vehicle-PBS. After 8 weeks HFD, the development of atherosclerosis was assessed in the aortic sinus. **(A)** Representative microimages show that mice with TNFα-sufficient (WT or IFNγ$^{-/-}$) B2 cells, but not TNFα-deficient (TNFα$^{-/-}$) B2 cells, have markedly increased for total intimal lesion area and Oil Red O-stained lipid accumulation in atherosclerotic lesions (n=7-10 per group) Original magnification X 60. **(B)** En face ORO staining of aorta show that WT B2 cells but not TNFα$^{-/-}$ B2 cells significantly increased atherosclerosis throughout the aorta (n=5 per group). (**C**) Comparable levels of WT B2, TNFα$^{-/-}$ B2 and IFNγ$^{-/-}$ B2 cells were found in the spleen. (**D**) Body weight and (**E**) plasma lipid levels were not significantly affected in all groups. Data represent (mean±SEM). * P<0.05 compared to vehicle-PBS, # P<0.05 compared to WT B2.


A
% TNFα Stained Area
PBS
B2
TNFα-/- B2
IFNγ-/- B2


B
TNFα
mRNA Fold increase


C
% IL1β Stained Area
PBS
B2
TNFα-/- B2
IFNγ-/- B2

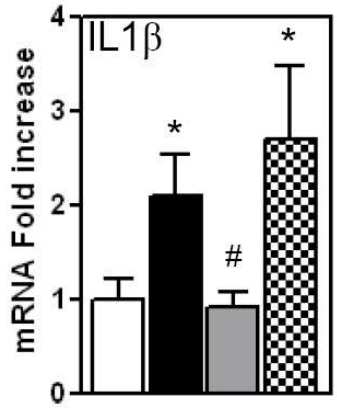

D
IL1β
mRNA Fold increase

Figure 3 B2-derived TNFα promotes expression of TNFα and IL1β in the atherosclerotic aorta.

Immunohistochemical peroxidase staining was carried out to measure the expression of TNFα and IL1β in atherosclerotic lesions. Expression of **(A)** TNFα and **(C)** IL1β in atherosclerotic lesions were significantly increased in lymphocyte-deficient $ApoE^{-/-}$ $Rag2^{-/-}\gamma c^{-/-}$ mice that received WT B2 cells or $IFN\gamma^{-/-}$ B2 cells but not $TNF\alpha^{-/-}$ B2 cells. Original magnification X 160. Quantitative Realtime-PCR was also carried out to assess arterial mRNA expression of TNFα and IL1β. $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice transferred with WT B2 cells or $IFN\gamma^{-/-}$ B2 cells showed increased levels of **(B)** TNFα and **(D)** IL1β aortic mRNA transcripts compared to control mice. Mice that received $TNF\alpha^{-/-}$ B2 cells did not exhibit increased arterial expression of TNFα and IL1β. Data represent (mean±SEM) and n=7 per group. * $P<0.05$ compared to vehicle-PBS; # $P<0.05$ compared to WT B2.

Figure 4

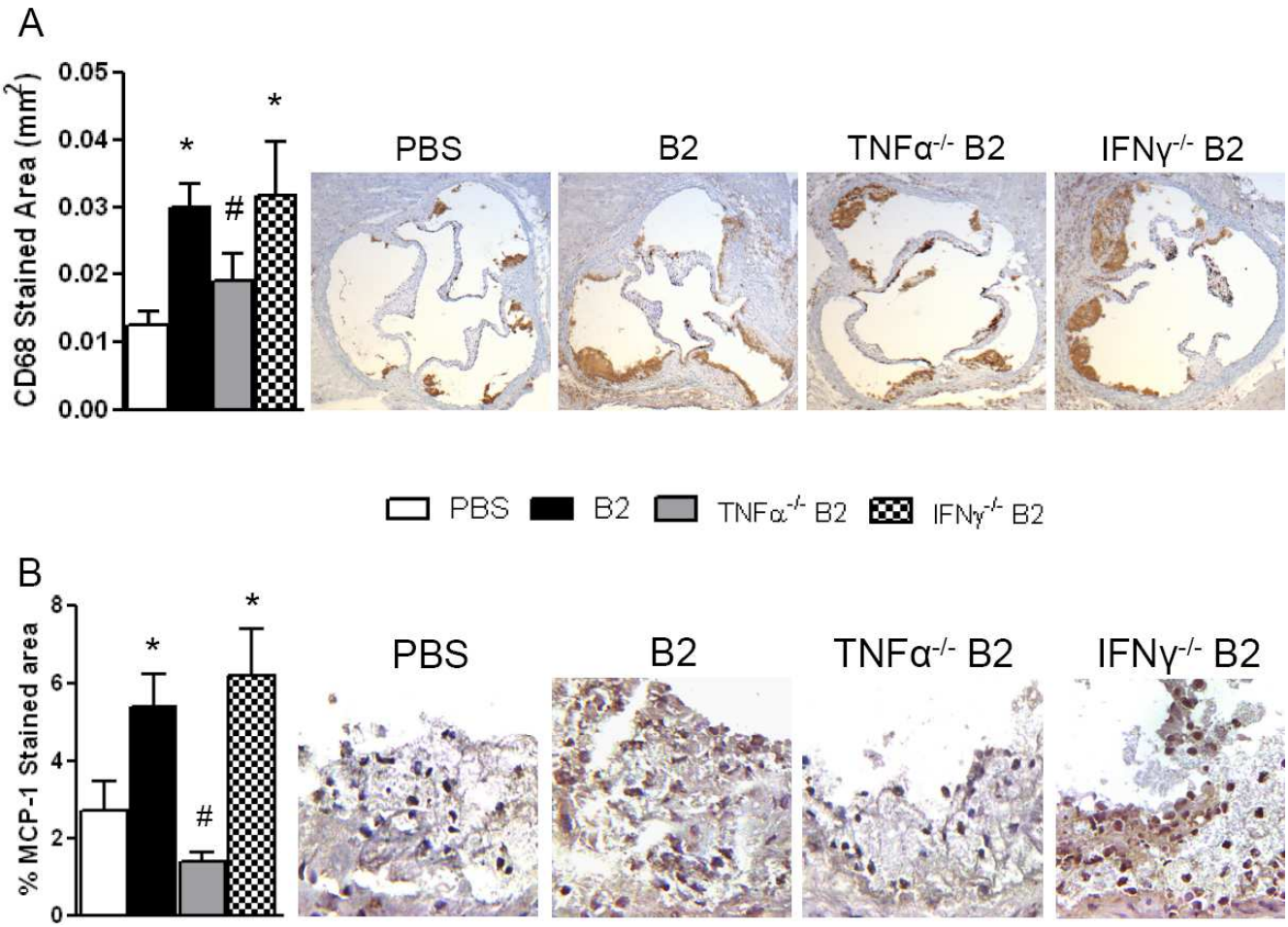

A
CD68 Stained Area (mm²)
0.05
0.04
0.03
0.02
0.01
0.00
*
*
#
PBS
B2
TNFα-/- B2
IFNγ-/- B2
PBS
B2
TNFα-/- B2
IFNγ-/- B2
B
% MCP-1 Stained area
8
6
4
2
0
*
*
#
PBS
B2
TNFα-/- B2
IFNγ-/- B2

Figure 4 B2-derived TNFα promotes inflammation and accumulation of macrophages in atherosclerotic lesions

(A) Immunohistochemical peroxidase staining was performed to measure $CD68^{+}$ macrophage content in lesions. $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice that received WT B2 cells or $IFN\gamma^{-/-}$ B2 cells, but not $TNF\alpha^{-/-}$ B2 cells, had significantly higher accumulation of macrophages in lesions compared to control mice that received PBS-vehicle. Original magnification X 60. **(B)** MCP1 in atherosclerotic lesion was also assessed by immuohistochemical staining. Similarly, the expression of MCP1 relatively to lesion area was significantly increased in mice transferred with WT B2 cells or $IFN\gamma^{-/-}$ B2 cells but not $TNF\alpha^{-/-}$ B2 cells. Original magnification X 160. Data represent (mean±SEM) and n=7 per group. * $P<0.05$ compared to vehicle-PBS; # $P<0.05$ compared to WT B2.

Figure 5

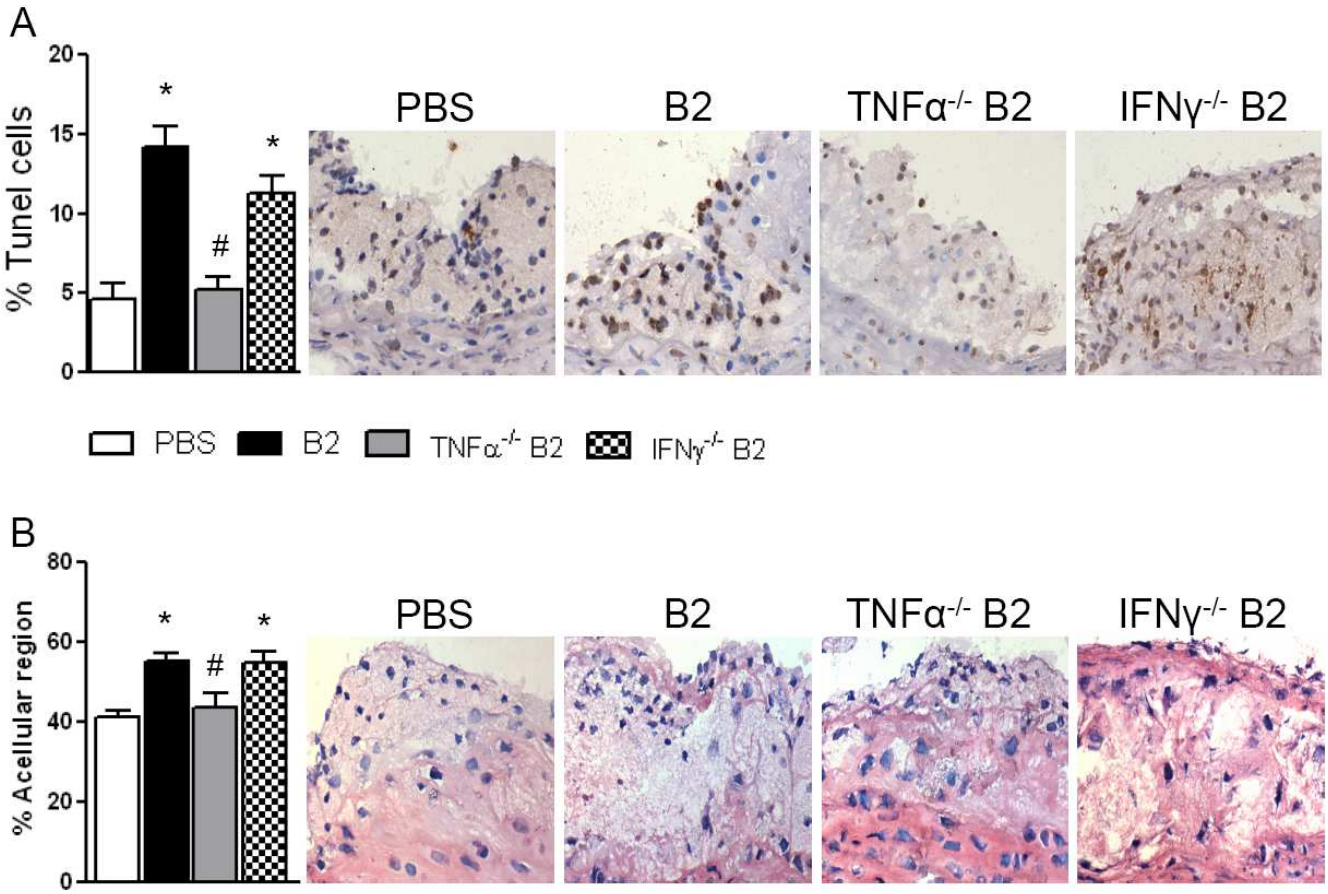

A
% Tunel cells
20
15
10
5
0
*
#
*
PBS
B2
TNFα-/- B2
IFNγ-/- B2
PBS
B2
TNFα-/- B2
IFNγ-/- B2
B
% Acellular region
80
60
40
20
0
*
#
*
PBS
B2
TNFα-/- B2
IFNγ-/- B2

Figure 5 Atherosclerotic lesions in mice that received TNFα-deficient B2 cells have decreased apoptotic cells and necrotic cores. (**A**) Representative microimages show more $TUNEL^{+}$ apoptotic cells in the lesions of mice that received TNFα-sufficient (WT and $IFN\gamma^{-/-}$) B2 cells but not in mice that received TNFα-deficient ($TNF\alpha^{-/-}$) B2 cells compared to PBS-vehicle control mice. (**B**) Representative microimages stained with H&E show significantly larger necrotic cores in atherosclerotic lesions of mice that received TNFα-sufficient (WT and $IFN\gamma^{-/-}$) B2 cells but not in mice that received TNFα-deficient ($TNF\alpha^{-/-}$) B2 cells compared to control mice. Original magnification X 160. Data represent (mean±SEM) and n=7-10. * $P<0.05$ compared to vehicle-PBS; # $P<0.05$ compared to WT B2.

Figure 6

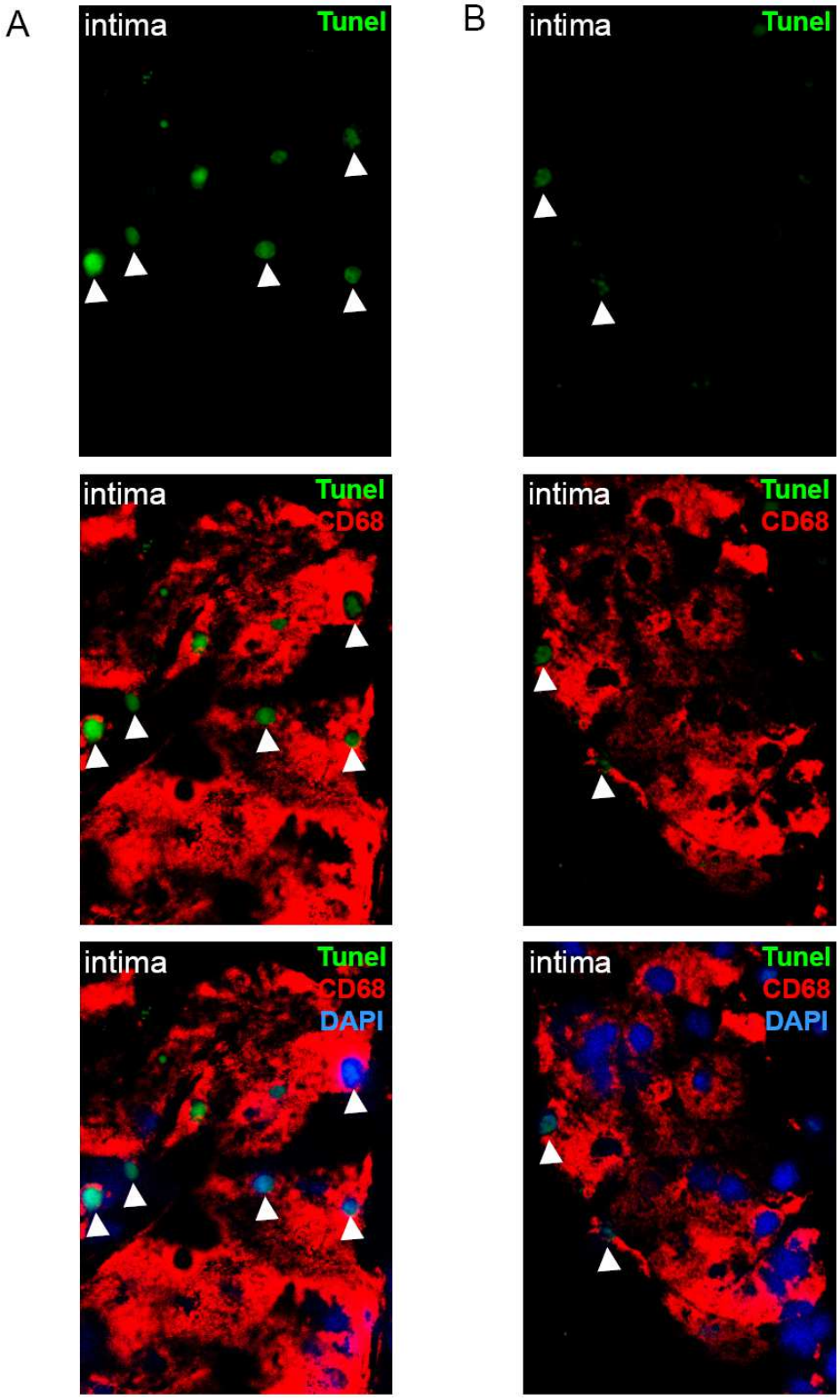

A
intima
Tunel
intima
Tunel
CD68
intima
Tunel
CD68
DAPI
B
intima
Tunel
intima
Tunel
CD68
intima
Tunel
CD68
DAPI

Figure 6 Atherosclerotic lesions in mice that received TNFα$^{-/-}$ B2 cells have less apoptotic macrophages in lesions. Atherosclerotic lesions of lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice that received (**A**) WT B2 cells and (**B**) TNFα$^{-/-}$ B2 cells were stained with anti-CD68 antibody to identify macrophages (red) and TUNEL (green) to identify apoptotic cells. Double immunofluorescence staining show more apoptotic macrophages in lesions of mice that received WT B2 cells compared to mice that received TNFα$^{-/-}$ B2 cells. Representative images show co-localization of apoptotic cells (TUNEL-positive; GREEN), macrophages (CD68-positive; RED) and DAPI (Nucleus; BLUE) n=3 per group.

Figure 7

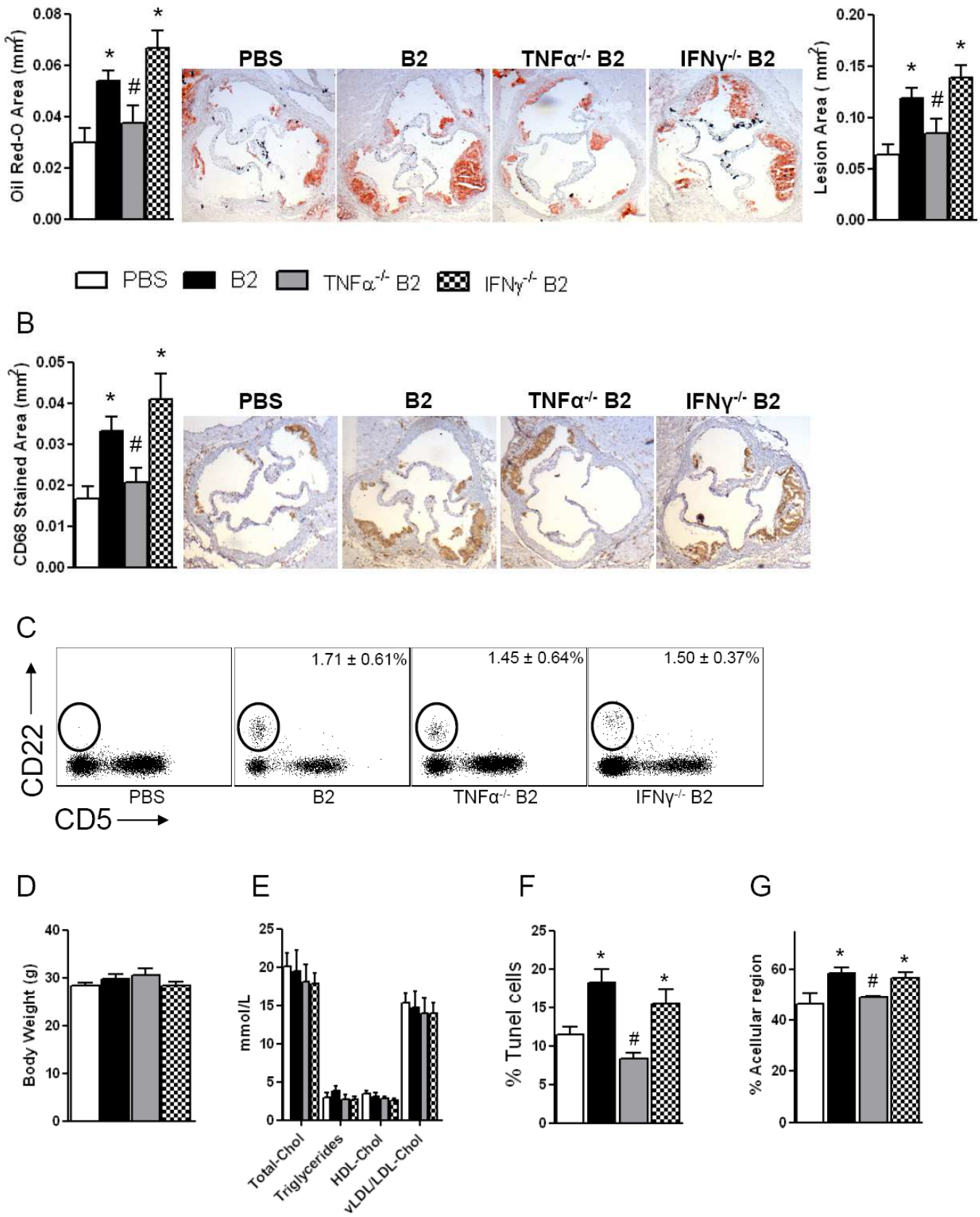

A
Oil Red-O Area (mm²)
0.08
0.06
0.04
0.02
0.00
*
#
*
PBS
B2
TNFα-/- B2
IFNγ-/- B2
Lesion Area (mm²)
0.20
0.15
0.10
0.05
0.00
PBS
B2
TNFα-/- B2
IFNγ-/- B2
B
CD68 Stained Area (mm²)
0.05
0.04
0.03
0.02
0.01
0.00
PBS
B2
TNFα-/- B2
IFNγ-/- B2
C
CD22
CD5
1.71 ± 0.61%
1.45 ± 0.64%
1.50 ± 0.37%
PBS
B2
TNFα-/- B2
IFNγ-/- B2
D
Body Weight (g)
40
30
20
10
0
E
mmol/L
25
20
15
10
5
0
Total-Chol
Triglycerides
HDL-Chol
vLDL/LDL-Chol
F
% Tunel cells
25
20
15
10
5
0
G
% Acellular region
60
40
20
0

Figure 7 TNFα-deficient B2 cells do not promote atherosclerosis in B cell-deficient μMT ApoE$^{-/-}$ mice. B cell-deficient ApoE$^{-/-}$μMT mice were adoptively transferred with WT, TNFα$^{-/-}$ or IFNγ$^{-/-}$ B2 cells and fed HFD for 8 weeks. Development of atherosclerosis was assessed in the aortic sinus. (**A**) ORO-stained atherosclerotic lesions in B cell-deficient ApoE$^{-/-}$μMT mice that received WT or IFNγ$^{-/-}$ B2 cells showed increased lipid accumulation in lesions compared to mice that received TNFα$^{-/-}$ B2 cells or PBS. A similar result was obtained for total lesion area. Original magnification X 60. (**B**) Representative microimages show that compared to control mice macrophage accumulation in atherosclerotic lesions, as assessed by immunoperoxidase staining with anti-CD68 antibody, was increased in mice that received WT B2 cells or IFNγ$^{-/-}$ B2 cells but not in mice that received TNFα$^{-/-}$ B2 cells compared to control mice. Original magnification X 60. (**C**) Representative dot-plots from spleen FACS analysis show that CD22^{+}CD5^{-} B2 cells were reconstituted at similar levels, albeit low compared to ApoE$^{-/-}$ mice fed a HFD for 8 weeks. (**D**) Body weight and (**E**) plasma lipid levels were not significantly affected in all groups. TNFα-sufficient (WT and IFNγ$^{-/-}$) B2 cells but not TNFα-deficient (TNFα$^{-/-}$) B2 cells increased (**F**) TUNEL^{+} apoptotic cell numbers and (**G**) necrotic cores in atherosclerotic lesions of ApoE$^{-/-}$μMT mice. Data represent (mean±SEM) and n=9-10 per group, *; $p<0.05$ compared to PBS group, #; $p<0.05$ compared to WT B2 group.

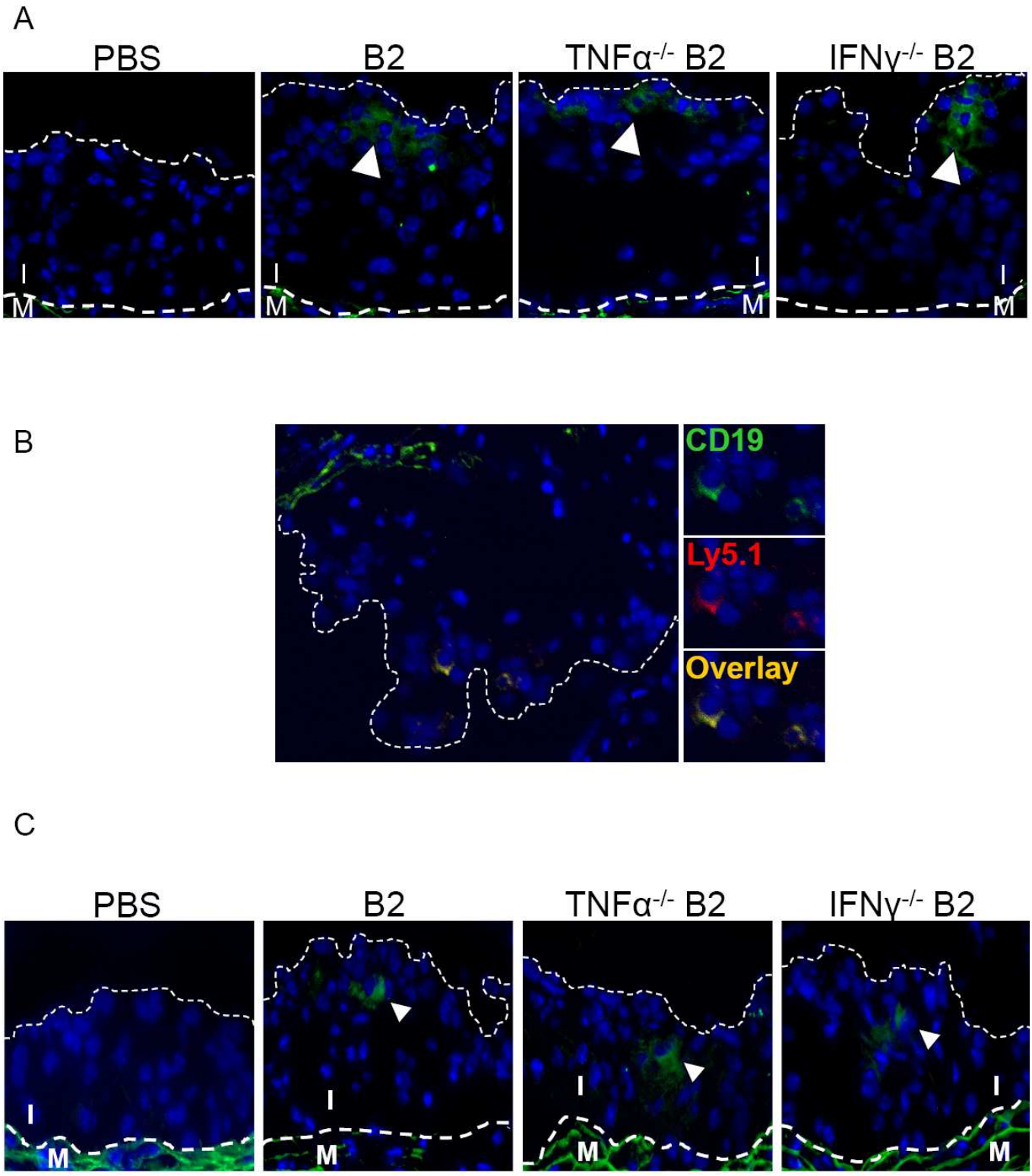

A
PBS
B2
TNFα-/- B2
IFNγ-/- B2
I
M
B
CD19
Ly5.1
Overlay
C
PBS
B2
TNFα-/- B2
IFNγ-/- B2
I
M

Figure 8 Adoptively transferred B2 cells are found in the atherosclerotic plaques of recipient mice. WT, TNFα$^{-/-}$ or IFNγ$^{-/-}$ B2 cells were adoptively transferred into lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice. After 8 weeks HFD, anti-CD19 immunofluorescence staining was carried out to detect the transferred B2 cells in lesions. **(A)** Representative microimages show CD19^{+} (green) B2 cells in atherosclerotic lesions of the respective recipient mice. Moreover, B2 cells were isolated from Ly5.1 congenic mice and transferred into B cell-deficient ApoE$^{-/-}$μMT. Original magnification X 160. **(B)** After 8 weeks HFD, CD19^{+} (green) Ly5.1^{+} (red) B2 cells were found in the atherosclerotic lesion by immunofluorescence staining. **(C)** Adoptively transferred CD19^{+} (green) WT B2, TNFα$^{-/-}$ B2 and IFNγ$^{-/-}$ B2 cells were also present in the lesions of the respective recipient ApoE$^{-/-}$μMT mice. Original magnification X 160. n=3 per group. I, tunica intima; M, tunica media; white arrow indicates CD19^{+} B2 cells

Chapter 6 Integrated discussion and summary

6.1 Discussion and Future Directions

The importance of determining the specific roles of B cell subsets in atherosclerosis cannot be over emphasized. Early studies suggested that B cells were atheroprotective. $LDLR^{-/-}$ mice that were depleted of B cells by bone marrow transplantation from μMT mice showed increased atherosclerosis (Major, Fazio et al. 2002) while the adoptive transfer of unfractionated splenic B cells into splenectomised $ApoE^{-/-}$ mice markedly reduced atherosclerosis (Caligiuri, Nicoletti et al. 2002). However, the notion that B cells have an atheroprotective role is only true to a certain extent. The B cell population consists of multiple B cell subsets which are phenotypically and functionally distinct. In this thesis, I have shown major differential roles for B1a and B2 B cells in atherosclerosis. B1a cells were found to be atheroprotective whilst B2 cells were found to be atherogenic and were major contributors to the development and progression of atherosclerosis (Kyaw, Tay et al. 2010, Kyaw, Tay et al. 2011). I have also shown that atherosclerosis can be reduced by selectively depleting B2 cells while sparing B1a cells (Kyaw, Tay et al. 2012).

In contrast to the previous paradigm of atheroprotective B cells, depletion of B cells by anti-CD20 antibody treatment suppressed the development of atherosclerosis and also intervened the progression of atherosclerosis in $ApoE^{-/-}$ mice (Kyaw, Tay et al. 2010). Treatment with anti-CD20 antibody reduced both B1a and B2 cells. These results bode well for rituximab as a clinical therapy to remit atherosclerosis. Indeed, short-term treatment with rituximab reduces rheumatoid arthritis-associated atherosclerosis (Kerekes, Soltesz et al. 2009, Benucci, Saviola et al. 2013). Further studies are required to determine the long-term effects of rituximab on atherosclerosis.

One major reason that hinders a large scale clinical trial is the difficulty of recruiting subjects with hypercholesterolemia and various levels of atherosclerosis which are largely asymptomatic.

Over the years, rituximab has proven well in safety terms. Reactions ranging from hypotension to fever and rash can be adequately controlled without compromising patient health and therapeutic efficacy (Mease 2008). There were initial concerns about adverse opportunistic infections following the depletion of the entire B cell population. Fortunately, such cases have rarely occurred (Mease 2008). This is likely due to the absence of CD20 expression in immature pro-B cells and antibody-secreting plasma cells. CD20 is only expressed from the pre-B cell stage to the mature B cell stage (Townsend, Monroe et al. 2010). It is a membrane-spanning protein of 297 amino acids (33 – 35kDa) and is assembled to form a homo-tetrameric complex (~140kDa), which is deemed to serve as a calcium conduit for the influx of extracellular calcium during the activation of B cells (Einfeld, Brown et al. 1988, Bubien, Zhou et al. 1993, Polyak, Li et al. 2008). By preserving pro-B cells and long-lived plasma cells, mature B cells can reconstitute post-treatment and humoral responses remain competent as shown by the normal antibody levels in rituximab-treated patients (Tsokos 2004). Once bound to CD20, rituximab instigates cell-death by one of four possible actions. These are antibody-dependent cellular cytotoxicity (ADCC), complement-dependent lysis (CDL), survival stimuli blockade and apoptosis (Tsokos 2004). The extent of these effects may depend on the population of B cells that is targeted. For instance, unlike CDL-prone B cells in the lymphoid organs, B cells in the peripheral blood are more susceptible to ADCC (Tsokos 2004).

Nevertheless, caution is still needed when treating patients with rituximab. Recent studies have shown that rituximab may increase the likelihood of infections, such as hepatitis B and *Pneumocystis* pneumonia, which can result in serious complications (Gea-Banacloche 2010). This may preclude the use of rituximab to treat atherosclerosis and highlights the possibility of selectively depleting B2 cells but not B1a cells which are the first line of defense against infections. Moreover, rituximab may not be suitable for patients who are already on statins, given that statins alter the conformation of CD20, potentially reducing the ability of rituximab to deplete B cells effectively (Winiarska, Bil et al. 2008).

A strategy that selectively targets atherogenic B cells is highly desired. The key is to avoid perturbing atheroprotective B cells and other subsets of B cells. In doing so, the effectiveness of therapeutic B cell depletion is expected to increase significantly. I assessed the roles of the two main subsets of B cells, namely B1a cells and conventional B2 cells. This approach was adopted in light of the atheroprotective effects of natural IgM, a principal product of B1a cells, and the pathogenic effects of B2 cells reported in other immune disorders such as rheumatoid arthritis. Indeed, atherosclerosis was increased in lymphocyte-deficient $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice that received adoptive transfer of B2 cells but not in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice that received B1a cells (Kyaw, Tay et al. 2010). A similar outcome was obtained in B cell-deficient $ApoE^{-/-}\mu MT$ mice, confirming an atherogenic role for B2 cells (Kyaw, Tay et al. 2010). Of note, the results in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice suggest that B2 cells can promote atherosclerosis independently of other lymphocytes and their trademark of producing IgG antibodies. The localisation of adoptively transferred B2 cells in atherosclerotic lesions proposes that B2 cells may exert atherogenic responses locally

within lesions. These findings are crucial for tailoring therapies against a specific function or subpopulation of B2 cells that is atherogenic.

While B1a cells do not aggravate atherosclerosis, it is possible that these cells protect against atherosclerosis. The splenectomised ApoE$^{-/-}$ model is an ideal platform to ascertain whether B1a cells protect against atherosclerosis. Splenectomised ApoE$^{-/-}$ mice have reduced peritoneal B1a cells and exhibit increased atherosclerosis (Kyaw, Tay et al. 2011). Remarkably, the partial reconstitution of B1a cells not only repressed the aggravation of atherosclerosis caused by splenectomy but also suppressed the development of atherosclerosis almost entirely (Kyaw, Tay et al. 2011). However, these effects did not occur in mice that were given B1a cells deficient in secretory IgM. On further investigation, the lesions of mice that received wild type B1a cells were found to have increased IgM deposition, decreased oxLDL accumulation, decreased apoptotic cell numbers and decreased necrotic cores (Kyaw, Tay et al. 2011). It is conceivable that natural IgM antibodies perform multiple atheroprotective roles simultaneously which render them highly potent in suppressing atherosclerosis. They may do so directly within atherosclerotic lesions by facilitating the disposal of oxLDL antigens and apoptotic cells and systemically through the activation of Th2 responses. Studies have shown that the Th2 cytokines – IL-5 and IL-33 stimulate B1a cells to secrete oxLDL-specific IgM and reduce atherosclerosis (Binder, Hartvigsen et al. 2004, Miller, Xu et al. 2008). As such, B1a cells may have a vital relationship with Th2 cells in modulating the development of atherosclerosis which is mainly driven by Th1 responses. IL-5 and IL-33 are certainly potential therapeutic candidates for expanding atheroprotective B1a cells. Future studies ought to examine the possibility of reversing established atherosclerosis by administering these cytokines or their

agonists. Studies are also required to determine the role of IL-10-producing B1a cells in atherosclerosis. These cells were found to modulate the activity of macrophages and dampen inflammation (Popi, Lopes et al. 2004). Hence, it is likely that B1a cells suppress atherosclerosis by producing IL-10 in addition to natural IgM.

B1a cells are known to migrate from the peritoneal cavity to inflammatory sites. They respond mainly to the chemokines CXCL12 and CXCL13 (Balabanian, Foussat et al. 2002). The responsiveness of B1a cells to CXCL12 and CXCL13 is regulated by their autocrine production of IL-10 (Balabanian, Foussat et al. 2002). Of interest, CXCL12 and CXCL13 are highly expressed in atherosclerotic plaques and are believed to be important in stabilizing established plaques (Smedbakken, Halvorsen et al. 2012, Akhtar, Gremse et al. 2013). On this account, B1a cells may be mobilised by chemotactic signals to infiltrate atherosclerotic lesions to deposit atheroprotective IgM. In this event, the local complement system may mediate the activity of B1a cells in lesions. This is conceived from reports of perturbed production of natural IgM antibodies by B1a cells deficient in complement receptors 1 and 2 (CD21/CD35) (Fleming, Shea-Donohue et al. 2002, Reid, Woodcock et al. 2002, Carroll 2004). Furthermore, the complement protein, C1q, has been implicated in the selection and maintenance of B1a cells and anti-apoptotic natural IgM. Mice deficient in C1q have a compensatory increase in B1a cells and IgM antibodies against antigens that are sequestered intracellularly in healthy cells but exposed on dying cells (Ferry, Potter et al. 2007). The binding of C1q to IgM forms complexes that are essential for efficient clearance of apoptotic cells. Absence of either C1q or IgM results in the build up of apoptotic cells (Ferry, Potter et al. 2007). Apoptotic cells that are not efferocytosed are likely to become necrotic. The uncontrolled leakage of intracellular material from

necrotic cells enhances inflammation and results in the enlargement of necrotic cores in atherosclerotic lesions which eventually become unstable. Hence, it is reasonable to postulate that the abundance of apoptotic cells calls for B1a cells to traffick to the site of lesions. This could spell a direct role for B1a cells in regulating inflammation and efferocytosis within atherosclerotic lesions. They may return to the peritoneal cavity upon secreting IgM and after apoptotic cells subside in lesions. This pathway can be exploited therapeutically to hamper the growth of developing lesions and maintain the stability of established lesions to prevent them from rupture.

Human B1a cells have yet to be clearly characterized. In mice, $CD19^{+}CD5^{+}CD11b^{+}$ cells are identified as B1a cells. They vary in their expression of certain surface molecules as they migrate from one location to another. For instance, B1a cells cease to express CD5, CD11b and CR3 after they exit the peritoneal cavity (Fagarasan S 2002, Kawahara, Ohdan et al. 2003). This poses a challenge to track the movement of B1a cells in vivo. One potential solution is to reconstitute B cell-deficient mice with fluorescent-labelled or congenic / transgenic B1a cells. This could provide additional insights into the dynamics of B1a cells in regulating inflammatory processes. The expression of CD5 is believed to be a good indicator of B1a responses. CD5 negatively regulates the activation of B1a cells (Bikah, Carey et al. 1996). Upon binding to cognate antigens, the B cell receptor associates with CD5 which subsequently induces apoptosis. In the absence of CD5, however, activated B1a cells expand and secrete increased amounts of IgM antibodies (Bikah, Carey et al. 1996, Berland and Wortis 2002). Taken together, it is highly plausible that atheroprotective B1a cells are stimulated within inflamed lesions where there is a high presence of

oxLDL antigens and apoptotic cells rather than in the peritoneal cavity where CD5 expression is turned on.

To validate the atheroprotective role of B1a cells and atherogenic role of B2 cells in a more physiologically relevant model, the development of atherosclerosis was assessed in ApoE$^{-/-}$ mice that lacked B cell activating factor receptor (BAFF-R). The BAFF : BAFF-R signalling system is crucial for the survival of mature B2 cells but not B1a cells (Sasaki, Casola et al. 2004). With the selective reduction of only B2 cells, ApoE$^{-/-}$BAFF-R$^{-/-}$ mice displayed smaller atherosclerotic lesions compared to control ApoE$^{-/-}$ mice (Kyaw, Tay et al. 2012). A similar result was obtained in a separate study that examined BAFF-R deficiency in LDLR$^{-/-}$ mice (Sage, Tsiantoulas et al. 2012). These findings strongly assert that B2 cells are atherogenic. Importantly, the reduction in atherosclerosis was accompanied by reduced inflammation in the lesions of ApoE$^{-/-}$ BAFF-R$^{-/-}$ mice. Future studies can consider expanding or stimulating B1a cells in ApoE$^{-/-}$BAFF-R$^{-/-}$ mice to further inhibit the development of atherosclerosis. This may encourage the design of combined therapies to deplete atherogenic B2 cells and expand atheroprotective B1a cells. Meanwhile, more attention can be placed on blocking BAFF : BAFF-R interaction. This strategy has recently gained traction with our report of reduced mature B2 cells and attenuated atherosclerosis in ApoE$^{-/-}$ mice treated with anti-BAFF-R antibody (Kyaw, Cui et al. 2013), a viable alternative to rituximab.

BAFF is a member of a larger family of cytokines known as the B Lymphocyte Stimulator (BLyS). The BLyS system is mainly comprised of two ligands – BAFF and A Proliferation-inducing Ligand (APRIL) and three receptors – BAFF-R,

Transmembrane Activator and Calcium modulator and cyclophylin ligand Interactor (TACI) and B cell maturation antigen (BCMA) (Tangye, Bryant et al. 2006, Vital and Emery 2008). In humans, BLyS receptors are differentially expressed among the B cell subsets. Early-stage transitional and naïve B2 cells only express BAFF-R. TACI and BCMA are mainly found on memory B2 cells and early plasmablasts whereas terminally differentiated plasma cells do not express BLyS receptors. Furthermore, BAFF signals are only crucial for the survival but not activation of human B2 cells (Vital and Emery 2008).

BAFF binds strongest to BAFF-R amongst all the receptors while APRIL primarily binds to TACI and BCMA (Vital and Emery 2008). Thus far, BAFF : BAFF-R is known to be the most critical for the maturation of B2 cells. It also appears that the effect of BAFF on B2 cells is dependent on its cellular source. For example, BAFF produced by CD40L- and IFNγ-stimulated dendritic cells is particularly important for the induction of isotype switching in activated B2 cells (Vital and Emery 2008). This may provide opportunities to specifically nullify the relevant atherogenic responses of B2 cells as opposed to depleting B2 cells. BAFF is certainly a door to many potential therapies. More work is required to uncover the functions of APRIL, TACI and BCMA which are still largely undefined.

Several BLyS-based therapies are currently undergoing clinical trials for the treatment of autoimmune disorders. Atacicept, otherwise known as TACI-Immunoglobulin, has shown promising results against rheumatoid arthritis in a phase 1 trial. It is a humanised antibody that binds to BAFF and APRIL and reduces mature B cell and immunoglobulin levels in the lymphoid organs and blood (Tak, Thurlings et al. 2008).

Other anti-BLyS agents include Belimumab, which is an anti-BAFF antibody, and a soluble decoy receptor for BAFF (Vital and Emery 2008). If successful, these treatments can be emulated to combat atherosclerosis.

Although there is compelling evidence of B2 cells playing an atherogenic role, the atherogenic action of B2 cells is not known. According to earlier findings, B2 cells can exacerbate the development of atherosclerosis in the absence of all other lymphocytes (Kyaw, Tay et al. 2010). This suggests that at least one atherogenic action of B2 cells is independent of T cells and independent of IgG antibody production. Hence, one viable mode of action by atherogenic B2 cells is the production of cytokines. Cytokine-producing B cells are important regulators of immunity. They do not account for the absolute amounts of cytokines secreted in vivo but are believed to have significant influence by closely interacting with the major cytokine producers, such as macrophages and Th1 and Th2 cells, through paracrine and autocrine manners (Lund 2008). Upon activation, B2 cells can become specialised in releasing either pro-inflammatory cytokines or anti-inflammatory cytokines. The earlier is more likely to occur for atherogenic B2 cells.

The failure of TNFα-deficient B2 cells, but not TNFα-sufficient B2 cells, to exacerbate atherosclerosis suggests that the production of TNFα by B2 cells has an atherogenic effect. Moreover, this was demonstrated in the lymphocyte-deficient $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ model. This underscores the high potency of TNFα-producing B2 cells in promoting the development of atherosclerosis without help from T cells and other lymphocytes. For this to occur, there is an increased likelihood that resident B2 cells in the aorta inflict atherogenic effects directly on developing lesions by releasing

TNFα. This is supported by a significant percentage of aortic B2 cells that express TNFα in mice and humans (Hamze, Desmetz et al. 2013). It is plausible that B2-derived TNFα complements the activities of other inflammatory cytokines and perhaps those of its own to amplify atherogenesis. This has been reported in other inflammatory settings (Paludan 2000, Dorner 2006).

One cytokine that is commonly found to synergize with TNFa to promote inflammation is IL-1β (Andersen, Larsen et al. 2000, Sutton, Brereton et al. 2006, Pugazhenthi, Zhang et al. 2013). IL-1β is a member of the IL-1 family and is mainly produced by macrophages. The secretion of IL-1β only occurs upon stimulation by inflammatory signals (Vicenova, Vopalensky et al. 2009). Like TNFα, IL-1β has been shown to be highly atherogenic. $ApoE^{-/-}$ mice deficient in IL-1β were reported to have significantly reduced atherosclerosis (Kirii, Niwa et al. 2003). IL-1β can promote atherosclerosis in several ways. Increasing inflammation within lesions (Nicklin, Hughes et al. 2000), inducing the expression of adhesion molecules for recruitment of immune cells into lesions (Kirii, Niwa et al. 2003) and diminishing the stability of lesions (Shah, Falk et al. 1995) are some examples of IL-1β-mediated atherogenic responses. This presents a possible synergy between B2-derived TNFα and IL-1β in promoting the development of atherosclerosis.

The increase in MCP-1 expression as well as number of apoptotic cells in the lesions of mice with TNFα-sufficient B2 cells, but not TNFα-deficient B2 cells, may be well due to the combined effects of TNFα and IL-1β. As a consequence, there is increased accumulation of macrophages and formation of necrotic cores in lesions. This could explain the potent atherogenic effect of TNFα-producing B2 cells in spite of them not

present in abundance in atherosclerotic lesions. In this regard, B2-derived TNFα may be a chief stimulator that ignites a chain of atherogenic responses. Unless TNFα-producing B2 cells express a distinct phenotype that can be targeted to reduce their presence in the aorta or atherosclerotic lesions, more work is required to identify the mechanisms that antagonise the atherogenicity of B2-derived TNFα and the manner which atherogenic B2 cells are activated to produce TNFα. Strategies to impede the traffic of B2 cells to the site of lesions, perhaps by blocking specific chemokines or chemokine receptors, may also be of significance.

In summary, B1a cells and B2 cells have opposing roles in atherosclerosis. B1a cells are atheroprotective while B2 cells are atherogenic. By coincidence, both cell types can act T-independently in their respective roles. B1a cells spontaneously produce natural IgM antibodies that stem the development of necrotic cores in lesions. On the other hand, B2 cells promote inflammation in lesions in a TNFα-dependent fashion without help from other lymphocytes and independently of IgG antibody production.

6.2 References

Akhtar, S., F. Gremse, F. Kiessling, C. Weber and A. Schober (2013). "CXCL12 promotes the stabilization of atherosclerotic lesions mediated by smooth muscle progenitor cells in Apoe-deficient mice." Arterioscler Thromb Vasc Biol **33**(4): 679-686.

Andersen, N. A., C. M. Larsen and T. Mandrup-Poulsen (2000). "TNFalpha and IFNgamma potentiate IL-1beta induced mitogen activated protein kinase activity in rat pancreatic islets of Langerhans." Diabetologia **43**(11): 1389-1396.

Balabanian, K., A. Foussat, L. Bouchet-Delbos, J. Couderc, R. Krzysiek, A. Amara, F. Baleux, A. Portier, P. Galanaud and D. Emilie (2002). "Interleukin-10 modulates the sensitivity of peritoneal B lymphocytes to chemokines with opposite effects on stromal cell-derived factor-1 and B-lymphocyte chemoattractant." Blood **99**(2): 427-436.

Benucci, M., G. Saviola, M. Manfredi, P. Sarzi-Puttini and F. Atzeni (2013). "Factors correlated with improvement of endothelial dysfunction during rituximab therapy in patients with rheumatoid arthritis." Biologics **7**: 69-75.

Berland, R. and H. H. Wortis (2002). "Origins and functions of B-1 cells with notes on the role of CD5." Annu Rev Immunol **20**: 253-300.

Bikah, G., J. Carey, J. R. Ciallella, A. Tarakhovsky and S. Bondada (1996). "CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells." Science **274**(5294): 1906-1909.

Binder, C. J., K. Hartvigsen, M. K. Chang, M. Miller, D. Broide, W. Palinski, L. K. Curtiss, M. Corr and J. L. Witztum (2004). "IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis." J Clin Invest **114**(3): 427-437.

Bubien, J. K., L. J. Zhou, P. D. Bell, R. A. Frizzell and T. F. Tedder (1993). "Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes." J Cell Biol **121**(5): 1121-1132.

Caligiuri, G., A. Nicoletti, B. Poirier and G. K. Hansson (2002). "Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice." J Clin Invest **109**(6): 745-753.

Carroll, M. C. (2004). "The complement system in B cell regulation." Mol Immunol **41**(2-3): 141-146.

Dorner, T. (2006). "Crossroads of B cell activation in autoimmunity: rationale of targeting B cells." J Rheumatol Suppl **77**: 3-11.

Einfeld, D. A., J. P. Brown, M. A. Valentine, E. A. Clark and J. A. Ledbetter (1988). "Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains." EMBO J **7**(3): 711-717.

Fagarasan S, W. N., Honjo T (2002). Experimental models of autoimmune hemolytic anemia. The molecular pathology of autoimmune diseases T. A. Bona CA. New York, Taylor and Francis**:** 839-873.

Ferry, H., P. K. Potter, T. L. Crockford, A. Nijnik, M. R. Ehrenstein, M. J. Walport, M. Botto and R. J. Cornall (2007). "Increased positive selection of B1 cells and reduced B cell tolerance to intracellular antigens in c1q-deficient mice." J Immunol **178**(5): 2916-2922.

Fleming, S. D., T. Shea-Donohue, J. M. Guthridge, L. Kulik, T. J. Waldschmidt, M. G. Gipson, G. C. Tsokos and V. M. Holers (2002). "Mice deficient in complement receptors 1 and 2 lack a tissue injury-inducing subset of the natural antibody repertoire." J Immunol **169**(4): 2126-2133.

Gea-Banacloche, J. C. (2010). "Rituximab-associated infections." Semin Hematol **47**(2): 187-198.

Hamze, M., C. Desmetz, M. L. Berthe, P. Roger, N. Boulle, P. Brancherau, E. Picard, C. Guzman, C. Tolza and P. Guglielmi (2013). "Characterization of resident B cells of vascular walls in human atherosclerotic patients." J Immunol **191**(6): 3006-3016.

Kawahara, T., H. Ohdan, G. Zhao, Y. G. Yang and M. Sykes (2003). "Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells." J Immunol **171**(10): 5406-5414.

Kerekes, G., P. Soltesz, H. Der, K. Veres, Z. Szabo, A. Vegvari, G. Szegedi, Y. Shoenfeld and Z. Szekanecz (2009). "Effects of rituximab treatment on endothelial dysfunction, carotid atherosclerosis, and lipid profile in rheumatoid arthritis." Clin Rheumatol **28**(6): 705-710.

Kirii, H., T. Niwa, Y. Yamada, H. Wada, K. Saito, Y. Iwakura, M. Asano, H. Moriwaki and M. Seishima (2003). "Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice." Arterioscler Thromb Vasc Biol **23**(4): 656-660.

Kyaw, T., P. Cui, C. Tay, P. Kanellakis, H. Hosseini, E. Liu, A. G. Rolink, P. Tipping, A. Bobik and B. H. Toh (2013). "BAFF receptor mAb treatment ameliorates development and progression of atherosclerosis in hyperlipidemic ApoE(-/-) mice." PLoS One **8**(4): e60430.

Kyaw, T., C. Tay, H. Hosseini, P. Kanellakis, T. Gadowski, F. MacKay, P. Tipping, A. Bobik and B. H. Toh (2012). "Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation." PLoS One **7**(1): e29371.

Kyaw, T., C. Tay, A. Khan, V. Dumouchel, A. Cao, K. To, M. Kehry, R. Dunn, A. Agrotis, P. Tipping, A. Bobik and B. H. Toh (2010). "Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis." J Immunol **185**(7): 4410-4419.

Kyaw, T., C. Tay, S. Krishnamurthi, P. Kanellakis, A. Agrotis, P. Tipping, A. Bobik and B. H. Toh (2011). "B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions." Circ Res **109**(8): 830-840.

Lund, F. E. (2008). "Cytokine-producing B lymphocytes-key regulators of immunity." Curr Opin Immunol **20**(3): 332-338.

Major, A. S., S. Fazio and M. F. Linton (2002). "B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice." Arterioscler Thromb Vasc Biol **22**(11): 1892-1898.

Mease, P. J. (2008). "B cell-targeted therapy in autoimmune disease: rationale, mechanisms, and clinical application." J Rheumatol **35**(7): 1245-1255.

Miller, A. M., D. Xu, D. L. Asquith, L. Denby, Y. Li, N. Sattar, A. H. Baker, I. B. McInnes and F. Y. Liew (2008). "IL-33 reduces the development of atherosclerosis." J Exp Med **205**(2): 339-346.

Nicklin, M. J., D. E. Hughes, J. L. Barton, J. M. Ure and G. W. Duff (2000). "Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene." J Exp Med **191**(2): 303-312.

Paludan, S. R. (2000). "Synergistic action of pro-inflammatory agents: cellular and molecular aspects." J Leukoc Biol **67**(1): 18-25.

Polyak, M. J., H. Li, N. Shariat and J. P. Deans (2008). "CD20 homo-oligomers physically associate with the B cell antigen receptor. Dissociation upon receptor engagement and recruitment of phosphoproteins and calmodulin-binding proteins." J Biol Chem **283**(27): 18545-18552.

Popi, A. F., J. D. Lopes and M. Mariano (2004). "Interleukin-10 secreted by B-1 cells modulates the phagocytic activity of murine macrophages in vitro." Immunology **113**(3): 348-354.

Pugazhenthi, S., Y. Zhang, R. Bouchard and G. Mahaffey (2013). "Induction of an inflammatory loop by interleukin-1beta and tumor necrosis factor-alpha involves NF-kB and STAT-1 in differentiated human neuroprogenitor cells." PLoS One **8**(7): e69585.

Reid, R. R., S. Woodcock, A. Shimabukuro-Vornhagen, W. G. Austen, Jr., L. Kobzik, M. Zhang, H. B. Hechtman, F. D. Moore, Jr. and M. C. Carroll (2002). "Functional activity of natural antibody is altered in Cr2-deficient mice." J Immunol **169**(10): 5433-5440.

Sage, A. P., D. Tsiantoulas, L. Baker, J. Harrison, L. Masters, D. Murphy, C. Loinard, C. J. Binder and Z. Mallat (2012). "BAFF receptor deficiency reduces the development of atherosclerosis in mice--brief report." Arterioscler Thromb Vasc Biol **32**(7): 1573-1576.

Sasaki, Y., S. Casola, J. L. Kutok, K. Rajewsky and M. Schmidt-Supprian (2004). "TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology." J Immunol **173**(4): 2245-2252.

Shah, P. K., E. Falk, J. J. Badimon, A. Fernandez-Ortiz, A. Mailhac, G. Villareal-Levy, J. T. Fallon, J. Regnstrom and V. Fuster (1995). "Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture." Circulation **92**(6): 1565-1569.

Smedbakken, L. M., B. Halvorsen, I. Daissormont, T. Ranheim, A. E. Michelsen, M. Skjelland, E. L. Sagen, L. Folkersen, K. Krohg-Sorensen, D. Russell, S. Holm, T. Ueland, B. Fevang, U. Hedin, A. Yndestad, L. Gullestad, G. K. Hansson, E. A. Biessen and P. Aukrust (2012). "Increased levels of the homeostatic chemokine CXCL13 in human atherosclerosis - Potential role in plaque stabilization." Atherosclerosis **224**(1): 266-273.

Sutton, C., C. Brereton, B. Keogh, K. H. Mills and E. C. Lavelle (2006). "A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis." J Exp Med **203**(7): 1685-1691.

Tak, P. P., R. M. Thurlings, C. Rossier, I. Nestorov, A. Dimic, V. Mircetic, M. Rischmueller, E. Nasonov, E. Shmidt, P. Emery and A. Munafo (2008). "Atacicept in patients with rheumatoid arthritis: results of a multicenter, phase Ib, double-blind, placebo-controlled, dose-escalating, single- and repeated-dose study." Arthritis Rheum **58**(1): 61-72.

Tangye, S. G., V. L. Bryant, A. K. Cuss and K. L. Good (2006). "BAFF, APRIL and human B cell disorders." Semin Immunol **18**(5): 305-317.

Townsend, M. J., J. G. Monroe and A. C. Chan (2010). "B-cell targeted therapies in human autoimmune diseases: an updated perspective." Immunol Rev **237**(1): 264-283.

Tsokos, G. C. (2004). "B cells, be gone--B-cell depletion in the treatment of rheumatoid arthritis." N Engl J Med **350**(25): 2546-2548.

Vicenova, B., V. Vopalensky, L. Burysek and M. Pospisek (2009). "Emerging role of interleukin-1 in cardiovascular diseases." Physiol Res **58**(4): 481-498.

Vital, E. M. and P. Emery (2008). "The development of targeted therapies in rheumatoid arthritis." J Autoimmun **31**(3): 219-227.

Winiarska, M., J. Bil, E. Wilczek, G. M. Wilczynski, M. Lekka, P. J. Engelberts, W. J. Mackus, E. Gorska, L. Bojarski, T. Stoklosa, D. Nowis, Z. Kurzaj, M. Makowski, E. Glodkowska, T. Issat, P. Mrowka, W. Lasek, A. Dabrowska-Iwanicka, G. W. Basak, M. Wasik, K. Warzocha, M. Sinski, Z. Gaciong, M. Jakobisiak, P. W. Parren and J. Golab (2008). "Statins impair antitumor effects of rituximab by inducing conformational changes of CD20." PLoS Med **5**(3): e64.