Therapeutic strategies in management of atherosclerosis by manipulation of anti-atherogenic B1a lymphocytes

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Notice 1

Notice 2

Table of Contents

List of figures:

Abbreviations

Ab	Antibody
AC	Apoptotic cell
APC	Antigen Presenting Cell
ApoE	Apolipoprotein E
ApoB-100	Apolipoprotein B-100
Apobec-1	Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-1
BAFF	B cell Activation Factor
BAFF-R	B cell Activation Factor Receptor
BCR	B cell receptor
BLyS	B-lymphocyte Stimulator
BM	Bone marrow
CCR	CC Chemokine Receptor
CVD	Cardiovascular Disease
DC	Dendritic cell
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
Fc	Constant region of immunoglobulin
FO	Follicular
FoxP3	Foxhead Box P3
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HMGB-1	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
IRF-4	Interferon regulatory factor-4
LDL	Low-density Lipoprotein
LDLR	Low-density Lipoprotein Receptor

LFA-1	Lymphocyte Function-associated Antigen 1
mAb	Monoclonal Antibody
mRNA	Messenger Ribonucleic acid
MDA	Malondialdehyde
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
MZ	Marginal Zone
NK	Natural Killer
OD	Optical Density
OxLDL	Oxidized Low-density Lipoprotein
PALS	Peri-arteriolar Lymphoid Sheath
PC	Phosphatidylcholine, Peritoneal Cavity
PCL	PhosphatidylcholineLiposome
PCR	Polymerase Chain Reaction
PRDM-1	PR domain zinc finger protein-1 also known as BLIMP-1
PRR	Pattern Recognition Receptor
RA	Rheumatoid Arthritis
PS	Phosphatidylserine
PSL	Phosphatidylserine liposome
RNA	Ribonucleic acid
RT-PCR	Real-time Polymerase Chain Reaction
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
TIM-1	T cell Ig and mucin-1
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor

Treg	T regulatory
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen 4

List of Publications

1) Kyaw T, Tay C, **Hosseini H**, Kanellakis P, Gadowski T, MacKay F, Tipping P[1], Bobik A[1], Toh BH[1].

   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

2) Kyaw T, Winship A, Tay C, Kanellakis P, **Hosseini H**, Cao A, Li P, Tipping P[1], Bobik A[1], Toh BH[1].

   Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation*. 2013; 127(9):1028-39

3) Kyaw T, Cui P, Tay C, Kanellakis P, **Hosseini H**, Liu E, Rolink AG, Tipping P[1], Bobik A[1], Toh BH[1].

   BAFF receptor mAb treatment ameliorates development and progression of atherosclerosis in hyperlipidemic ApoE(-/-) mice. *PLoS One*. 2013; 8(4):e60430.

4) Li Yi[1], Kelly To[1], Peter Kanellakis, **Hamid Hosseini**, Virginie Deswaerte, Peter Tipping, Mark J Smyth, Ban-Hock Toh[1], Alexander Bobik[1], Tin Kyaw[1]

   CD4+ Natural Killer T Cells Potently Augment Aortic Root Atherosclerosis by Perforin- and Granzyme B-Dependent Cytotoxicity. Circulation Research. 2015 Jan 16;116(2):245-54.

5) **Hamid Hosseini**, Yi Li, Peter Kanellakis, Christopher Tay, Anh Cao, Peter Tipping, Alex Bobik[1], Ban-Hock Toh[1], Tin Kyaw[1]

   Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes (In press)

6) **Hamid Hosseini**, Yi Li, Peter Kanellakis, Christopher Tay, Anh Cao, Edgar Liu, Peter Tipping, Alex Bobik[1], Ban-Hock Toh[1], Tin Kyaw[1]

TLR4 and MyD88 Are Essential for Atheroprotection by B1a B cells in Hyperlipidemic ApoE$^{-/-}$ Mice (Submitted)

7) **Hamid Hosseini**, Peter Kanellakis, Yi Li, Christopher Tay, Anh Cao, Peter Tipping, Alex Bobik[1], Ban-Hock Toh[1], Tin Kyaw[1]

   Anti-TIM-1 Monoclonal Antibody (RTM1-10) Ameliorates Atherosclerosis development and progression by Expansion of Atheroprotective B1a Cells (In preparation)

8) C Tay, Yu-Han Liu, **H Hosseini,** P Kanellakis, A Cao, P Tipping, A Bobik[1], BH Toh[1], T Kyaw[1]

   Tumor necrosis factor α produced by B2 B lymphocytes promotes atherosclerosis by augmenting lesion inflammation and cell death (Submitted)

1-equal contribution

List of Conferences

1) **Hamid Hosseini**, Tin Kyaw, Christopher Tay, Peter Kanellakis, Peter Tipping, Alex Bobik, Ban-Hock Toh (2012)

   In-vivo expansion of atheroprotective B1a cell by apoptotic cells decreases atherosclerosis by reducing inflammation and necrotic core in atherosclerotic lesions. Australian Vascular Biology Society 20th National Scientific Conference (Poster Presentation)

2) **Hamid Hosseini**, Tin Kyaw, Christopher Tay, Peter Kanellakis, Peter Tipping, Alex Bobik, Ban-Hock Toh (2013)

   B1a B Cells Require Toll-like Receptor 4 and MyD88 to Suppress Development of Atherosclerotic Lesions

   Australian Society for Immunology (ASI) Conference 2013 (Poster Presentation)

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PART A: General Declaration

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 1 original paper published and 3 unpublished publications in peer reviewed journals. The main theme of the thesis is the therapeutic roles of B cells in atherosclerosis. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of me, 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 2-4 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Peritoneal B1a lymphocytes Require Toll-like Receptor 4/MyD88 Dependent Activation to Produce IgM and Suppress Atherosclerosis Development in ApoE-/- Mice	In press	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 90%
3	Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a cells	Submitted	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 90%
4	Anti-TIM-1 Monoclonal Antibody (RTM1-10) Ameliorates Atherosclerosis development and progression by Expansion of Atheroprotective B1a Cells	In pre-submission preparation	Study design, experimentation, data analysis, result interpretation, preparation of manuscript; 90%

[* 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: [redacted] Date: 13/02/15

Abstract

Atherosclerosis is a chronic inflammatory disease of large elastic and muscular arteries in which lesions are characterized by the deposition of cholesterol, leukocyte influx, smooth muscle cell proliferation, cell debris and collagen accumulation in the intima. Inflammatory cells of the innate and adaptive immune systems contribute to lesion development and progression. Cardiovascular diseases such as heart attacks and strokes are the leading causes of death globally with underlying of atherosclerosis. Their current treatment is restricted to lipid-lowering statins which are not sufficient in preventing and remitting atherosclerosis. Therefore new additional anti-inflammatory therapeutic strategy is required in the management of atherosclerosis.

Earlier studies showed a protective role for total B cells; however further studies by our group and others showed a pro-atherogenic role for B2 cells and atheroprotective role for B1a cells. B cells have been shown to accumulate in atherosclerotic lesions in very low numbers. Understanding the role of B cells in atherosclerosis is rather complex as there are two main subsets of B cells, B1 and B2 B cells, each with differing origin, development, differentiation, localization and effector functions. B1a cells mainly produce Natural IgM antibodies while B2 cells mainly produce IgG antibodies. Splenectomy of Apolipoprotein E knockout mice ($ApoE^{-/-}$) resulted in a preferential depletion in peritoneal B1a cell population and enhanced atherosclerosis. Our group showed that reconstitution of B1a cells by adoptive transfer rescued the aggravated atherosclerosis in splenectomised $ApoE^{-/-}$ mice. In another study we showed that B2 cells are atherogenic as adoptive transfer of B2 cells into lymphocyte-deficient $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice and in B cell-deficient $ApoE^{-/-}\mu MT$ mice exacerbated atherosclerosis. These studies established opposing roles of B cell subsets in atherosclerosis development in $ApoE^{-/-}$ atherogenic mouse model. B2 cells were identified as atherogenic whereas B1 cells were atheroprotective. Atherogenicity of B2 cells was confirmed by further studies from our laboratory on BAFF-R deficient $ApoE^{-/-}$ mice that are

selectively deficient in B2 but not B1 cells. BAFF-R knockouts were selected for study because BAFF is a growth and maturation factor for B2 cells but not B1 cells. Additional confirmation was provided when we used monoclonal antibody to BAFF-R to selectively deplete B2 cells and spare B1a cell. This antibody not only attenuated the development of atherosclerosis but also suppressed the established atherosclerosis.

To further extend these observations the studies presented in this thesis explores therapeutic strategies to expand B1a cells to suppress atherosclerosis development. These findings may have potential for clinical translation.

Firstly I investigated the role of toll-like receptors (TLRs) and CD40 in activating atheroprotective B1a cells. Peritoneal B1a cells were depleted by splenectomy from ApoE$^{-/-}$ mice, then mice adoptively received B1a cells from C57Bl/6 wild type (WT), TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, MyD88$^{-/-}$ and CD40$^{-/-}$ mice and fed a high fat (HFD) diet for 8 weeks. B1a cells from WT, TLR2$^{-/-}$, TLR9$^{-/-}$ and CD40$^{-/-}$ mice suppressed atherosclerosis whilst B1a cells from TLR4$^{-/-}$ or MyD88$^{-/-}$ were without affect. Adoptively transferred B1a cells from WT but not TLR4$^{-/-}$ or MyD88$^{-/-}$ mice elevated IgM in plasma and atherosclerotic lesions. This was associated with reductions in lesion oxLDL, macrophages, T cells and proinflammatory cytokines. Also lesion apoptotic cell numbers were reduced by B1a cells from WT but not TLR4$^{-/-}$ or MyD88$^{-/-}$ mice and lesion anti-inflammatory cytokines IL-10 and TGF-β1 were increased. Polyclonal natural IgM produced by TLR-stimulated B1a cells during development of atherosclerosis not only neutralised oxLDL and facilitated removal of lesion apoptotic cells but also bound to leukocytes, CD3 and CD4 T cells and reduced local inflammation in atherosclerotic lesions.

Next, I examined whether activating B1a cells with apoptotic cells (AC) as well as phosphatidylserine liposomes (PSL) could enhance their protective actions during atherosclerosis. Intraperitoneally administration of both ACs and PSL attenuated atherosclerosis in ApoE$^{-/-}$ mice and the effect was dependent on B1a cells as splenectomy abrogated these effects. Atheroprotection was associated with reductions in lesion

macrophages and CD4+ and CD8+ T cell accumulation was well as reductions in proinflammatory cytokines and increases in anti-inflammatory cytokines. ACs and PSLs also increased B1a cell numbers including TIM-1+ B1a cells without affecting other lymphocyte populations. Total plasma IgM, anti-leukocyte, anti-CD3, anti-CD4 and anti-oxLDL IgM were also significantly elevated. The elevated IgM in developing atherosclerotic lesions were associated with reductions in lesion MDA-LDL (oxLDL), apoptotic cell numbers and lesion necrotic core size.

Thirdly I explored the role of TIM-1 receptor, a receptor for phosphatidylserine (PS) expressed on regulatory B cells. I examined the capacity of anti-TIM-1 RMT1-10 low affinity agonist monoclonal antibody (mAb) to expand B1a cells to inhibit progression and development of established atherosclerosis. Six-week old male ApoE$^{-/-}$ mice were treated with anti-TIM-1 RMT1-10 mAb and fed a HFD for 8 weeks. B1a TIM-1+IgM+ B cells and B1a TIM-1+IgM+IL-10+ B cells were selectively expanded. These effects reduced lesion size and markedly increased plasma and lesion IgM and decreased lesion oxidatively modified LDL (oxLDL). This was associated with reduction of CD4+ and CD8+ T cells, macrophages and monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), proinflammatory cytokines expression, apoptotic cell numbers and necrotic cores in atherosclerotic lesions. Splenectomy indicated that these effects were B1a cell-dependent. B1a cell stimulation *in-vitro* with the anti-TIM-1 mAb promoted dose-response B1a cell proliferation and IgM production. In an intervention study to determine whether anti-TIM-1 mAb treatment could attenuate developed atherosclerosis progression, 6 week-old ApoE$^{-/-}$ mice were fed a HFD for 6 weeks, and treated with anti-TIM-1 mAb for another 8 weeks while continuing the HFD. Treatment also increased B1a TIM-1+IgM+ B cells, B1a TIM-1+IgM+IL-10+ B cells and IgM levels and greatly attenuated atherosclerosis progression.

Taken together, my findings provide persuasive data for the expansion of atheroprotective B1a B cells in the prevention of atherosclerosis development and the suppression of already established atherosclerosis. The latter finding suggests potential for clinical translation.

Chapter 1: General Introduction

1.1 Pathogenesis of Atherosclerosis

Atherosclerosis as a chronic inflammatory disease of medium and large sized arteries remains the main cause of heart attacks, strokes and death [1-3]. Initially atherosclerosis was considered for many years to be solely a lipid storage disease with passive accumulation of lipids in the intima of vessels, but now it is recognized that inflammation contributes to its pathogenesis [4]. Atherosclerosis characterized by accumulation of modified lipids, macrophages and other immune cells in the intima of the arterial wall [5] and the identification of immune cells such as macrophages, monocytes, dendritic cells and lymphocytes in atherosclerotic lesions supports the involvement of immune mechanisms [6, 7]. Atherosclerosis develops slowly over decades in humans and can even be present in early childhood as pre-atheromatous fatty streaks. More advanced atherosclerotic plaques can increase in size to overtime narrow the lumen, restricting blood flow to heart muscle resulting in stable exercise induced angina. Advanced plaques are also fragile and normally associated with neovascularization from adventitia and can rupture, resulting in thrombus formation and occlusion of arteries, frequently stopping flow to major organs. Hypertension, hyperlipidemia, diabetes, rheumatoid arthritis, and many other autoimmune diseases are well known risk factors for atherosclerosis [8, 9]. Elastic and muscular arterial vessels most affected by atherosclerosis consist of a number of distinct layers containing different cell types with various functions. From the luminal side, a mono-layer of endothelial cells (ECs), the most internal layer of the vessel and form an interface between the bloodstream and blood vessel. As they are in

direct contact with blood, they are affected by the hemodynamic forces of blood-flow. Alterations in blood-flow characteristics can influence the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, and increase the adherence of monocytes to the endothelium, both key initiating events in atherogenesis [10, 11]. ECs also release factors that modulate vascular tone in response to changes in blood flow, such as nitric oxide (NO), a potent vasodilator. Not only does NO control smooth muscle cells (SMCs) relaxation and proliferation, it is also important for inhibiting leukocyte adhesion [12, 13], and preventing platelet aggregation [14], both of which influence atherosclerosis. Increased oxidative stress, the consequence of increased reactive oxygen species such as superoxide anion, which are produced by macrophages in atherosclerotic lesions, reduce the bioavailability of NO, and impair the ability of ECs to protect against atherosclerosis [15]. This, along with other factors that damage the endothelium such as hypertension, and biochemical injury caused by hypercholesterolemia and chronic inflammation, leads to substantial endothelial dysfunction. Endothelial dysfunction is one of the very early changes detected in the pathogenesis of atherosclerosis, and is considered an important initiating factor [16]. Changes to the physical structure and properties of the endothelium may enable leukocytes from the circulation to enter the vessel more easily. The intima, the region from the endothelium to the internal elastic lamina is the most inner layer of arterial wall (IEL) [17] and in humans this layer contains SMC, extracellular matrix (ECM), and vascular-associated lymphoid tissue (VALT) consisting of resident macrophages, occasional T cells and dendritic cells (DC) [18]. The size of the intima increases with age in humans [19], but not in mice, particularly in normal arteries. In humans, atherosclerosis may be initiated by resident intimal

cells responding to modified lipids, whilst in mice, atherosclerosis is associated with the migration and retention of vascular cells in the intimal region. The IEL separates the intima from tunica media, and during lesion formation, separates the contents in the intima from the media. Multiple layers of tightly packed SMCs that possess a contractile phenotype form the media. These cells relax or contract to modulate vascular tone. SMCs can migrate from the media through the IEL and into the intima where they alter their contractile phenotype to a synthetic phenotype. These synthetic SMCs produce extracellular matrix proteins including proteoglycans, elastin, and collagen. The latter provides tensile strength to developing atherosclerotic lesions, and helps maintain plaque stability. The adventitia is the outermost layer of the vessel, and is separated from the tunica media by the external elastic lamina. Lymphocytes can be found residing in the adventitia of non-atherosclerotic aortas of mice [20, 21]. During progression of atherosclerosis the vasa vasorum within the adventitia vascularize the lesions and facilitate leukocyte infiltration. Leukocytes within the adventitia form adventitial aortic tertiary lymphoid organs and cells of this origin are able to migrate to the intima [20, 22-24].

1.2 Historical Perspectives of Atherosclerosis

Atherosclerosis is a disease that is found in human bodies and considered to be due to degenerative arterial changes in arteries of Egyptian mummies dating from 1500BC to 250AD [25]. Later studies indicated that these arteries exhibited regions of lipid accumulation [26]. For the first time in 1755, Albrecht von Haller used the term “atheroma” to describe atherosclerotic lesion and subsequently, inflammation was detected in atheroma by Joseph Hodgson [27]. Later studies revealed that atherosclerosis lesions developed when rabbits were fed with egg and milk or

cholesterol and cholesterol excess was the major cause of atherosclerosis [27]. Russell Ross revealed mechanistic insights into atherosclerosis and he proposed that endothelial injury caused by factors such as mechanical injury, modified LDL and hypertension initiated atherosclerosis [28].

Subsequently, Jonasson and colleagues reported the presence of macrophages and lymphocytes in atherosclerotic plaques [29] and other histological studies of ruptured plaques from patients with acute coronary syndrome confirmed the presence of macrophages, T cells as well as mast cell at rupture sites [30]. Further histopathological studies in young patients with no clinical symptoms confirmed the presence of macrophages, dendritic cells, MHC class-II and activated T cells in atherosclerotic lesions [30]. These studies and studies over the last decade suggest that immune cells are important contributors to atherosclerosis and both innate and adaptive immune systems participate in the development of atherosclerosis lesions.

1.3 Epidemiology of Atherosclerosis

Atherosclerosis is the underlying cause of heart attacks and strokes and is the leading cause of death in western and developing countries [1] as World Health Organization (WHO) report showed a significant increase of death caused by atherosclerosis complications because more developing countries adopted western habits during last decades [31, 32]. Cardiovascular disease (CVD) is the leading cause of death. According to the latest estimates, the number of deaths from CVD stands at an astonishing 17.3 million worldwide. This is expected to reach 23.6 million by the year 2030 [32]. National Heart Foundation of Australia in 2006 reported that cardiovascular disease remains the largest single cause of mortality by 34% and 39% in males and females respectively [33].

1.4 Risk Factors and Theories on the Pathogenesis of Atherosclerosis

There are many risk factors associated with coronary heart disease and stroke. Some risk factors such as family history, ethnicity and age cannot be changed but other risk factors such as tobacco exposure, high blood pressure (hypertension), high cholesterol, obesity, physical inactivity, diabetes, unhealthy diets, and harmful use of alcohol can be treated or changed. As inflammation is involved in atherosclerosis and atherosclerosis is an inflammatory disease, there are several factors that lead to inflammation including injury to the endothelium. Elevated and modified LDL, free radicals from smoking, hypertension, diabetes mellitus, genetic abnormalities and infections are possible causes of injury to the endothelium.

1.4.1 Atherosclerosis as a response to injury

The response to injury hypothesis was proposed by Russel Ross [34]. It was proposed that the development of atherosclerosis is the result of damage primarily to the endothelium and is based on observations that platelet derived growth factor stimulated the proliferation of arterial SMCs leading to development of an arterial intima [34]. The loss of endothelium exposes the underlying collagen layer and SMCs to platelet-derived growth factor (PDGF) from platelets that adhere to the exposed sub-endothelial connective tissue. The infiltration of platelets, leads to migration and proliferation of SMC medial layer. The proliferation of SMC in the intima forms new connective tissues ultimately results in intracellular and extracellular lipid deposition. The uncontrolled migration and proliferation of SMCs

was thought to eventually cause artery occlusion. Repetition and amplification of this process leads to the development of complex lesions. Ross acknowledged that the endothelium plays a more active role in the pathogenesis of atherosclerosis. It was recognized that endothelial cells are in an activated state and promote cell adhesion and invasion of monocytes, secrete oxygen free radicals oxidized LDL (oxLDL); they also synthesize and secrete PDGF-like protein stimulating medial SMC migration and proliferation [35]. The mechanism of macrophage accumulation in the intima was not discussed in this hypothesis. Subsequently, the hypothesis was modified to include injury to the endothelium caused by chronic hyperlipidemia, infections, mechanical factors and immunological injury that lead to endothelial dysfunction. Endothelial dysfunction due to endothelial injury increased the trapping of lipoprotein in branch point of the arterial tree and up regulation of adhesion molecules and chemokines on the surface of the endothelium [8, 36]. These adhesion molecules and chemokines attracted monocytes while attach to the endothelium and migrate into the intima. Monocytes then differentiate into macrophages. These macrophages remove extracellular lipids by taking up lipoproteins and develop into foam cells and subsequently release inflammatory chemokines and cytokines and growth factors to promote and become fatty streak and atheroma development [8].

1.4.2 Response to Hypercholesterelmia

Hypercholeterolemia plays a critical role in atherosclerosis. The response to retention hypothesis was proposed in 1995 by William et al [37]. It proposed that the extracellular trapping of cholesterol-rich atherogenic lipoprotein within the arterial intima is responsible for atherosclerosis. Studies shown that only elevated levels of lipoproteins containing apolipoprotein (Apo) B can develop atherosclerosis in

humans and animals even in the absence of other risk factors [5]. In early studies thought that the extracellular matrix of the subendothelium, particularly proteoglycans play an important role in the retention of atherogenic lipoproteins [38]. The interaction between atherogenic lipoproteins and proteoglycans involves in interaction between basic amino acids in apoB100 and negatively charged sulphate groups on the proteoglycans [39]. Skalen and colleagues revealed a link between the atherogenicity of ApoB-containing low-density lipoproteins (LDL) and their affinity for artery wall proteoglycans. They showed that mice expressing proteoglycan-binding-defective LDL protected against development of atherosclerosis compared to mice expressing wild-type control LDL and they concluded that subendothelial retention of apoB100- containing lipoprotein is an early step in atherogenesis [40]. Mouse LDL often contains ApoE, but ApoB100 is the sole apolipoprotein on human LDL. Thus, bridging molecules are probably less important than a direct interaction between apoB100 and proteoglycans for subendothelial retention of atherogenic lipoproteins in humans. Retained lipoproteins can directly or indirectly provoke all known features of early lesions and, by stimulating local synthesis of proteoglycans, can accelerate further retention and aggregation [37]. Atherogenic lipoproteins enter the arterial wall and are retained through interactions with proteoglycans secreted by SMCs. Hypercholesteremia, results in a rapid retention of lipoproteins [41] which are subsequently modified by oxidation or enzymes [42] and captured by macrophages resulting in foam cell formation [43-45]. The study suggested that atherosclerosis is initiated by sub-endothelial retention of atherogenic lipoprotein. OxLDL initiates inflammation by increasing the expression of adhesion molecules by the endothelium and secretion of chemokines and proinflammatory cytokines by both macrophages and the endothelial cells [46].

1.4.3 Atherosclerosis and Inflammation

In 1999 Ross proposed that atherosclerosis is an inflammatory disorder of blood vessels modifying his earlier “Response to Injury” hypothesis [47]. Damage to the endothelium was caused by agents such as modified LDL, dyslipidemia, elevated plasma homocysteine concentrations, hypertension, diabetes, and pathogens. They stimulated the endothelial layer to lose its normal function and increase its permeability to lipoproteins and other plasma constituents and increase the expression of adhesion molecules and growth factors which promote the adherence and migration of monocytes, macrophages and T cells. These cells migrate through the endothelial layer and reside at the sub-endothelial layer. Macrophages accumulate lipid and develop into foam cells, which then trigger the release of growth factors and cytokines that promote migration and proliferation of SMCs resulting in fatty streak and the continuation of these processes results in the formation of fatty streaks. Inflammation plays a fundamental role in mediating all stages of atherosclerotic lesion development from the initiation, progression to the destabilisation of atheromas. However, early stages of lesion formation such as fatty streaks can be reversible, by removing the cause of injury, or halting early inflammatory processes. There are several inflammatory mediators involved in development and progression of atherosclerosis. It includes IL-6, TNF-α, IL-1β, MCP-1, VCAM-1, ICAM-1, and C-reactive protein (CRP) all of which have been associated with coronary heart disease (CHD) [27, 48-51]. CRP is a sensitive, unspecific marker for inflammation. In bacterial infection, CRP levels increases dramatically within 24 hours of infection [52]. Elevated CRP levels are known to be present in over 65% in patients with unstable angina [4]. CRP is capable of binding

and activating complement, inducing expression of adhesion molecules, mediate LDL uptake by macrophages and induce recruitment of monocytes and production of MCP-1 [3, 4, 7]. There is also direct association of IL-6 and CVD, where IL-6 levels were elevated in patients with unstable angina [53]. However, its role in atherosclerosis, hearth attack and stroke is still controversial and more studies need to be done.

1.4.3.1 Hypertension

Hypertension is an another important risk factor for atherosclerosis and as angiotensin II (AII) is an important hypertensive agent and vasoconstrictor, AII can activate T cells [54] and increase the expression of inflammatory cytokines and chemokines such as IL-6 and MCP-1 by SMCs and VCAM-1 by endothelial cells [55, 56].

1.4.3.2 Diabetes

Hyperglycemia affects the endothelium to increase the production of free radicals which in turn reduces nitric oxide, a chemical important in blood vessel dilation and reducing inflammation. Diabetes aggravates atherosclerosis by complex yet to be fully defined the mechanism. It can interrupt the function of the enzyme extracellular signal-regulated kinase 5 (ERK-5) [57]. ERK-5 activates endothelial nitric oxide synthase (eNOS) which produces nitric oxide and dilates blood vessels. Patients with diabetes also produce high levels of advanced glycation products (AGEs) [58]. AGEs promote production of free radicals and inflammation and also attenuate the

atheroprotective role of ERK-5. Attenuation of ERK-5 function promotes the adhesion of pro-inflammatory leukocytes and secretion of proinflammatory cytokines [58].

1.4.3.3 Obesity

Obesity is a risk factor for atherosclerosis and may lead to development of insulin resistance and diabetes; it can also contribute to dyslipidaemia. Obesity is associated with endothelial dysfunction, partly due to reduced bioavailability of nitric oxide [59]. In addition, adipose tissue can release inflammatory cytokines such as TNF-α, lL-6, complement factor C3, angiotensin II and plasminogen activator inhibitor-1 (PAI-1), factors that promote atherosclerosis [60]. Circulating TNF-α is markedly elevated in obese patients [61]. Leptin secreted by adipocytes also plays a role in obesity-related endothelial dysfunction [62]. It stimulates in endothelial cells the production of superoxide to increase oxidative stress in endothelial cells, CCL2 expression and endothelin-1; it can also promote the migration and proliferation of SMCs [63, 64]. Treatment of $ApoE^{-/-}$ mice with recombinant leptin also promotes atherosclerosis and thrombosis [65].

1.4.3.4 Infections

Chronic infections can also contribute to the development of atherosclerosis [66] and associations between atherosclerosis and microbial infections has been widely documented [67, 68]. This is not surprising as an active immune response to microbial pathogens could also act at the site of the vessel wall and contribute to lesion progression, especially if the pathogen is present in the arterial wall. Numerous pathogens have been implicated in atherosclerosis. *Chlamydia pneumonia* [69], and *herpes viruses* [70] are two pathogens that have been detected

in atherosclerotic lesions. Infection by *C. pneumonia* in human plaques releases endotoxin and heat shock protein which activate vascular endothelial cells, SMCs and macrophages to enhance inflammation during atherosclerosis progression [71]. The presence of *C. pneumonia* in atherosclerotic lesion has been reported in a number of studies, and the presence of *C. pneumonia*-specific T cells primarily the $CD4^+$ Th1 subtype has been demonstrated. These T cells maintain inflammatory responses due to infection and augment progression of atherosclerosis [72-74]. *C. pneumonia* infection in $ApoE^{-/-}$ mice accelerates the development of atherosclerosis [75]. This pathogen can multiply within the cells of atherosclerotic lesions, including endothelial cells and macrophages. LPS from the cell walls of bacteria and other toll-like receptor (TLR) ligands can enhance macrophage foam cell formation by promoting cholesteryl ester accumulation [76]. Activation of TLRs has been shown to increase the expression of fatty acid binding proteins in macrophages, and also inhibit efflux of cholesterol by macrophages via inhibition of liver X receptor signaling [77, 78]. Also after T and B cell function, heat shock proteins (HSPs) have been implicated in the development of atherosclerosis, especially during the initial stages. Micro-organisms can express heat shock proteins that show close homology to those in the host species, and the immune response generated against these pathogens can cross-react with self-antigens by molecular mimicry, causing tissue damage. Antibodies against cytomegalovirus-derived proteins, and HSPs on *Escherichia coli*, *Chlamydia pneumonia*, and *Mycobacterium tuberculosis* can cross-react with HSP60 expressed by vascular endothelial cells, augmenting endothelial injury and dysfunction to initiate atherosclerosis [79-82]. Immunization of animals with HSP65 leads to accelerated lesion development in which T cells play a major role [83, 84]. Lymphocytes reactive to HSP65 are strongly associated with atherosclerosis,

promote fatty streak formation [85, 86]. In contrast, mice fed HSP65, oxLDL and subsequently immunized with the same antigen to induce oral tolerance, developed smaller lesions compared to control mice [87-90]. In-vitro studies indicate that monocytes infected with *C.pneumonia* transmit the pathogen to endothelial cells [91], thereby up regulating the expression of adhesion molecules and secretion of proinflammatory cytokines. Infection of endothelial cells triggers production of IL-6 and IL-8. Infected macrophages secrete pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and MCP-1, MIP-1 and IL-12 [92, 93]. An example of molecular mimicry that provides the host protection against atherosclerosis has been observed between *Streptococcus pneumonia* and oxLDL. Mice immunized with *S. pneumonia* show expansion of T15 IgM-secreting B1 B cells, which bind to pathogen or oxLDL, reducing the extent of atherosclerosis. Plasma from these immunized mice can also block the uptake of oxLDL by macrophages [94]. While a B cell response (generation of antibodies) against oxLDL has generally been shown to be beneficial, T cell responses generally aggravate atherogenesis. Injection of oxLDL in neonatal mice to induce tolerance by deletion of reactive T cells decreases atherosclerosis [95]. Malondialdehyde (MDA)-LDL, another form of modified lipid, is strongly associated with atherosclerosis. Immunization with this antigen has a similar effect to immunization with oxLDL (i.e. reduced atherosclerosis) [96, 97].

1.4.4 Autoimmune Diseases

Autoimmunity is an immune response to self-antigens that involves autoantibody or self-reactive T cells in pathogenesis of disease. Autoimmune diseases are also a major risk factor for atherosclerosis. Several autoimmune rheumatic conditions,

including rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) enhance atherosclerosis and subsequently contribute to higher cardiovascular morbidity and mortality rates [98]. Rheumatoid arthritis is a chronic inflammatory disease and more interesting both atherosclerosis and RA have similarities in their inflammatory pathways and patients with RA have increased risk of cardiovascular events and RA has been considered an independent risk factor for atherosclerosis [98, 99]. Dyslipidemia is a common risk factor observed in RA which is associated with low levels of HDL and high levels of triglyceride [100]. High levels of proinflammatory cytokines in RA have also been associated with the development of atherosclerosis. The chronic systemic inflammation that occurs in RA can contribute to endothelial dysfunction and oxidative stress to promote atherosclerosis [101]. TNF-α and interleukin-1 expressed in joints affected by RA, may act on other tissues and promote atherogenesis. C-reactive protein (CRP), a marker for inflammation, levels are also elevated in RA patients and this marker for inflammation has also been implicated in atherosclerosis [101]. SLE is a complex multisystem inflammatory disease that predominantly affects young women [102] and atherosclerosis develops early in the course of this disease. Risk factors that could contribute to atherosclerosis development are CRP, fibrinogen, interleukin-6, CD40/CD40L, adhesion molecules; immunological factors: αCL, anti-B2GPI, and anti-oxLDL; abnormal coagulation factors; fibrinogen, plasminogen activator inhibitor-1, and homocycteine and the lipoprotein HDL [103]. Wick postulated that the inflammatory immunological processes characteristics of very early stages of atherosclerosis are initiated by humoral and cellular immune reactions against heat-shock proteins 60 (HSP60) [104]. HSP60 is expressed by cells under conditions of stress such as heat

and toxins. HSP60 have been identified as an autoantigen present during development of atherosclerosis [105-107].

1.5 Cholesterol and Atherosclerosis

Cholesterol is only slightly soluble in water. Since cholesterol is also insoluble in blood, it is transported in the circulatory system within lipoproteins, complex discoidal particles that have an exterior composed of amphiphilic proteins and lipids whose outward-facing surfaces are water-soluble and inward-facing surfaces are lipid-soluble; triglycerides and cholesterol esters are carried internally. In addition to providing a soluble means for transporting cholesterol through the blood, lipoproteins have cell-targeting signals that direct the lipids they carry to tissues. For this reason, there are several types of lipoproteins in blood, called, in order of increasing density, chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The more lipids and less protein a lipoprotein has, the less dense it is.

1.5.1 Very-low-density lipoprotein

Very-low-density lipoprotein (VLDL) is one of the five major groups of lipoprotein made by the liver [108] that enable fats and cholesterol to move within the water-based solution of the bloodstream. VLDL is assembled in the liver from triglycerides, cholesterol, and apolipoproteins and transports endogenous triglycerides, phospholipids, cholesterol, and cholesteryl esters in the blood stream. VLDL also serves for long-range transport of hydrophobic intercellular

messengers[109]. VLDL is converted in the bloodstream to low-density lipoprotein (LDL). VLDL changes during circulation as when was released from the liver contains apolipoprotein B100, apolipoprotein C1 (ApoC1), apolipoprotein E (ApoE), cholesterol, cholestryl esters and triglycerides. As it circulates in blood, it picks up apolipoprotein C-II (ApoC-II) and additional ApoE donated from high-density lipoprotein (HDL). At this point VLDL becomes a mature VLDL. Once VLDL passes through capillaries, it will come in contact with lipoprotein lipase (LPL) in the organs. Then, triglycerides will be removed by LPL from VLDL for storage or energy production. Now, VLDL meets up with HDL where ApoC-II is transferred back to HDL. HDL also transfers cholesteryl esters to VLDL in exchange for phospholipids and triglycerides via cholesterylester transfer protein (CETP). As more and more triglycerides are removed from VLDL, the composition of the molecule changes, and it becomes intermediate-density lipoprotein (IDL) [110]. Around half of IDLs are recognized by receptors in the hepatocytes because of their ApoB-100 and Apo E content. The other 50% of IDL lose ApoE. When their cholesterol content becomes greater than the content of triglyceride, they become LDL, with apoB-100 as their primary apolipoprotein. LDL is taken into a cell via the LDL receptor to be stored. This LDL can be used for cell membrane structure or converted into other products such as steroid hormones or bile acids [111].

1.5.2 Low-density Lipoprotein

Low-density lipoprotein (LDL) is a spherical molecule 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 B100 (ApoB100) [112]. 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 sub-endothelialspace of arteries [40, 113]. 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) [114]. There are copious derivatives of oxidized LDL (oxLDL) ranging from minimally to highly oxidized LDL. LDL is susceptible to oxidation mostly at polyunsaturated fatty acid chains of esterified cholesterol and triglycerides and the sterol group of cholesterol [112, 113]. When these LDL components are modified by free radicals, a cascade of reactions occurs and further oxidative modifications ensue.

1.5.3 High-density lipoprotein

High-density lipoprotein (HDL) is one of the five major groups of lipoproteins and the smallest one. These lipoproteins are complex particles containing multiple proteins which transport, lipids in the bloodstream and extracellular fluid. Unlike the larger lipoproteins which deliver fat molecules to cells, HDL remove fat molecules such as cholesterol, phospholipids, and triglycerides from cells through the bloodstream, back to both LDL particles and to the liver for disposal from the body. High level of HDL is strongly associated with decreasing accumulation of atherosclerosis within the intima [115]. HDL particles are sometimes referred to as good cholesterol because they can transport cholesterol and triglycerides out of artery walls, reduce macrophage accumulation and prevent progression of atherosclerosis and as a result help

prevent CHD and stroke. In contrast, LDL particles are often called bad cholesterol or unhealthy cholesterol, because they deliver fat molecules to macrophages in the wall of arteries [116].

1.5.4 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 ApoB100 [117, 118]. Mild oxidation of LDL does not affect recognition by the LDL-receptor (LDLR) [114, 117]. Mildly oxidized LDL stimulates the production of cAMP in endothelial cells. In doing so, it increases the expression of several inflammatory agents of which P-selectin, monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (MCSF) have been identified in-vivo [119-123]. 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 [114, 124].

1.5.5 Highly-oxidized LDL

LDL is extensively modified when its oxidized lipid components disintegrate into smaller molecules comprising mostly of ketones and aldehydes. These lipid byproducts can form covalent cross-links with other lipids as well as protein adducts with ApoB100 [117]. One example is the association between malondialdehyde (MDA) and the ε-amino ends of exposed lysine and histidine residues on ApoB100 [114, 125]. The resultant Schiff bases are also very reactive as they bring about further lipid and peptide conjugation [112]. Consequently, ApoB100 loses its

conformation and breaks down into secondary fragments that can fuse with the phosphorylcholine head groups of LDL phospholipids and reconfigure them to reveal cryptic epitopes [126, 127]. 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 [112, 114, 128].

1.5.6 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 sera of mice and humans with established atherosclerosis [129, 130]. Recent efforts have taken great steps to clarify 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 suppression of atherosclerosis while IgG antibodies are associated with aggravation of atherosclerosis [131]. This idea has sparked a keen interest to develop vaccines against atherosclerosis. Since oxLDL particles are mostly confined within atherosclerotic lesions, this poses the question on their route of access to peripheral lymphoid organs where they activate immune cells. The heterogeneity of dendritic cells (DC) 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 [132]. Alternatively, antigens from inflamed tissues can directly track to draining lymph nodes through lymphatic conduits [133]. These could be the means which B cells and other immune cells are exposed to oxLDL antigens.

1.6 Development of Atherosclerotic Lesions

The normal human arterial wall consist of three main regions, the innermost region tunica intima, middle region called media and an outer region, the adventitia which embeds the vessel in its surrounding (Figure 1.1A). The intima is defined as the region commencing at the endothelium surface and extending to the luminal margin of the media defined by the IEL (internal elastic lamina) [17]. It is composed of proteoglycan [134], occasional macrophages and SMCs. This layer increases in thickness with increasing age as well as in subjects who exhibit increased susceptibility to atherosclerosis. The endothelium layer not only covers the intima but also serves as a barrier between circulating molecules and cells in blood. The endothelium synthesizes signalling molecules that can regulate both vascular tone and structure, inhibit platelet adhesion and aggregation, leukocyte adhesion and migration, vascular smooth muscle cell proliferation and migration [34, 135]. Functional and structural changes in endothelial cells can contribute to the pathogenesis of atherosclerosis. Under the endothelial monolayer, the sub-endothelial intima of human arteries is populated by predominantly SMCs, some immune cells and extracellular matrix [136, 137]. The number and morphology of cells especially SMCs in an atherosclerotic lesion increases substantially compared to normal intima [136]. At the site of atherosclerotic lesion, the stellate cell population increases substantially and appear to have a synthetic phenotype which is rich in rough endoplasmic reticulum. During atherogenesis, SMCs are thought to migrate

from the medial layer through the fenestrations in the internal elastic lamina to the intima with response to injury, and then proliferate and produce extracellular matrix which contributes to the increase in plaque size. In-vitro studies have suggested that the arterial media might be composed of phenotypically and functionally diverse subpopulations of SMCs [138]. A SMC subpopulation with unique characteristics such as enhanced growth capabilities, increased proliferation and contraction ability probably contribute to pathological conditions. Originally it also was thought that SMC in atherosclerotic lesions were solely derived from the media, but recently it has been suggested that bone marrow progenitor cells infiltrate the intima and probably differentiate in-vivo to form SMCs [139]. There is also evidence from human studies that after bone marrow transplant, SMCs of donor origin are increased in coronary atherosclerotic plaque [140]. The internal elastic lamina separates the media from the intima. Media is the thickest layer of normal vessels and contains 20% of SMCs of both contractile and synthetic phenotype, and 60% of collagen and elastin which provides the contractile ability for the vessel. The medial lamellar unit is both the structural and functional unit of most arteries. Medial lamellar units are oriented in concentric layers or lamellar units with smooth muscle cell and collagen in between in a fairly uniform composition and are constant in size regardless of species and vessel size [141]. The media layer contains collagen to maintain the integrity of the vessel wall which includes mainly type 1 collagen [142] and medial SMCs can proliferate and migrate into the sub-endothelial intima and contribute to the development of atherosclerosis in response to endothelial injury. In-vitro and in-vivo studies suggest that changes in media occur in response to shear stress alterations caused by arterial stenosis [143]. In order to normalize lumen diameter and shear stress, extracellular matrix in the medial layer remodels enabling expansion of the

artery and the repetition of such remodelling results in medial thinning [143]. The outermost layer of the vessel, the adventitia is separated from the media by an external elastic lamina which consists of extracellular matrix composed of collagen and fibroblasts. The human adventitial fibroblast population includes mesenchymal progenitor cells that can differentiate into adipocytes, osteoblast, myofibroblast and SMCs [144, 145]. This layer changes function and can contribute to pathology of atherosclerosis [146]. There is evidence for a role of lymphocytes in the adventitia in vessel disease [20, 146]. In the setting of murine hyperlipidaemia, lymphocytes are present in clusters within the adventitia of atherosclerotic abdominal aorta [20, 21]. Both T and B cells have been identified within the adventitia of atherosclerotic lesions [147, 148]. Complex lymphoid-like structures or nodules within the adventitia of advanced lesion mostly associated with the abdominal aorta are composed of primarily of B cells surrounded by macrophages and T cells [149]. It is thought that these are centres for antigen-derived B cell maturation to generate antibodies against inflammatory antigens generated by advanced lesions [20]. These lymphoid-like structures also appear to contain lymphatic vessels and drain local inflammatory cells and cytokines to the lymph nodes and these structures ensures a constant delivery of active inflammatory cells and cytokines to promote chronic inflammation within the vessel [150].

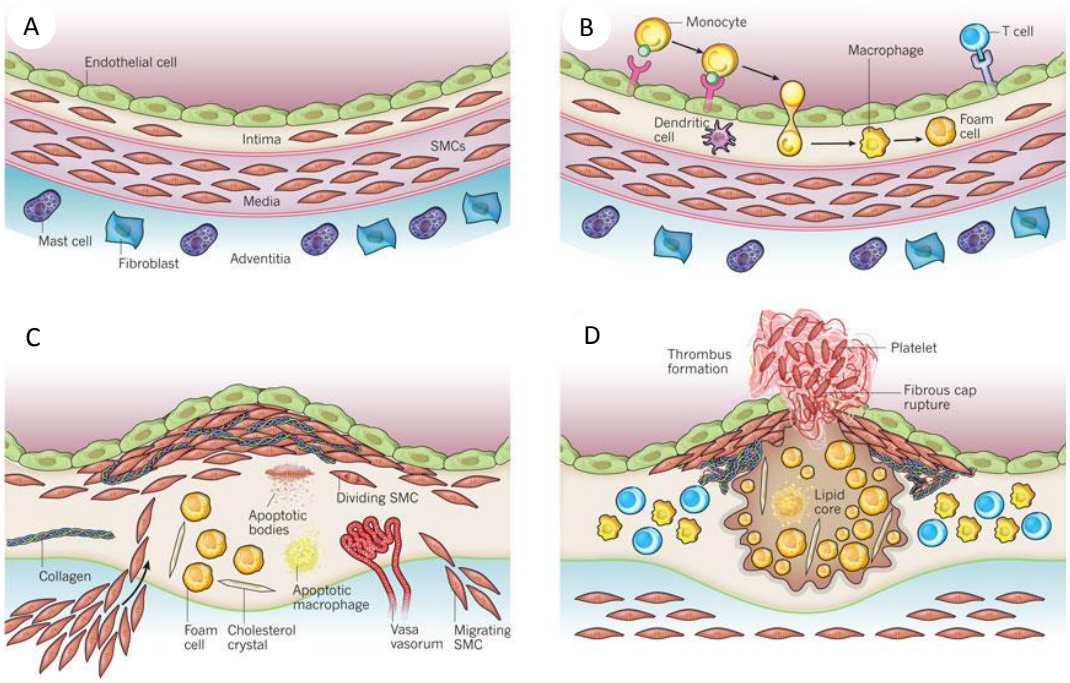

Figure 1.1. Normal artery wall and development of atherosclerotic lesions, from Nature [151].

(A) Three layers of normal artery are: 1. An inner layer containing a monolayer of endothelial cells that is in contact with blood stream. The middle layer contains SMCs surrounded by extracellular matrix and collagens. The adventitia or the outer layer contains some leukocytes, nerve endings and micro vessels. **(B)** Damage to vascular endothelium from shear stress, especially at arterial branch points, triggers expression of adhesion molecules and chemokines. These facilitate leukocyte and lipid accumulation at the site and extravasation into the vessel intima. Endothelial damage also reduces nitrous oxide production, thereby promoting accumulation of monocytes, lipids, and platelets. Lipid within the arterial wall, predominantly LDL, is oxidised to oxLDL and malondialdehydemodified LDL (MDA-LDL). Upon leaving the circulation, monocytes differentiate into macrophages which are induced to express scavenger receptors that uptake the modified lipid to become 'foam cells' characteristic of atherosclerosis. **(C)** With lesion progression, additional

macrophages and T lymphocytes are attracted to the site. Vascular smooth muscle cells (SMCs) migrate from the media, proliferate, and lay down collagen which contributes to fibrous cap formation in advanced lesions. Along with endothelial cells (ECs) they release growth factors and cytokines, and ingest modified lipid to become foam cells. A necrotic core of lipid and apoptotic cells forms and expands as the lesion progresses, and calcification may occur. **(D)** Plaques more likely to rupture, termed ‘vulnerable’, have features of degraded extracellular matrix, increased cell apoptosis, large necrotic cores, reduced smooth muscle cells and thin fibrous caps. Plaque rupture exposes thrombogenic factors to the circulation and triggers rapid formation of a platelet-fibrin thrombus which occludes the lumen. Dendritic cells, other leukocytes, and low numbers of B cells are also present in atherosclerosis [151].

1.7 Sites of atherosclerosis development

Atherosclerosis occurs at specific sites within the vasculature, mainly in large elastic and medium sized muscular arteries including the abdominal aorta and coronary arteries [152]. These highly susceptible atherosclerotic sites are exposed to low shear stress, oscillatory flow and turbulent flow, normally in areas that have high vessel curvatures and at branching sites. Sites where atherosclerotic lesions develop include coronary arterial bed, major branches of arch, visceral branches of abdominal aorta, and terminal abdominal aorta and its branches (Figure 1.2). Endothelium respond to changes in blood flow particularly changes to hemodynamic shear stress [153].These cells align according to the axis of laminar flow, and this alignment is disrupted in areas of interrupted flow. Endothelial cells express genes that are regulated by flow rate including cell-surface adhesion molecules (vascular cell adhesion molecule- 1 [VCAM-1] and intracellular adhesion molecule-1 [ICAM-1]), and antioxidant enzymes (NO synthase, superoxidase dismutase). Cultured human endothelial cells up-regulate VCAM-1 and ICAM-1 when exposed to oscillatory flow effects mediated by nuclear factor-κB (NF-κB) [10, 154]. Nitric oxide (NO), a product of conversion of L-arginine into L-citrulline by nitric oxide synthase (NOS) plays a major role in modulating vascular tone and is regulated by laminar shear stress. Oscillatory shear stress down-regulates expression of endothelial nitric oxidase synthase (eNOS) [155]. Hypercholesterolemia down-regulates bio-availability of endothelium-derived NO which in turn promotes neutrophil adherence to the endothelium [156]. A deficiency in NO also augments smooth muscle proliferation, platelet aggregation and adhesion [157]. $ApoE^{-/-}$ mice which are deficient in eNOS and fed a Western diet exhibit an increase in development of atherosclerosis [158].

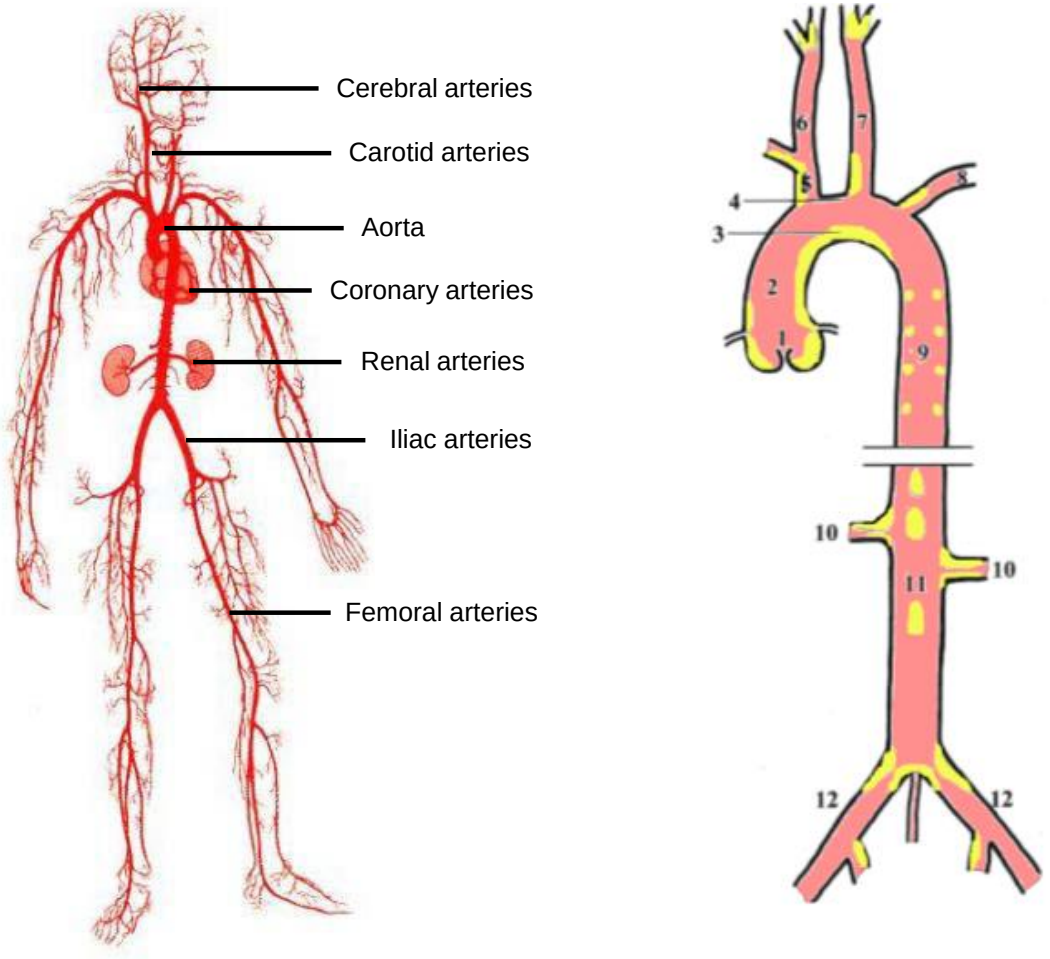

Figure 1.2. Common sites of atherosclerosis formation in humans and C57Bl/6 mouse strain, from Arterioscler Thromb Vasc Biol [152].

(A) Schematic shows common sites of atherosclerotic plaque formation in human vasculature. (B) Schematic shows the major arterial vasculature distribution of atherosclerosis (yellow) in the vasculatures of LDL receptor–deficient mice fed a high-fat atherogenic diet. 1, Indicates aortic sinus; 2, ascending aorta; 3, lesser curvature of aortic arch; 4, greater curvature of aortic arch; 5, innominate artery; 6, right common carotid artery; 7, left common carotid artery; 8, left subclavian artery; 9, thoracic aorta; 10, renal artery; 11, abdominal aorta; and 12, iliac artery [152].

1.8 Atherosclerotic lesions

Atherosclerotic lesions have been classified into a number of subtypes by the American Heart Association (AHA). “Early” implies that these lesions are followed by “latter” (advanced) lesions. It also implies that they can be found early in life. Neither implication is necessarily true. Early atherosclerotic lesions can be classified into types I, II and III.

1.8.1 Type I lesions

Type I lesions (the initial lesion) are the earliest microscopically identifiable lesions, and can even be found in infants and children. They are defined as lesions containing lipid deposits, macrophages, and foam cells [159]. They are initiated by lipoprotein particles becoming trapped in the intima by extracellular matrix proteins such as proteoglycans [40], and then undergoing oxidative modification, by enzymes such as lipoxygenases, NADPH oxidases, myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS). Oxidized LDL (OxLDL) and other modified forms of LDL are thought to initiate atherogenesis by activating endothelial cells, which in turn upregulate their expression of adhesion molecules on their luminal cell surface and produce chemotactic molecules that attract monocytes and lymphocytes [160-162]. Expression of adhesion molecules such as E- and P-selectin enables leukocytes from the circulation to roll along and then firmly adhere to the endothelium. These events involve molecules from the Ig gene superfamily such as VCAM-1 and ICAM-1; deficiency of these molecules in mice reduces the severity of atherosclerosis [163-165]. Activated endothelial cells can also produce molecules such as monocyte chemotactic protein-1 (MCP-1), which recruits leukocytes, mainly monocytes, into the vessel wall [121] (Figure1.3). A deficiency of MCP-1 in mice also reduces lesion

development [166]. OxLDL itself is also chemotactic, and attracts monocytes to the intima [167]. Lightly oxidized LDL induces macrophage activation and proliferation, whereas extensively oxidized LDL, or high concentrations of oxLDL causes usually cell death [168] (Figure 1.5).

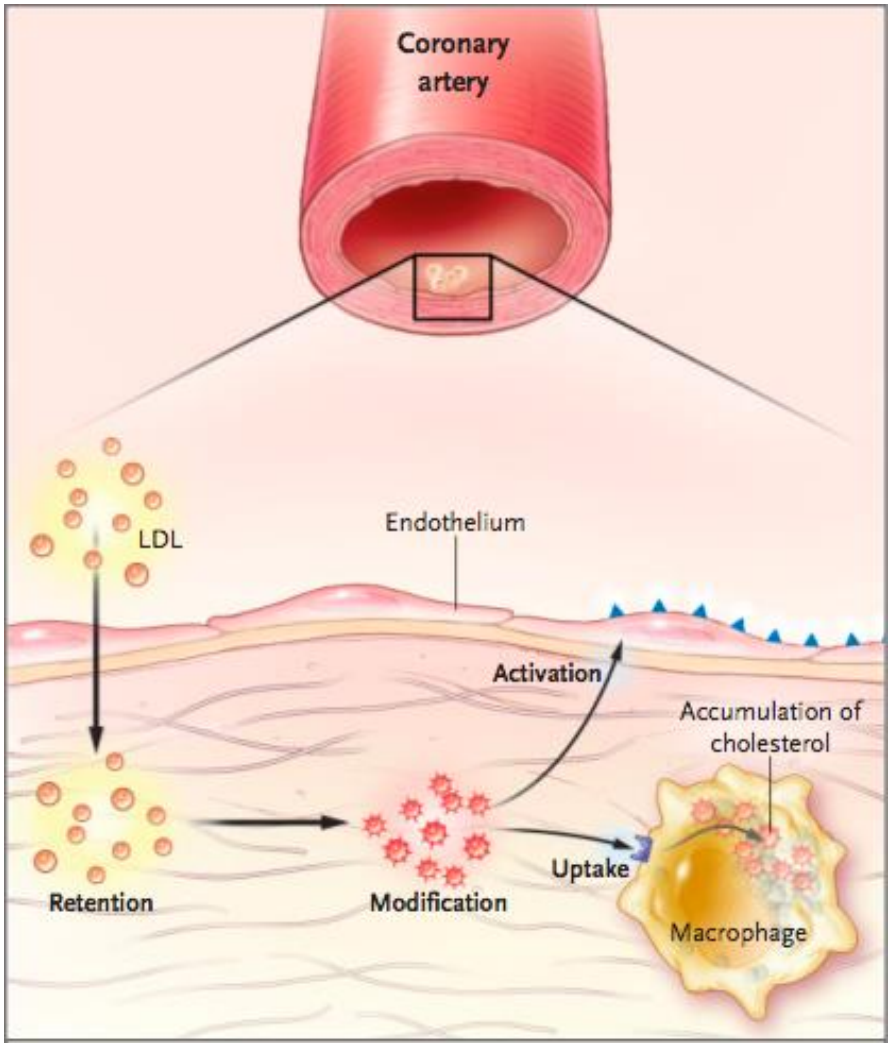

Figure 1.3. Endothelial cell activation causing immune cell infiltrate and oxLDL uptake, from N Engl J Med [124].

The process of LDL infiltration and retention occurs under conditions of hyperlipidaemia, systemic inflammation and mechanical stress. Accumulated LDL undergoes enzymatic and oxidative modification within the intima. The product of this is OxLDL, which activates ECs to upregulate expression of cellular adhesion molecules for leukocyte recruitment. Leukocyte recruitment is also facilitated by the expression of cytokines and chemoattractant molecules such as monocyte chemotactic protein-1 (MCP-1) [124].

1.8.2 Type II lesions

Type II lesions (the fatty streak) are the first grossly visible atherosclerotic lesions. They are characterised by stratified layers of macrophage foam cells, lipid-containing intimal SMCs, T cells, small numbers of mast cells, and small amounts of extracellular lipid droplets [159] (Figure 1.1B & 1.4).

1.8.3 Intermediate lesions

Type III lesions (the intermediate lesion or pre-atheroma) are the progressive form of fatty streaks (Figure 1.4). They contain increased extracellular lipid droplets and foam cells, which remain the major cell type in the lesions, and also contain accumulated SMCs and T cells. As the intima enlarges to encroach on the lumen, the vessel can compensate by outward remodeling to preserve the lumen size. Beyond this stage, the progression of atherosclerosis is often difficult to reverse. Depletion of monocytes before establishment of lesions reduces the extent of lesion development, but depletion of monocytes in mice with established lesions has no effect on lesion size or characteristics [169].

1.8.4 Advanced complicated lesions

Type IV lesions (the atheroma) are characterised by a lipid core that disrupts the organisation of the intima (Figure 1.4). Over time, atherosclerotic lesions progress to become type V lesions.

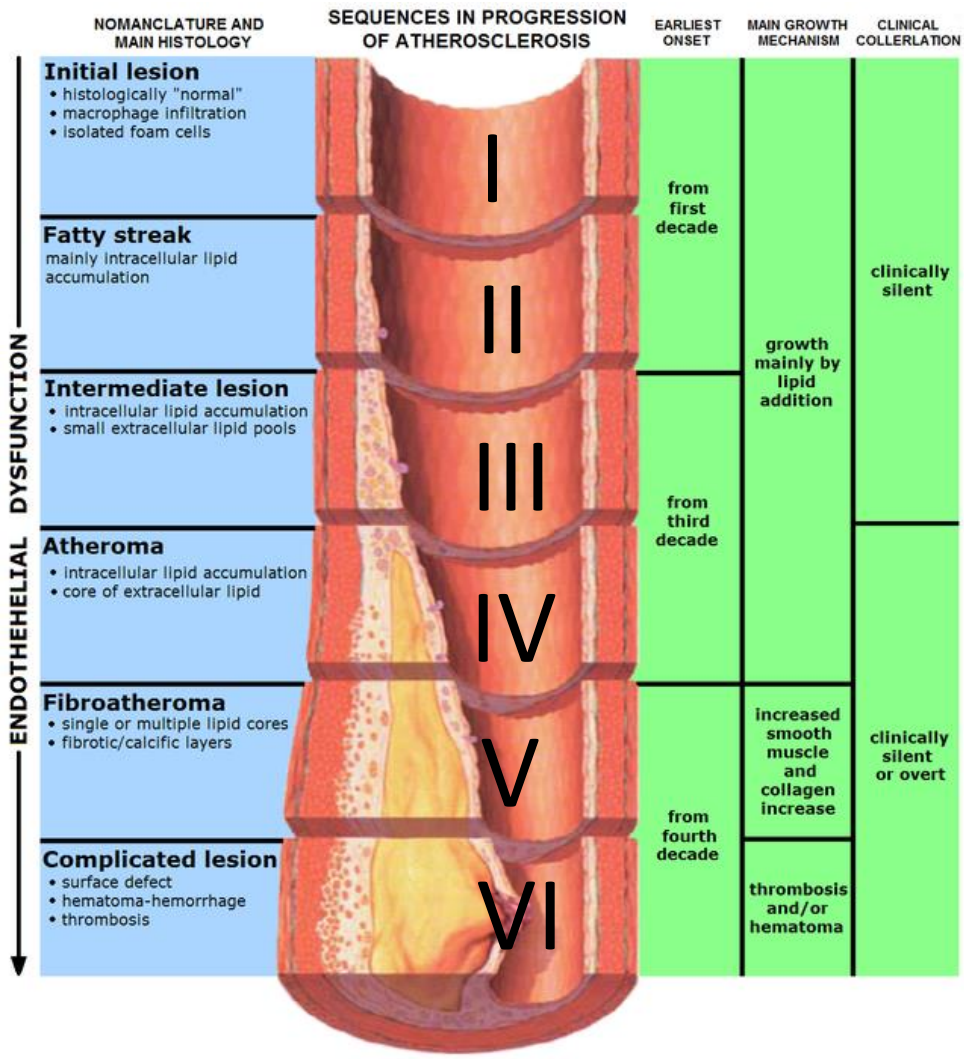

Figure 1.4. The diagram shows atherosclerosis development of human atherosclerotic lesions, from circulation [170].

Top, the section of artery showing the “timeline” of human atherogenesis from normal artery (1) to thrombosis or stenosis (5, 6). Bottom, Cross sections of artery during atherosclerosis development. 1, Normal artery; the artery wall contains three layers, intima, media and adventitia. The intima of human arteries is more developed than other species and contains resident smooth muscle cells at early stage of life. 2, fatty streak; lesion initiation occurs with recruitment of lipids and inflammatory leukocytes such as monocytes and T lymphocytes in hyperlipidemic environment. 3, Intermediate lesion or fibrofatty stage; with lipid taken by macrophages and foam cell

formation with secretion of inflammatory cytokines from resident and recruited immune cells cause more leukocyte recruitment and SMCs migration and proliferation. 4, Atheroma; inflammatory mediators cause expression of tissue factor, a potent procoagulant, and of matrix-degrading proteinases that weaken fibrous cap of plaque. 5, Fibroatheroma; Lesion progression involves migration of SMCs from the media to the intima, proliferation of resident intimal SMCs and media-derived SMCs, and heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and micro vessels. 6, Complicated lesion or thrombosis, the ultimate complication of atherosclerosis, often follows physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow [170].

1.8.5 Type V lesions

Type V lesions (the fibroatheroma) contain large numbers of inflammatory cells, lipids, and increased amounts of fibrous tissue [171] (Figure 1.1C & 1.4). Deposition of ECM proteins, mainly collagen, forms a fibrous cap over the lipid core and is essential for stability of the lesion. Intimal SMCs are the main producers of this collagen, and selective apoptosis of SMCs reduces collagen in fibrous caps [172] (Figure 1.5). Matrix metalloproteinases (MMPs) produced by macrophages can also reduce collagen within fibrous caps via degradation. Reductions in the amount of collagen are associated with thinning of the fibrous cap, increasing the risk of plaque rupture. Clinical manifestations of atherosclerosis, such as strokes and heart attacks, are frequently due to rupture of such lesions. Collagen degradation pathways, involving enzymes such as matrix metalloproteinase, cathepsins, and their respective inhibitors are important regulators of collagen levels. Degenerative enzymes can promote the breakdown of elastic lamina [173], allowing smooth muscle cell migration from the media into the intima, which may ultimately lead to increased collagen deposition in lesions. MMPs are also major participants in intimal collagen degradation and contribute to plaque instability. [174-176]. MMPs are also involved in cytokine and chemokine activation [177] and initiation of cell proliferation [178, 179]. Observations of the effects of MMPs on atherosclerosis showed complex effects which are mostly likely attributable to the multi-functional role of MMPs [173, 180, 181]. There is no consensus on the role of cysteine proteases, cathepsins K and S, which can also degrade proteins including collagen in atherosclerosis [182-184] (Figure 1.4).

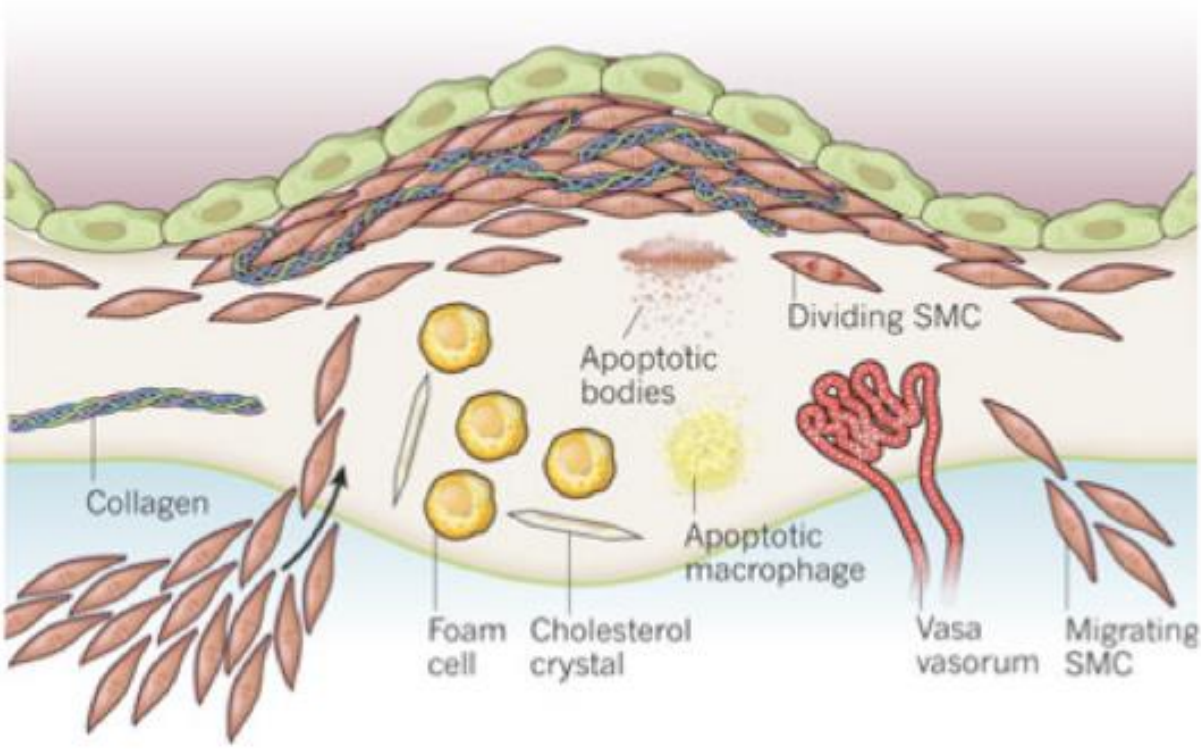

Figure 1.5. Lesion development with SMC infiltration causing lesion progression and plaque destabilization, from Nature [151].

Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and micro vessels [151].

1.8.6 Type VI lesions

Type VI lesions (the complicated lesion) develop from type IV or V lesions with one or more additional features that include hematoma formation, haemorrhage, and thrombosis [171] (Figure 1.1D & 1.4). These lesions consist of large necrotic cores. Although apoptosis is generally thought not to elicit an immune response, the exposure of phosphatidylserine (PS) on the cell surface, which serves to signal phagocytes and neighbouring cells that the cell requires removal, can serve as a substrate for the generation and activation of the potent coagulant, thrombin [185]. Apoptosis of vascular ECs can also make them procoagulant through loss of their anti-coagulant membrane components [186]. Inadequate efferocytosis, the clearance of apoptotic cells, leads to post-apoptotic secondary necrosis, which account for the necrotic core present in many advanced lesions. During progression to secondary necrosis, oxidized LDL may be released from cells, leading to inhibition of binding and uptake of apoptotic cells by macrophages, further contributing to inflammation and an enlarged necrotic microenvironment [187]. Mice with defective Mer tyrosine kinase (Mertk) receptor gene, which is involved in efferocytosis, do indeed have increased apoptotic cells in their lesions, which contribute to necrotic areas as atherosclerotic plaques develop [188]. Increased levels of tissue factor (TF), primarily produced by activated macrophages in lesions but also by apoptotic cells [189], increases the procoagulant properties of plaques, and together with fragile fibrous caps commonly found in advanced lesions, greatly increases the risk of plaque rupture and localised thrombosis following plaque rupture. Disruption of the fibrous cap leads to TF interacting with coagulation proteins from the circulation, and formation of a thrombus. This is the most common mechanism of thrombus induction. The resulting thrombus may either occlude the area where rupture has

occurred, or be carried to smaller vessels and embolize. Non-fatal and repeated plaque ruptures can also increase stenosis, leading to angina.

1.9 Animal models of atherosclerosis

To date, many animal models of atherosclerosis have been used to further our understanding of mechanisms involved in the development and progression of this condition. These initially included mice [190-192], chickens [193, 194], rabbits [195, 196], pigs [197, 198], and non-human primates [199, 200]. The development of genetically modified mice in the early 1990's greatly advanced research in atherosclerosis. Due to the ability to manipulate genes potentially involved in atherosclerosis, mice are now the most extensively used animal for studies in atherosclerosis. Mouse models of atherosclerosis closely mirror atherogenesis in humans [201]. The C57BL/6J strain of mouse is most commonly used as they are most susceptible to atherosclerotic lesion development. They exhibit inflammatory Th1-biased immune responses as opposed to other mouse strains, such as BALB/c, which predominantly exhibit Th2-like immune responses [202]. The apolipoprotein E (ApoE)-deficient mouse [191, 203] and low density lipoprotein receptor (LDLR)-deficient mouse [192] developed on the C57BL/6J background have both been extensively characterized for lesion development and morphology, and are now most commonly used in atherosclerosis research [204-206]. These mice are deficient in genes important for clearance of lipids from the plasma. LDLR removes intermediate density lipoprotein (IDL) and low density lipoprotein from the plasma [207] by binding to lipoproteins particles containing ligands such as ApoE, a high affinity ligand found on LDL, or ApoB100, a lower affinity ligand found on LDL. As a result, mice lacking

either the LDLR or ApoE gene develop hypercholesterolemia, especially when fed a Western type diet containing moderate to high amounts of fat and cholesterol (typically 21% fat, 0.15% or 1.25% cholesterol). Prolonged feeding on this diet and sustained elevation of plasma cholesterol levels leads to spontaneous development of atherosclerotic lesions in sites similar to those seen in humans, which include the coronary, carotid, renal, and iliac arteries. However, mice also develop extensive atherosclerosis at the aortic sinus, which is uncommon in humans (Figure 1.4). Despite the usefulness of mouse models in the study of atherosclerosis, certain limitations exist when using this species. Apart from some differences in vascular sites where lesions are prone to develop, another notable difference in atherosclerosis between humans and mice is the development of unstable, rupture-prone lesions. In humans, atherosclerotic lesions become vulnerable over time, and rupture, resulting in complications such as myocardial infarctions and strokes. Mice, on the other hand, develop atherosclerotic lesions that are more resistant to rupture, making it difficult to model end-stage effects of atherosclerosis in these animals. As most studies in mice are relatively short in duration, this maybe one of the reason as to why plaque rupture in mice is relatively infrequent.

C57BL/6 mice or wild type (WT) mice have normal plasma cholesterol, plasma triglyceride levels; and High Density Lipoprotein (HDL) is the predominant lipoprotein with only small amounts of VLDL and LDL [208]. WT mice develop small lesions when fed western diet over a long period of time. Knockout and transgenic mice on C57BL/6 background are extensively used to study atherosclerosis. Both $ApoE^{-/-}$ mice and $LDLR^{-/-}$ mice are the most commonly studied mouse models of atherosclerosis.

1.9.1 ApoE-deficient mice

ApoE$^{-/-}$ mice are commonly used to study atherosclerosis, and were created by homologous recombination in embryonic stem cells. These mice developed hypercholesterolemia when on a normal chow diet and developed atherosclerotic lesions are similar to humans [191]. The knockout model was generated by inactivating ApoE gene [203]. The lack of a functional ApoE gene prevents these mice from producing apolipoprotein E, a glycoprotein that is essential for the transport and metabolism of lipids. Both human and mice lacking ApoE exhibit hypercholesterolemia and near normal triglyceride leads decreased HDL levels and elevated LDL and VLDL levels [204]. ApoE is a 34 kD glycoprotein synthesized in liver, brain, intestine, lung and macrophages in both human and mice [209]. The function of ApoE is to facilitate binding of lipoproteins to cell surface proteins, which enhances the transfer of components such as cholesterol ester and triglycerols to or from cells [208]. It has a high affinity ligand for ApoB and ApoE receptor and chylomicron remnant receptor which are used to facilitate uptake of ApoE-containing particles by liver [204]. ApoE also serves as an immune modulator; macrophages express ApoE. Activation of T cells by macrophages induces secretion of IFN-γ, which in turn inhibit the expression of ApoE on macrophages [210]. ApoE also down regulates TH1 immune responses [211] to maintain feedback regulation and reverse cholesterol transport. When compared with WT mice, ApoE$^{-/-}$ mice are healthy, have no difference in body weight or litter size, reproduce normally but exhibit hyperlipidaemia with increased total plasma cholesterol, cholesterol ester and free cholesterol levels and plasma triglycerides and phospholipids. HDL levels are reduced to one third the levels seen in normal WT mice. The major lipoprotein particles in ApoE$^{-/-}$ mice are VLDL and IDL compared to WT where HDL is the

predominant lipoprotein [204, 208]. ApoE$^{-/-}$ mice fed a normal chow diet develop fatty streaks in the aortic wall by 3 months of age, which then progresses to advanced atherosclerotic lesions by 8 months [204]. Lesions develop in aortic root and throughout the aorta. Lesions develop first in the aortic root, followed by lesser curvature of aortic arch, branches of brachiocephalic artery, branches of superior mesenteric artery, both renal arteries, aortic bifurcation and pulmonary artery [206]. In older mice, lesions are present in the descending thoracic, lower abdominal, proximal coronary, common iliac, femoral arteries. Upon feeding ApoE mice a high fat diet (HFD), lesions first appear as small yellowish-white nodules [206]. Mononuclear cell adhesions to endothelium and foam cells are also apparent. At 15 weeks of age ApoE$^{-/-}$ mice fed a western diet, exhibit early fibrous plaques which contain a small necrotic core covered by a fibrous cap [206]. ApoE$^{-/-}$ mice can also develop complex atherosclerosis even when fed a chow diet [191, 204].

1.9.2 LDLR$^{-/-}$ mice

The LDLR knockout (LDLR$^{-/-}$) mouse was created by homologous recombination in embryonic stem cells [192]. The LDL receptor is a glycoprotein receptor located at the surface of cells and binds to LDL increasing its entry into cells. The receptor has high affinity for LDL and is able to cycle cholesterol in and to the tissues, while keeping plasma LDL low; it is an important regulator of plasma cholesterol levels [207]. LDLR$^{-/-}$ mice exhibit delayed clearance of VLDL and LDL from plasma. Both female and male homozygous mice deficient in LDL receptors were viable and fertile. The total plasma cholesterol levels are twofold higher than in WT mice. They also exhibit a seven fold increase in intermediate density lipoprotein (IDL) and LDL without significant change in HDL. Plasma triglycerides levels are normal in LDLR-/-

mice [192]. Therefore, this knockout mouse is given a cholesterol diet to elevate the plasma cholesterol in studies of atherosclerosis [192]. $LDLR^{-/-}$ mice fed a cholesterol diet (1.25% cholesterol, 7.5% cocoa butter and 7.5% casein and 0.5% sodium chlorate) [212]), exhibit marked hypercholesterolemia with total cholesterol levels of 1500 mg/dl after 2 weeks on diet compared to wild type C57BL/6 mice, 160mg/dl [192]. $LDLR^{-/-}$ mice develop atherosclerosis within the aortic root similar to $ApoE^{-/-}$ mice [213]. These mice when fed a high fat diet develop atherosclerotic lesions more slowly compared to $ApoE^{-/-}$, however lesions reach the same level of complexity after 30 weeks [214].

1.9.3 ApoE3-Leiden mice

Familial dysbetalipoproteinemia in humans is a hereditary disease caused by mutation in ApoE3-Leiden gene [215, 216]. It is characterized by a duplication of codons 120 to 126 [217]. Transgenic mice with over expression of the ApoE3-Leiden gene exhibited an increase in plasma cholesterol and triglycerides when fed a chow diet. ApoE-Leiden mice on high fat/high cholesterol diet have increased levels of cholesterol and ApoE levels when compared to WT mice fed a similar diet. Hyperlipidemia can be enhanced in ApoE3-Leiden mice when fed a diet in saturated fats and cholesterol. These mice are highly susceptible to diet-induced atherosclerosis and their atherosclerotic lesions are predominantly lipid-laden foam cells developed in the aortic root [218] and along the entire vascular tree with lesions similar to human pathology [216]. In these mice, DNA synthesis occurs at early stage of atherosclerotic lesions and apoptosis peaks at late stage of atherosclerotic lesions and mostly confined to macrophage-derived foam cells.

1.10 The immune system in atherosclerosis

Atherosclerosis is considered a chronic inflammatory disease, and as such, its pathogenesis greatly depends on involvement of the innate and adaptive immune systems. Initiation and acceleration of atherosclerosis development are both correlated with immune activity. For example, induction of an inflammatory response such as by injection of lipopolysaccharide (LPS) significantly increases the size of atherosclerotic lesions [219]. Almost every cell type of the immune system and the factors they produce (e.g. cytokines and chemokines) have been implicated in atherosclerosis, and many of these have been shown to influence development of atherosclerosis (Figure 1.6).

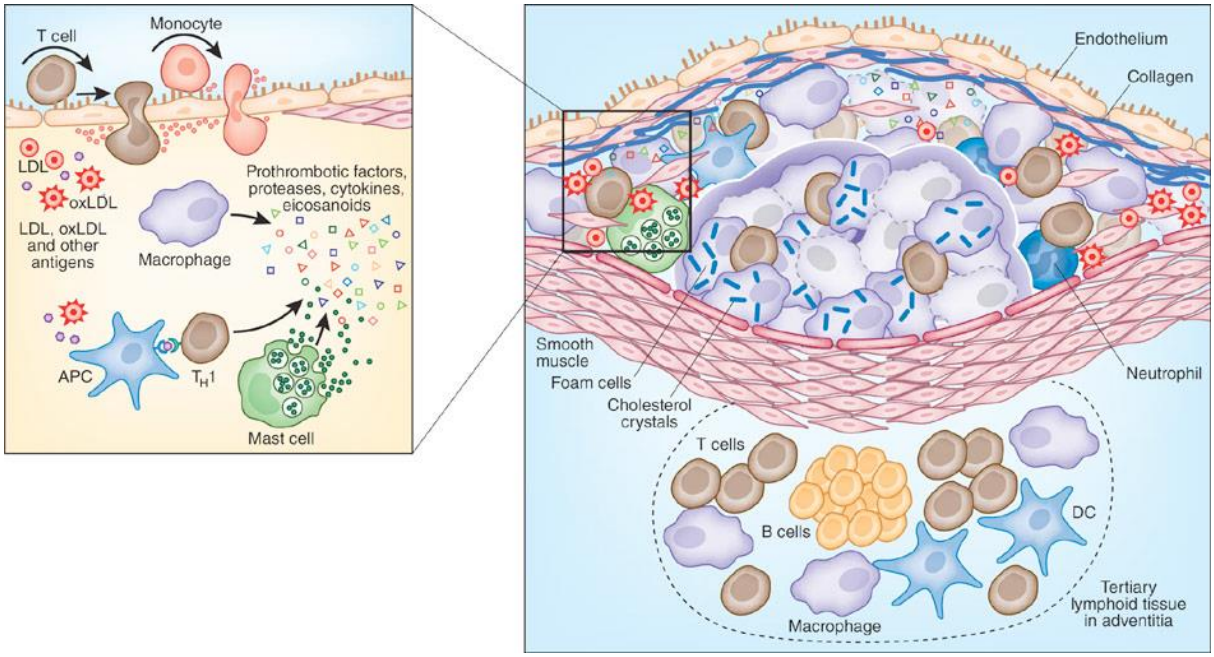

Figure 1.6. Coordination of the innate and adaptive immune system in forming the atheroma, from Nat Immunol [2].

Inflammatory cells, including lipid-laden macrophage foam cells, accumulate in the intima owing to the persistent influx of new cells, particularly monocytes, neutrophils, dendritic cells and natural killer cells. Moreover, apoptotic macrophages are not efficiently cleared and so they undergo secondary necrosis. This process contributes to formation of the necrotic core, which promotes plaque disruption [2].

1.10.1 Cytokines

Cytokines are proteins and glycoproteins produced by hematopoietic and non-hematopoietic cells that can have varied effects depending on the cell and environment in which the cytokine is acting upon. Both Th1 and Th2 cytokines have been implicated in atherosclerosis. Typical Th1 cytokines include interleukin (IL)-2, IL-12, IL-18, granulocyte macrophage colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), while Th2 cytokines include IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 cytokines are generally proatherogenic, and Th2 cytokines anti-atherogenic, with exceptions [27, 220], such as IL-4 being a pro-atherogenic cytokine [221]. The use of genetically modified mice has allowed the importance of these molecules in atherosclerosis to be elucidated. IFN-γ is one of the main Th1 proinflammatory cytokines implicated in atherogenesis. It can prime macrophages for activation and inhibits the growth and collagen synthesis by SMCs, thus creating a more vulnerable plaque phenotype [222]. Mice deficient in IFN-γ develop smaller lesions with increased collagen content [223, 224], while mice administered recombinant IFN-γ have increased atherosclerosis [225]. Similarly, reduced atherosclerosis is observed in mice deficient in other pro-atherogenic Th1 cytokines such as IL-12 [221], which is also involved in recruitment of T cells in atherosclerotic lesions [226], IL-18 [227], GM-CSF [228], and TNF-α [229, 230], although some studies on TNF-α in atherosclerosis have been conflicting. Mice that lack the TNF-α receptor, p55 (TNFR1), have been shown to develop larger [231], and smaller [232] atherosclerotic lesions in different studies. Additionally, mice lacking TNF-α have also been reported to have unaltered lesion development, while lymphotoxin-α (LT-α), a cytokine with homology to TNF-α was shown to be pro-atherogenic in the same study [233]. Like TNF-α the effect of GM-CSF on

atherogenesis is also varied, with one study reporting increased atherosclerosis in GM-CSF$^{-/-}$ ApoE$^{-/-}$ mice [234]. Conversely, mice injected with recombinant Th1 cytokines GM-CSF [24], IL-2 [235], IL-12 [236], or IL-18 develop larger atherosclerotic lesions, although the effects of IL-18 appear to be dependent on the presence of IFN-α [237]. Other pro-inflammatory cytokines also appear to play a pro-atherogenic role. These include IL-1 [238, 239], and the stimulating factors granulocyte colony stimulating factor (G-CSF) [24] and macrophage colony stimulating factor (M-CSF) [240-242], which promotes the differentiation of haematopoietic cells into granulocytes and macrophages, respectively.

Anti-inflammatory cytokines that are atheroprotective include transforming growth factor-beta (TGF-β), interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1ra). Administration of IL-1ra to mice reduced atherosclerosis [243]. TGF-β is a potent anti-inflammatory cytokine that inhibits the expression of adhesion molecules on vascular endothelial cells [244], formation of foam cells [245], and SMC migration and proliferation [246, 247]. It is one of the most potent stimulators of collagen synthesis [222], and is required to maintain T cells in an inactivated state. Abrogation of TGF-β signaling in atherosclerosis-prone mice sees changes in the size and composition of atherosclerotic lesions. These lesions are larger, contain more activated T cells, and less collagen, indicative of a more vulnerable phenotype [248-252]. Interleukin-10, another anti-inflammatory cytokine also has protective effects in atherosclerosis. Total deficiency of IL-10 in mice results in greatly increased atherosclerosis [253, 254], and hematopoietic cell-derived IL-10 is important for attenuating atherosclerosis [255]. Specific up regulation of IL-10 by macrophages is sufficient to attenuate atherosclerosis. IL-10 induces scavenger receptor expression, increases modified LDL uptake, and increases cholesterol efflux in macrophages

[256]. Overexpression of IL-10 by T cells also reduces atherosclerosis by shifting the atherosclerotic lesion to a Th2 environment, and reducing macrophage activation and apoptosis [257]. Although IL-4 is classically considered an anti-inflammatory Th2 cytokine, and promotes the differentiation of Th2 cells, it can also have some pro-inflammatory and pro-atherogenic effects. Studies of IL-$4^{-/-}$ mice in atherosclerosis have produced conflicting results. Weekly IL-4 injections to mice greatly reduce atherosclerotic lesion size [258]. Conversely, atherosclerosis-susceptible $ApoE^{-/-}$ or $LDLR^{-/-}$ mice with total IL-4 deficiency or IL-4 deficiency in hematopoietic cells, respectively, develop less extensive atherosclerosis in mostly the aortic arch and thoracic aorta [221, 259], but in another study, IL-4 deficient C57BL/6 mice develop lesions similar to those in wild-type mice [260]. Some effects of IL-4 that may contribute to their pro-atherogenic role include induction of IFN-α production in NK and NKT cells [261], increased production of reactive oxygen species (ROS) and MCP-1 expression by ECs [262]. Other Th2 cytokines, IL-5 and IL-6 appear to be atheroprotective. Transfer of IL-$5^{-/-}$ bone marrow to mice increases atherosclerosis [97], while IL-6-deficient mice develop increased atherosclerosis, possibly due to disruptions in cholesterol homeostasis from IL-6 deficiency [263, 264]. However, one study found injection of recombinant IL-6 to mice increased systemic levels of pro-inflammatory cytokines, and exacerbated lesion development [265]. Whether the level of systemic IL-6 used in the study occurs naturally to cause such an effect is not known. IL-6-deficiency also had no effect on atherosclerosis development in another study (Figure1.7) [266].

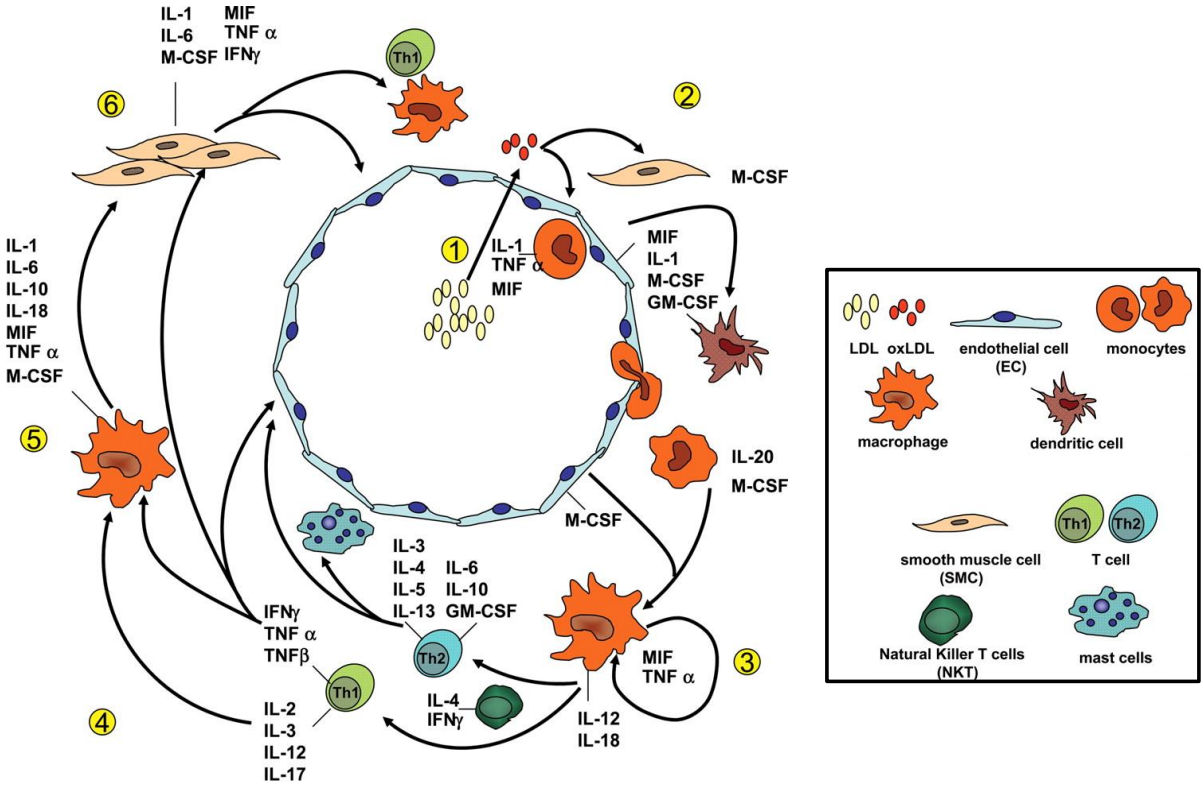

Figure 1.7. Cytokines involved in atherogenesis and their cellular source and targets, from Cardiovasc Res [267].

(1) LDL units infiltrate into the endothelial cells (EC) and are oxidized (oxLDL) in the intima. (2) OxLDL-released lipids act as pro-inflammatory elements and induce expression of cytokines in EC (e.g. IL-1, MIF, M-CSF, and GM-CSF) and SMCs (M-CSF). Chemotactic factors MIF and M-CSF recruit monocytes and T-cells. Differentiation and accumulation of DC is regulated by GM-CSF in atherosclerotic lesions. (3) Macrophages augment inflammation in lesions through autocrine secretion of TNF-α and MIF. IL-12 and IL-18 secreted by macrophages promote differentiation of naive T cells into Th1 cells and IL-4 secreted by NKT cells promotes differentiation of naive T cells into Th2 cells. IL-12 and IL-18 also induce IFN-γ secretion by NKT cells. (4) T_H1 cells promote inflammation by macrophages, SMCs and EC activation through production of proinflammatory cytokines such as IL2, IL3,

IL17, IFN- γ and TNF- α while Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 with opposite effect on macrophages, T cells and ECs. On the other hand, IFN-γ inhibits Th2 cell development. Also, IL-4, IL-5 and IL-13 secreted by Th2 are involved in B cell differentiation and antibody production (not shown). (5) Further secretion of IFN-γ, IL-1 and TNF-α by activated macrophages through Th1 cell interaction and stimulation result in augmentation of the inflammation. (6) Cytokines secreted from macrophages and T cells stimulate SMCs to produce and secrete a range of proinflammatory cytokines such as IL-1, TNF-α, and IFN-γ [267].

1.10.2 Chemokines

Chemokines are small cytokines with chemotactic properties that influence the migration of cells. They can be divided into two main families, the CC and CXC chemokines that are defined by positions of the first two of four conserved cysteines. They are involved in migration of different leukocytes while CXC chemokines usually recruit neutrophils, CC chemokines more commonly attract mononuclear cells to the sites of inflammation or injury. Many chemokines have been detected in atherosclerotic lesions, and again, their influence on atherogenesis has been examined using genetically modified mice. Of these, MCP-1 has been shown to be important in the initiation and progression of atherosclerosis due to its chemotactic properties on circulating monocytes [166, 268]. Macrophage migration inhibitory factor (MIF) is a cytokine with chemotactic properties which attracts and initiates the transmigration of monocytes and T cells to lesions by interacting with CXCR2 and CXCR4, respectively [269], and promotes cell survival [270], consequently further contributing to accumulation of cells within lesions [271, 272]. Deficiency or neutralization of MIF in mice reduces macrophage and T cell accumulation in lesions [269, 273], and also reduces cell proliferation and collagenolytic activity in lesions [274]. Deletion or antagonism of chemokine receptor CXCR3 in atherosclerosis is associated with an immuno-suppressive plaque phenotype, and reduced atherosclerotic lesion size [275, 276]. CCL5 is another chemokine that attracts monocytes and T cells to the sites of inflammation [277] and interacts with chemokine receptors CCR1 [278], CCR3 [279], and CCR5 [280]. Antagonism of CCL5 [281] or deletion of its receptor CCR5 reduces atherosclerosis [282-284]. However, deletion of another CCL5 receptor, CCR1 increases atherosclerosis [284, 285]. Decreased atherosclerotic lesion size was generally observed in the absence

of other chemokines or chemokine receptors, such as CXCR2 [286], platelet factor-4 [287], CX3CL1 [288] and its receptor CX3CR1 [289, 290]. The differential effect of deletion of CXCL16 or its receptor CXCR6 in atherosclerosis demonstrates the multi-functional capacity of chemokines. While deficiency of CXCR6 in mice reduces macrophage and T cell accumulation in lesions, and reduces lesion size [291], deletion of CXCL16 increases lesion area, possibly due to loss of the atheroprotective scavenger receptor activity mediated by CXCL16 [292]. Combined deletion of more than one chemokine or its receptor in mice, such as in $ApoE^{-/-}$ x $CX3CL1^{-/-}$ x $CCR2^{-/-}$ mice [293], or $ApoE^{-/-}$ x $CCL2^{-/-}$ x $CX3CR1^{-/-}$ x $CCR5^{-/-}$ mice [294], results in even greater reductions in atherosclerosis, and illustrates that chemokines and their receptors have independent and additive effects in atherosclerosis (Figure 1.8).

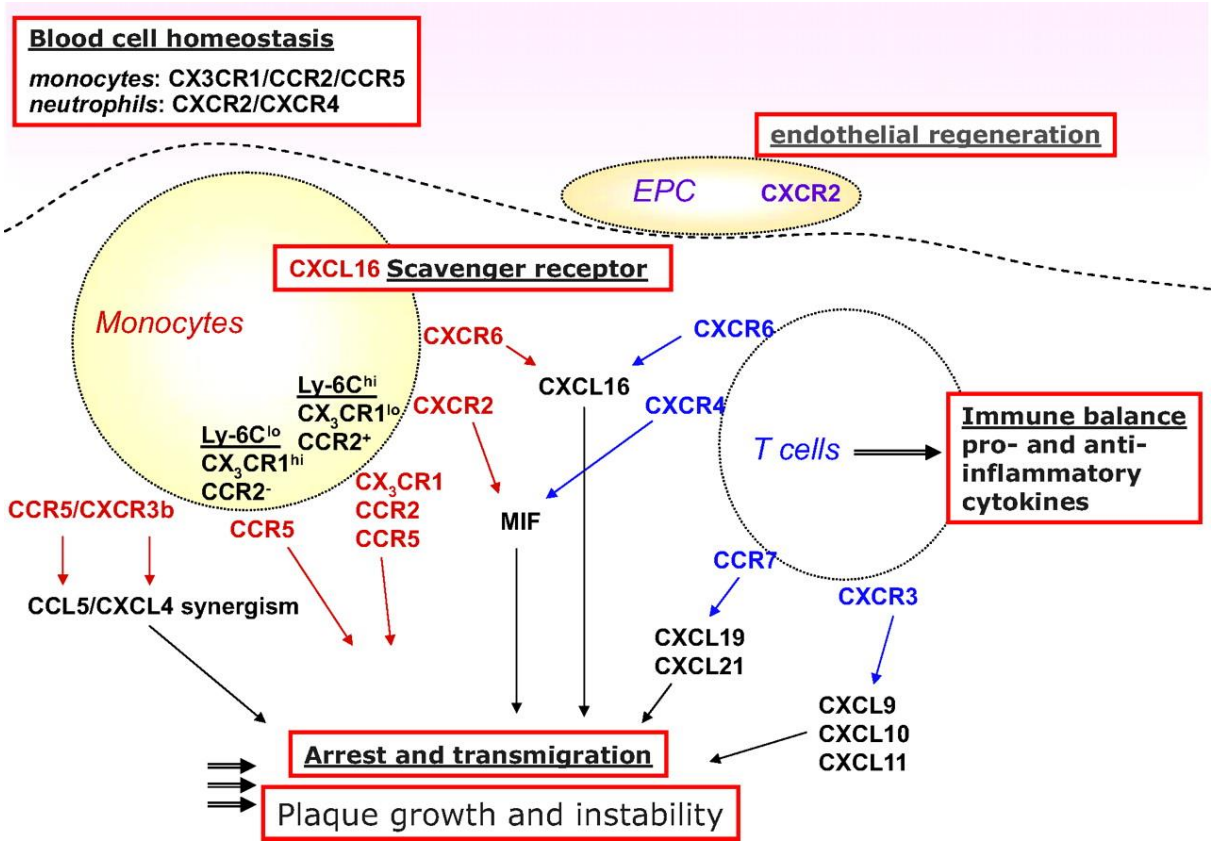

Figure 1.8. Involvement of chemokines and their receptors in atherosclerotic lesion formation, from Arteriosclerosis, Thrombosis and Vascular Biology [295].

This figure shows chemokine's involvement in atherosclerotic lesions, interaction with their receptors and recruitment of different monocyte subsets and lymphocytes during atherosclerosis development [295].

1.10.3 The innate immune system and atherosclerosis

The innate immune system plays a major role in atherosclerosis. Most leukocyte populations from the innate immune system, including monocytes, mast cells, basophils, eosinophils, neutrophils, and natural killer (NK) cells have been implicated in atherosclerosis. A disturbance to the normal functioning of the innate immune system reduces atherosclerosis. Deficiency of toll-like receptor-4 (TLR-4) or myeloid differentiation factor 88 (MyD88) and TLR2 involved in innate immune system signaling, attenuates atherosclerosis in mice [296-298] and in contrast TLR9 deficiency resulted in aggravation of atherosclerosis in ApoE$^{-/-}$ mice [299]. However, the contribution of each cell population to atherogenesis, and the level of understanding of their role in this disease are varied.

1.10.3.1 The role of Neutrophils in atherosclerosis

Neutrophils are the most prominent cell type in the circulation, and are one of the first cells to respond to a pathological or physiological insult, yet they have not been extensively studied in atherosclerosis. They have been identified in atherosclerotic lesions, especially those considered unstable and have ruptured or eroded [300, 301] and appear to promote atherosclerosis [302], but their precise role in disease is still unclear.

1.10.3.2 The role of Monocytes and Macrophages in atherosclerosis

Monocytes are bone marrow-derived leukocytes that are recruited to tissues in response to inflammatory signals, and differentiate into tissue macrophages. Macrophages produce a wide range of proteins, including cytokines, chemokines, growth factors, and proteases, and perform a variety of functions including

propagation of inflammatory responses, recruitment of cells involved in tissue repair, degradation of extracellular matrix, and phagocytosis of foreign and apoptotic bodies. Macrophages express a range of receptors, including TLRs and scavenger receptors, which recognize pathogen-associated molecular patterns (PAMPs) on foreign antigens. Encounter of macrophages with antigens containing PAMPs leads to activation of these cells and induction of an inflammatory response or endocytosis of foreign particles followed by lysosomal degradation. Macrophages also take up oxLDL particles and present them to T cells, resulting in T cell activation and proliferation [303, 304]. They are detected in arterial walls from the very early stages of atherosclerosis, and are found throughout all stages of lesion development. Circulating monocytes enter the intima in response to hypercholesterolemia and chemokines produced by vascular cells and differentiate into macrophages [305, 306] (Figure 1.9). This influx of monocytes is continuous throughout the progression of atherosclerosis [305]. There, uptake of modified lipids by macrophages via scavenger receptors leads to the formation of foam cells characteristic of atherosclerotic lesions [307, 308]. Chronic peripheral depletion of monocytes or prevention of monocyte chemotaxis into the intima significantly reduces atherosclerosis [166, 169, 268]. Monocytes can be differentiated into subsets based on the expression of Ly6C, $Ly6C^{hi}$ and $Ly6C^{lo}$. $Ly6C^{hi}$ monocytes (also designated $Gr\text{-}1^{+}CCR2^{+}CX3CR1^{lo}$ cells in mice or $CD14^{hi}CD16^{-}$ in humans) were found to be preferentially recruited to sites of inflammation, while the $Ly6C^{lo}$ subset (also phenotypically $Gr\text{-}1^{-}CCR2^{-}CX3CR1^{hi}$ cells in mice or $CD14^{+}CD16^{+}$ cells in humans) were found to enter into non-inflamed tissues under homeostatic conditions [309, 310]. Recently it was shown that the $Ly\text{-}6C^{hi}$ subset of monocytes, which are involved in pro-inflammatory immune responses, increased in numbers in the

circulation and spleen during hypercholesterolemia. Ly-6C^{hi} monocytes are the major monocyte subset in atherosclerotic lesions and subsequently differentiate into macrophages in the intima [311]. Ly6C^{lo} monocytes also enter into atherosclerotic lesions, although at a much lower frequency than Ly6C^{hi} cells, and are more likely to differentiate into CD11c^{+} cells [312]. The chemokine receptors, CX3CR1 and CCR2, distinguishing Ly6C^{hi} from Ly6C^{lo} monocytes interact with their ligands CX3CL1 (fractalkine) and MCP1, respectively. Both of these chemokines are highly implicated in the exacerbation of atherosclerosis [268, 288], and along with preferential adherence of Ly6C^{hi} monocytes to the activated endothelium [311], contributes to the higher influx of Ly6C^{hi} monocytes into lesions.

Death of macrophages by apoptosis seems to be beneficial in early atherosclerosis, but harmful in more advanced stages [313], as excess accumulation of apoptotic cells can lead to increased atherosclerosis [314]. The cytokines and growth factors produced by macrophages can further contribute to lesion development and affect the vessel microenvironment, and MMPs produced by activated foam cells can degrade ECM components, and cause complications during the later stages of atherosclerosis. Plaque macrophages besides phagocytosing oxLDL, have a secondary role in removing dying/dead cells called efferocytosis, is a process by which apoptotic and dead cells are removed by phagocytic cells. Plaque macrophages are the main professional efferocytes in plaques [315, 316]. Efferocytosis takes place efficiently during early atherosclerosis but is not efficient in the later phases when conditions are remained severe inflamed [317, 318] and extensive necrosis occurs in advanced lesions from defective disposal of apoptotic cells. The uncontrolled exposure of toxic intracellular factors (e.g. High mobility group box protein-1; HMGB1 and heat shock proteins; HSP) from necrotic cells can cause

extensive damage on atherosclerotic lesions and thinning of the fibrous cap towards unstable and vulnerable plaque, thrombosis, arterial occlusion and as a result heart attack or stroke [319]. Our previous study showed that HMGB1 is a potent atherogenic cytokine that increased the recruitment of macrophages and stimulation of inflammatory mediators in plaques [320]. The exact reason behind the regression of efferocytosis remains unclear; however some studies suggested that a decline in the number of functional efferocytes could be the result of oxLDL-induced macrophage death and reduced recognition of apoptotic cells due to the disruption of efferocytic ligands such as phosphatidylserine in advanced plaques [188, 318, 321].

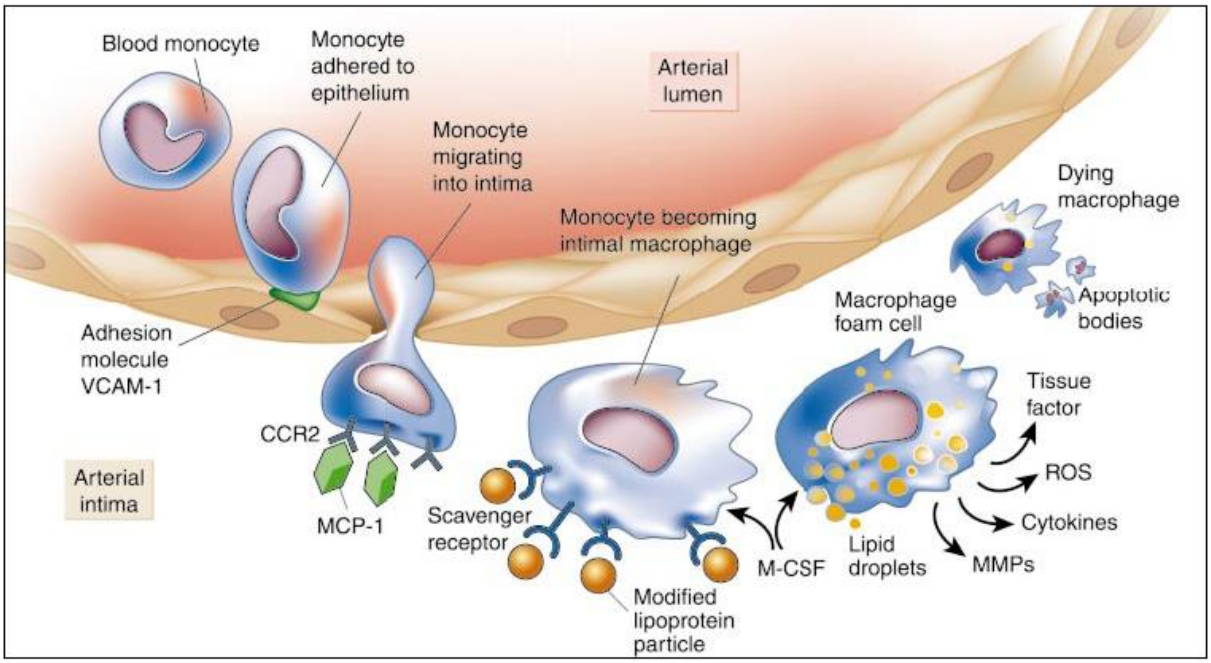

Figure 1.9. Macrophage recruitment in atherosclerotic lesion formation, from Circulation [4].

Following endothelial dysfunction, vascular cell adhesion molecule-1 (VCAM-1) is overexpressed on endothelial cells resulting in enhanced binding of monocytes to endothelium. Monocytes in the blood traverse the endothelial layer entering the vascular intima. The chemokine thought to direct migration of monocytes across endothelium into intima is Monocyte chemotactic protein-1 (MCP-1) also known as CCL2. In the intima, these monocytes mature into macrophages. Macrophages utilize their scavenger receptors to bind to modified lipoprotein particles (oxLDL) and develop into foam cells. The foam cells release substances such as Reactive Oxygen Species (ROS), Matrix metalloproteinases (MMPs), cytokines and tissue factor. MMPs break down collagen during atherosclerotic lesion formation whilst pro-inflammatory cytokines and ROS trigger further inflammation and recruitment of other leukocytes and lymphocytes. By means of apoptosis and necrosis, dying foam cells form the necrotic core of the atherosclerotic plaque [4].

1.10.3.3 The role of Dendritic cells in atherosclerosis

Dendritic cells are professional antigen presenting cells (APCs) that take up free- or cell-associated protein or lipid molecules, process them intracellularly and present the fragmented molecules on major histocompatibility complex (MHC) or CD1 molecules to T cells to initiate an adaptive immune response. DCs are present in the intima of non-atherosclerotic arteries in humans and in atherosclerotic lesions of mice [322-324], and accumulate with the progression of atherosclerosis [325] (Figure 1.10). They can be found co-accumulated with T and NKT cells, especially in the rupture-prone shoulder regions of atherosclerotic plaques, indicating interactions between these cells in situ [325, 326]. Their ability to produce pro-inflammatory cytokines, and expression of co-stimulatory molecules is impaired, leading to reduced induction of Th1-responses [327]. However, one study found that DCs fully retain their function and are equally effective in priming T cells under hypercholesterolemic conditions [328], while other studies showed that oxLDL can actually push monocytes to differentiate into mature DCs with increased capacity to induce T cell proliferation [329, 330].

Two subsets of dendritic cells, the myeloid and plasmacytoid dendritic cells (mDCs and pDCs), have been identified in atherosclerotic lesions [331]. The more common mDCs primarily recognize bacterial fragments via their cell surface TLRs and produce IL-12, IL-6 and TNF-α, while pDCs respond primarily to viral DNA and RNA, but also to nucleotides from dying cells and produce large amounts of type-1 IFN. Mature mDCs can activate T cells and promote Th1-type immune responses via IL-12 secretion. Type-1 IFN produced by pDCs can upregulate TLR-4 expression on

mDCs and sensitize them towards TLR-4 ligands [332], and also magnify the cytotoxic function of T cells by up regulating TRAIL expression on $CD4^+$ T cells in lesions [333]. Together, DCs may promote atherosclerosis by activating pro-inflammatory and cytotoxic T cells, and contribute to plaque instability.

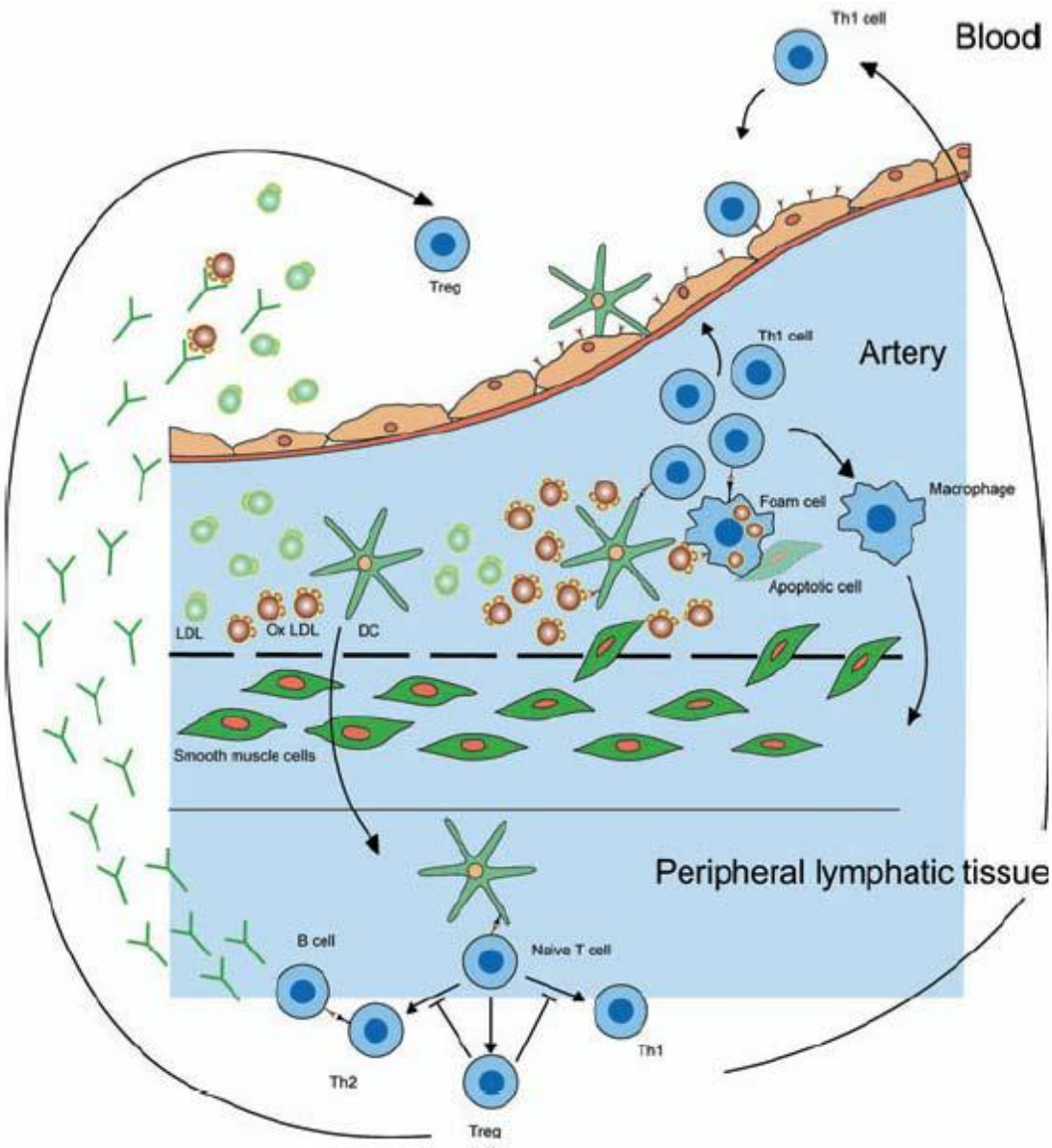

Figure 1.10. Dendritic cells in atherosclerotic lesion formation, from J Intern Med [334].

DCs take up oxLDL and become mature as they migrate to peripheral lymphatic tissue where they present antigens (oxLDL) to naïve T cells and activate them. T cells differentiate into Th1, Th2 or Treg cells according to different cytokine environment. Th1 cells go back to intima to produce and release pro-inflammatory cytokines. Tregs are involved in suppression of atherosclerotic progression [334].

1.10.3.4 The role of Natural killer cells in atherosclerosis

Natural killer cells are bone marrow-derived innate lymphocytes, primarily involved in cytotoxic responses against abnormal “non-self” cells such as tumor or virally infected cells. They mediate their effects by a number of mechanisms, including the Fas/Fas ligand (FasL) pathway that is more associated with immune regulatory processes, or perforin/granzyme-mediated killing of target cells, especially those harboring intracellular pathogens. NK cells expressing perforin can be found in atherosclerotic aortic aneurysms and atherosclerotic lesions from carotid arteries of humans and mice [190, 335, 336]. In a study using Ly49A transgenic mice, which have reduced numbers of NK cells in the periphery [337], found a pro-atherogenic role for NK cells. Transfer of bone marrow from Ly49A transgenic mice, which contain NK cells deemed non-functional, to $LDLR^{-/-}$ mice led to a significant reduction in lesion size [190]. In humans, NK cells can be divided into subsets based on the expression of CD16 and CD56 [338]. The majority of NK cells are $CD16^{+}CD56^{dim}$, which exhibit strong cytotoxic activity. $CD16^{-}CD56^{bright}$ NK cells on the other hand have reduced cytotoxicity, but are more effective at producing cytokines such as IFN-γ [339, 340]. In mice, a population of thymic-derived NK cells, distinguished by the expression of Gata3 and CD127, is functionally similar to $CD56^{bright}$ human NK cells [341]. Mature $CD11b^{+}$ NK cells can also be subset into $CD27^{high}$ and $CD27^{lo}$ NK cells. $CD11b^{+}CD27^{high}$ NK cells proliferate and exhibit significantly greater cytotoxic function and IFN-γ secretion compared to $CD11b^{+}CD27^{lo}$ NK cells [342]. In a very recent study in our group, we have shown that Treatment of $ApoE^{-/-}$ mice with anti-Asialo-GM1 antibodies depleted NK cells without affecting other lymphocytes, including natural killer T cells, and greatly attenuated atherosclerosis. These effects were independent of plasma lipids. To confirm the atherogenicity of NK cells, wild-

type NK (WT-NK) cells were adoptively transferred into lymphocyte-deficient ApoE$^{-/-}$Rag2$^{-/-}$IL-2rg$^{-/-}$mice and resulted doubled lesion size, confirming a pro-atherogenic role for NK cells. To determine whether their atherogenicity was dependent on production of interferon-γ (IFN-γ) or cytotoxins, we compared the transfer of NK cells deficient in IFN-γ, perforin, and granzyme B with the transfer of wild-type NK cells. Transfer of IFN-γ-deficient NK cells increased lesion size in the lymphocyte-deficient ApoE$^{-/-}$ mice as wild-type NK cells. However, granzyme B- and perforin-deficient NK cells did not affect lesion size. Only wild-type NK cells increased necrotic core size, whereas perforin- and granzyme B-deficient NK cells did not and we concluded that NK cells are atherogenic and their production of perforin and granzyme B contributes to atherosclerosis and the expansion of necrotic cores [343].

1.10.3.5 The role of other innate immune cells in atherosclerosis

Other minor populations of innate leukocytes have been detected in atherosclerotic lesions and contribute to lesion formation. These include mast cells, basophils, and eosinophils. Mast cells are less studied in atherosclerosis, but have been shown to be involved in development of atheromata. Activated mast cells containing proteases and TNF-α have been detected in the shoulder regions of human atherosclerotic lesions [344-346] Degranulation and release of proteases such as chymase and tryptase by mast cells can lead to activation of MMPs, and indeed shoulder regions of lesions containing larger numbers of degranulated mast cells also have higher levels of MMP-1 and -3 [345]. Systemic activation of mast cells leads to development of larger atherosclerotic lesions, while specific activation of adventitial mast cells does not alter lesion size, but increases intra-plaque hemorrhage, macrophage

apoptosis, and leukocyte recruitment [347] (Figure 1.11). A study by Sun *et al;* revealed that the pro-atherogenic effects of mast cells did not require TNF-α, but involved IL-6 and IFN-γ [348]. Histamine released by basophils, and other leukocytes can affect a number of cells involved in atherogenesis, including endothelial cells, SMCs, and macrophages, to affect vascular permeability to lipids, cell proliferation, cytokine production and MMP expression [349, 350]. OxLDL abundant in atheromatous lesions not only recruits monocytes, but has been shown to induce chemotaxis of eosinophils [351]. Indeed, eosinophils are present in atherosclerotic lesions of monkeys [352], but their role in disease development is unclear.

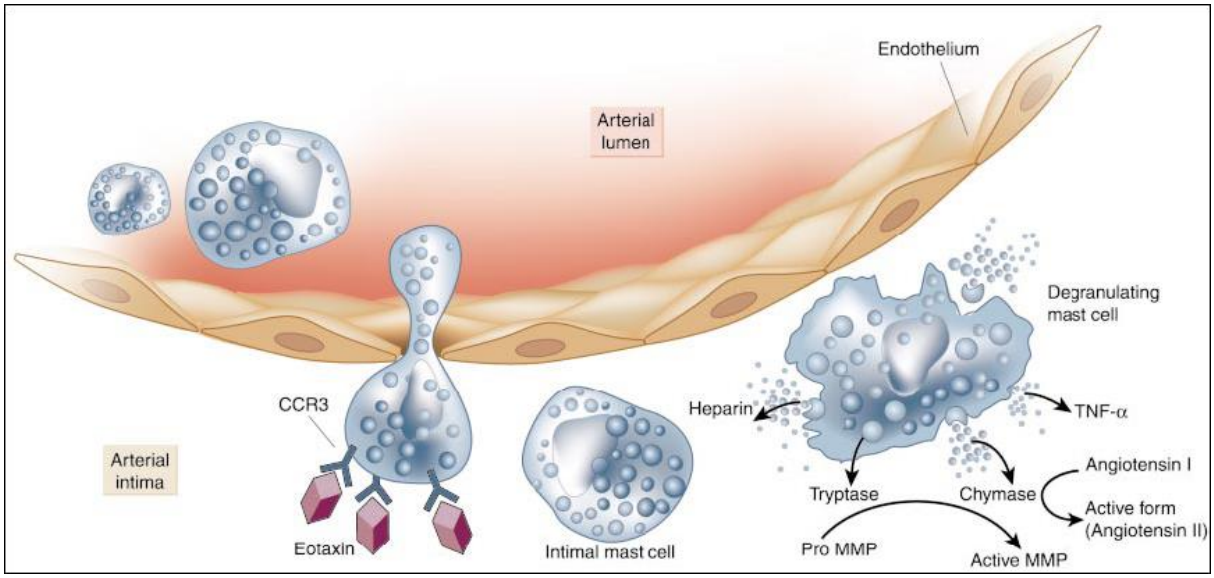

Figure 1.11. Recruitment and function of Mast cells in atherosclerosis, from Nature [9].

Mast cells recruited into atherosclerotic lesion via eotaxin. In the intima, these activated mast cells release molecules such as heparin, proteases, TNF-α. Heparin inhibits VSMC proliferation. Proteases (trypase and chymase) activate MMPs and chymase convert Angiotensin-I to active form, angiotensin-II resulting in hypertention. TNF-α enhances inflammatory responses [9].

1.10.4 The adaptive immune system and atherosclerosis

Although the innate immune system plays a central role in atherogenesis, the adaptive immune system also contributes to the development of atherosclerosis. The adaptive immune system consists of T and B cells that recognize specific antigens via their T cell receptors (TCRs) and immunoglobulins (Igs), respectively. T helper cells can promote cellular or humoral response, and also contribute to enhanced innate immune responses. They can be found in atherosclerotic lesions throughout all stages of disease [147], while antibodies against antigens found in lesions can be detected in animals and patients with atherosclerosis. $Rag^{-/-}$ mice, which are deficient in T and B cells, show delayed development of atherosclerosis when fed a Western diet for a short duration [353, 354] (Figure 1.12).

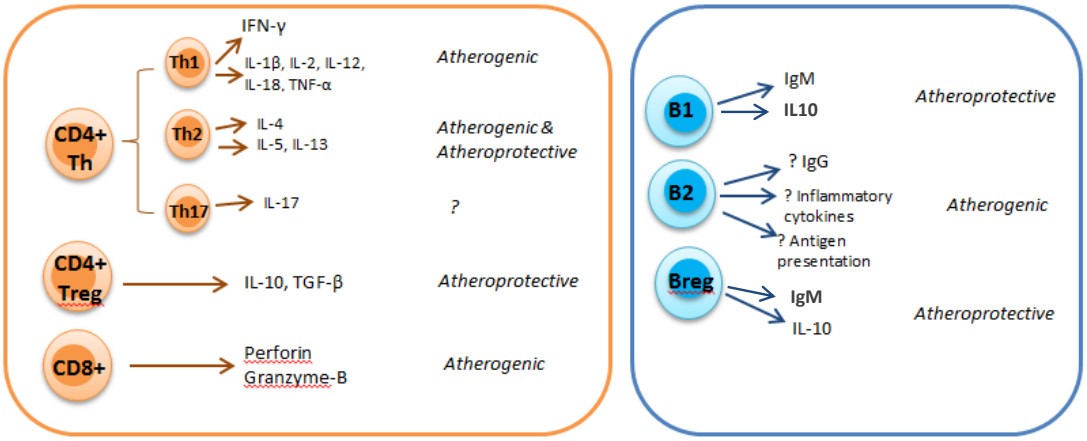

Figure 1.12. T and B lymphocyte subsets make diverse contributions to atherosclerosis pathophysiology.

A) $CD4^+$ T helper (Th) cells polarise into subsets with different effector functions. Th1 cells are atherogenic via production of pro-inflammatory cytokines, the main one being IFN-γ, and account for a high proportion of pathogenic lesional T cells [355-357]. Th2 cells may be atherogenic via IL-4 production, however they also produce IL-5 and IL-13 which are atheroprotective [358]. Th2 cells can also dampen inflammation via suppression of IFN-γ and inhibiting Th1 polarisation [359]. Th17 cells produce IL-17, the role of which in atherosclerosis remains controversial [360-365]. $CD4^+$ regulatory T cells (Tregs) perform immunomodulatory functions in atherosclerosis through production of the anti-inflammatory cytokines IL-10 and TGF-β [366]. $CD8^+$ T cells have recently been characterised as atherogenic via release of cytolytic enzymes perforin and granzyme-B [367]. **B)** As with T cells, B cells can be both atherogenic and atheroprotective [368]. B1 B cells are atheroprotective through their release of IgM. B2 B cells have been shown to be atherogenic in mice, yet the precise mechanism by which this occurs remains to be established. Regulatory B cells (Breg), sometimes termed B10 cells in reference to their production of atheroprotective IL-10, may or may not be a committed subset.

1.10.4.1 The role of T cells in atherosclerosis

A number of T cell subtypes perform important and varied roles during the development of the atherosclerotic lesion, from fatty streaks to the advanced complicated lesions [2, 369]. 10-15% of the lesion cells are TCR (T cell receptor) positive in contrast to peripheral blood, where only 1-2% cells are TCR positive [370]. T cells migrate into intima by binding to chemokines such as inducible protein-10 (IP-10), IFN-inducible T cell chemoattractant (ITAC) and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) [3]. Transfer of oxLDL-reactive T cells from atherosclerotic ApoE-/- to ApoE-/- scid/scid mice abrogates the natural resistance of lesion development, implying the pro-atherogenic role of T cells [371] (Figure 1.13).

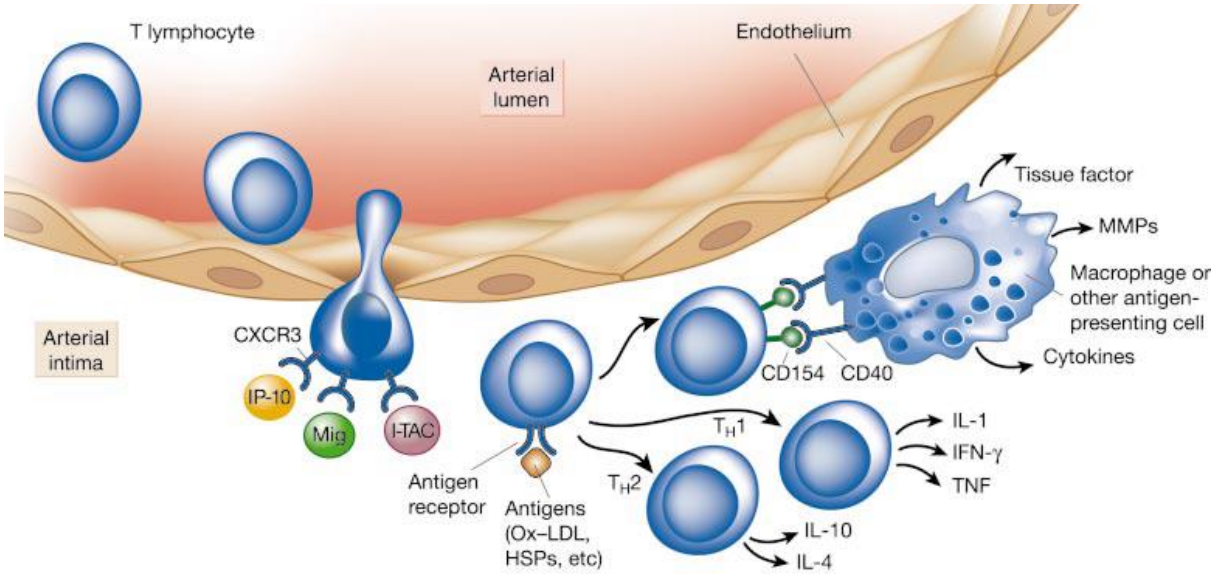

Figure 1.13. CD4+ T lymphocyte recruitment in atherosclerotic lesion and their contribution in formation and progression of atherosclerotic lesions, from Nature [9].

Chemotactive substances such as monokine induced by Interferon-γ (Mig), Inducible protein-10 (IP-10) and IFN-inducible T cell α-chemoattractant (I-TAC) recruit T lymphocytes into vascular intima. Binding of autoantigens such as oxLDL and Heat-shock proteins (HSP) to these T cells may activate their development into Th1 or Th2 cells. Th1 cells secrete pro-inflammatory cytokines such as IL-1, IFN-γ and TNF and on the contrary, Th2 cells able to secrete anti-inflammatory cytokines such as IL-10 and IL-4 and skew the immune response to an antibody secreting hormonal response. Activated T lymphocytes can interact with antigen presenting cells such as macrophages and enhance the production of inflammatory cytokines, MMPs and tissue factor [9].

1.10.4.1.1 The role of CD4+ T cells in atherosclerosis

T cells are detected in atherosclerotic lesions of humans and animals, and T cells reactive against OxLDL can be isolated from atherosclerotic plaques [372-374]. Earlier studies have mainly examined the pro-atherogenic effects of T cells, as adoptive transfer of $CD4^+$ T cells into immuno-deficient $ApoE^{-/-}$ mice accelerates atherogenesis [371]. Hence transfer of $CD4^+$ T cells reactive against MDA-LDL or HSP65 results in mice forming larger lesions compared to those transferred with naïve T cells, or T cells from mice immunized with irrelevant antigen [85, 86, 375]. Therefore as a whole, T cells were regarded as proatherogenic, although there may be some differences in their role in different vascular beds [376].

More recently, the influence of different T cell subpopulations on atherosclerosis has been investigated. When naïve T cells become activated, they differentiate into T helper (Th) cells that direct the immune system to produce the most appropriate immune response to fight infections or other insults. Their actions are mediated primarily through cytokines; the cytokines produced and the immune response elicited is in part determined by co-stimulatory signals from APCs, and eventual T cell differentiation. A number of different $CD4^+$ T cell subtypes exist, each with different cytokine production and immunological roles. T helper type 1 (Th1) cells are generally associated with inflammatory responses, and are believed to promote atherosclerosis. Patients with acute coronary syndromes (ACS) often have proportionally higher levels of T cells secreting the Th1 cytokine, IFN-γ [377]. They also produce, and are more effective at inducing MMP expression in macrophages, compared to naïve T cells or Th2 cells [378]. Blockade of Th1 cell differentiation by deletion of the transcription factor T-β leads to a more pronounced Th2 response, and reduction of atherosclerosis [379]. In atherosclerosis, severe and chronic

hypercholesterolemic conditions can induce a switch from Th1 to Th2 responses [380]. T helper type 2 (Th2) cells are mostly associated with anti-inflammatory responses and promote antibody production by B cells. The major cytokine secreted by Th2 cells is IL-4, which can provide signals to B cells to produce antibodies. However, in atherosclerosis, a Th2 response can go both ways. B cells induced by Th2 cells to produce antibodies reactive to HSP65 does not provide any atheroprotective effect, but rather can react with HSP60 on stressed endothelial cells, and cause cellular damage [381], while immunization with ApoB100 peptide sequences leads to increased Th2-specific anti-ApoB100 IgG1 antibodies and reduced atherosclerosis [382]. Immunization of mice with MDA-LDL also induces Th2 cell differentiation and expansion that results in increased IL-5 production and increased natural atheroprotective IgM antibodies [97]. Hypercholesterolemia not only switches immune responses from a Th1 to a Th2 as mentioned before, but can also elevate TGF-β levels, the main effector cytokine of T helper type 3 (Th3) cells [383]. Widespread and uncontrolled T cell activation due to disrupted TGF-β signaling leads to reductions in collagen maturation, which has implications on plaque stability [251]. T helper type 17 (Th17) cells are another subset of T cells which mainly involved in mucosal immunity and their role in atherosclerosis is currently unclear. In one study, IL-17 receptor deficiency in bone marrow-derived cells reduced atherosclerosis in $LDLR^{-/-}$ mice [365]. In contrast, another study found increased levels of IL-17 to be atheroprotective, an outcome possibly attributable to reductions in endothelial VCAM-1 expression and T cell recruitment [363].

1.10.4.1.2 The role of Regulatory T (Treg) cells in atherosclerosis

Regulatory T (Treg) cells are a subpopulation of T cells that were originally termed suppressor T cells. They are a heterogeneous population, consisting of naturally occurring and acquired regulatory T cells, which can inhibit the functions of different leukocytes, including CD8+ T cells [384, 385], macrophages [386, 387], NK cells [388-390], NKT cells [391], and B cells [392, 393]. Since leukocytes are major participants in atherogenesis, and regulatory T cells can inhibit the activation or actions of leukocytes, they are likely to be atheroprotective. Monocytes co-cultured with Tregs proliferate less, and produce reduced amounts of TNF-α IL-6 and IFN-γ. Expression of CD40, HLA-II, C80 and CD86 were also decreased on these monocytes, which impeded their ability to activate $CD4^{+}CD25^{-}$ T cells [387]. Tregs also inhibited the formation of foam cells from macrophages [386]. In humans, regulatory T cells have been detected in atherosclerotic lesions in low numbers [394]. Transfer of Tregs which secrete IL-10 and TGF-β attenuates atherosclerosis in ApoE mice [395], while depletion of Tregs increases atherosclerosis [396-398]. Expansion of Tregs by injection or oral administration of anti-CD3 antibodies also reduces atherosclerosis by a mechanism dependent on TGF-β [399, 400]. Oral tolerance to otherwise pro-atherogenic peptides such as oxLDL and HSP60 results in a reduction in atherosclerosis that in part is attributable to expansion of Tregs [87, 88]. However, the function of Tregs may generally be compromised in atherosclerosis as Treg numbers decline with the development of atherosclerosis, and oxLDL has been shown to reduce their suppressive properties [396]. Therefore higher numbers of Tregs would be required to exert the same suppressive effects seen in the absence of oxLDL. Patients with acute coronary syndromes (ACS) have less peripheral Tregs with compromised suppressive capacity compared to patients with stable angina (Figure 1.14) [401].

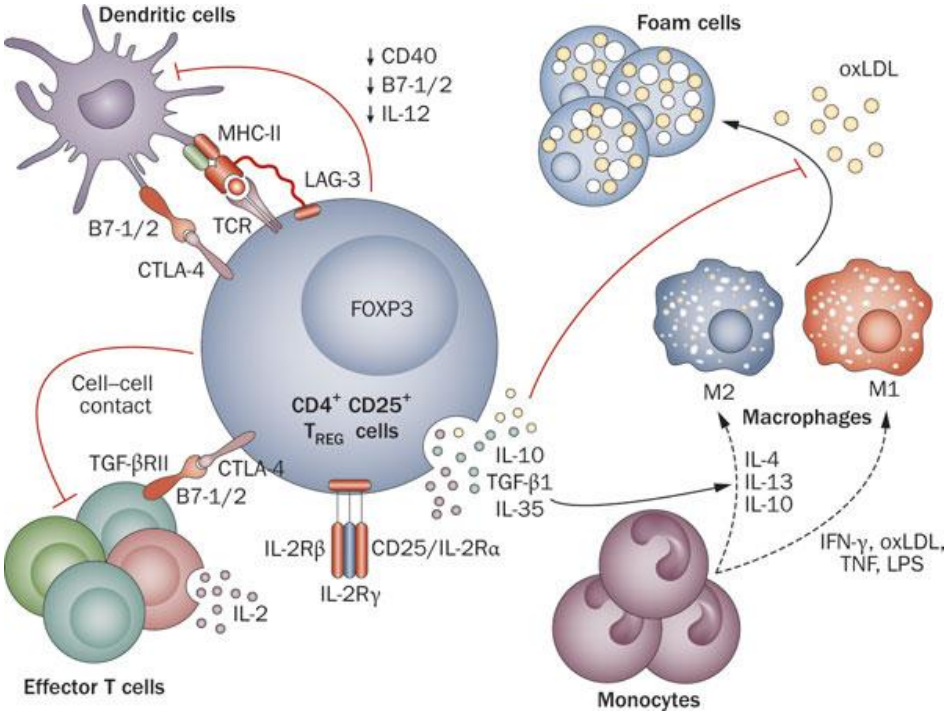

Figure 1.14. Tregs in atherosclerosis, from Nat Rev Cardiol [402].

Tregs can supress the development of atherosclerosis in different ways. They might have direct contact with effector T-cell and limit their activity or secretion of anti-inflamatory cytokines (such as IL-10, TGF-β, and IL-35), or by consuming most of the IL-2 required for T-cell proliferation. Tregs are also able to suppress the activation and antigen presentation of dendritic cells via CTLA-4 and LAG3. Cytokines secreted by Tregs can inhibit the formation of foam cells and induce the differentiation of monocytes into anti-inflammatory M2 macrophages instead of proinflammatory M1 macrophages. Abbreviations: B7-1/2, CD80/CD86 CTLA-4, cytotoxic T-lymphocyte antigen 4; IL-2R, IL, interleukin; IL-2 receptor; IFN-γ, interferon γ; LAG-3, lymphocyte activation gene 3 protein; LPS, lipopolysaccharide; MHC, major histocompatibility complex; oxLDL, oxidized LDL; TCR, T-cell receptor; TGF, transforming growth factor; TGF-β RII, TGF-β receptor II; TNF, tumour necrosis factor; Treg cells, T-regulatory cells [402].

1.10.4.1.3 The role of CD8$^+$ T cells in atherosclerosis

CD8$^+$ T cells and γδ-T cells are minor T cell populations implicated in atherosclerosis. CD8$^+$ T cells can be detected in human atherosclerotic lesions [403], particularly in those containing Chlamydia pneumoni [404]. We recently showed that CD8$^+$ T cells promote the development of vulnerable atherosclerotic plaques by perforin- and granzyme B–mediated apoptosis of macrophages, smooth muscle cells, and endothelial cells that, in turn, leads to necrotic core formation and further augments inflammation by TNF-α secretion [367]. CD8$^+$ T cells have been found to express IFN-γ in lymph nodes draining the aortic root in mice [405], and are also activated by antigen-pulsed DCs in lesions [21]. γδ -T cells are a small subset of T cells that also possess cytolytic properties, and are mostly found in skin epithelia and the gut mucosa [406]. They represent a small proportion of T cells in human atherosclerotic lesions [18, 407]. γδ -T cells have been found to respond to intracellular bacterial pathogens such as *Listeria monocytogenes* [408] and mycobacterial HSP60/65 [409]. Since HSP60/65 is believed to be a protein important in the initiation of atherosclerosis, their stimulatory effect on γδ-T cells may be additive to their pro-atherogenic effects on other cells involved in atherogenesis.

1.10.4.1.4 The role of Natural killer T cells in atherosclerosis

Natural killer T (NKT) cells are a population of CD1d-restricted T cells that bridge the innate and adaptive immune systems. They are activated early in an immune response, and rapidly release cytokines that aid in the priming and activation of adaptive immune cells. NKT cells are a heterogeneous cell population identified by their cell surface expression of T cell receptor and reactivity to CD1d/α-galactosylceramide (α-GalCer) tetramers [410]; their subsets differentiated by

surface expression of CD4 and CD8. NKT cells are typically found in all organs where other lymphocytes are found for example in thymus, spleen, lymph nodes, bone marrow, liver, and peripheral blood. NKT cells are most abundant in the liver, consisting of 15-40% of hepatic lymphocytes. They account for 20-30% of bone marrow T cells, 10-20% of mature thymocytes, 0.5-1% of splenocytes, and 0.1-0.5% of cells in peripheral lymph nodes [411].

1.10.4.2 B cells and atherosclerosis

B cells are detected in the intima with lower number compare to macrophages and T cells, but become the major cell population in adventitia where their accumulation leads to the formation of tertiary lymphoid like structures [412]. Early animal studies suggest the B cells may be atheroprotective [413, 414]. However, complete pictures of differential effects of two distinct subsets of B cells in atherosclerosis have recently been determined.

B cells are classically divided into two main subpopulations: B1 B cells that reside in pleural and peritoneal cavities and also found in the spleen in smaller numbers, and B2 B cells also called conventional B cells that populate secondary lymphoid organs. B1 B cells develop mostly in foetal liver, whereas B2 B cells are constantly produced in the adult bone marrow [415]. As immature B cells leave the bone marrow, they enter the spleen where they differentiate into transitional type-1 (T1) and type-2 (T2) B cells that are then selected to become either marginal zone B cells, which are mostly immobile, or follicular B cells, which recirculate between lymphoid organs and populate lymph nodes [416]. B1 B cells are larger than B2 B cells, and they are defined by a distinctive combination of cell surface markers, specifically high

expression of IgM and low expression of IgD. B1a B cells are a subset of B1 B cells defined by expressing the pan-T cell marker CD5 which is distinct from the other B1 sister population B1b [415]. A third small subpopulation of B cells with regulatory properties (Bregs) was recently reported. Their surface markers have not been clearly defined. They include B10 cells because they regulate the immune response via IL-10 production [412]. In atherosclerosis, B cell-derived autoantibodies recognizing epitopes from OxLDL, particularly malondialdehyde-lysine (MDA-lysine), have been detected in the plasma and atherosclerotic lesions from humans and animals, their levels increasing with development of atherosclerosis, and even more so when animals are immunized with MDA-LDL or plaque homogenates to protect them against atherosclerosis [96, 97, 125, 129, 417-419]. Antibodies against epitopes on modified lipids may delay atherosclerosis by inhibiting lipoprotein uptake by macrophages, thereby reducing foam cell formation [420-422]. Deficiency of B cells has been shown to reduce total serum antibody and anti-OxLDL antibody levels, and increase atherosclerosis in LDLR-/- mice [423]. Additionally, splenectomy of mice results in the aggravation of atherosclerosis, which is reversed upon transfer of B, but not T cells, further confirming a protective role for B cells [413]. On the other hand, immunization of mice with HSP60/65 or β2-glycoprotein-I (β2-GPI) accelerates atherosclerotic lesion development. High antibody titers against HSP60/65 has been correlated with increased atherosclerosis [424], and injection of IgG from serum of mice immunized with HSP65 increases fatty streak formation in recipient mice [85]. Furthermore, IgG autoantibodies containing Fc domains can promote the uptake of oxLDL by macrophages by binding to their Fc receptors (Figure 1.15) [44].

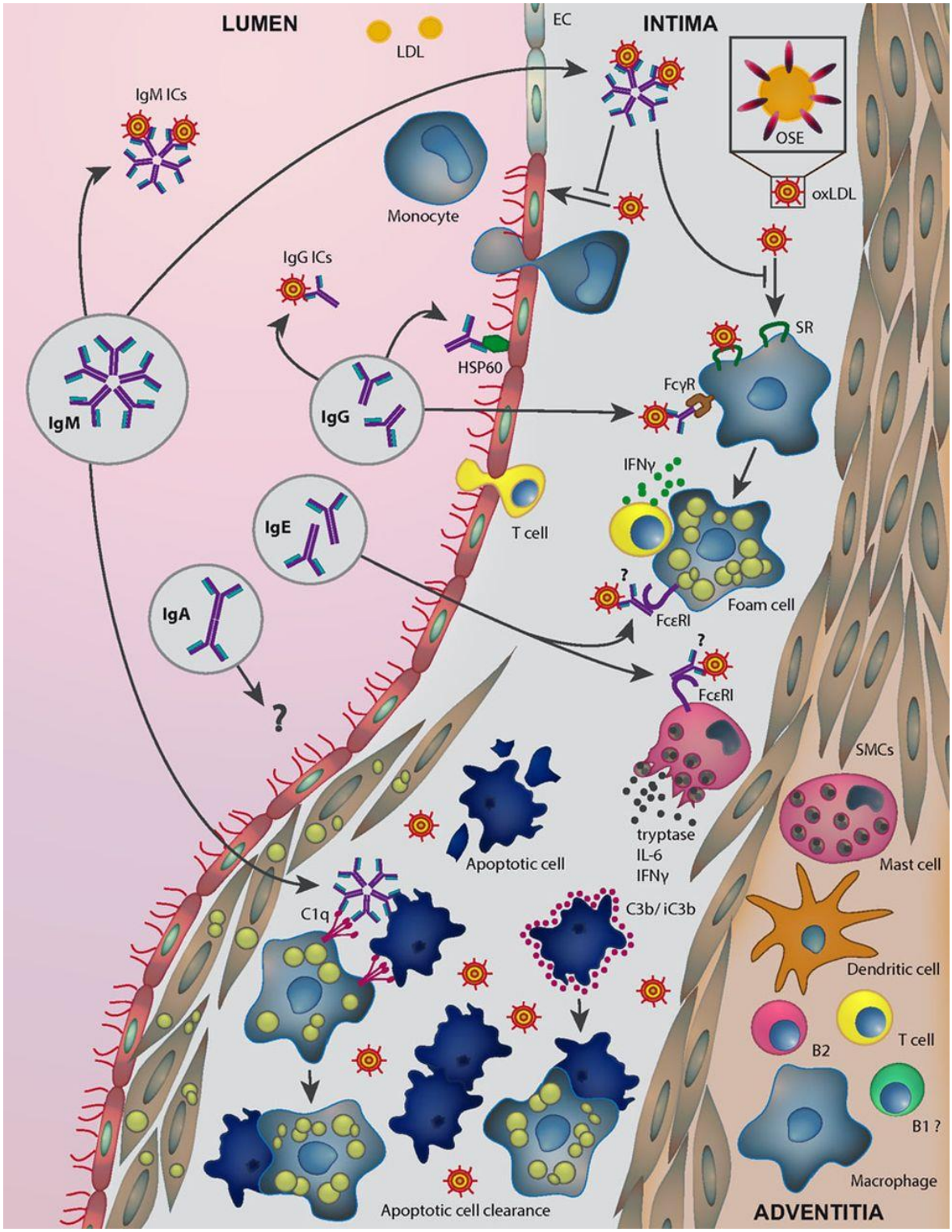

Figure 1.15. Role of B cells and immunoglobulins in atherosclerotic lesion development, from Sirc Res [425].

Studies detected different kind of immunoglobulin antigens such as HSPs and oxLDLs. IgM as an atheroprotective antibody mediate atheroprotection by

neutralizing oxLDL, removing apoptotic cells and inhibiting oxLDL uptake by macrophages. Recognition of oxidation-specific epitopes (OSEs) presented on both oxLDL and apoptotic cells by IgM might be the mechanism. On the other hand, oxLDL-specific IgG promotes atherosclerosis by activation of macrophages. Immune complexes of IgM and IgG with OSEs in the blood also promote clearance of pro-atherogenic LDL particles. HSP60/65-specific IgG recognize stressed endothelial cells and induce damage via antibody-dependent cellular cytotoxicity. IgE antibodies may be involved in plaque destabilization via activation of mast cells and macrophages. The role of IgA antibodies in atherosclerosis is still not clear. EC indicates endothelial cells; IC, immune complexes; IFN-γ interferon-γ; IL-6, interleukin-6; SMCs, smooth muscle cells; and SR, scavenger receptors [425].

1.10.4.2.1 The role of Conventional B2 B cells in atherosclerosis

Conventional B2 B cells are essential to adaptive immune responses. Random gene rearrangement and non-template N-nucleotide addition confer the B2 B cell population with a highly polymorphic BCR repertoire. B2 B cells elicit multiple responses that are highly restricted to their target antigens. They secrete antibodies that are strictly antigen-specific and activate CD4 T cells with corresponding antigen-specificity to mount concerted and sustained actions against the antigens. Rituximab as a chimeric anti-CD20 monoclonal antibody 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 Erythematous (SLE) [426, 427]. Much of the success owes to the depletion of pro-inflammatory B2 B cells as shown in experimental animals [428-430]. This strongly appeals for the design of B2 B cell-centric immunotherapy, an undertaking that is only achievable not without first understanding the precise mechanistic actions of B2 B cells in each disease. The ultimate aim is to abolish only the particular responses of B2 B cells that are damaging rather than deleting the entire population of B2 B cells. The development of B2 B 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 B cells, mature B2 B cells exit the bone marrow, complete their development in the spleen and patrol the periphery to carry out their immune duties. The peripheral pool of B2 B cells is continually streamlined 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 [431]. T1 B2 B cells are the earliest B2 immigrants in the spleen. They enter the red pulp of the

spleen and migrate to the outer peri-arteriolar lymphoid sheath (PALS) where the T cell:B cell interface lies. T2 and T3 B2 B cells co-localize in the splenic follicle [432].

The fate of transitional B2 B cells hangs 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 B 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 B cells [433, 434].

1.10.4.2.1.1 Marginal Zone (MZ) B2 B cells

MZ B2 B cells share similarity to B1a B 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 [415, 435, 436]. MZ B2 B cells are mostly confined within the splenic MZ. After capturing antigens, MZ B2 B cells shuttle from the MZ to the FO zone to transfer captured antigens to follicular dendritic cells [437-439]. MZ B2 B cells are retained in the MZ through the integrin–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 B cells from the splenic MZ [440]. However up to now, their roles in atherosclerosis have not been studied.

1.10.4.2.1.2 Follicular (FO) B2 B cells

FO B2 B cells undergo additional selection for their responsiveness to CD4 T cell help [441]. This ensures that FO B2 B cells are appropriate to collaborate effectively with CD4 T-helper cells. Unlike MZ B2 B cells, FO B2 B cells are free to recirculate between the blood and the lymphatic system [442, 443]. Presently, there is no known interaction between MZ B2 B cells and FO B2 B cells. Recent studies showed that B2 B cells are major aggressors of atherosclerosis in their own capacity and possibly also synergistically with other cells. The earliest lead came about when the depletion of B2 B cells in ApoE$^{-/-}$ mice significantly suppressed the development and progression of atherosclerosis [444, 445]. In addition, lymphocyte- and B cell-deficient ApoE$^{-/-}$ mice had increased atherosclerosis after receiving adoptive transfers of B2 B cells [444]. 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 B cells but not B1a B cells [446]. Hence, ApoE$^{-/-}$BAFF-R$^{-/-}$ mice have diminished B2 and normal B1a populations. Indeed, the hypothesis on anti-atherogenic B1a B cells and pro-atherogenic B2 B 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 [447]. A similar outcome was observed in chimeric LDLR$^{-/-}$ mice that were selectively deprived of BAFF-R in B2 B cells [448]. It is beyond any doubt that B2 B cells are viable therapeutic targets for combating atherosclerosis. B cell depletion studies have suggested that B2 B cells are an atherogenic B cell subset. In support of this finding, adoptive transfer of 5×10^6 splenic B2 B cells from mice with a C57BL/6 background, aggravated atherosclerosis in B cell deficient μMT ApoE$^{-/-}$ mice fed 6 weeks of Western diet

[444]. The mechanisms by which B2 B cells can aggravate atherosclerosis are poorly understood. Anti-CD20 treatment was associated with an increase in the percentage of IL-17^{+} T cells (Th17 cells), and IL-17A neutralization abrogated anti-CD20 attenuation of atherosclerosis [445]. These results suggest that IL-17 may mediate B2 B cell aggravation of atherosclerosis[449]. However, the role for IL-17 in atherosclerosis remains controversial. In addition to increasing Th17 cells, anti-CD20 treatment was also associated with a decrease in CD4 T cell secretion of the Th1 cytokine IFNγ, and reduced proliferation and activation of splenic CD4 T cells [445, 448]. Several pro-atherogenic roles have been identified and reviewed for Th1 lymphocytes [450-453]. Depletion of B2 B cells was also associated with decreased T cells in the atherosclerotic plaque [445, 447, 448], suggesting that B2 B cells may aggravate atherosclerosis by regulating T cells in the aorta as well as the spleen and further investigations are required to unveil the mechanistic atherogenic functions of B2 B cells.

1.10.4.2.1.3 IgG production by B cells and atherosclerosis

IgG is the hallmark isotype of immunoglobulins produced by B2 B cells during an immune reaction. There are multiple IgG subclasses – IgG1, IgG2a, IgG2b and IgG3. Each is distinguished by the constant region on the γ heavy chain (Fcγ). The same applies to the Fcγ receptors (e.g. FcγRI, FcγRIIa and FcγRIIb) which differ by the strength of binding to IgG molecules [454, 455]. The overall impact of IgG antibodies on atherosclerosis remains controversial with several studies providing distinct views [456, 457]. 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 [458-460]. Deposition of oxLDL-IgG within atherosclerotic lesions is also evident [461]. Despite these indications, there are claims that oxLDL-specific IgG antibodies are clear biomarkers of atherosclerosis and are only suitable for diagnostic and prognostic purposes. Increasing oxLDL-IgG titers through vaccination has been shown to suppress atherosclerosis in animal models [456, 462]. 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 [417]. 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 although 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 [463]. Following the success in rabbits, confirmation of the atheroprotective effect of increasing oxLDL-IgG levels was attained in LDLR-/- and ApoE-/- mice [382, 418, 419, 464]. 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 possibility 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 [380]. Amongst all IgG subtypes, IgG1 binds strongest to FcγReceptors and the advantage of this is the rapid repossession of IgG1 immune complexes by macrophages. On this account, oxLDL-IgG1 antibodies may reduce the retention and

oxidation 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 [465]. 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 [456]. 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 ApoB100 peptides (p2, p45 and p210) have been identified to protect against atherosclerosis [419]. Apobec-1$^{-/-}$LDLR$^{-/-}$ mice that were passively immunized with human IgG against MDA-modified ApoB100 p45 showed a significant reduction in atherosclerosis [466]. B2 B cell depletion was associated with a reduction in total serum IgG including IgG1, IgG2a, IgG2c, as well as IgG1 and IgG2a in the atherosclerotic plaque. Furthermore, B2 B cell depletion resulted in a reduction in serum IgG against modified lipids, oxLDL and malondialdehyde LDL (MDA-LDL). Consistent with the predominant depletion of B2 B cells and not B1a B cells, there were only modest decreases in total IgM and IgM against MDA-LDL and oxLDL [444, 445, 447, 448]. Interestingly, univariate analysis revealed that serum levels of IgG and IgM to oxLDL have divergent associations with coronary artery disease in humans. IgM to oxLDL was inversely associated with coronary artery disease while IgG was positively associated [467]. Mechanisms whereby adaptive

immunoglobulins might regulate plaque development are poorly understood. Downstream of antibody production, B cells may indirectly regulate atherosclerosis in an antigen-independent manner when IgG immune complexes bind to Fc gamma receptors (FcγR). Activating FcγRs have been implicated as being pro-atherogenic [468] and inhibitory FcγRIIb anti-atherogenic [457, 469]. Additionally, IgE and its Fc receptor present on mast cells, FcεR1α, are pro-atherogenic [470].

1.10.4.2.1.4 BAFF and BAFF receptor in B cell homeostasis in atherosclerosis

The TNF family member B cell-activating factor (BAFF) [471], also known as BLyS, TALL-1, zTNF4, or THANK) regulates B cell survival, generation and differentiation, and determines the size of peripheral mature B cell pool and may drive B cell-dependent autoimmunity. BAFF is expressed on a variety of cells of myeloid and other origin [472], and its expression pattern may control the compartmentalization of B cells in the peripheral immune system. BAFF specially up regulates survival of the immature transitional type 2 (T2) B cells, and when combined with a B cell receptor (BCR) stimulus, results in differentiation of T2 subset to mature B cells [473]. BAFF transgenic mice display increased T2, follicular and marginal zone B cell subsets of the spleen as well as germinal centre formation [473, 474]. Genetic disruption of the BAFF gene (BAFF-deficient mice) leads to a profound decrease in mature B2 B cells, whereas B1 B cells are not affected [475]. Both $CD4^+$ and $CD8^+$ T cells can be activated by recombinant BAFF to increase CD25 expression [476] and BAFF-mediated human T-cell proliferation can be blocked with BAFFR-Ig [477]. However, blocking BAFF in some T-cell dominant and little B cell involved diseases was not

efficacious [478]. BAFF can be produced by myeloid DCs in response to type I interferons (IFNs) and activates immunoglobulin (Ig) class switching and plasma cell differentiation [479, 480]. Many lines of BAFF transgenic mice develop spontaneous autoimmune diseases with significant B cell hyperplasia, hypergammaglobulinaemia and production of various circulating autoantibodies and immune complexes [474, 481]. It has been demonstrated that many patients suffering from SLE, RA and Sjogren's syndrome (SS) display elevated levels of soluble BAFF in their plasma [482-484]. However the overexpression of BAFF may not trigger the autoimmune response [485]. Transgenic mice engineered to overexpress BAFF show increased numbers of peripheral B cells and elevated serum Ig levels, including anti-DNA Abs, rheumatoid factor, and circulating immune complexes, and develop autoimmune symptoms resembling human systemic lupus erythematosus and Sjögren's syndrome as they age [474, 486].

BAFF can bind to three receptors of the TNFR family: transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI); B cell maturation Ag (BCMA); and BAFF-R (also known as BLyS receptor 3 or BR3). BAFF-R is predominately expressed on all mature peripheral B cell subsets in secondary lymphoid organs and peripheral blood. TACI is the predominant receptor on T2, marginal zone and B1 B cells [485]. Mice lacking BAFF-R expression display a strong reduction in late transitional and mature B2 B cell numbers [487, 488]. In contrast, mice with individual or combined deficiencies of TACI and BCMA contain normal or even increased populations of mature B cells [489]. This genetic evidence suggests that BAFF-R is the receptor that mediates BAFF-dependent B cell survival. In addition to B cells, BAFF-R expression has been demonstrated on naïve and memory T cells and particularly high on $CD4^{+}CD25^{+}$ Regulatory T cells [490]. BAFF-

R is essential for mediating BAFF induced survival signals of B2 B cell maturation past the transitional type 1 (T1) stage [491]. Mature B cells express both BAFF-R and TACI but require BAFF-R alone to survive in-vivo and BAFF-R KO mice have significantly decreased peripheral B cell numbers [485].

Treatment of mice with soluble BAFF-R-Ig (BR3-Fc) and TACI-Ig (Atacicept) fusion proteins depleted non-recirculating B cells through block of survival signals [492] and suppressed disease in animal models of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) [493-495]. Rituximab (anti-CD20) deplete recirculating B cells through an Fc receptor dependent manner. BR3-Fc is currently in pharmaceutical development. Belimumab (trade name Benlysta), a fully human IgG1 monoclonal antibody specific for soluble BAFF, was recently approved by FDA for treatment of SLE and has also undergone phase II clinical trials for RA. Overexpression of BAFF and APRIL has recently been found in human atherosclerotic plaques [496]. Recently Kyaw and Sage demonstrated atherosclerotic lesions are significantly attenuated in BAFF-R deficient mice. Macrophage accumulation and IgG deposition are significantly decreased in lesion and the expression of adhesion molecule and proinflammatory cytokines are also dramatically reduced [447, 448].

1.10.4.3 B1 B cells and other Regulatory B cells

B1 B cells (IgM^{hi} $B220^{lo}$ IgD^{lo} $CD23^{-}$) [497] are a minor population of B cells that are mostly found in the peritoneal and pleural cavities with very low numbers in the blood and spleen [430, 498] consisting of three subtypes namely, B1a, B1b and B1c [499]. In contrast to B2 B cells, B1 B cells are long-lived and self-renewing [500-502]. They

are really rare in peripheral lymph nodes and only account for about 5% of B cells in the spleen [500, 503]. They are larger in size than B2 B cells [498]. They also express IL-5 receptor and CD43 surface marker on their surface [430, 498, 500, 504]. As pleural and peritoneal cavities are the main sites of residence for B1 B cells, their numbers in these sites are about 10-15% of B cells [503, 505].

1.10.4.3.1 B1a B cells development

B2 and B1 B cells are two main populations of B lymphocytes from different origins (Figure 1.16). B1 B cells are part of the innate immune system which produce immunoglobulins against pathogens. Their roles in providing immunity to specific pathogens have been reviewed broadly [506-508]. B2 B cells are present in secondary lymphoid organs such as spleen, LN and thymus and are involved in adaptive immunity. B2 B cells are divided into two sub populations, a predominant population of follicular (FO) and a minor population of marginal zone (MZ) B cells, both of which can undergo Ig class switching and differentiate into memory cells [509, 510]. B1 B cells resident in serous cavities are divided into two subpopulations, B1a B cells are $sIgM^+$ $CD11b^+$ $CD5^+$ cells, whereas B1b B cells are $sIgM^+$ $CD11b^+$ $CD5^-$ [511] and different combination of cell surface markers need to be used to detect them in other lymphoid organs such as spleen [415, 512].

Soon after the description of B1 B cells, two different models of origins arose for B1 B cells. The ‘‘selection model’’ proposed that the decision of an Ig expressing B lymphocyte to become a B1 or B2 cell was driven by the response to particular antigens [500, 513]. In contrast, Herzenbergs proposed the ‘‘layered immune system hypothesis’’ showing that the B2 B cells and B1 B cells belong to separate heredities and derive from separate progenitors developing at different times during

development [514]. To define the origin of both these two cells, transplantation of neonatal liver and adult bone marrow into irradiated recipients were done by Hayakawa and colleagues and the result showed that both recipients produced B2 B cells but, B1 B cells were reconstituted just in neonatal liver cells of recipients [515]. This suggested that B1a B cells were derived from fetal progenitors. Studies done in the early 1970s and later showed that B lymphocyte lineage cells are present in fetal tissues of liver, bone marrow, spleen, placenta and blood [516]. Fetal liver transplantation was shown to reconstitute B1 B cells in irradiated recipients [511], and the fetal omentum demonstrated to be a selective source of B1 B cells [517]. These observations supported the existence of a separate B1 B cell progenitor [516]. Hematopoiesis is not as simple as we think, a linear process originating in the fetus and continuing in the adult. Fetal and adult HSCs have different patterns of gene expression that exhibit differences in proliferative and developmental potential [518, 519]. Evidence showed that the adult immune system consists of cells generated in layered, developmental programs. HSCs in bone marrow differentiate into early lymphoid progenitors and then common lymphoid progenitors, pro-B, pre-B, and finally immature B cells. [520-522] indicated that the precursor lymphoid cells express B220 at pro-pre B cell transition stage, but CD19 expression does not occur until these cells have matured into pro-B cells (Figure 1.16).

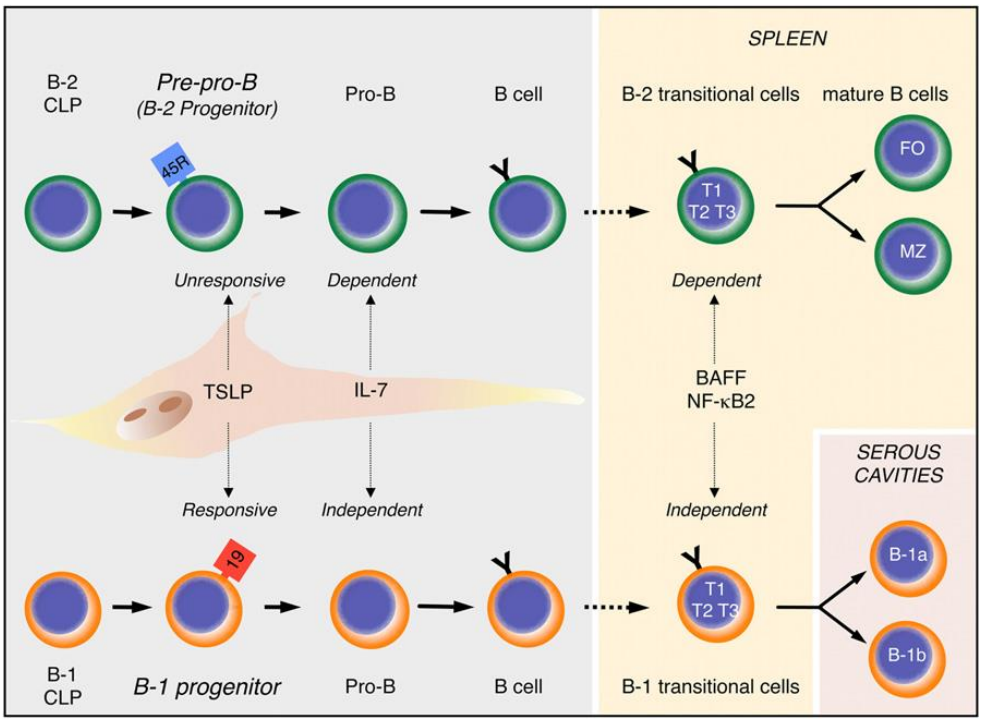

Figure 1.16. B1 and B2 B cells Development, from Nat Immunol [516].

Top flow; B2 B cells are formed in the bone marrow after birth from common lymphoid progenitors (CLPs), B2 cell progenitors (pre-pro-B), pro-B, and pre-B (not shown) and then, cell intermediates develop into immature sIgM$^+$ B cells (in figure shown as ‘‘B cell’’). Immature sIgM$^+$ cells migrate to the spleen, to develop through B2 cell transitional 1 (T1), 2 (T2) and 3 (T3) stages into follicular (FO) or marginal zone (MZ) B cells.

Bottom flow; Mature B1 B cells are derived from B1 B cell-specified CLPs that sequentially differentiate through B1 B cell progenitor, pro-B, pre-B (not shown), and immature sIgM$^+$ B1 B cell (in figure shown as ‘‘B cell’’). Then, sIgM$^+$ B1 B cells migrate to spleen for maturation through the transitional cell stages. Finally, mature B1 B cells as two B1a and B1 B cell phenotypes migrate to serous cavities. The figure also shows that B1 B cell progenitors are TSLP responsive and B1 B cell but not B2 cell expansion and survival is independent on IL-7, BAFF and NF-kB2 [516].

Later, Montecino-Rodriguez et al. identified a population of lineage negative (Lin^-) $CD93^+B220^{-/lo}CD19^+$ progenitors whose existence was not predicted by the Hardy scheme [523, 524]. These cells were present in high numbers in fetal liver and then declined their total number in the weeks after birth. They also reported the presence of similar cell phenotype at a low number in the bone marrow of young adults [525], and De Andre had also identified low numbers of $B220^{-/lo}$ $CD19^+$ B cell progenitors in the fetal liver of embryos at day 11 of gestation [526]. The in-vitro culture of Lin^- $CD93^+B220^{-/lo}CD19^+$ progenitors showed up regulation in expression of B220 and matured into $sIgM^+$ B cells. These cells co-expressed CD11b later on and *in-vivo* transplantation of them showed their differentiation into both B1a and B1b B cells in the peritoneal cavity of recipients, but do not differentiate into B2 B cells [524]. They also express Ig heavy chain genes and secreted Igs with reactivity toward the B1 antigen phosphorylcholine. Taken together, these data and further studies supported the existence of distinct B1 B cell progenitors which support the lineage model [527-530].

B cell production takes place in three different waves during development (Figure 1.17). The first wave initiates in the yolk sac where non-HSC derived predecessors with the potential to generate B1 B cell progenitors arise, whereas the second wave initiates during mid-gestation in fetal liver, fetal bone marrow, and fetal spleen [528]. Studies revealed that most of B1 B cell progenitors that appear in this second wave are HSC derived. Finally, during the third wave, which occurs near the end of fetal life, the production of B1 B cell progenitors declines and the B2 developmental program becomes increasingly well established. Further studies have shown that the timing of B1a and B1b cells appearance during ontogeny also indicate that they arise

separately [530, 531] and additional B1 B cell progenitors and waves of development may exist.

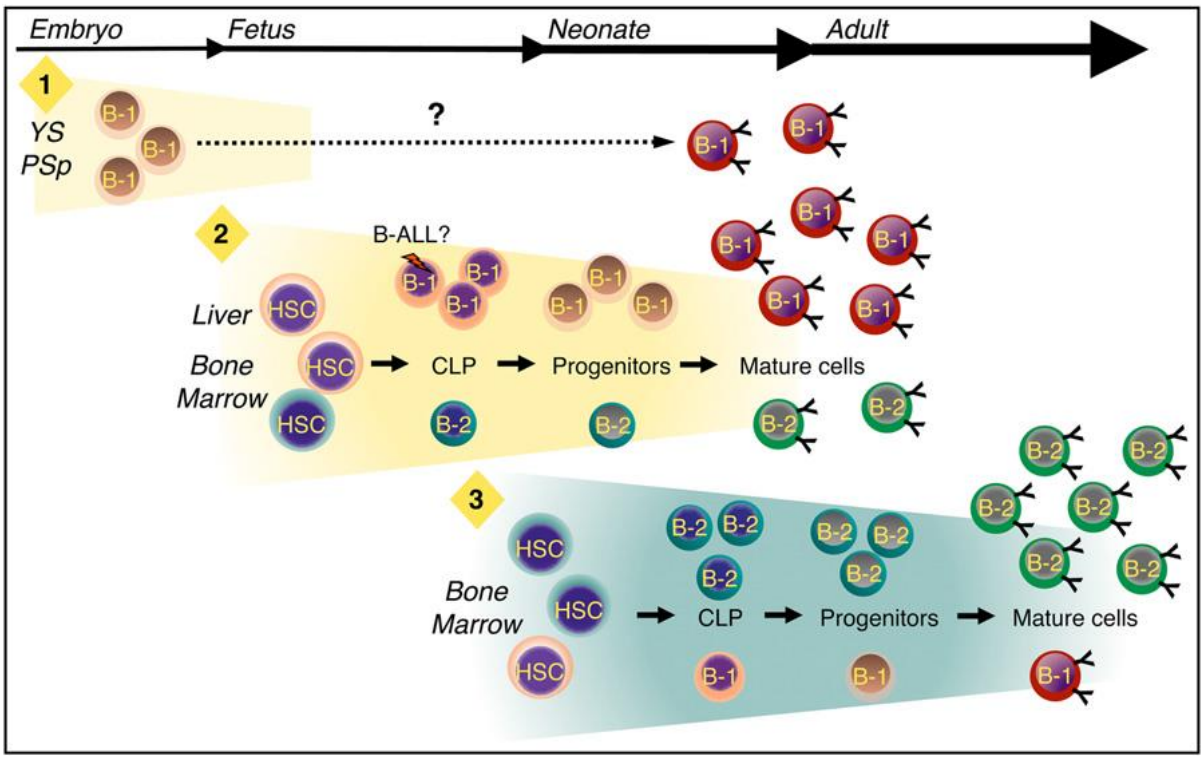

Figure 1.17. B1 and B2 B cells development happen in three waves of development, from Nat Immunol [516].

The first wave (1) initiates in the yolk sac (YS) and para-aortic splanchnopleura (PSp) region before the appearance of hematopoietic stem cells (HSCs). During the second wave (2), which initiates in the fetal liver and fetal bone marrow, HSCs generate B1 B cell progenitors from which mature B1 B cells are derived. Although B2 B cell development initiates during this second phase, B1 B cell production predominates. Whether or not B1 and B2 cell CLPs and progenitors are produced from a single type of HSC or multiple B1 and B2 cell-specified stem cells exist, as indicated by the different coloured HSCs, is not known. B1 B cell production peaks during late embryogenesis and then begins to decline just before birth. The third wave (3) of B cell development takes place in the bone marrow and results primarily in the production of B2 B cells. B1 B cells can be produced during this third wave, but the efficiency with which this occurs in comparison to wave two is substantially reduced [516].

1.10.4.3.2 Development and function of regulatory B cells

B1 B cells are likely related to regulatory B cells but their precise relationship to these cells is not currently known. Regulatory B cells (Bregs), like B1 B cells share the common property of expressing interleukin-10 (IL-10) as an immunoragulatory cytokine. A number of overlapping phenotypes are currently used to describe these regulatory B cells. In mice, the following cells are credited as Bregs, $CD5^{+}CD1d^{hi}$ B10 B cells, $CD21^{hi}CD23^{hi}CD24^{hi}$ transitional type 2 marginal zone precursors (T2-MZP) Breg cells, and TIM-1^{+} B cells all which have regulatory function in autoimmune and infection diseases and also in transplantation [532-534]. Although, T2-MZP Breg cells might be the precursors to B10 Breg cells, the relation of these subsets to each other and to TIM-1^{+} Breg cells is not clear [533]. All these cells express Pax5 in spleen at a stage of development before differentiation into plasma cells [535]. Shen and colleagues reported that IL-10- and IL-35-expressing Breg cells were predominantly found within the $CD138^{+}$ plasma cell pool in experimental autoimmune encephalitis (EAE) and murine Salmonella infection [536] which control autoimmune inflammation by inhibiting dendritic cell activation in the draining lymph nodes of mice with EAE [537]. IRF-4 (Interferon regulatory factor-4) and PRDM-1 (PR domain zinc finger protein-1 also known as BLIMP-1) are two genes which control plasma cells differentiation and deficiency in these two genes resulted in development of more severe disease than control mice. Moreover, IRF-4 transcription has been reported to be important for the promotion of Breg cell IL-10 transcription because IRF-4 binds to a promoter region of the IL-10 gene [537].

Studies have also shown Breg cells develop their function in derange LNs in the absence of spleen [537, 538]. Splenectomy did not affect the severity of the disease or the generation of $CD138^{+}CD44^{hi}$ plasmablasts in the derange LNs. However, in mice lacking B cells that expressed CD62L, a receptor that allows B cell entry to LNs, plasmablasts were undetectable in the derange LNs and these mice were unable to resolve EAE. Similarly, adoptive transfer of $CD62L^{-/-}$B cells to B cell-deficient mice was unable to inhibit EAE because, B cells could not enter the derange LNs in the absence of CD62L expression and no regulatory plasmablasts were generated [533, 535]. In particular, transfer of splenic B10 Breg cells has been reported to suppress EAE [533] indicating that splenic B10 B cells require migration to derange LNs to differentiate into plasmablasts and it is possible that B10 Breg cells have regulatory activity at the stage before developing into antibody-secreting cells [537] or they achieve their full regulatory potential only after differentiating into plasmablasts. In a human study, stimulation of $IL\text{-}10^{+}$ plasmablasts induced IgM secretion [537]. Both B10 and T2-MZP Breg cells in mice, and $CD24^{hi}CD38^{hi}$Breg cells in humans, express high levels of IgM and could be the direct precursors of these regulatory plasmablasts (Figure 1.18) [539].

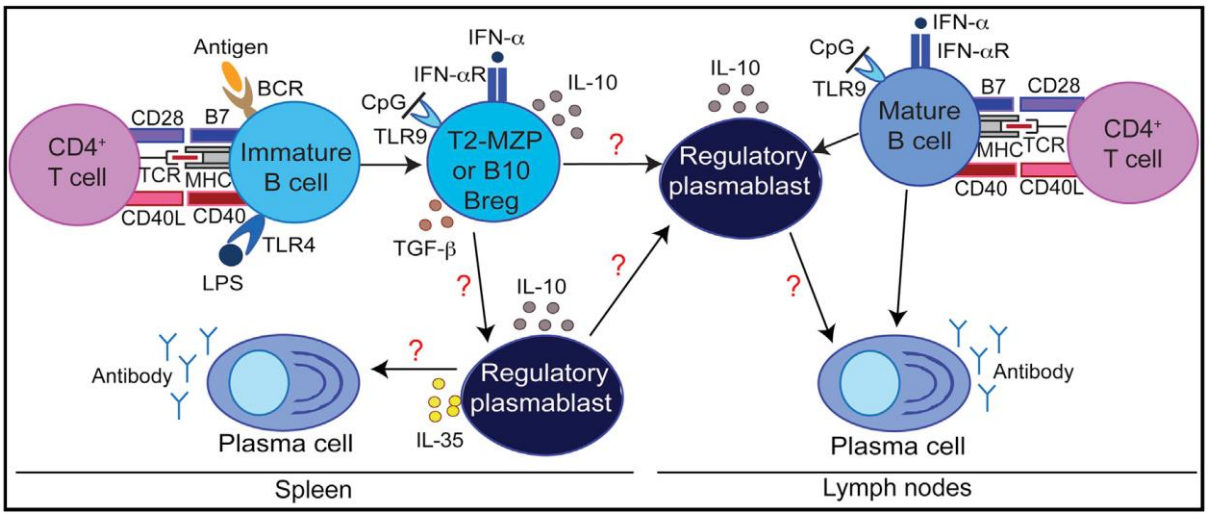

Figure 1.18. Theoretical Development Pathways of Breg Cells, from Immunity [539].

Development of IL-10^{+} T2-MZP or B10 Breg cells from the immature B cell pool in the spleen after CD40 and TLR stimulation with additional IFN-α and TLR9 stimulation in further development to become regulatory plasmablasts in the spleen or after migration to lymph nodes. On the other hand, regulatory plasmablasts might develop from mature B cells in the lymph nodes during inflammation without the need for a regulatory B cell precursor [539].

1.10.4.3.3 B10 cells and their regulatory function in immune system

Conventional B2 B cells positively regulate immune responses through antibody production and T cell activation. However, B1a B cells and regulatory B cells negatively regulate immune responses in mouse autoimmunity and inflammation models. The lack or loss of regulatory B cells has been confirmed by aggravated symptoms in EAE, chronic colitis, contact hypersensitivity, collagen-induced arthritis, and non-obese diabetic mouse models. Regulatory B cells such as B1, MZ or T2–MZ precursor B cell subsets exert their regulatory role through IL-10 production. The majority of IL-10-producing regulatory B10 cells express $CD1d^{hi}CD5^{+}$ which share their surface markers with both B1 and MZ B cells. B10 cells only produce IL-10 and are responsible for most IL-10 production by B cells [540]. B10 cells represent a small number of regulatory B cell subsets are present in the spleens of naive wild type mice which are responsible for majority of IL-10 production by B cells. Studies revealed that B10 cell subsets able to inhibit T cell proliferation and T cell-dependent inflammatory responses in-vivo [504].

Tedder and colleagues demonstrated that $CD19^{-/-}$ mice only have approximately 20% reduced numbers of splenic B cells [541] and their $CD1d^{hi}B220^{+}$ MZ B cell subset is significantly reduced [542]. In addition, the majority of peritoneal B1 B cells in $CD19^{-/-}$ mice are $CD11b^{+}CD5^{-}$ B1b cells [542]. $CD19^{-/-}$ B cells produce less IL-10 compared with wild type B cells [543]. By contrast, B cells from transgenic mice that express both mouse and human CD19 on their cell surface (hCD19Tg) are hyper-responsive to LPS, proliferate at higher levels to mitogens, generate elevated humoral immune responses to T-dependent Ags, and spontaneously produce IgG autoantibodies as they age [544, 545]. hCD19Tg mice have normal B1a B cell numbers, but significant reduction in the number of follicular B cells, B1b and MZ B

cell subsets [542]. Moreover, IL-10 production by B cells was significantly higher in hCD19Tg mice when compared with wild type B cells, while B cell IL-10 production is modest in $CD19^{-/-}$ mice, providing evidence of a negative regulatory role for IL-10-producing B cells in this model [542].

Some spleen B cells and peritoneal $CD5^{+}$ B1a B cells are known to produce IL-10 [546-548]. Splenocyte $CD21^{hi}CD23^{-}$ MZ B cells produce IL-10 in response to CpG [549] or apoptotic cell [548] stimulation and $CD21^{hi}CD23^{+}IgM^{hi}$ B cells with a T2–MZ precursor phenotype also produce IL-10 and inhibit CIA [550]. IBD generates $CD1d^{hi}CD21^{int}CD23^{+}$ mesenteric lymph node B cells with IL-10-dependent regulatory properties [551]. Spleen $CD5^{+}$B cells also produce IL-10 following IL-12 stimulation, while $CD5^{-}$B cells do not [552]. Thereby, IL-10-producing B cells share some phenotypic markers with $CD5^{+}$ B1a B cells, $CD1d^{hi}CD23^{+}$ T2–MZ precursor cells and $CD1d^{hi}CD21^{hi}$ MZ B cells. To identify IL-10-producing naive B cells, Tedder and colleagues used wild type, $CD19^{-/-}$, and hCD19Tg mice to collect purified B cell subclasses and then stimulated them to clearly distinguish the $IL\text{-}10^{+}$ B cell subsets by intracellular cytokine staining with flow cytometry analysis, and used B cells from $IL\text{-}10^{-/-}$ mice as negative controls for IL-10 staining [504]. They showed that spleen hCD19Tg B cells produced large amount of cytoplasmic IL-10 compared with naïve and $CD19^{-/-}$ B cells. In wild type mice, 7–8% of peritoneal B cells produced IL-10, while B cells from blood, peripheral and mesenteric lymph nodes, and Peyer's patches exhibited little. They also revealed that IL-10-producing B cells represent a distinct subset that is dramatically reduced in $CD19^{-/-}$ mice, but preferentially expanded in hCD19Tg mice. B10 cells from naïve and hCD19Tg mice belong to $CD1d^{hi}CD5^{+}CD19^{hi}$ B cell subset which do not express CD23 and are unlikely to be belong to the T2–MZ precursor B cell subset [504]. The functional and lineage

relationships between spleen B10, B1a, and MZ B cells, and peritoneal B1a, B1b, and peritoneal IL-10-producing B cells are unknown, because they share similar phenotypic markers. It is possible that B10 cells and B1a B cells come from a common lineage, because the number of both B10 and B1a B cells accumulate in hCD19Tg mice and are reduced in $CD19^{-/-}$ mice [542, 544]. By contrast, the number of B1b cells is increased in $CD19^{-/-}$ mice [542] and on the other hand only approximately 50% of B10 cells exhibit the $CD21^{hi}$ phenotype of MZ B cells, demonstrating a different origin for these subset of cells. Each B cell subset has different functions, with B10 cells generated during the course of immune responses auto regulating T-cell function, autoimmune disease, and inflammation through IL-10 production, while B1a B cells produce natural autoantibodies, B1b cells produce adaptive immune responses to T cell-independent antigens [542], and MZ B cells provide protection early during pathogen challenge [435].

B10 cells express both CD1d and CD5. $CD1d^{+}$ B cells and other Ag-presenting cells are capable of presenting microbe-derived glycolipids to invariant NKT (iNKT) cells through CD1d. Human and mouse iNKT cells can provide direct help for B cell proliferation and antibody production through CD1d- and CD40-restricted mechanisms [553, 554]. Therefore, B10 cells may influence the function of iNKT cells, and/or iNKT cells may influence B10 cell function. B10 cell expression of CD5 is also puzzling, because it can either positively or negatively regulate the intracellular strength of BCR signals, and play a key role in B cell selection as well as the generation and maintenance of tolerance [555]. Moreover, the selective expression of CD5 by B1a and B10 cells suggests that they may be regulated through common pathways. In the clinic, the selective depletion of mature B cells while sparing B10 cells could offers multiple potent and potential therapeutic

approaches. Moreover, the in-vivo or in-vitro expansion of B10 cells may offer new strategies for treating patients with autoimmune or inflammatory diseases. The direct regulation of B10 B cells in atherosclerosis have not yet been studied, however IL10 cytokine is well known as an atheroprotective cytokine.

1.10.4.3.4 B1 B cell subclasses in peritoneal cavity

In peritoneal cavity, B cells are divided into 4 different subpopulations identified on the basis of the expression of the CD11b and CD5 antigens [499, 514, 556, 557]. B cells expressing neither of these antigens are referred to as B2 B cells. They correspond to conventional B cells, the main B cell population outside body cavities. The second peritoneal B cell sub-populations are the B1b cells which express CD11b but not CD5. Peritoneal B2 B cells like circulating B2 cells are derived from bone marrow precursors. The third subpopulation, the B1a B cells, express CD11b and CD5 and have the unique property of self-renewal in the peritoneal cavity, independent of bone marrow–derived progenitors. The forth subpopulation, the B1c cells express CD5 but not CD11b are a small subpopulation of B1 B cells [499]. The main sites of residence for B1a B cells are the pleural and peritoneal cavities. Within these compartments, B1a B cells comprise 10-15% of B cells [503, 505]. B1a B cells seem to be directly derived from fetal liver precursors [515] and to persist from birth in body cavities as an autonomous cell population; the repopulation of immune-deficient mice with peritoneal B cells restores only the B1a subpopulation [415, 558, 559].

1.10.4.3.5 B1a B cells and their role in natural IgM production

Studies focused on the mechanistic actions of oxLDL-specific IgM in atherosclerosis for many years and evidence showing that oxLDL-IgM contribute to limiting the development of atherosclerosis through several mechanisms at the local and systemic levels. Given its importance in pathology of atherosclerosis, measurements of oxLDL-IgM in plasma and IgM deposit in atherosclerotic lesions are routinely used in atherosclerotic studies. For instance, increased oxLDL-IgM is believed to modulate inflammation in atherosclerotic plaques by preventing 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 [421, 560].

1.10.4.3.6 IL-5 and B1a B cells

The mechanisms by which B1a B cells are retained, survive, and expand in body cavities are not completely understood, but the production of a limited set of cytokines appears to be required. Mice transgenic for interleukin-9 (IL-9) display considerable expansion of the peritoneal B cell compartment because of the accumulation of B1 B cells and, to a lesser extent, of B1a B cells [561]. Mice lacking the IL-5 or IL-5R gene display transient and partial depletion of peritoneal B1a B cells [562, 563]. The link between B1a B cells and Th2 cells is supported by IL-5. Deficiency in IL-5 or administration of IL-5 depleting antibody and also deficiency in α-chain of IL-5 receptor (IL-5Rα), a receptor expressed in B1a B cells showed a reduction in the numbers of B1a B cells [562, 564, 565]. On the other hand, over expression of IL-5 in mice increased B1a B cells and serum IgM [566, 567]. The mechanism of how IL-5 is involved is still not completely clear. IL-5 is only important for the survival of B1a B cells but not B2 B cells and B2 B cell number is not affected

by the lack of IL-5 and IL-5Rα [564]. However, IL-5 is involved in B2 B cell differentiation into antibody-secreting plasma cells [565]. Study by Moon and colleagues have shown that IL-5Rα$^{-/-}$ mice treated with Lipopolysaccharide have decreased IgG1 but normal IgG2a levels after treatment, an indication that they are tending toward provoking Th1 responses [564]. IL-5 secretion by Th2 cells act as a bridge between innate and adaptive immune responses and immunization of LDLR$^{-/-}$ mice with MDA-LDL showed an expansion of Th2 cells against MDA-LDL which initiate a protective response. Indeed, MDA-LDL-specific Th2 cells produced large quantities of IL-5, even more than IL-4, IL-10 and IL-13 [97, 419]. In-vitro and in-vivo experiments showed that B1a B cells secrete atheroprotective oxLDL-binding natural IgM in the presence of IL-5 [97]. Similar results were found in ApoE$^{-/-}$ mice treated with IL-33, a member of the IL-1 family and a Th2-inducing cytokine [568]. IL-4, IL-5 and IL-13 in the blood and lymph nodes were increased in IL-33 treated mice and in these mice IL-5 production was dominant and that was accompanied with a higher level of oxLDL-specific IgM in plasma and reduced atherosclerosis. However, anti-IL-5 blocking antibody treatment abrogated the atheroprotective effects of IL-33 [568]. Further study showed that LDLR$^{-/-}$ recipients of IL-5$^{-/-}$ bone marrow cells had significantly lower oxLDL-natural IgM levels and larger atherosclerotic lesions compared to LDLR$^{-/-}$ recipients of IL-5$^{+/+}$ bone marrow cells [97]. Sampi et all. revealed an atheroprotective function for IL-5, when shown a positive correlation between IL-5 and oxLDL-specific IgM levels and negatively with atherosclerosis in humans [569]. Natural IgM production by B1a B cell stimulation spared production of atherogenic IgG2a by B2 B cells are the advantages of using an IL-5 agonist over MDA-LDL immunization [97, 564]. 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 [97]. IL-5 induces the expansion of B1a B cells in-vitro and in-vivo, [570, 571] and mice transgenic for IL-5 produce autoantibodies [566]. It is unknown whether IL-5 and IL-9 are produced in body cavities, and their potential sources at these sites have not been identified.

1.10.4.3.7 IL-10 and B1a B cells

IL-10 is another cytokine that may be involved in B1a self-renewal. The neutralization of IL-10 by anti–IL-10 monoclonal antibody (mAb) administration depletes B1a B cells but not B2 cells in normal mice [572] and prevents autoimmunity in NZB/W F1 mice, a model of systemic lupus erythematosus in which the B1a-lymphocyte compartment is abnormally expanded [573]. In-vivo administration of IL-10 activates peritoneal B1a B cells and stimulates autoantibody production in predisposed mice [571]. Because B1a B cells produce IL-10 [574], this cytokine may act as an autocrine growth factor for these cells. IL-10 production by B1a B cells is another explanation for the relationship between natural IgM and Th2 cells. B1a B cells are the major source of natural IgM and also constitutively secrete IL-10 cytokine [575, 576], a potent Th2 cytokine that modulates inflammation and involve in B1a B cells proliferation [571]. B1a B cells are the largest producers of IL-10 amongst all B cells in the peritoneal cavity [574] and peritoneal cavity is one of the major location of IL-10 production. B1a-derived IL-10 is involved in immune-regulatory responses by maintaining the expression of FoxP3 in Treg cells [577] and Treg-derived IL-10 is expected to lead to B1a-derived IL-10 in most inflammatory settings. Tregs are

divided into three subgroups, FoxP3^{+}IL-10^{-} Tregs, FoxP3^{+}IL-10^{+} Tregs and FoxP3^{-}IL-10^{+} Tregs. FoxP3^{+}IL-10^{-} Tregs mostly reside in the lungs, liver and secondary lymphoid organs which can secrete other regulatory cytokines such as TGF-β [578]. Within these regions, B1a B cells could be the main source of IL-10 which is critical to immune homeostasis.

1.10.4.3.8 B1a B cells activation

B1a B cells respond rapidly and strongly to pathogen-encoded signals, such as lipopolysaccharide and phosphorylcholine that constitute pathogen-associated molecular patterns (PAMPs). Strong selective pressure for recognition and responses to PAMPs are largely dependent on toll-like receptors (TLRs) [579]. To date, 14 TLRs have identified [579] with some such as TLR2 and TLR4 expressed on cell membranes and others such as TLR3, TLR7, TLR8 and TLR9 expressed endosomally [580, 581]. TLRs, with the exception of TLR3 utilise MyD88, an adaptor protein to activate the transcription factor NF-κB [580-583]. Toll-like receptors (TLRs) are a family of pattern-recognition receptors that detect bacteria, viruses, and certain parasites and also their elements and products. Studies have been shown that they play a vital role in the induction of immune responses [584]. TLR-dependent antigen recognition through TLRs expressed on innate immune cells, such as DCs, prompts their maturation leading to initiation of antigen-specific adaptive immune responses through T cell activation. Furthermore, direct signals through TLRs expressed on B cells to T-dependent antigens activate B cells and their antibody production [585]. Adaptive immune responses need an adequate time for a sufficient response, which is too much of delay to fight quickly against rapid replicating microorganisms. To

combat against these microorganism rapidly, innate-like B cells including MZ and B1 B cells evolutionarily developed [586, 587] and act as a bridge between the innate and adaptive immune responses and their optimal involvement in antibody production and antigen clearance. Indeed, B1 B cells are well recognised to participate in an early T-independent stage of immune responses against microorganisms [435, 542, 588]. In a study, Côrte-Real and colleagues showed that NOD B1a B cells express high level of TLRs and had an obviously increased pattern of activation and responded to TLR stimulation in-vitro. These activated B1a B cells had high capacity of proliferation and secretion of anti-type-1-diabetes-related IgM, but they produced lower amounts of IL-10 [589]. A unique characteristic of B cells is the expression of BCR in conjunction with the expression of one or more members of TLRs. TLRs are a family of pattern recognition receptor (PRR) that recognizes a wide range of microbial ligands [590, 591] and use Toll–IL-1 receptor (TIR) domain-containing adaptors, such as myeloid differentiation primary response protein-88 (MyD88) and TIR domain-containing adaptor inducing IFN-β (TRIF), to induce activation of transcription factors, including NF-κB, MAP kinases, and IFN regulatory factors [580]. The dual expression of these two recognising receptors on B cells permits them to uniquely integrate both antigen-specific signals and danger signals via these key receptor systems. TLR activation in B lymphocytes up-regulate their proliferation, cytokine and immunoglobulin secretion, and Blimp-1 expression and terminal differentiation into plasma cells [585, 592-594]. B1a B cells express and respond to activation by multiple TLRs, and in particular TLR2, TLR4 and TLR9 [580, 595]. Pharmacological stimulation of these receptors initiates proliferation of peritoneal B1 B cells and differentiation into plasma cells [595, 596]. TLR9 activation

appears essential for protecting against atherosclerosis [299], and depending on conditions, TLR4 can either protect [597] or exacerbate atherosclerosis [296].

1.10.4.3.9 Natural IgM and its property

Natural IgM is a well-known atheroprotective immunoglobulin mainly secreted by B1a B cells. IgM is the largest antibody in the human circulatory system and is the first antibody to appear in response to initial exposure to antigen. IgM forms polymers multiple immunoglobulins are covalently linked together with disulfide bonds, mostly as a pentamer but also as a hexamer. IgM has a molecular mass of approximately 970 kDa (in its pentamer form) [598, 599]. Because each monomer has two antigen binding sites, a pentameric IgM has 10 binding sites. Typically, however, IgM cannot bind 10 antigens at the same time because the large size of most antigens hinders binding to nearby sites [598]. Natural antibodies are mostly IgM isotype, and can bind to a particular antigen or pathogen, even if the host has never been exposed to it [600-602]. Little is known about the function of natural IgM autoantibodies; natural antibodies arise independently of known antigenic stimulation, are mostly IgM, polyreactive, and are generally encoded by V genes in germ line configuration. The variable regions of natural IgM antibodies are germ line programmed and highly well-maintained [603]. Natural IgM antibodies are non-specific antibodies and recognize a wide range of molecules such as phospholipids and glycoproteins with common structure. Natural IgM develop under selective pressure from endogenous neo-antigens such as apoptotic ligands and commensal pathogens [604, 605]. Natural IgM antibodies do not undergo somatic hypermutation and affinity maturation [606-608]. IgM antibodies with the structure of pentamers or sometimes hexamers have high affinity to several antigens and particles [598, 599]. In essence, natural IgM

antibodies are constructed to interact promptly and simultaneously with multiple antigens. The variable regions of natural IgM antibodies are germ line encoded and highly conserved [603]. They evolve under selective pressures from endogenous neo-antigens such as apoptotic ligands and commensal pathogens [604, 605]. Natural IgM antibodies do not undergo somatic hypermutation and affinity maturation [606-608]. Natural IgM antibodies are configured to interact promptly and simultaneously with multiple antigens. An additional consequence of the lack of somatic mutations in natural IgM V region segments is that natural IgM tends to have rather low antigen-binding affinities compensated for, to some extent, by the pentameric nature of secreted IgM. Moreover, its multimeric structure makes IgM a strong complement activator; a single bound IgM pentamer can trigger the classical pathway of complement activation and can lyse a red blood cell, while approximately a thousand IgG molecules are required to accomplish the same [609].

1.10.4.3.10 B1a B cells and their IgM production

B1a B cells play a critical role in innate immunity [415, 514, 588, 610]. B1a B cells are the principal source of natural IgM antibodies. B1a B cells constitutively produce natural IgM without T-cell help and supply the majority of the entire IgM pool [611]. Studies have shown that mice raised in a germ-free environment had a normal plasma IgM level spontaneously produced by B1a B cells [612]. Plasma IgM is restored in immune-deficient $Rag1^{-/-}$ mice following the adoptive transfer of B1a B cells [613, 614]. B1a B cells expanded when exposed to antigens and generated increased amounts of IgM [606, 615]. B1 B cells in young mice provide a first line of defense against common pathogens. As the mice grow older, antigen experience

becomes increasingly involved in influencing the B1 population. B1a B cells are also involved in the production of autoantibodies [616-618].

1.10.4.3.11 B1 B cells in human

CD5 is expressed on the surface of some human and murine B cell malignancies and normal B cells [511, 619]. A subpopulation of $CD5^+$ B cells are found in different human tissues which produce autoantibody and expand in some autoimmune diseases [620-622]. It is not clear that CD5 is a reliable marker of the B1 B cell population across species. B2 B cell populations in the human system express CD5 (including transitional, pre-naïve and activated B cells) [623, 624]. Further, both $CD5^-$ and $CD5^+$ B cells can produce IgM autoantibodies [625-627]. It has been impossible to accurately study B1 B cells during health and illness because the nature of human B1 B cells has not been successfully defined. Because of that the existence of human B1 B cells and their identification and characterisation is controversial. To address this issue, Griffin and colleagues determined the phenotype of human B1 B cells based on three fundamental B1 B cell functions on mouse studies: spontaneous IgM production, efficient T cell stimulation and tonic intracellular signalling [628]. They found that a small population of $CD20^+CD27^+CD43^+$ cells are present in both umbilical cord and adult peripheral blood with all the above criteria which express B cell receptors. These B cells do not express CD69 and CD70, the markers which are up-regulated after activation of naive ($CD20^+CD27^-CD43^-$) and memory ($CD20^+CD27^+CD43^-$) B cells. They identified human B1 B cells as $CD20^+CD27^+CD43^+CD70^-$ and determined that B1 B cell number declines with age [628]. These cells are approximately 5% to 10% of B cells in cord or adult blood [629].

Further studies by Griffin et al. revealed two kinds of B1 B cells in both umbilical cord and adult peripheral blood, a $CD11b^+$ subset that constitutes approximately 5-10% of B1 B cells and a $CD11b^-$B1 B cell subset. $CD11b^-$B1 B cells spontaneously secrete IgM. In contrast, $CD11b^+$B1 B cells express more CD86, and are more potent in stimulating $CD4^+$T cell expansion [630]. The frequency of these $CD11b^+$B1 B cells with high expression of CD86 is significantly raised in lupus patients and was associated with increased T cell stimulating activity in disease [630].

Although such a discovery may have been expected to be welcomed by human B cell experts, in keeping with the historical controversies that have determined murine B1 B cells, the veracity of this initial study has been vigorously debated [631, 632]. For example, an alternative explanation for some of these data was that human $CD20^+CD27^+CD43^+$ B cells actually correspond to plasmablast type cells [632]. This possibility has now been addressed [633].

Covens and colleagues by employing a phenotypic, functional, gene profiling, and in-vitro culture approach indicated that $CD20^+CD27^+CD43^+$ B cells are more likely to correspond to activated B2 cells undergoing the early stages of plasma cell lineage and thus are nominated preplasmablasts rather than belonging to B1 B cell lineage [633]. They have shown that $CD20^+CD27^+CD43^+$ B cells not only secrete spontaneously IgM, but produce spontaneously IgG, and IgA. $CD20^+CD27^+CD43^+$ B cells also secret IgG specific for the TD tetanus toxoid following booster vaccination, and that gene expression profiling revealed them to be more similar to plasmablasts than naive or memory B cells [633]. There is controversy that $CD20^+CD27^+CD43^+$ B cells are B1 B cells, because B1 B cells would not be predicted to produce class-switched Ig nor to elicit enhanced recall responses following booster immunization with TD antigen [508, 511, 516, 629]. The $CD20^+CD27^+CD43^+$ B cells also exhibit

other features of plasma cells $CD38^{hi}CD20^{lo}$ phenotype, PRDM1 (encoding the transcription factor Blimp-1) and BCMA1 [629, 631, 632]. As only 5-10% of $CD20^{+}CD27^{+}CD43^{+}$ B cells spontaneously secrete Ig, it may suggest that $CD20^{+}CD27^{+}CD43^{+}$ B cells are a mixed population comprising both preplasmablasts and B1 B cells. Further studies are required to characterise and distinguish the phonotype of equivalent mouse B1a cells in humans.

1.10.4.3.12 IgM and atherosclerosis

IgM is the most evolutionary conserved antibody isotype which is present in all vertebrates and it is also the earliest isotype to be expressed during immune development. Polyreactive IgM natural antibodies are produced by mainly B1 B cells which account for most of the B cell repertoire in the fetus and neonate. Although endowed with self-reactivity, natural antibodies also bind exogenous antigens. This spontaneously produced antibody has also been detected in human cord blood and in antigen-free mice [612, 634]. Natural antibody appears in the absence of apparent antigenic stimulation, and is secreted by the long-lived, self-renewing B1 subset of B cells [635]. B1 B cells differ from the conventional B2 B by their differentiation during fetal and neonatal development and their characteristic cellular localization in pleural and peritoneal cavities in the adult [415, 511]. A large proportion of the natural antibodies are polyreactive to phylogenetically conserved structures, such as nucleic acids, heat shock proteins, carbohydrates, and phospholipids [415, 511]. In the pathogenesis of atherosclerosis all immune cells interact in the atherosclerotic plaque and have important influence in progression of the lesions and removal of apoptotic plaque cells in the atherosclerotic lesion, a process known as efferocytosis, is one of them [636]. Macrophages in the plaque apart from phagocytosing oxLDL

have a secondary role in removing dying cells since they are the main professional efferocytes in plaques especially at early stages of plaque formation [315, 316].

1.10.4.3.13 Clearance of apoptotic cells in the lesions by natural IgM

The immune system is regulated at several checkpoints during lymphocyte differentiation by mechanisms that clear up pathogenic elements. These checkpoints serve to prevent the development of autoimmune disease and auto-reactive antibodies can play important roles in tissue homeostasis. The majority of natural IgM antibodies are low affinity antibodies and can display polyreactivity for a range of ligands [625], however another type of natural antibodies can display great mono-specificity for their binding self-ligand. One of the most fundamental functions of the immune system is the recognition and removal of the large number of dying cells that are continually generated in our bodies. Even in adults, hundreds of billions of cells die each day and are continually replaced. Cells dying from apoptosis, a form of programmed cell death, undergo an active energy-dependent cascade that is highly controlled, resulting in cell shrinkage, protein cleavage, DNA breakdown, and extensive plasma membrane changes specific for apoptosis. These apoptotic cells are cleared by phagocytosis by macrophages in a process that has been termed efferocytosis, for removing the dead cells [637]. This mechanism is required for normal tissue homeostasis as it prevents the accumulation of cells that would otherwise progress to secondary necrosis and the release of auto-antigens and pro-inflammatory factors such as the high-mobility group box-1 (HMGB1) protein [638], uric acid, heat-shock proteins, and S100 proteins [638-640]. While apoptotic cells do not themselves pose a risk to the host, their cell membranes contain components

that can be anti-inflammatory. Hence, in-vivo treatment with apoptotic cells have been shown to inhibit inflammatory responses in murine models [548, 641, 642]. Apoptotic cells express two oxidation-associated neo-determinants, the head group of oxidized lipids, phosphorylcholine (PC), and the small oxidation-associated determinant, malondialdehyde (MDA) which are not displayed on healthy cells [613]. These two chemically active elements of apoptotic cells are recognized by splenic IgM-secreting cells [643]. Natural IgM has the capacity for specific immune recognition of phosphorylcholine (PC) containing antigens presented on apoptotic cell membranes, in oxidized LDL and in the pneumococcal bacterial cell wall polysaccharide [127, 421, 613]. Most natural antibodies to PC utilize the VHS107.1 gene which is highly dominant in the B1 B cells. As a consequence, mice deficient in this single VH gene segment have highly impaired immune responses to the PC determinants on both apoptotic cells and on bacteria [643, 644]. B1 B cells produce antibody due to signaling thresholds and the BCR mediated responses. Encounter of a B1 B cell precursor with its related self-antigen may result in a positive selection process that leads to clonal selection and expansion, while in conventional follicular B cell result in activation associated cell death [645]. Some B1 B cell derived natural IgMs are inherently polyreactive, but other B1 B cells secrete and display highly selective binding antibodies and the repertoire of IgM natural auto antibodies is not randomly generated [646].

In advanced atherosclerotic plaques, removal of apoptotic cells is not efficient and as a result cell necrosis increases and toxic intracellular factors such as HMGB1 and HSPs leakage from necrotic cells. HMGB1 and HSPs as athrogenic elements can cause recruitment of macrophages, stimulation of inflammatory mediators in plaques [320], enlargement of necrotic core and the thinning of fibrous cap and massive

damage on atherosclerotic lesions [647] and as a result unstable plaque. Studies suggested that efferocytosis has an important influence on hemostasis of atherosclerotic lesions. Study by Lewis and colleagues reported that sIgM$^{-/-}$LDLR$^{-/-}$ mice have significantly elevated plaque apoptosis in the absence of natural IgM compared to LDLR$^{-/-}$ mice [648] and the result strongly suggested defective removal of apoptotic cells in mature atherosclerotic plaques that lack IgM. This is an important finding showing that natural IgM serves as scavengers of apoptotic cells in atherosclerotic plaques [649]. Apoptotic cells display a range of 'eat me' signals and ligands such as phosphatidylserine and phosphorylcholine of oxidized phospholipids which are attracted by natural IgM [606]. Phosphatidylserine is a membrane phospholipid that is located on the cytosolic side in healthy cells but becomes exposed on the outer surface of the cells at apoptosis stage [650]. IgM binding of apoptotic cells decreases markedly in the presence of oxLDL or MDA-LDL but not native LDL antigens and apoptotic markers and oxLDL antigens are partner candidates for the intrinsic selection of natural IgM [651]. The essential role of IgM for the clearance of apoptotic cells has been well established [642, 643, 652] and incubation with polyclonal IgM has been demonstrated to also promote clearance of apoptotic micro particles released from dying cells [653] as well as enhance the phagocytosis of apoptotic cells in the lungs by alveolar macrophages [654, 655]. Indeed, sIgM deficient mice have defects in the clearance of apoptotic cells [642, 656] and have increased susceptibility to develop a lupus-like syndrome with expression of IgG autoantibodies to nuclear antigens [657]. Importantly, recent studies have shown that there are certain IgM clones that are essential for mediating the immunoregulatory effects of apoptotic cells [642, 643]. Natural IgM has also recently been reported to enhance apoptotic cell phagocytosis in the marginal zone

of the spleen and promote IL-10-secreting B and T cells that restrain the development of inflammation [656]. Further studies are required to disclose the downstream signals of IgM-assisted efferocytosis.

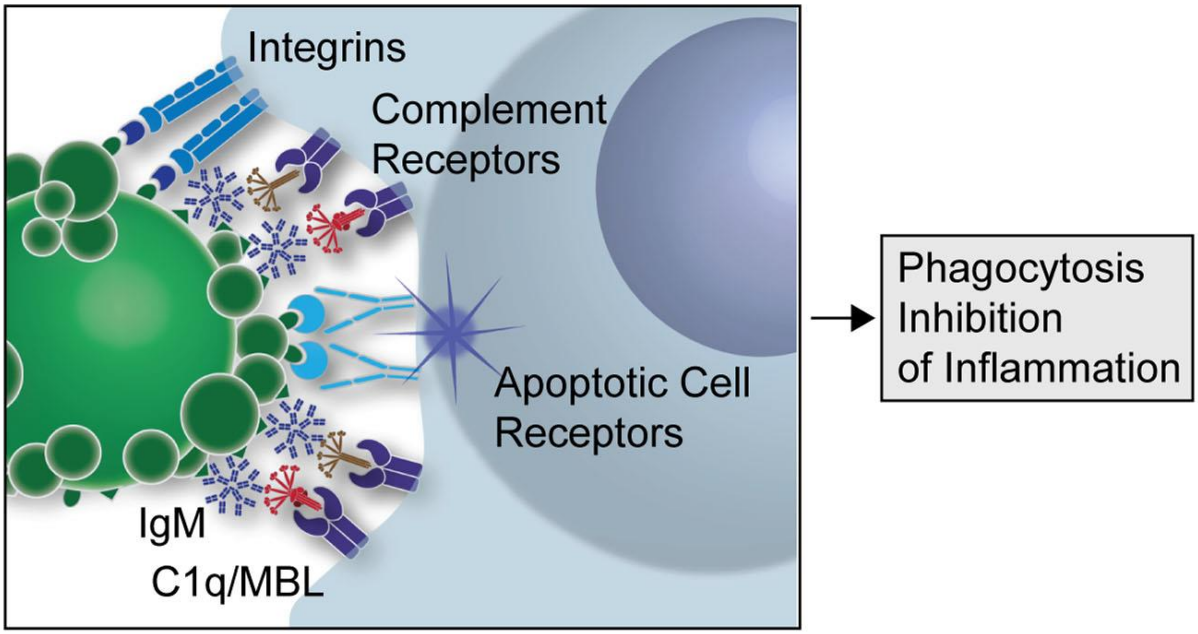

Figure 1.19. Involvement of natural IgM in clearance of apoptotic cell, from Front Immunol [606].

Diagram shows the engagement of natural IgM in apoptotic cell removal. Natural IgM facilitate the formation of synapse between the apoptotic cell and the phagocyte through some complement recognition factors such as C1q and MBL [606].

1.10.4.3.14 Natural IgM requires complements for efferocytosis

An immune complex, formed between antigen, IgM and activated complement component C3 (Ag–IgM–C`) can dramatically augment a B cell antibody response [658, 659]. Indeed, the simultaneous administration of antigen and exogenous antigen-specific IgM often [660], but not always [661] yields an enhanced antibody response. For the study of IgM under physiological conditions, mice that are specifically deficient in secreted IgM (sIgM) were generated. In both independently generated strains of sIgM-deficient mice, increased B1 B cell numbers were reported. B1 B cells produce a major portion of sIgM, which indicates a role for IgM in feedback regulation of these cells [599, 662]. To find out the mechanistic actions of oxLDL-specific IgM in atherosclerosis have been under investigation for many years and studies have shown that oxLDL-IgM restricted the development of atherosclerosis through various immunosuppressive mechanism at the local and systemic levels. For instance, increased oxLDL-IgM is believed 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 [421, 560]. Studies revealed that IgM required complement system such as C1q and mannose-binding lectin (MBL) for completion of apoptotic efferocytosis [606, 650]. This was supported by Ogden and colleagues, who showed an increased deposition of the complement protein, C3, on apoptotic cells in $sIgM^{-/-}$ mice but not $sIgM^{-/-}C1q^{-/-}$ mice when they treated these mice with IgM antibodies [652]. $C1q^{-/-}sIgM^{-/-}LDLR^{-/-}$ and $sIgM^{-/-}LDLR^{-/-}$ mice showed similar atherosclerotic plaque size but larger compared to $C1q^{-/-}LDLR^{-/-}$ and $LDLR^{-/-}$ mice on HFD [648]. Perhaps, the same mechanism involved in apoptotic removal in atherosclerotic lesions by IgM and C1q acts as a co-factor for IgM to

initiate efferocytosis in the atherosclerotic plaque. MBL, a protein analogous to C1q, is another complement which is involved in phagocytosis of apoptotic cells by IgM [606, 642]. Although IgM antibodies bind to apoptotic cells at both early and late apoptosis stages and provide a platform for C1q and MBL to be involved in apoptotic cells clearance, but both C1q and MBL, by themselves are also involved in the clearance of apoptotic cells at late apoptosis stage (Figure 1.18) [606, 642, 663].

Studies by Chen and colleagues revealed that natural IgM antibodies with the association of complement proteins recognize cells dying from apoptosis. Apoptotic cell infusion inhibited the activation of macrophages and DCs and decreased the levels of pro-inflammatory cytokines and chemokines and resulted in anti-inflammatory effects in naïve mice and this suppression was greater when combined with natural IgM infusion [642]. The binding of IgM complex with C1q/MBL to apoptotic cells provide a stable connection of apoptotic cells with phagocytes and prevents their proinflammatory activities [606]; this could be the mechanism involved in anti-inflammatory function of IgM. In-vitro study also support that anti-apoptotic natural IgM-C1q/MBL complex required mitogen activated protein kinase (MAPK) signal [606, 664].

1.10.4.3.15 Natural IgM regulate Immune response towards Th2 responses

Natural IgM also regulate immune response by governing the differentiation of effector CD4 T cells toward Th2 rather than Th1. Natural IgM targeted immunization of mice revealed Th2 immunity [97] and $sIgM^{-/-}$ mice showed reduction in production of the Th2 cytokines, IL-5 and IL-9 without affecting production of IFNγ [604]. GATA-

3, a GATA family of transcription factors, been shown to promote the secretion of IL-4, IL-5, and IL-13 from Th2 cells, and induce the differentiation of effector CD4 T cells towards Th2 cells while suppressing their differentiation towards Th1 cells [665]. GATA-3 Activation by IgM-antigen complex stimulates Th2 characteristics by down regulating the expression of IFNγ and up regulating the expression of IL-4 and IL-5 [666]. Zhou and colleagues revealed that plasma oxLDL-IgM levels increased in ApoE-/- mice fed a HFD with established atherosclerosis and that was associated with spontaneous switching of Th1 to Th2 cells [380]. However, the contribution of Th2 over Th1 dominance is not clear in to the progression of atherosclerosis as one study showed a decrease in the size of mature plaques in $ApoE^{-/-}IL\text{-}4^{-/-}$ mice compared to $ApoE^{-/-}$ mice [221, 667] and another study showed controversial result in $LDLR^{-/-}$ mice deficient in the Th1 transcription factor, T-bet. $LDLR^{-/-}T\text{-}bet^{-/-}$ mice reduced atherosclerosis together with increased Th2 responses and natural IgM [379].

1.11 Aims of thesis

Atherosclerosis is a chronic inflammatory disorder with the lipids deposition in intima of arterial wall. 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 and our previous studies revealed that B1a cells play a major atheroprotective role. The focus of this thesis is to find out a therapeutic strategy in treatment of atherosclerosis by manipulation of atheroprotective B1a B cells in favor of natural IgM production.

1. To determine atheroprotective functions of B1 cells and mechanisms by which naïve B cells are activated in atherosclerosis.
2. To study the activation of peritoneal B1a cells through TLRs dependent stimulation of IgM and/or IL-10 secretion.
3. To investigate the activation of atheroprotective B1a cells through CD40 dependent IgM and/or IL-10 secretion.
4. To study the role of apoptotic cells (ACs) and their unique surface marker phosphatidylserine in suppression of atherosclerosis by activation of B1a B cells.
5. To study the role of TIM-1 receptor using anti-TIM-1 antibody agonist (anti-TIM-1 RMT1-10 mAb) in suppression of atherosclerosis by activation of B1a B cells via mechanisms involving Tim-1 receptor in natural IgM and IL10 production.

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Chapter 2. TLR4 and MyD88 Are Essential for Atheroprotection by B1a B cells in Hyperlipidemic ApoE-/- Mice

2.1 *Short Introduction*

B1a cells are atheroprotective B cells and slenectomy has been shown to deplete B1a cells and aggravate atherosclerosis. Here, I investigated the role of toll-like receptors (TLRs) and CD40 in activating atheroprotective B1a cells in hyperlipidemic environment. ApoE$^{-/-}$ mice, depleted of peritoneal B1a cells by splenectomy, were adoptively transferred with B1a cells from wild-type or TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, MyD88$^{-/-}$ and CD40$^{-/-}$ C57BL/6 mice, fed a high fat diet for 8 weeks and examined for atherosclerotic lesions, lesion immune cells and cytokines and IgM. Remarkably, the reconstitution of WT, TLR2$^{-/-}$, TLR9$^{-/-}$ and CD40$^{-/-}$ 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 TLR4$^{-/-}$ or MyD88$^{-/-}$ B1a cells did not exhibit any modulatory effect. WT-B1a cells but not TLR4$^{-/-}$ or MyD88$^{-/-}$ B1a cells elevated plasma total IgM, oxLDL IgM, anti-leukocyte IgM, anti-CD3 IgM and anti-CD4 IgM and also lesion IgM deposits associated with reduction of atherosclerotic lesion size, apoptotic cells, necrotic cores and local inflammation.

2.2 *Declaration for Thesis Chapter*

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 concept and design, Experimentation and handling, Data analysis, results interpretation, Preparation of manuscript	90

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
Peter Kanellakis	Experimentation	
Yi Li	Experimentation	5%
Christopher Tay	Experimentation	
Anh Cao	Experimentation	
Edgar Liu	Experimentation	
Peter Tipping	Study concept, Study design, Results interpretation	
Alex Bobik	Study concept, Study design, Results interpretation, Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Results interpretation, Preparation of manuscript	
Tin Kyaw	Study design, Experimentation, results 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		**Date 13/02/15**
Main Supervisor's Signature	[redacted]	**Date 13/02/15**

*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.

2.3 *Submission Letter*

Hamid Hosseini

From: [redacted]

Sent: Wednesday, 28 January 2015 12:03 PM
To: Tin Soe Kyaw
Cc: [redacted]

Subject: Manuscript submitted - TH-15-01-0080

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Dear Dr. Tin Kyaw,

Thank you for the submission of your manuscript TH-15-01-0080 "TLR4 and MyD88 Are Essential for Atheroprotection by B1a B cells in Hyperlipidemic ApoE-/- Mice" for publication in Thrombosis and Haemostasis.

Your manuscript has been forwarded to the Managing Editor to be checked for completeness. If complete, it will be sent immediately to the Editor-in-Chief who will then distribute it to the Section Editor and Referees for review.

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2.4 *Manuscript*

Thrombosis and Haemostasis

TLR4 and MyD88 Are Essential for Atheroprotection by B1a B cells in Hyperlipidemic ApoE-/- Mice

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Keywords:	Atherosclerosis, Inflammation, Immunity

TLR4 and MyD88 Are Essential for Atheroprotection by B1a B cells in Hyperlipidemic ApoE$^{-/-}$ Mice

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Short title: TLR4 activates atheroprotective B1a B cells
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References: 47; No more than 50 references

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Subcategory – atherosclerosis

Thrombosis and Haemostasis

Summary

B1a lymphocytes are atheroprotective by secreting natural IgM. However, whether B1a lymphocytes require toll-like receptors (TLR) and MyD88 for atheroprotection is not known. To address this question, we adoptively transferred B1a B cells from wild-type C57BL/6 mice or from mice deficient in TLR2, TLR4, TLR9, MyD88 or CD40 into ApoE$^{-/-}$ mice depleted of peritoneal B1a B cells by splenectomy and fed the mice a high fat diet for 8 weeks. B1a B cells from wild-type, TLR2$^{-/-}$, TLR9$^{-/-}$ and CD40$^{-/-}$ mice suppressed atherosclerosis development. Atherosclerosis suppression was associated with reduced lesion apoptotic cells, necrotic cores, oxLDL, macrophages, CD4 T cells, CD8 T cells, MCP-1, VCAM-1, IFN-γ, TNF-α, IL-1β and IL-18 and increased IL-10 and TGF-β. Plasma total IgMs were elevated as were anti-oxLDL IgM, anti-leukocyte IgM, anti-CD3 IgM, anti-CD8 and anti-CD4 IgMs. These reduced effects in lesions and elevated circulating IgMs were not observed in mice that received B1a B cells deficient in TLR4 or MyD88. *In vitro* stimulation of B1a cells by the TLR4 ligand LPS promoted their differentiation into CD138 expressing plasma cells and significantly augmented their secretion of IgM. Our study indicates that whilst B1a B cells are known to express multiple TLRs, including TLR2, TLR4 and TLR9 whose activation greatly increase IgM secretion, only expression of TLR4-MyD88 are essential for B1a B cell mediated suppression of atherosclerotic lesions and associated lesion apoptosis and inflammation.

Introduction

Atherosclerosis is a chronic inflammatory disease of medium and large arteries responsible for heart attacks and strokes that remain leading causes of global mortality (1). The inflammation is driven by immune responses following lipid entry into the arterial wall (2). Among immune cells, conventional B2 cells are atherogenic (3-5) and B1a B cells are atheroprotective (6).

B1a B cells are innate-like B cells expressing CD5 that arise from fetal liver and found mainly in peritoneal and pleural cavities (7). The spleen is required for their survival and maintenance (8). B1a B cells are long-lived self-replenishing B cells that produce most of the circulating natural low-affinity IgM in the absence of T cell help (9). B1a B cell repertoire is selected by recognition of self-antigen that results in evolutionarily important antibody specificities responding to pathogen-related signals, crucial for providing immediate, early humoral protection against pathogens (10). We identified B1a B cells as an atheroprotective B cell subset, demonstrating that adoptive transfer of wild-type (WT) but not IgM deficient B1a lymphocytes ameliorates atherosclerosis (6). Atheroprotection was accompanied by increases in lesion IgM deposits and reductions in apoptotic cells, oxLDL, and necrotic core size. We suggested that IgM scavenges apoptotic debris and oxLDL, attenuating inflammation and reducing necrotic cores in atherosclerotic lesions, effects consistent with scavenger properties of natural IgM (11-14).

B1a B cells respond rapidly and strongly to pathogen-encoded signals that constitute pathogen-associated molecular patterns (PAMPs). Strong selective pressure for recognition and responses to PAMPs are largely dependent on toll-like receptors (TLRs) (15). Fourteen TLRs have been identified (15) with some such as TLR2 and TLR4 expressed on cell membranes and others such as TLR3, TLR7, TLR8 and TLR9 expressed endosomally (16,17). TLRs, with exception of TLR3 utilise MyD88, an adaptor protein to activate transcription factor NF-κB (16-19). B1a B cells express and respond to activation of multiple TLRs, and in particular TLR2, TLR4 and TLR9 (16,20). Pharmacological stimulation of these receptors initiates proliferation of peritoneal B1 B cells and differentiation into plasma cells (20,21). TLR9 activation appears essential for protecting against atherosclerosis (22), TLR2 has been reported to modulate atherosclerosis (23) and depending on conditions, TLR4 can either protect (24) or exacerbate atherosclerosis (25).

While TLR9, 4 and 2 have been reported to have roles in atherosclerosis, whether these TLRs are implicated in the atheroprotective action of B1a cells is not known. Therefore, we set out to determine whether these TLRs have roles in the atheroprotection mediated by B1a cells. To this end, we examined the effects of adoptively transferring B1a B cells deficient in TLR9, TLR4 or TLR2 or deficient in MyD88 into $ApoE^{-/-}$ mice rendered B1a B cell-deficient by splenectomy. Our findings indicate that B1a B cell expression of TLR4-MyD88 but not TLR2 or TLR9 is absolutely essential for these cells to suppress atherosclerosis. The suppressive effects are dependent on these cells secreting natural IgMs that have multiple targets during development of atherosclerosis, including apoptotic cells, oxidatively modified lipids, leukocytes and T-cells.

Methods

Animals

All mice used in experiments were on C57BL/6 background. $ApoE^{-/-}$, WT, $TLR2^{-/-}$, $TLR4^{-/-}$, $TLR9^{-/-}$ and $CD40^{-/-}$ mice were maintained at the Alfred Medical Research, and Education Precinct (AMREP), Prahran, Melbourne, Australia. $MyD88^{-/-}$ mice were from the Walter and Eliza Hall Institute, Melbourne, Australia. $ApoE^{-/-}$ mice (6-8 week-old male) were subjected to splenectomy and fed a high-fat diet (HFD) containing 21% fat and 0.15% cholesterol

(Specialty Feeds, Glen Forrest, Western Australia) and sterile water. All animal procedures and study protocols were approved by the Animal Ethics Committee, AMREP.

Splenectomy

Splenectomies were performed on 6-8 week-old male ApoE$^{-/-}$ mice under aseptic conditions (6). Briefly, under anaesthesia induced by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg), spleen identified through a 10-mm left flank incision was removed via diathermy. After confirming no intra-abdominal bleeding, the peritoneum and skin were closed using a 2-0 monofilament suture, atipamezole HCl (anti-sedan 100mg/kg) was administered subcutaneously as recovery agent and mice were placed in 37°C recovery chambers before returned to their cages.

Cell Isolation and Adoptive Cell Transfers

Peritoneal fluid collected from donor mice as described previously (6), were stained with fluorochrome-labelled anti-CD3, anti-CD19 and anti-CD5 antibodies. CD3^{-} CD19^{+} CD5^{+} B1a cells were then isolated using FACS Aria Cell Sorter (BD Biosciences) and after assessing cell viability (>95%), 10^5 B1a cells were adoptively transferred via tail vein injection into splenectomised ApoE$^{-/-}$ mice during weeks 1, 4, and 7 after splenectomy (6), whilst feeding them a high fat diet.

***In-vitro* Cell Culture**

In *in-vitro* studies, peritoneal B1a cells ($8x10^4$ cells) were cultured in U-bottomed 96-well plate at 37°C 5% CO_2 for 72 hours. Cells were suspended in 200µl of complete 10%FBS-RPMI containing 50µM 2-mercaptoethanol, IL-4 (200 U/ml) and IL-5 (150U/ml). LPS, a ligand for TLR4, at 1µg/ml, 5µg/ml and 10µg/ml was included to stimulate B1a cells. Supernatants were collected after 72 hours to determine IgM level by ELISA (26,27). In some experiments, cells collected after 72 hours were stained with APC-conjugated anti-CD138 and FITC-conjugated anti-CD19 antibodies (eBioscience) to determine plasma cells that differentiated from B1a cells. Plasma cells defined as CD19^{-}CD138^{+}B cells were presented as percentage of total cells.

Plasma anti-leukocyte IgM detection using splenic leukocytes

In a modified protocol to detect anti-leukocyte IgM antibody (28,29), splenocytes from C57Bl/6 mice ($2x10^6$ cells/0.2 ml of complete 10%FBS-RPMI per well) were initially activated with 10µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 hours at 37°C in 5% CO2. After Fc blocking for 30 minutes, splenocytes were incubated with plasma samples (diluted at 1:300) at 37°C for 2 hours using U-bottomed plates previously blocked with 1% BSA. After washing for three times, cells were incubated with HRP-conjugated goat anti-mouse IgM antibody (ICL, Portland USA) again for 1 hour, followed by washing cells again. Colour development was done using TMB substrate for colour development. The OD at 450 nm was read by ELISA reader.

Plasma anti-lymphocyte IgM detection using recombinant proteins (CD3 and CD4)

In a modified protocol to detect anti-lymphocyte IgM antibodies (28), recombinant CD3 and CD4 extracellular domain proteins (Life technology, USA) were coated in flat-bottomed 96-well ELISA plates (50µl at 5µg/ml 1xPBS/well) at 4°C for 18 hours. Wells were blocked with 1% BSA for 2 hours and plasma diluted at 1:300 in 1%BSA were added and incubated for 2 hours at room temperature. HRP-conjugated goat anti-mouse IgM antibody (ICL, Portland USA) was added into the wells, followed by addition of TMB substrate for colour development. The OD at 450 nm was read by ELISA reader.

Tissue Collection

At the end of experiments, peritoneal fluids were collected for lymphocyte analysis. Plasma and thoracic aortas were kept at -80C freezer for further analysis and aortic sinuses embedded in OCT for histological and immunohistochemical staining.

Flow Cytometry
B lymphocytes and non-B-lymphocyte populations in the peritoneal cavity were analysed with fluorochrome conjugated antibodies (BD Pharmingen, San Diego, CA) on a FACS Canto-II (BD Biosciences) as described (3,5,6). For B cells, PE-conjugated anti-CD19, APC-conjugated anti-CD5, and APC-Cy7–conjugated anti-CD11b antibodies were used. For non-B lymphocyte populations, Pacific Blue-conjugated anti-CD4, PerCP-conjugated anti-CD8a, FITC-conjugated anti-TCR-b, and PE-Cy7–conjugated anti-NK1.1 antibodies were used.

Atherosclerosis Assessment and Histological Staining
Frozen aortic sinus sections of 6 µm in thickness were stained with Oil-Red O (ORO) and total intimal lesion areas and ORO-stained lipid accumulation were measured as described previously (6). To determine necrotic core areas in atherosclerotic lesions, acellular areas from H&E stained atherosclerotic lesions were measured as described previously (6).

Immunohistochemical Staining
Frozen aortic sinus sections were stained using different antibodies. Macrophages were stained with anti-CD68 antibody (Serotec, Raleigh, NC); CD4 and CD8 T cells with anti-CD4 and CD8 antibodies respectively (BD Biosciences), Macrophage chemoattractant protein-1 (MCP-1) with anti-MCP-1 antibody (BD Pharmigen), vascular cell adhesion protein-1 (VCAM-1) with anti-VCAM-1 antibody (BD Pharmigen), immunoglobulin M with anti-IgM antibody (BD Pharmigen), oxLDL with MDA-oxLDL antibody (Abcam, UK) as described before (3,30). Apoptotic cells identified by terminal dUTP nick end-labelling (TUNEL) under light microscopy were expressed per lesion areas as described previously (3,30).

Total and OxLDL-specific Immunoglobulins
An enzyme-linked immunosorbent assay (ELISA) was used to determine plasma levels of total immunoglobulins (total Ig, IgG and IgM) and oxLDL-specific immunoglobulins (total Ig, IgG and IgM) levels at end point as described previously (6).

Lipid Profiles
Cholesterol profiles (total cholesterol, high-density lipoprotein cholesterol, very-low-density lipoprotein/LDL cholesterol, and triglycerides) in plasma were measured as described previously (6).

Real-time PCR Analysis
Total RNAs were extracted from thoracic aortas using RNeasy fibrous tissue mini kit (Qiagen) as described (3). Expression of targeted gene in 20µg total RNA was carried out using one-step QuantiFast SYBR Green RT-PCR kit (Qiagen) on 7500 Fast Real-Time PCR system (Applied Biosystem) to determine the expression of each gene. The comparative cycle threshold (Ct) method was used to determine target-gene expression with housekeeping gene 18S (Applied Biosystems) as an endogenous control. Relative amounts of each mRNA for each of the genes in lesions from control and test mice were calculated using comparative CT values (31). The sequences of oligonucleotides used were as below;

TNF-α: Sense (S), 5'-TATGGCCCAGACCCTCACA-3'
Anti-sense (AS), 5'-TCCTCCACTTGGTGGTTTGC-3'
IFN-γ: S, 5'-TCCTCAGACTCATAACCTCAGGAA-3'
AS, 5'-GGGAGAGTCTCCTCATTTGTACCA-3';
IL-1β: S, 5'-CCACCTCAATGGACAGAATATCAA-3'
AS, 5'-GTCGTTGCTTGGTTCTCCTTGT-3';
IL-18: S, 5'GATCAAAGTGCAGTGAACC-3'
AS, 5'-AACTCCATCTTGTTGTGTCC-3';
MCP-1: S, 5'-CTCAGCCAGATGCAGTTAACG-3'
AS, 5'-GGGTCAACTTCACATTCAAAGG-3';

VCAM-1:S, 5'-AGAACCCAGACAGACAGTCC-3'
AS, 5'-GGATCTTCAGGGAATGAGTAGAC-3,
TGF-β:S, 5'-AGCCCTGGATACCAACTATTGC-3'
AS, 5'-TCCAACCCAGGTCCTTCCTAA-3';
IL-10: S, 5'GAAGACAATAACTGCACCCA-3'
AS, 5'-CAACCCAAGTAACCCTTAAAGTC-3';

Statistical Analysis

Results are expressed as means±SEM. Comparisons between groups were carried out using Student T test or Mann-Whitney U test, depending on whether the data was normally distributed, as assessed using the Kolmogorov-Smirnov test. For multiple comparisons, results were analysed using one-way ANOVA (after confirming normality of distribution) followed by Bonferroni post-test. A value of $P<0.05$ was considered statistically significant.

Results

TLR4 and MyD88 are Required for B1a B cells to Suppress Atherosclerosis Development

To investigate the role of TLRs in atheroprotection conferred by B1a B cells, ApoE$^{-/-}$ mice were first subjected to splenectomy to deplete peritoneal B1a B cells (6), whilst not affecting peritoneal B1b cells (8) or sham operation. Then one week later the mice received B1a B cells isolated from WT, TLR2$^{-/-}$, TLR4$^{-/-}$ or TLR9$^{-/-}$ donor mice and fed a HFD for 8 weeks. After 8 weeks HFD, body weights and plasma cholesterols did not differ among the mouse groups ($P>0.05$; Table 1); lymphocyte populations in the peritoneal cavity were similar and not affected by transfer of different B1a B cells ($P>0.05$; Table 2). Transfer of WT B1a B cells attenuated atherosclerosis to levels seen in sham-operated mice, measured as total lesion area or Oil Red O lesion area ($P<0.05$; Figure 1A&B). Transfer of B1a B cells deficient in TLR2 and TLR9 also attenuated lesions to a similar extent as WT B1a B cells with reductions averaging 35% of total lesion area and 45% of Oil Red O stained lesion areas ($P<0.05$; Figure 1A&B). Macrophage accumulation was also reduced after transfer of WT or TLR2 or TLR9-deficient B1a B cells ($P<0.05$; Figure 1C). In contrast, B1a B cells deficient in TLR4 did not attenuate atherosclerosis or macrophage accumulation; lesion size in mice that received TLR4-deficient B1a B cells was similar to those that received PBS, measured as total or Oil Red O stained lesion areas ($P>0.05$; Figure 1A&B) macrophage accumulation was also unaffected ($p>0.05$, Figure 1C). Survival of B1a B cells deficient in the different TLRs could not account for these effects as their numbers in the peritoneal cavity level were similar to those after transfer of wild type B1a B cells at the end of the studies (Table 1).

TLR4 can signal via MyD88-dependent and -independent mechanisms (32). To determine the role of MyD88 for TLR4 activation of B1a B cells we compared the effects of adoptively transferring B1a B cells from WT mice and mice deficient in MyD88. Similar to effects seen with TLR4-deficient B1a B cells, MyD88-deficient B1a B cells also failed to attenuate atherosclerosis in splenectomised ApoE$^{-/-}$ mice, measured as total lesion area or Oil Red O stained area ($P>0.05$; Figure 1D&E); macrophage accumulation was also unaffected ($P>0.05$; Figure 1F). The extent of B1a B cell reconstitution was similar in mice receiving WT, TLR4- or MyD88-deficient B1a B cells averaging 20-24%, expressed as B1a B cells/total peritoneal B cell population, levels that were three times greater than in splenectomised mice (6-8%) that received PBS ($P<0.05$). As CD40 is also a B1a B cell activator that can interact with TLR4 (33) and CD40L is highly expressed in mouse lesions (34), we examined whether B1a B cell CD40-CD40L interaction might also contribute to atheroprotection. CD40 expression by B1a B cells does not confer atheroprotection as atherosclerosis was unaffected by transfer of CD40-deficient B1a B cells ($P>0.05$; Figure 1D-F). Thus B1a B cell activation via the TLR4-MyD88 pathway appears to be a major mechanism by which B1a B cells protect against atherosclerosis.

TLR4 expression by B1a B cells is required for their secretion of IgM

B1a B cells protect against atherosclerosis by secreting natural IgMs that remove apoptotic cells and accumulated oxLDL (6). We asked if failure of TLR4 or MyD88 deficiency in B1a B cells to reduce atherosclerosis is related to their production of natural IgMs. Thus we compared plasma IgM levels between splenectomised mice that received vehicle, WT-B1a B cells, B1a B cells deficient in TLR4 or MyD88 and B1a B cells deficient in TLR2 and TLR9 during development of atherosclerosis. At the end of 8 weeks HFD, total IgM levels were reduced by 58% and 45% respectively in splenectomised mice that received $TLR4^{-/-}$ or $MyD88^{-/-}$ B1a B cells compared to those that received WT B1a B cells; levels in mice that received TLR2 or TLR9 deficient B1a B cells were similar to WT B1a B cells ($P<0.05$; Figures 2A). Plasma levels of MDA-oxLDL IgM were reduced by approximately 62% and 30% in splenectomised mice that received $TLR4^{-/-}$ B1a B cells or $MyD88^{-/-}$ B1a B cells respectively compared with mice receiving WT-B1a B cells or B1a B cells deficient in TLR2 or TLR9 ($P<0.05$; Figure 2B). As natural IgMs also include anti-leukocyte autoantibodies that are increased in inflammatory disorders (28), we examined whether these were also affected by TLR4-MyD88 activation in B1a B cells. Anti-leukocyte IgM antibodies increased by 6 times following transfer of WT B1a B cells but were unaffected after transfer of TLR4 or MyD88-deficient B1a B cells ($P<0.05$; Figure 2C) Similarly, IgMs binding to CD3, CD4 and CD8 exhibited an identical pattern of expression and dependence on TLR4-MyD88 ($P<0.05$; Figure 2D, E and F) whilst effects of B1a B cells deficient in TLR2 and TLR9 were similar to WT-B1a B cells. Thus TLR4-MyD88 activation is critical for B1a B cells to secrete multiple IgM antibodies that can attenuate atherosclerosis.

TLR4 Activation of B1a B cells by LPS *in vitro* promotes plasma cell differentiation and IgM production

To confirm that TLR4 activation is important for IgM secretion by B1a B cells, we examined the effects of TLR4 activation *in-vitro* by the TLR4 ligand LPS on the ability of B1a B cells to secrete IgM and IgG and differentiate into $CD138^{+}$ plasma cells. TLR4 activation of B1a B cells by LPS promotes B1a B cell differentiation into antibody-secreting plasma cells (20). LPS activated B1a B cells markedly increased their secretion of IgM ($P<0.05$; Figure 2F); smaller increases in IgG were also apparent ($P<0.05$; Figure 2G). FACS analysis of the B1a B cell population stimulated with LPS for 72 hours indicated a doubling of a small population of CD138-expressing plasma cells, averaging 4.3% compared to 2.1% (vehicle) respectively ($P<0.05$; Figure 2H). Because stimulating TLR4 on B1a B cells also increased IgG secretion we assessed whether such effects were significant *in-vivo*. Measurement of IgG levels in $ApoE^{-/-}$ mice that received WT B1a B cells or B1a B cells deficient in TLR4 or MyD88 indicated comparable plasma levels of Ig and IgG (both total and oxLDL-specific) antibodies ($P>0.05$; Figure S1A-D), indicating minimal contribution of B1a B cells to IgG and oxLDL-specific IgG during atherosclerosis development.

TLR4 and MyD88 expression by B1a B cells is required for IgM deposition in Atherosclerotic Lesions

To determine whether TLR4 and MyD88 expression by B1a B cells affects IgM accumulation in atherosclerotic lesions, we compared IgM accumulation following adoptive transfer of WT B1a B cells with TLR4 or MyD88-deficeint B1a B cells. IgM in atherosclerotic lesions of splenectomised $ApoE^{-/-}$ mice were barely detectable (Figure 3A&B) and markedly increased (4-5-fold) following transfer of WT B1a B cells ($P<0.05$; Figure 3A&B). Following transfer of B1a B cells deficient in either TLR4 or MyD88, this increase was attenuated by ~60% compared to WT-B1a B cells ($P<0.05$; Figure 3A&B). As IgM promote clearance of oxidatively modified LDL (oxLDL), we examined MDA-LDL levels in atherosclerotic lesions. MDA-LDL in lesions was reduced by approximately 50% after transfer of WT B1a B cells but after transfer of B1a B cells deficient in either TLR4 or MyD88 reduction was not significant ($P >0.05$; Figure 3C&D).

TLR4 expression byB1a B cells is required for reduction of lesion apoptotic cells and necrotic cores and increase in IL10 and TGF anti-inflammatory cytokine expression

IgM facilitates removal of apoptotic cells by binding with membrane lysophosphatidylcholine together with complement activation, facilitating phagocytosis by macrophages and production of anti-inflammatory cytokines (14,35). Thus we examined the extent to which TLR4 expressed by B1a B cells influenced lesion apoptotic cell numbers, necrotic core and lesion anti-inflammatory cytokines. Adoptive transfer of WT B1a B cells into splenectomised mice reduced lesion apoptotic cell numbers by nearly 60% ($P<0.05$ B1a B cells compared to PBS; Figure 4A&B) but apoptotic cell numbers were unaffected following transfer of TLR4 or MyD88-deficient B1a B cells. The reduction in lesion apoptotic cell number is accompanied by significant reductions in lesion necrotic core size ($P< 0.05$), reduced by approximately 58% following transfer of B1a B cells but not affected by TLR4 or MyD88-deficient B1a B cells ($P<0.05$ B1a B cells compared to PBS; Figure 4C&D). As phagocytosis of IgM bound apoptotic cells by macrophages elicits secretion of TGF-β and IL-10 (14), we also assessed effects on lesion transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10) expression. Transfer of B1a B cells into splenectomised mice but not TLR4 or MyD88-deficient B1a B cells increased TGF-β and IL-10 expression in atherosclerotic lesions ($P<0.05$; Figure 4E&F). Thus lesion TGF-β and IL-10 are also in part regulated via B1a B cell TLR4 and MyD88-dependent mechanisms.

TLR4 is required for B1a B cells to reduce CD4 and CD8 T cell accumulation and proinflammatory IFN-γ, TNF-α, IL-1β and IL-18 cytokines and adhesion molecules MCP-1 and VCAM-1 in atherosclerotic lesions

Natural IgM inhibit T-cell activation and chemotaxis (36). To determine whether B1a B cell derived IgM exerted similar effects in atherosclerosis; we compared the effects of WT and TLR4-deficient B1a B cells on lesion $CD4^+$ and $CD8^+$ T cell accumulation. WT but not TLR4-deficient B1a B cells, suppress lesion $CD4^+$ and $CD8^+$ T cell accumulation ($P<0.05$; Figure 5A&B). As IgM can also inhibit proinflammatory cells from proliferating and producing proinflammatory cytokines (29), we examined whether TLR4 expression by B1a B cells was required to attenuate proinflammatory cytokine expression in lesions. Transfer of WT but not TLR4 deficient B1a B cells into splenectomised $ApoE^{-/-}$ mice reduced expression of proinflammatory cytokines IFN-γ, TNF-α, IL-1β and IL-18 in lesions by ~50%, 60%, 33%and 52% respectively ($P<0.05$; figure 5C&D) and, MCP-1 and VCAM-1 expression by 40% and 60% respectively ($P<0.05$; Figure 5E); reduction in IL-1β was not significant ($P>0.05$; Figure 5D).

Discussion

We have reported that B1a B cell protection against atherosclerosis is dependent on IgM secretion (6). Here we demonstrate that B1a B cell secretion of atheroprotective IgMs is dependent on TLR4 despite the low expression of this receptor compared with TLR2 or TLR9. We found that TLR4 deficient B1a B cells do not attenuate atherosclerosis or produce significant IgMs, contrasting with suppressive effects of TLR4 sufficient WT B1a B cells and B1a B cells deficient in TLR2, TLR9 or CD40. This effect of TLR4 deficiency was not a consequence of differences in B1a B cell survival and was dependent on MyD88. IgMs secreted by TLR4 expressing B1a B cells not only bound to oxLDL but also to leukocytes, and CD3 and CD4 T lymphocytes. Lesion apoptotic cell numbers, necrotic core development and lesion anti-inflammatory cytokine levels, TGF-β and IL-10 were also regulated by B1a B cells expressing TLR4-MyD88. Together the results indicate that TLR4-MyD88 expressing B1a B cells account for the atheroprotective effects of B1a B cells, acting via multiple mechanisms involving secreted IgMs.

B1a B cells express higher levels of TLR2 and TLR9 compared with TLR4 (20). TLR2 mediates *in-vivo* B1a B cell expansion in spleen and bone marrow (37) and its activation in vitro elevates secretion of IgM to levels observed following activation of TLR4 (20). Despite

these similar effects on IgM production in vitro, *in vivo* IgM production by B1a B cells during development of atherosclerosis was only dependent on B1a B cells expressing TLR4 as were effects on atherosclerotic lesions. Adoptive transfer of TLR2-deficient B1a B cells into splenectomised ApoE$^{-/-}$ mice augmented IgM production and attenuated atherosclerosis similar to WT B1a B cells. Whilst *in vitro* stimulation of TLR9 on B1a B cells elevates IgM secretion to levels seen after TLR4 activation (20), TLR9 expressed by B1a B cells also does not influence plasma IgM levels or atherosclerosis in splenectomised mice. Also whilst DNA complexes on the surface of apoptotic cells has been shown to induce tolerogenic IL-10 secreting cells B1a B cells via TLR9-dependent mechanisms (38), such a mechanism seems at best very minor in B1a B cell mediated suppression of atherosclerosis. Studies utilising global knockout TLR9 ApoE$^{-/-}$ mice indicates that this TLR protects against atherosclerosis by yet to be defined mechanisms (22). Rather, our studies on the different TLRs indicate that TLR4 is the major TLR receptor responsible for atheroprotection by B1a B cells. B1a B cells deficient in TLR4 are unable to attenuate atherosclerosis in splenectomised ApoE$^{-/-}$ mice. These cells do not differentiate into plasma cells and increase IgM secretion following *in-vitro* activation by the TLR4 ligand LPS; neither do they produce IgM or MDA-oxLDL IgM or anti-leukocyte or anti-T cell IgMs in atherosclerotic mice *in-vivo*. While TLR4 activated peritoneal B1a B cells can also increase their expression of IL-10 (33), its contribution to atheroprotection may be due to IL-10 promotion of activated B cells to secrete IgM (39,40). These studies are the first report of the essential requirement of TLR4-MyD88 expression by B1a cells for their atheroprotective action. The study also extend previous reports (6,41) of mechanisms by which B1a B cells are atheroprotective.

In addition to promoting the removal of apoptotic cells and oxLDL in atherosclerotic lesions, this study also indicates that IgMs can also potentially contribute to atheroprotection by interacting with lesion leukocyte and T cells. IgM is increased in inflammatory disorders (28). IgM inhibits immune cell activation and chemotaxis by interacting with CD3, CD4 CCR5 and CXCR4 expressing T cells, macrophages and dendritic cells (28) and inhibits cytokine production by activated splenocytes of prediabetic mice (42). IgM can also inhibit CD4^{+} T cell differentiation into Th1 and Th17 cells (29). Our findings suggest that B1a-derived IgMs may also provide atheroprotection by binding to leucocytes, CD3 and CD4 T cells and reducing lesion CD4 and CD8 T lymphocytes that have been reported to promote atherosclerosis (30,43). By reducing lesion apoptotic cell numbers, TLR4 expressing B1a B cells can also reduce post apoptotic necrosis, evidenced by effects on necrotic cores to attenuate necrotic cell mediated inflammation (41,42,44).

The present study also indicates that B1a-derived IgM promotes the production of the anti-inflammatory cytokines TGF-β and IL10. Our findings are consistent with the report that following phagocytic clearance of apoptotic cells initiated by interaction of macrophage surface receptors with apoptotic cell-bound pentameric IgM and complement proteins macrophages produce anti-inflammatory cytokines such as TGF-β and IL-10. TGF-β secretion is mediated through phosphatidylserine on apoptotic cells (45) whilst IL-10 secretion is via Homeodomain Proteins Pbx1 and Prep-1 (46). Our results showing that TLR4- and MyD88-deficient B1a B cells failed to increase arterial mRNA expression of TGF-β and IL-10 in the absence of IgM antibodies highlight a healing role of B1a-derived IgM antibodies in addition to their atheroprotective action.

Taken together our findings extend our earlier reports of the atheroprotective actions of B1a B cells via IgM (47), by demonstrating that IgM production and atheroprotection by these cells is critically dependent on TLR4-MyD88 expression by B1a cells. Activation of this pathway greatly increases secretion of IgMs that not only reduce lesion apoptotic and necrotic cells and oxLDL but also lesion pro-atherogenic T cells as well as promoting increases in lesion anti-inflammatory cytokines and reducing lesion proinflammatory cytokine levels to attenuate atherosclerosis. Understanding the factors responsible for activating

TLR4/MyD88 expressing atheroprotective B1a B cells may lead to novel strategies to ameliorate atherosclerosis.

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Disclosure

None declared.

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Figure legends:

Figure 1- TLR4 and MyD88 deficient B1a B cells failed to reduce splenectomy aggravated atherosclerosis.
Splenectomised ApoE$^{-/-}$ mice were adoptively transferred with peritoneal B1a B cells isolated from different donor mice. To maintain peritoneal B1a B cells in the absence of the spleen, 10^5 B1a B cells and PBS as control were transferred via tail vein injection at weeks 1, 4, 7 after splenectomy. Sham operated (SO) Apo E$^{-/-}$ mice did not receive any injection while fed a HFD. At the end of 8 weeks, atherosclerosis was assessed at the aortic sinus by (A) total intimal lesion areas and (B) ORO-stained lipid accumulation. Splenectomy aggravated atherosclerosis was reduced by TLR4-competent (WT, TLR2$^{-/-}$ and TLR9$^{-/-}$) B1a B cells but not by TLR4$^{-/-}$B1a B cells. Similar findings were observed in (C) CD68^{+} macrophage accumulation. MyD88$^{-/-}$ but not CD40$^{-/-}$B1a B cells also failed to reduce splenectomy-aggravated atherosclerosis assessed by (D) total intimal lesion areas and (E) ORO-stained lipid accumulation. Similar findings were also observed in (F) CD68^{+} macrophage accumulation. Representative microimages show total intimal lesion areas, ORO-stained lipid and CD68^{+} macrophage accumulation. Data represent mean±SEM. (TLRs study: SO: n=9, PBS: n=10, WT-B1a: n=11, TLR2$^{-/-}$B1a: n=12, TLR4$^{-/-}$B1a: n=11and TLR9$^{-/-}$ B1a: n=12) and (MyD88$^{-/-}$ and CD40$^{-/-}$ studies: SO: n=7, PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$B1a: n=10 and CD40$^{-/-}$B1a: n=10). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a and † = $p<0.05$ compared with SO.

Figure 2- IgM secretion by B1a B cells is dependent on TLR4 and MyD88 activation.
ELISA using plasma samples from splenectomy-B1a transfer studies showed TLR4 activation through MyD88 is required for B1a B cells to produce plasma (A) total IgM and (B) MDA-oxLDL specific IgM antibodies and (C) anti-leukocyte, (D) anti-CD3 and (E) anti-CD4 IgM antibodies. Data represent mean±SEM; (TLR study: PBS: n=10, WT-B1a: n=11, TLR2$^{-/-}$ B1a: n=12, TLR4$^{-/-}$B1a: n=11and TLR9$^{-/-}$ B1a: n=12) and (MyD88$^{-/-}$ and CD40$^{-/-}$ studies: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$B1a: n=10 and CD40$^{-/-}$B1a: n=10). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a. ELISA on supernatant collected at 72hours after *in-vitro* B1a B cells stimulation with LPS revealed TLR4$^{-/-}$ B1a B cells failed to produce (F) IgM and (G) IgG. Data represent mean±SEM; (n= 7-8 samples per LPS dose) *: $P<0.05$. LPS-stimulated B1a B cells (n=6) differentiated into (H) CD19- CD138+ plasma cells. *: $P<0.05$.

Figure 3- TLR4 and MyD88 activation of B1a B cells is required for lesion IgM deposits and lesion oxLDL removal.
Representative results and microimages of immunohistochemical analysis showed (A) TLR4$^{-/-}$ B1a B cells and (B) MyD88$^{-/-}$ B1a B cells failed to increase IgM deposits in atherosclerotic lesions. Representative results and images of MDA-oxLDL levels in intimal atherosclerotic lesions assessed by immunohistochmeical staining showed WT- but not (C) TLR4$^{-/-}$ and (D) MyD88$^{-/-}$ B1a B cells reduced MDA-oxLDL. Data represent mean±SEM; representative results of three experiments (TLR4 study: PBS: n=10, WT-B1a: n=11, TLR4$^{-/-}$ B1a: n=11) and (MyD88 study: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$ B1a: n=10). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a, one-way ANOVA with Bonferroni post-test.

Figure 4- TLR4$^{-/-}$B1a B cells failed to reduce apoptosis, necrotic cores and increase IL10 and TGF in splenectomy-aggravated atherosclerotic lesions.
TUNEL and H&E staining were used to determine numbers of apoptotic cells and necrotic cores in atherosclerotic lesions. Representative results and mircoimages of B1a B cells deficient in TLR4 and MyD88 failed to reduce (A-B) TUNEL^{+} apoptotic cells and (C-D) acellular necrotic cores. Real time-PCR analysis revealed (E-F) increased expression of IL-10 and TGF-β mRNA in aorta in mice that received WT-B1a B cells compared with those mice that received TLR4$^{-/-}$ or MyD88$^{-/-}$ B1a B cells or PBS. Data represent mean±SEM. (TLR4 study: PBS: n=10, WT-B1a: n=11, TLR4$^{-/-}$ B1a: n=11) and (MyD88 study: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$ B1a: n=10). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a.

Figure 5- TLR4$^{-/-}$ B1a B cells failed to reduce lesion CD4 and CD8 T cell infiltrates and inflammatory cytokines IFN-γ, TNF-α, IL-1β, and IL-18 and adhesion molecules MCP-1 and VCAM-1 in splenectomy-aggravated atherosclerotic lesions.
Immunohistochemical staining for (A) CD4^{+} T cells and (B) CD8^{+} T cells in atherosclerotic lesions showed that TLR4$^{-/-}$ B1a B cells failed to reduce lesion CD4^{+} T cells and CD8^{+} T cells. mRNAs extracted from aorta and real time-PCR showed mice that received WT but not TLR4$^{-/-}$ B1a B cells reduced expression of (C-D) inflammatory cytokines IFN-γ, TNF-α, IL-1β, and IL-18 and (E) adhesion molecules MCP-1 and VCAM-1, . Graphs represent mean±SEM; (PBS: n=8, WT-B1a: n=10 and TLR4$^{-/-}$ B1a: n=9). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a.

Tables:

Table 1: Lymphocytes population in peritoneal cavity of splenectomised ApoE$^{-/-}$ mice

Exp. mice	B2 cells (10^5)	B1a cells (10^5)	$CD4^+$T cells (10^5)	$CD8^+$T cells (10^5)	NKT cells (10^5)	NK cells (10^5)
SO	7.9 (±0.5)	0.8 (±0.12)	2.1 (±0.44)	0.9 (±0.09)	0.12 (±0.02)	0.65 (±0.06)
SX-PBS	7.8 (±1.5)	**0.09 (±0.01)***	2.5 (±0.44)	0.8 (±0.07)	0.14 (±0.03)	0.68 (±0.07)
SX-WT B1a	7.9 (±1.9)	0.6 (±0.1)	2.4 (±0.21)	0.75 (±0.13)	0.12 (±0.02)	0.64 (±0.08)
SX-TLR2$^{-/-}$ B1a	7.8 (±2.1)	0.57 (±0.09)	1.9 (±0.29)	0.72 (±0.11)	0.12 (±0.01)	0.6 (±0.04)
SX-TLR4$^{-/-}$ B1a	6.9 (±2)	0.58 (±0.09)	2.2 (±0.31)	0.78 (±0.07)	0.13 (±0.03)	0.59 (±0.05)
SX-TLR9$^{-/-}$ B1a	7.3 (±1.4)	0.68 (±0.08)	2.0 (±0.42)	0.72 (±0.14)	0.13 (±0.01)	0.59 (±0.08)

Data are represented as cell NO. (±SEM) of 10-12 mice in each group; * = $p<0.05$ compared with other groups

Table 2: Body weight and lipid profile of splenectomised ApoE$^{-/-}$ mice

Exp. mice	Body weight (gr)	Total Cholestrol (mmol/L)	VLDL/LDL Cholestrol (mmol/L)	Triglyceride (mmol/L)	HDL Cholestrol (mmol/L)
SO	24.8 (±1)	20.4 (±0.9)	15.1 (±0.5)	3.4 (±0.2)	3.2 (±0.2)
SX-PBS	25 (±1.3)	20.8 (±3.2)	15.8 (±2.4)	3.5 (±0.6)	3.4 (±0.5)
SX-WT B1a	26 (±0.8)	24.3 (±2.2)	18 (±1.6)	4.8 (±0.6)	4 (±0.4)
SX-TLR2-/- B1a	25 (±0.9)	21.13 (±2)	15.9 (±1.4)	4 (±0.7)	3.4 (±0.3)
SX-TLR4-/- B1a	25 (±0.6)	24.2 (±0.9)	18.1 (±0.70)	4.5 (±0.4)	4.1 (±0.2)
SX-TLR9-/- B1a	26 (±1.0)	24.5 (±1.7)	18.7 (±1.3)	4.4 (±0.4)	3.9 (±0.2)

Data are represented as body weight or lipid profile (±SEM) of 10-12 mice in each group.

Table 3: Lymphocytes population in peritoneal cavity of splenectomised ApoE$^{-/-}$ mice

Exp. mice	B2 cells (10^5)	B1a cells (10^5)	CD4+ T cells (10^5)	CD8+ T cells (10^5)	NK T cells (10^5)	NK cells (10^5)
SO	8.9 (±0.4)	0.92 (±0.02)	2.9 (±0.24)	1.4 (±0.19)	0.17 (±0.02)	0.52 (±0.09)
SX-PBS	9.3 (±1.9)	**0.11 (±0.01)***	2.6 (±0.26)	1.5 (±0.41)	0.14 (±0.02)	0.55 (±0.12)
SX-WT B1a	11 (±1.9)	0.71 (±0.12)	3.5 (±1.1)	1.4 (±0.36)	0.15 (±0.03)	0.59 (±0.09)
SX-MyD88$^{-/-}$ B1a	10 (±1.5)	0.77 (±0.11)	3.2 (±0.85)	1.2 (±0.18)	0.15 (±0.01)	0.57 (±0.12)
SX-CD40$^{-/-}$ B1a	8.6 (±0.85)	0.77 (±0.07)	3.8 (±0.26)	1.3 (±0.17)	0.13 (±0.02)	0.54 (±0.04)

Data are represented as cell NO. (±SEM) of 8-10 mice in each group * = $p<0.05$ compared with other groups.

Table 4: Body weight and lipid profile of splenectomised ApoE$^{-/-}$ mice

Exp. mice	Body weight (gr)	Total Cholestrol (mmol/L)	VLDL/LDL Cholestrol (mmol/L)	Triglyceride (mmol/L)	HDL Cholestrol (mmol/L)
SO	28.7 (±0.9)	28.5 (±1.9)	21.3 (±1.4)	5.2 (±0.4)	5.1 (±0.4)
SX-PBS	28.5 (±0.8)	28.5 (±2.2)	20.8 (±1.5)	5.6 (±0.5)	5.1 (±0.8)
SX-WT B1a	28.3 (±1)	27.6 (±2.6)	21.2 (±1.8)	5.1 (±0.5)	4.5 (±0.6)
SX-MyD88-/- B1a	27.5 (±0.8)	26.7 (±2.5)	20 (±1.9)	5.7 (±0.4)	5.1 (±0.4)
SX-CD40-/- B1a	27.4 (±1.1)	29.3 (±1.3)	21 (±0.9)	6.8 (±0.6)	5.3 (±0.2)

Data are represented as body weight or lipid profile (±SEM) of 8-10 mice in each group.

Thrombosis and Haemostasis

Figure 1 **16**

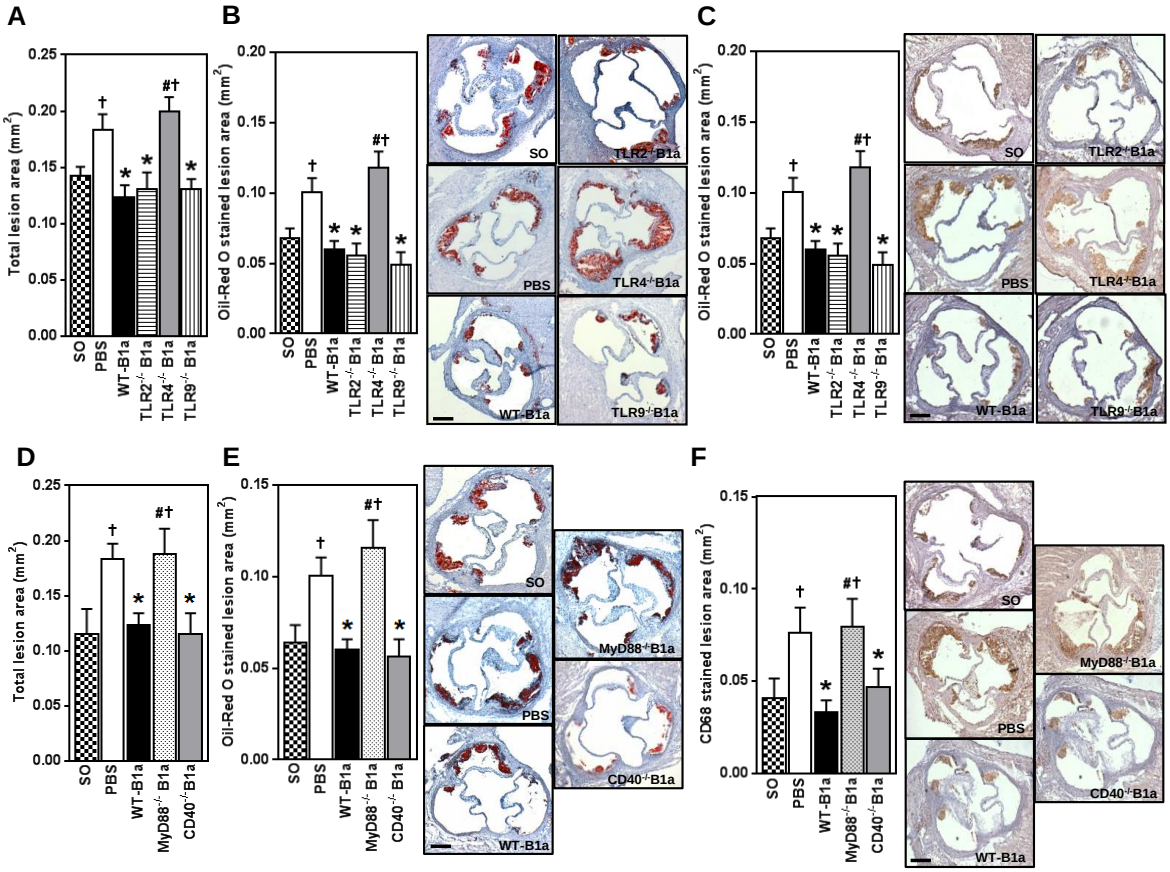

Figure 1- TLR4 and MyD88 deficient B1a cells failed to reduce splenectomy aggravated atherosclerosis.

Splenectomised ApoE$^{-/-}$ mice were adoptively transferred with peritoneal B1a cells isolated from different donor mice. To maintain peritoneal B1a cells in the absence of spleen, 10^5 B1a cells and PBS as control were transferred via tail vein injection at weeks 1, 4, 7 after splenectomy but sham operated (SO) Apo E$^{-/-}$ mice were not received any injection while fed a HFD. At the end of 8 weeks, atherosclerosis assessment at aortic sinus by (**A**) total intimal lesion areas and (**B**) ORO-stained lipid accumulation showed that TLR4$^{-/-}$B1a cells failed to reduce splenectomy aggravated atherosclerosis whilst TLR4-competent (WT, TLR2$^{-/-}$ and TLR9$^{-/-}$) B1a cells did. Similar finding was observed in (**C**) CD68^{+} macrophage accumulation. MyD88$^{-/-}$ but not CD40$^{-/-}$B1a cells also failed to reduce splenectomy-aggravated atherosclerosis assessed by (**D**) total intimal lesion areas and (**E**) ORO-stained lipid accumulation. Similar finding was also observed in (**F**) CD68^{+} macrophage accumulation. Representative microimages showed total intimal lesion areas, ORO-stained lipid and CD68^{+} macrophage accumulation. Data represent mean±SEM. (TLRs study: SO: n=9, PBS: n=10, WT-B1a: n=11, TLR2$^{-/-}$B1a: n=12, TLR4$^{-/-}$B1a: n=11and TLR9$^{-/-}$ B1a: n=12) and (MyD88$^{-/-}$ and CD40$^{-/-}$ studies: SO: n=7, PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$B1a: n=10 and CD40$^{-/-}$B1a: n=10). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a and † = $p<0.05$ compared with SO.

Figure 2

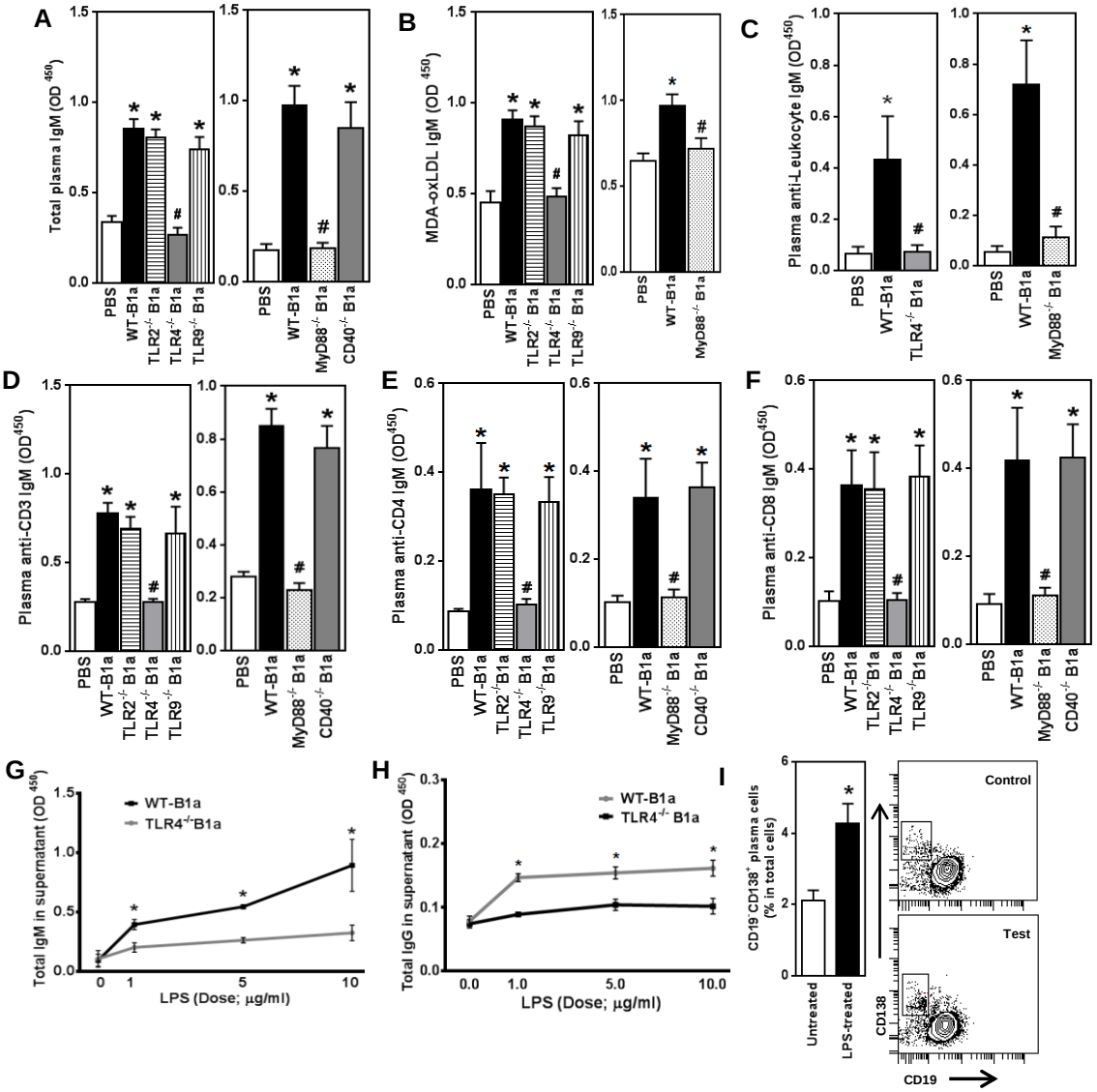

Figure 2- IgM secretion by B1a cells is dependent on TLR4 and MyD88 activation.
ELISA using plasma samples from splenectomy-B1a transfer studies showed TLR4 activation through MyD88 is required for B1a cells to produce plasma (**A**) total IgM and (**B**) MDA-oxLDL specific IgM antibodies and **(C)** anti-leukocyte, **(D)** anti-CD3 and (**E**) anti-CD4 IgM antibodies. Data represent mean±SEM; (TLRs study: PBS: n=10, WT-B1a: n=11, TLR2$^{-/-}$B1a: n=12, TLR4$^{-/-}$B1a: n=11and TLR9$^{-/-}$ B1a: n=12) and (MyD88$^{-/-}$ and CD40$^{-/-}$ studies: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$B1a: n=10 and CD40$^{-/-}$B1a: n=10). * = p<0.05 compared with PBS, # = p<0.05 compared with WT-B1a.
ELISA on supernatant collected at 72hours after *in-vitro* B1a cells stimulation with LPS revealed TLR4$^{-/-}$ B1a cells failed to produce **(F)** IgM and **(G)** IgG. Data represent mean±SEM; (n= 7-8 samples per LPS dose) *: P<0.05. LPS-stimulated B1a cells (n=6) differentiated into **(H)** CD19-CD138+ plasma cells. *: P<0.05.

Figure 3

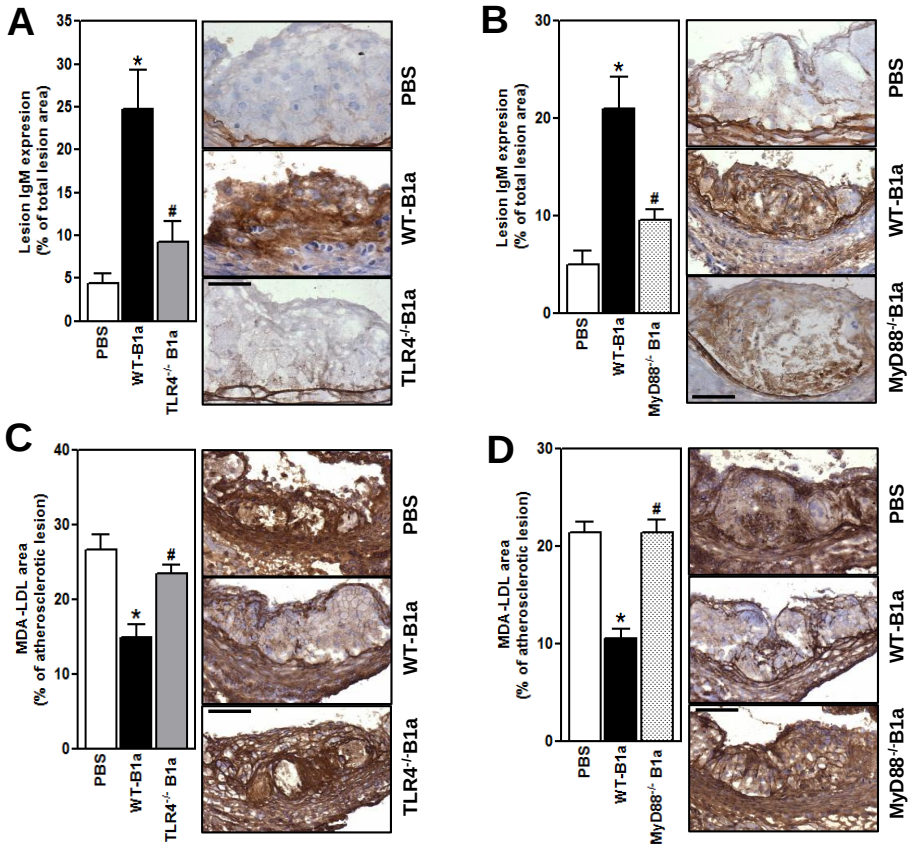

Figure 3- TLR4 and MyD88 activation of B1a cells is required for lesion IgM deposits and lesion oxLDL removal.

Representative results and microimages of immunohistochemical analysis showed (**A**) TLR4$^{-/-}$ B1a cells and (**B**) MyD88$^{-/-}$ B1a cells failed to increase IgM deposits in atherosclerotic lesions. Representative results and images of MDA-oxLDL levels in intimal atherosclerotic lesions assessed by immunohistochmeical staining showed WT- but not (**C**) TLR4$^{-/-}$ and (**D**) MyD88$^{-/-}$ B1a cells reduced MDA-oxLDL. Data represent mean±SEM; representative results of three experiments (TLR4 study: PBS: n=10, WT-B1a: n=11, TLR4$^{-/-}$ B1a: n=11) and (MyD88 study: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$ B1a: n=10). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a, one-way ANOVA with Bonferroni post-test.

Figure 4

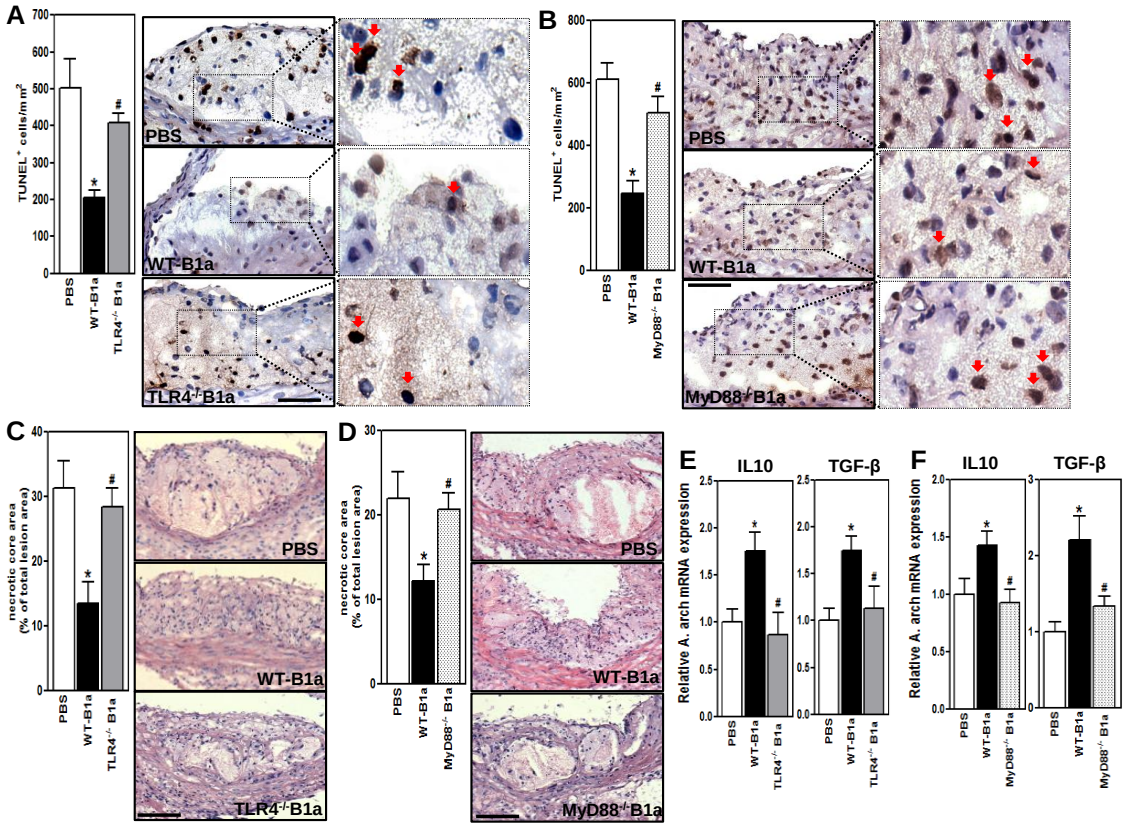

Figure 4- TLR4$^{-/-}$B1a cells failed to reduce apoptosis, necrotic cores and inflammation in splenectomy-aggravated atherosclerotic lesions.

TUNEL and H&E staining were used to determine numbers of apoptotic cells and necrotic cores in atherosclerotic lesions. Representative results and mircoimages of B1a cells deficient in TLR4 and MyD88 failed to reduce (**A-B**) TUNEL^{+} apoptotic cells and (**C-D**) acellular necrotic cores. Real time-PCR analysis revealed (**E-F**) increased expression of IL-10 and TGF-β mRNA in aorta in mice that received WT-B1a cells compared with those mice that received TLR4$^{-/-}$ or MyD88$^{-/-}$ B1a cells or PBS. Data represent mean±SEM. (TLR4 study: PBS: n=10, WT-B1a: n=11, TLR4$^{-/-}$ B1a: n=11) and (MyD88 study: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$ B1a: n=10). . * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a.

Thrombosis and Haemostasis

20

Figure 5

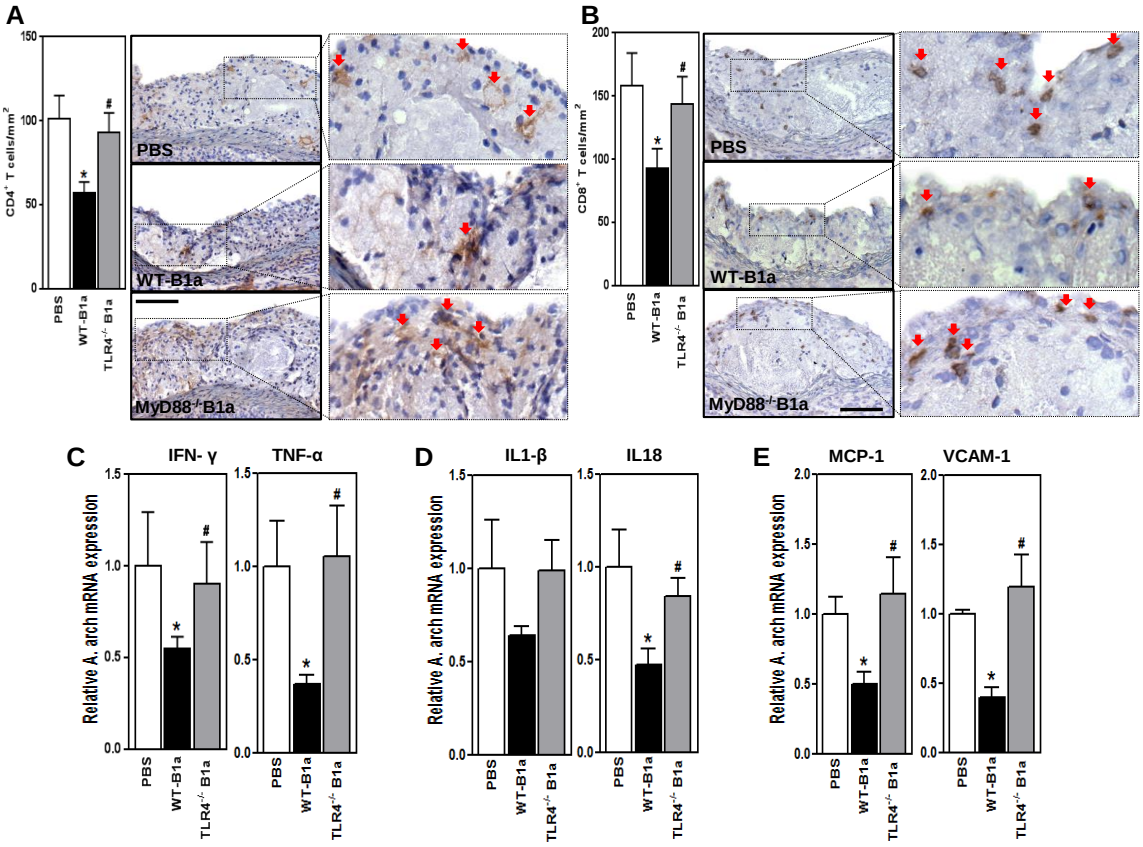

Figure 5- TLR4$^{-/-}$ B1a cells failed to reduce arterial inflammation in splenectomy-aggravated atherosclerotic lesions.

Immunohistochemical staining for (**A**) CD4^{+} T cells and (**B**) CD8^{+} T cells in atherosclerotic lesions showed that TLR4$^{-/-}$ B1a cells failed to reduce lesion CD4^{+} T cells and CD8^{+} T cells. mRNAs extracted from aorta and real time-PCR showed mice that received WT but not TLR4$^{-/-}$ B1a cells reduced expression of (**C-D**) inflammatory cytokines IFN-γ, TNF-α, IL-1β, and IL-18 and (**E**) adhesion molecules MCP-1 and VCAM-1, . Graphs represent mean±SEM; (PBS: n=8, WT-B1a: n=10 and TLR4$^{-/-}$ B1a: n=9). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a.

Figure S1

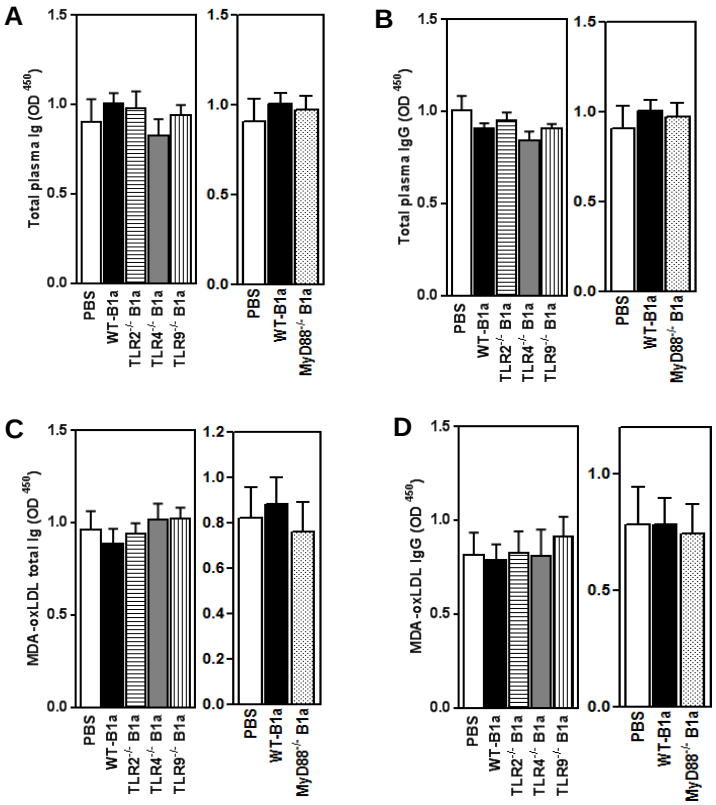

Figure S1- Plasma total Ig and IgG were not affected by lack of TLR4 and MyD88 in B1a B cells.

ELISA of plasma samples from TLR4- and MyD88-deficient B1a transfer studies showed that (**A**) total Ig, (**B**) total IgG, (**C**) total MDA-oxLDL Ig and (**D**) total MDA-oxLDL IgG were comparable among control and test groups. Data represent mean±SEM; (TLRs study: PBS: n=9, WT-B1a: n=9, TLR2$^{-/-}$B1a: n=11, TLR4$^{-/-}$B1a: n=10 and TLR9$^{-/-}$ B1a: n=10) and (MyD88$^{-/-}$ and CD40$^{-/-}$ studies: PBS: n=8, WT-B1a: n=8 and MyD88$^{-/-}$B1a: n=9 and CD40$^{-/-}$B1a: n=9). * = $p<0.05$ compared with PBS, # = $p<0.05$ compared with WT-B1a.

Chapter 3. Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes

3.1 Short Introduction

We have previously identified peritoneal B1a cells as protective against atherosclerosis development in ApoE-/- mice. Here, I examined whether activating B1a B cells with apoptotic cells (ACs) as well as phosphatidylserine liposomes (PSL) could enhance their atheroprotective actions. Intraperitoneally administered ACs attenuated atherosclerosis in ApoE-/- mice by 53% compared to controls, effects that were mimicked by administering PSLs and dependent on B1a cells. These effects were associated with reductions in lesion macrophages, CD4+ and CD8+ T cell accumulation was well as reductions in proinflammatory cytokines and increases in anti-inflammatory cytokines. ACs and PSLs also increased B1a cells including TIM-1+ B1a cells *in-vivo* and stimulated B1a cell proliferation *in-vitro*; other lymphocyte populations were unaffected. Total plasma IgM, anti-leukocyte, anti-CD3, anti-CD4 and anti-oxLDL IgM were also significantly elevated. The elevated IgM in the lesions was associated with reductions in lesion MDA-LDL (oxLDL), reduced apoptotic cell numbers and lesion necrotic core size. These many effects of activating B1a cells in lesions could be attributed to the actions of polyreactive IgM secreted by activated B1a cells, reducing inflammatory cytokines by lowering lesion oxLDL levels via anti-oxLDL IgM, reducing T-cell numbers via anti-leukocyte, anti-CD3 and anti-CD4 IgM, reducing apoptotic cell numbers and necrotic core size via IgM binding to apoptotic cells and enhancing phagocytosis, and elevating anti-inflammatory cytokine expression.

3.2 Declaration for Thesis Chapter

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 concept and design, Experimentation and handling, Data analysis, results interpretation, Preparation of manuscript	90

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
Peter Kanellakis	Experimentation	
Li Yi	Experimentation	5%
Christopher Tay	Experimentation	
Anh Cao	Experimentation	
Peter Tipping	Study concept, Study design, Results interpretation	
Alex Bobik	Study concept, Study design, Results interpretation, Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Results interpretation, Preparation of manuscript	
Tin Kyaw	Study design, Experimentation, results 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		**Date 13/02/15**
Main Supervisor's Signature	[redacted]	**Date 13/02/2015**

*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.

3.3 Acceptance Letter

Tin Soe Kyaw

From: [redacted] on behalf of [redacted]
Sent: Tuesday, 20 January 2015 9:37 AM
To: Tin Soe Kyaw
Subject: Editorial Decision on CVR-2014-1421

MS # CVR-2014-1421
Title: Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes Corresponding Author: Dr Tin Kyaw

Dear Dr. Kyaw,

The reviewers and the Editorial Team at Cardiovascular Research have evaluated your revised manuscript and are pleased to inform you that it has been accepted for publication.

The manuscript has been sent to the publisher and you will receive the page-proofs directly from the technical editor.

We wish to thank you for your valuable contribution to the journal.

Yours sincerely,

Karin R. Sipido
Editor-in-Chief
Cardiovascular Research

Deputy Editors
Paul Holvoet
Stefan Janssens
Aernout Luttun
Maurilio Sampaolesi

Reviewer #1: Almost all issues have been addressed and the paper has much improved. One minor point: Fig.1B panel contains a typo (BPS instead of PBS).

Reviewer #2: Thank you for your comments. No, further questions.

Reviewer #4: None

--

3.4 Manuscript

Cardiovascular Research

Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes

--Manuscript Draft--

Manuscript Number:	CVR-2014-1421
Full Title:	Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes
Short Title:	Liposomes targeting B cells in atherosclerosis
Article Type:	Original Article
Keywords:	B1a cells; IgM; apoptotic cells; phosphatidylserine liposomes; atherosclerosis
Corresponding Author:	Tin Kyaw, MBBS PhD BakerIDI heart and diabetes institute Melbourne, AUSTRALIA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	BakerIDI heart and diabetes institute
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	Anh Cao
	Peter Tipping
	Ban-Hock Toh
	Alex Bobik
	Tin Kyaw, MBBS PhD
Order of Authors Secondary Information:	
Abstract:	Aims: To investigate whether activation of atheroprotective peritoneal B1a cells by apoptotic cells or phosphatidylserine liposomes can enhance their protective actions during atherosclerosis development. Methods and Results: Male Apolipoprotein E-knockout (ApoE-/-) mice were treated with ACs or PSL at the beginning of 8 week-high fat diet. Intraperitoneally administered ACs attenuated atherosclerosis in hypercholesterolemic ApoE-/- mice by 53% and macrophage accumulation by 52%, effects mimicked by administering PSL and abolished by B1a cell depletion by splenectomy. These effects were associated with reduced lesion CD4+ and CD8+ T cells, mRNAs of MCP-1, VCAM-1, TNF-α, IL-1β, IL-12 and IL-18 while anti-inflammatory TGF-β mRNA levels doubled. ACs and PSL increased B1a lymphocytes including TIM-1+ B1a cells in-vivo and in-vitro while other lymphocyte populations were unaffected. Total plasma IgM, anti-leukocyte, anti-CD3, anti-CD4 and anti-oxLDL IgM were elevated. IgM in atherosclerotic lesions was also elevated and this was associated with reduced lesion MDA-LDL (oxLDL), apoptotic cells and necrotic core size. These effects of activating B1a cells could be attributed to B1a-derived polyreactive IgM deposited in lesions that reduce inflammatory cytokines by lowering lesion ox-LDL via anti-oxLDL IgM, T-cells via anti-leukocyte, anti-CD3 and anti-CD4 IgM, apoptotic cells and necrotic core size via IgM binding to apoptotic cells

	and enhancing phagocytosis, which also elevates anti-inflammatory cytokines. Conclusion: Targeting B1a cell activation by phosphatidylserine liposomes may be a potentially potent therapeutic strategy to attenuate atherosclerosis and reduce the incidence of atherosclerosis dependent myocardial infarction and stroke.
Suggested Reviewers:	
Opposed Reviewers:	

Phosphatidylserine Liposomes Mimic Apoptotic Cells to Attenuate Atherosclerosis by Expanding Polyreactive IgM Producing B1a lymphocytes

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[1]BakerIDI Heart and Diabetes Institute, Melbourne, Australia; [2]Centre for Inflammatory Diseases, Department of Medicine, Southern Clinical School and [3]Department of Immunology, Central Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Australia

Short title: Liposomes targeting B cells in atherosclerosis

*Equal contribution

Total words: 5486

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Abstract

Aims: To investigate whether activation of atheroprotective peritoneal B1a cells by apoptotic cells or phosphatidylserine liposomes can enhance their protective actions during atherosclerosis development.

Methods and Results: Male Apolipoprotein E-knockout (ApoE-/-) mice were treated with apoptotic cells or phosphatidylserine liposomes at the beginning of 8 week-high fat diet. Intraperitoneally administered apoptotic cells attenuated atherosclerosis in hypercholesterolemic ApoE-/- mice by 53% and macrophage accumulation by 52%, effects mimicked by administering phosphatidylserine liposomes and abolished by B1a cell depletion by splenectomy. These effects were associated with reduced lesion CD4+ and CD8+ T cells, mRNAs of MCP-1, VCAM-1, TNF-α, IL-1β, IL-12 and IL-18 while anti-inflammatory TGF-β mRNA levels doubled. Apoptotic cells or phosphatidylserine liposomes increased B1a lymphocytes including TIM-1+ B1a cells *in-vivo* and *in-vitro* while other lymphocyte populations were unaffected. Total plasma IgM, anti-leukocyte, anti-CD3, anti-CD4 and anti-oxLDL IgM were elevated. IgM in atherosclerotic lesions was also elevated and this was associated with reduced lesion MDA-LDL (oxLDL), apoptotic cells and necrotic core size. These effects of activating B1a cells could be attributed to B1a-derived polyreactive IgM deposited in lesions that reduce inflammatory cytokines by lowering lesion ox-LDL via anti-oxLDL IgM, T-cells via anti-leukocyte, anti-CD3 and anti-CD4 IgM, apoptotic cells and necrotic core size via IgM binding to apoptotic cells and enhancing phagocytosis, which also elevates anti-inflammatory cytokines.

Conclusion: Targeting B1a cell activation by phosphatidylserine liposomes may be a potentially potent therapeutic strategy to attenuate atherosclerosis and reduce the incidence of atherosclerosis dependent myocardial infarction and stroke.

Key Words: B1a cells; IgM; apoptotic cells; phosphatidylserine liposomes; atherosclerosis;

Introduction

Despite lipid-lowering statins, heart attack and stroke are atherosclerosis-related cardiovascular diseases remaining the leading cause of mortality worldwide[1]. Atherosclerosis is a chronic inflammatory disease of medium and large arteries characterised by accumulation of lipids and immune cells that modulate atherosclerotic lesion development and progression. Atherosclerosis becomes clinically significant upon severe lumen encroachment or thrombotic occlusion following lesion rupture[2]. B cells have been identified within atherosclerotic lesions and associated adventitia in humans[3] and in mice[4]. We[5, 6] and others[7, 8] reported that conventional B2 cells promote atherosclerosis. We also provided evidence that peritoneal B1a cells are atheroprotective[9, 10] by producing natural IgM that is required for protection against atherosclerosis[11]. We have reviewed the opposing roles of conventional B2 cells and peritoneal Bla cells in atherosclerosis development[12].

Administration of apoptotic cells protects mice from autoimmune inflammation by inducing regulatory B cells that secrete IL-10, through direct interaction of apoptotic cells with B cells[13]. DNA complexes expressed on the surface of apoptotic cells and their interaction with toll-like receptor 9 (TLR9) expressed by B cells at least in part accounts for the anti-inflammatory actions of B cells[14]. Phosphatidylserine (PS), normally sequestered on the inner leaflet of the plasma membrane is exposed on the outer leaflet in apoptotic cells[15]. As B1a cells express TIM-1 and TIM-4, receptors for PS[16], it is also likely that B1a cell activation by apoptotic cells involves TIM receptors. TIM receptors exhibit both phagocytic and costimulatory properties and antibody ligation of TIM-1 induces tolerogenic IL-4 and IL-10 producing B1a cells which promote long-term graft survival[16].

Given that apoptotic cells induce tolerance[17], suppress development of type I diabetes[18], augment bone marrow engraftment[19] and suppress inflammatory arthritis[20], the latter via an IgM dependent mechanism[20], we investigated the effects of apoptotic cell administration on atherosclerosis development and its dependency on phosphatidylserine. We demonstrate that administration of apoptotic cells significantly ameliorates atherosclerosis development. Phosphatidylserine liposomes (PSL) mimicked these effects and were associated with activation of B1a cells and production of polyreactive IgM antibodies targeting leukocytes, CD3 and CD4 T cells, oxidised LDL (oxLDL) and apoptotic cells. PSL treatment attenuates atherosclerosis development and changes the lesion proinflammatory cytokine milieu to anti-inflammatory.

Materials and Methods

Animals and Experimental Protocol

Six-week-old male ApoE-KO mice commenced on a HFD were treated every alternate week for eight weeks with i.p. injection with $30x10^6$ irradiated apoptotic thymocytes controlled by PBS injection or; with 0.5mg/mouse PSL controlled by 0.5 mg/mouse phosphocholine liposomes (PCL) injection. Mice were euthanized at 14 weeks of age and effects on atherosclerosis, lymphocytes and immune cells assessed.

All experimental procedures complied with national guideline for the care and use of laboratory animals were approved by institute animal ethics committee (AEC no: E/1277/2012/B) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication

No 85-23, revised 2011).

An expanded Materials and Methods section is available in the online-only Data Supplement.

Statistical Analyses

Results are expressed as means ± SEM. Comparisons between groups were carried out using Student t-test or Mann-Whitney U test, depending on whether the data was normally distributed, as assessed using the Kolmogorov-Smirnov test. For multiple comparisons, results were analysed using one-way ANOVA (after confirming normality of distribution) followed by Bonferroni post-test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Apoptotic Cells Attenuate Development of Atherosclerotic Lesions

Treatment of hyperlipidemic ApoE-/- mice intraperitoneally with irradiated apoptotic thymocytes every 2 weeks markedly attenuated development of atherosclerotic lesions. On average, lesion areas were reduced by 53% in mice treated with apoptotic cells ($P<0.05$; Figure 1A), whilst Oil Red O stained areas were reduced by 61% ($P<0.05$; Figure 1B); viable thymocytes did not affect lesion or lipid stained areas ($P> 0.05$; Figures 1A-B). Macrophage accumulation was also reduced, by 52% ($P<0.05$; Figure 1C) following administration of apoptotic cells but unaffected by viable thymocytes. Apoptotic cell treatment also reduced CD4+ and CD8+ T cell numbers in atherosclerotic lesions by 56% and 63% respectively ($P<0.05$; Figures 1D-E). Expression of inflammatory and adhesion molecules was also reduced. Expression of MCP-1 and VCAM-1 were reduced by 54% and 56% respectively ($P<0.05$; Figure 1F), expression of proinflammatory cytokines TNF-α, IL-1β, IL-12 and IL-18 were reduced by between 47% and 80% (all $P<0.05$; Figure 1G) and expression of anti-inflammatory cytokine TGF-β mRNA levels doubled ($P<0.05$; Figure 1H). Body weight and plasma cholesterol levels were unaffected by administering apoptotic cells ($P>0.05$; Supplementary Figure 1A-B).

Phosphatidylserine Liposomes Mimic the Effects of Apoptotic cells on Atherosclerotic Lesions

Multiple mechanisms can account for the reduced lesion size and inflammation mediated by apoptotic cells. Apoptotic cells can induce tolerogenic immunosuppressive B1a cells by activating TLR9 via extracellular DNA bound to apoptotic cells[14]. Suppressive B cells can be activated by apoptotic cells binding phosphatidylserine receptors expressed by B1a cells[16] or via other phagocytic immune cells[21]. To determine whether the effects are mediated by phosphatidylserine receptors expressed by B1a cells, we next assessed the effects of intraperitoneal phosphatidylserine liposome treatment on development of atherosclerosis; phosphatidylserine liposomes are free of extracellular bound DNA. Chronic treatment with phosphatidylserine liposome attenuated atherosclerosis by 42% ($P<0.05$; Figures 2A-B); PC liposomes were without affect ($P > 0.05$; Figures 2A-B). Macrophage accumulation was also reduced in PSL-treated mice, by 47% and CD4+ and CD8+ T cell numbers were reduced by 64% and 52% respectively (all $P<0.05$; Figures 2C-E). Like apoptotic cells, PSL also reduced expression of VCAM-1 and MCP-1 by 76% and 58% respectively (both $P<0.05$; Figure 2F) whilst anti-inflammatory cytokines TGF-β mRNA and IL-10 nearly doubled ($P<0.05$; Figure 2G) and proinflammatory cytokines IFN-γ, IL-17 and

IL-18 were all reduced by between 60% and 83% respectively (all P<0.05; Figure 2H). PSL as well as AC treatment resulted in a significant (~30%) increase in plasma IL-5 levels (P < 0.05; Supplementary Figure 1C). Body weight and plasma cholesterol levels were unaffected by PSL treatment (P>0.05; Supplementary Figure 1A-B).

Phosphatidylserine Liposomes and Apoptotic Cells Expand B1a cells during Development of Atherosclerosis

Apoptotic cells protect mice from inflammation by expanding regulatory B cells[13]. To determine whether apoptotic cells and PS liposomes exert similar effects on B1a cells during atherosclerosis development, we assessed peritoneal and spleen B1a cell numbers after treating hyperlipidemic ApoE-/- mice with apoptotic cells or PS liposomes; B1a B cells were defined as CD5+CD19+CD1d^{low}IgM+ that are CD43^{low} (Supplementary Figure 2A-B). Following treatment with apoptotic cells peritoneal B1a cell numbers increased by 76% (P<0.05; Figure 3A) whilst the increase in spleen cells was not statistically significant (P>0.05; Figure 3A). Treatment with PS liposomes also increased peritoneal B1a cells by 50% (P<0.05; Figure 3B) and spleen cells by 52% (P<0.05; Figure 3B). Both treatments increased TIM-1+B1a cells in the peritoneal cavity and spleen (P<0.05; Figure 3C-E). Other immune cell types were unaffected by the treatments including follicular, marginal zone, transitional (T1 and T2) B cells as well as CD4+, CD8+, NK and NKT cells (all P>0.05; Supplementary Figures 3 and 4). To determine whether this increase in B1a cell numbers was due to direct interaction of PS liposomes with B1a cells, we compared the effects of phosphatidylserine and phosphatidylcholine liposomes on B1a cell proliferation *in-vitro*. Compared to phosphatidylcholine liposomes, phosphatidylserine liposomes significantly stimulated B1a cell proliferation (P<0.05; Figure 3F) and their IgM production (data not shown).

Phosphatidylserine liposomes and Apoptotic Cells Increase Polyreactive IgM levels and Reduce local Inflammation

Since PS liposomes stimulated the expansion of B1a cells, we next examined whether apoptotic cells and PS liposomes increased natural IgM antibody secretion during atherosclerosis development. Treatment with either PS liposomes or apoptotic cells increased plasma IgM levels by 67% and 115% respectively (both P<0.05; Figure 4A). Anti-oxLDL and anti-leukocyte IgM antibodies were also significantly increased by both treatments, as were anti-CD3 and anti-CD4 IgM antibodies (all P<0.05; Figure 4B-E). IgM accumulation in atherosclerotic lesions also increased following treatment with PS liposomes or apoptotic cells, by 46% and 42% respectively (P<0.05; Figure 4F-G). These increases in IgM were associated with reductions in accumulated lesion oxidised LDL (MDA-LDL), by 42% and 28% after PS liposomes and apoptotic cell treatments (both P<0.05; Figure 4H-I). Since IgM facilitates removal of apoptotic cells[11, 22], we also compared the effects of the two treatments on lesion apoptotic cell numbers and necrotic core size. Lesion apoptotic cell numbers and necrotic core size were reduced by 52% and 20% respectively after treatment with apoptotic cells whilst following treatment with PS liposomes, apoptotic cells were reduced by 57% and necrotic cores by 34% (all P<0.05; Figure 5A-D).

Splenectomy Abolished the Anti-Atherosclerotic Effects of PS Liposomes and Apoptotic Cells

To confirm the dependency of anti-atherosclerotic effects of PS liposomes and apoptotic cells on peritoneal B1a cells we splenectomised ApoE-/- before treating with PS liposomes or apoptotic cells; as splenectomy specifically deletes B1a cells from the peritoneal

cavity[10]. Splenectomised ApoE-/- mice fed a high fat diet for 8 weeks exhibited larger lesions than sham operated mice ($P < 0.05$; Figures 6A-B) and treating with either PS liposomes or apoptotic cells lesions size was unaffected and identical to mice that received vehicle, measured as total intimal or Oil Red O stained lesion areas ($P>0.05$; Figure 6 A&B). Peritoneal B1a cells were on average reduced by nearly 64% in splenectomised mice ($P<0.05$; Figure 6C) affecting CD5+CD19+CD1d^{low}IgM+ B1a cells (Supplementary Figure 2 C). Similar findings of B1a cells in blood and lymph nodes were observed (all $P<0.05$; supplementary figure 5A-B), whilst splenectomy did not affect lymphocyte populations, dendritic cells, macrophages and monocytes in PC (all $P>0.05$; Figure 6C), blood and lymph nodes (Supplementary Figure 5A-B). Body weights and plasma cholesterol levels were also unaffected ($P>0.05$; Figure 6D-E).

Discussion

Our findings indicate that targeting peritoneal B1a cells with apoptotic cells is highly effective in attenuating atherosclerosis development, an effect mimicked by PS liposomes. B1a cell activation with either apoptotic cells or PS liposomes induces B1a cell expansion together with increased secretion of polyreactive IgM antibodies, accounting for much of the immunosuppression by apoptotic cells and PS liposomes on atherosclerosis.

Apoptotic cells can mediate immunosuppressive effects via multiple ligands on their cell surface. Annexin A1, a cytosolic protein that translocates to the surface of early stage apoptotic cells acts as an inhibitory effector molecule that prevents induction of inflammatory dendritic cells and facilitates development of tolerogenic dendritic cells that mediate immunosuppression[23]. DNA and PS are also abundant on the surface of apoptotic cells. DNA complexes on the surface of apoptotic cells interact with toll-like receptor 9 (TLR9). Such interactions of DNA with TLR9 expressed by B1a B cells results in their differentiation to IgM producing plasma cells [24] as well as induction of tolerogenic IL-10 secreting B cells, which suppress experimental autoimmune encephalitis[14], whilst PS can interact with PS receptors encoded by TIM to regulate both innate and adaptive immunity[25]. Our findings that partial deletion of B1a B cells by splenectomy prevented the attenuation of atherosclerosis by apoptotic cells and PS liposomes indicate an important role for spleen dependent B1a B cells in regulating atherosclerosis. Earlier studies have suggested at least two populations of peritoneal B1a B cells, CD5+B220^{DULL} and CD5+B220+[26]. Hox11-/- spleenless mice only possess CD5+B220+ B1a B cells in the peritoneal cavity whilst C57Bl6 mice possess two populations, CD5+B220^{DULL} (major population) and CD5+B220+ (minor population), indicating that only the CD5+B220^{DULL} population is dependent on the presence of a spleen. Whilst we used a different gating strategy to detect peritoneal B1a b cells, it is very likely that both CD5+ populations (CD5+B220^{DULL} and CD5+B220+) are included in the CD5+CD19+IgM+ peritoneal population of ApoE-/- mice. Presumably the latter CD5+B220+ population whose survival is not spleen depended is not responsive to either apoptotic cells or liposomes. Whilst our study indicates that apoptotic cells are anti-inflammatory and attenuate atherosclerosis, an earlier report indicated apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory[27]. The difference between our study and the previous study may be due to different methods of inducing cell apoptosis; only previously stressed apoptotic cells appear to be proinflammatory[28]. Our study indicating that apoptotic cells can be anti-inflammatory during development of atherosclerosis are in accord with other studies demonstrating that their administration can induce tolerance in

other inflammatory disorders[17-19], protect against autoimmune inflammation[13] and suppress inflammatory arthritis[20].

Our finding that PS liposomes mimic the suppressive effects of apoptotic cells on atherosclerosis indicates a major role for PS in attenuating development of atherosclerosis. TIM-1 is highly expressed on regulatory B cells[16] and we demonstrate high TIM-1 expression on B1a cells, suggesting a role for their activation by PS in attenuating atherosclerosis. Deletion of B1a cells by splenectomy prevented the attenuation of atherosclerosis by both apoptotic cells and PS liposomes. Direct B1a cell activation by PS *in-vitro* stimulated expansion of B1a cells including the TIM-1^{+} B1a cell population together with increased secretion of polyreactive IgM. The findings suggest that PS interacts with TIM-1 to initiate B1a cell expansion and secretion of polyreactive IgM antibodies. TIM-1 may act as a membrane signaling receptor. TIM-1 on B cells interacts with the kinase Fyn resulting in TIM-1 phosphorylation which is increased when TIM-1 is activated[29]; Fyn promotes B cell proliferation mediated by T-independent antigens. Whilst TIM-1 expressed by B1a cells is likely responsible for the suppressive effects of PS on atherosclerosis, we cannot exclude a role for other PS receptors expressed by B1a cells, e.g., term-like transcript 2 (TLT2) receptors[30, 31] or a role for macrophages in also activating peritoneal B1a B cells. The small (~30%) increase in plasma IL-5 levels after apoptotic cell and PS liposome treatment suggests some involvement of indirect stimulation of B1a B cells by macrophages. B1aB cells can be activated to produce IgM by IL-5[32], a cytokine secreted by macrophages[33]. Apoptotic cell engulfment by macrophages activates liver X receptor (LXR) signalling[34]and LXR activation in macrophages induces IL-5 expression[33].

Antibody production by B1a cells appears critically dependent on their location within body cavities, including the peritoneal cavity[35]. Their activation in the peritoneal cavity by either apoptotic cells or PS liposomes markedly increases secretion of polyreactive IgM antibodies, including anti-leukocyte, anti-CD3 and anti-CD4 IgM antibodies. IgM antibodies were elevated in both plasma and within atherosclerotic lesions of treated atherosclerotic mice. Natural anti-leukocyte as well as anti-CD3 and anti-CD4 IgM antibodies inhibit both T-cell activation and chemotaxis[36]. Our findings of reduced numbers of CD4+ and CD8+ T cells within atherosclerotic lesions of mice treated with apoptotic cells or PS liposomes is consistent with such inhibitory effects of IgM antibodies on T cell activation and migration. Also, the reduction in oxidised LDL (MDA-LDL) accumulation in lesions indicates an important role for MDA-oxLDL IgM antibodies in removing oxLDL from atherosclerotic lesions. OxLDL enhances pro-inflammatory responses of macrophages including their secretion of TNF-α, IL-1β and MCP-1[37] in addition to promoting macrophage apoptosis. Our findings of reductions in lesion proinflammatory cytokines are consistent with the IgM mediated reduction in oxLDL accumulation. In addition to IgM antibodies affecting T-cell and macrophages proinflammatory responses, IgM also recognises and promotes phagocytosis of apoptotic cells as well as apoptotic microparticles[38], attenuating accumulation of post apoptotic necrotic cells in developing lesions. Our finding of significant reductions in lesion necrotic core size is consistent with such an effect of IgM within atherosclerotic lesions, together with increased expression of anti-inflammatory cytokines IL-10 and TGF-β in lesions of treated mice. Phagocytosis of apoptotic cells by macrophages markedly increases expression of both IL-10[39] and TGF-β1[40]. Thus PS activated B1a cell derived IgM markedly also alters the cytokine milieu within developing atherosclerotic lesions from one that is predominantly proinflammatory to anti-inflammatory.

Our findings indicate that B1a cells can be specifically activated by PS to attenuate atherosclerosis. Administration of PS in liposomes activates and expands peritoneal B1a cells augmenting secretion of polyreactive IgM which in turn profoundly dampens inflammation in atherosclerotic lesions. Polyreactive IgM secreted by PS stimulated B1a cells include T-cell targeting IgM antibodies, anti-oxLDL IgM antibodies that attenuate inflammation, and IgM antibodies interacting with apoptotic cells that promotes their clearance, reducing necrotic core size and increasing anti-inflammatory cytokine expression. Given these atheroprotective effects of PS from stimulated B1a cells, B1a cell activation by PS may be a useful therapeutic strategy to reduce the morbidity and mortality of atherosclerosis associated with myocardial infarction and stroke.

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Conflict of interest

None declared

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Figure 1

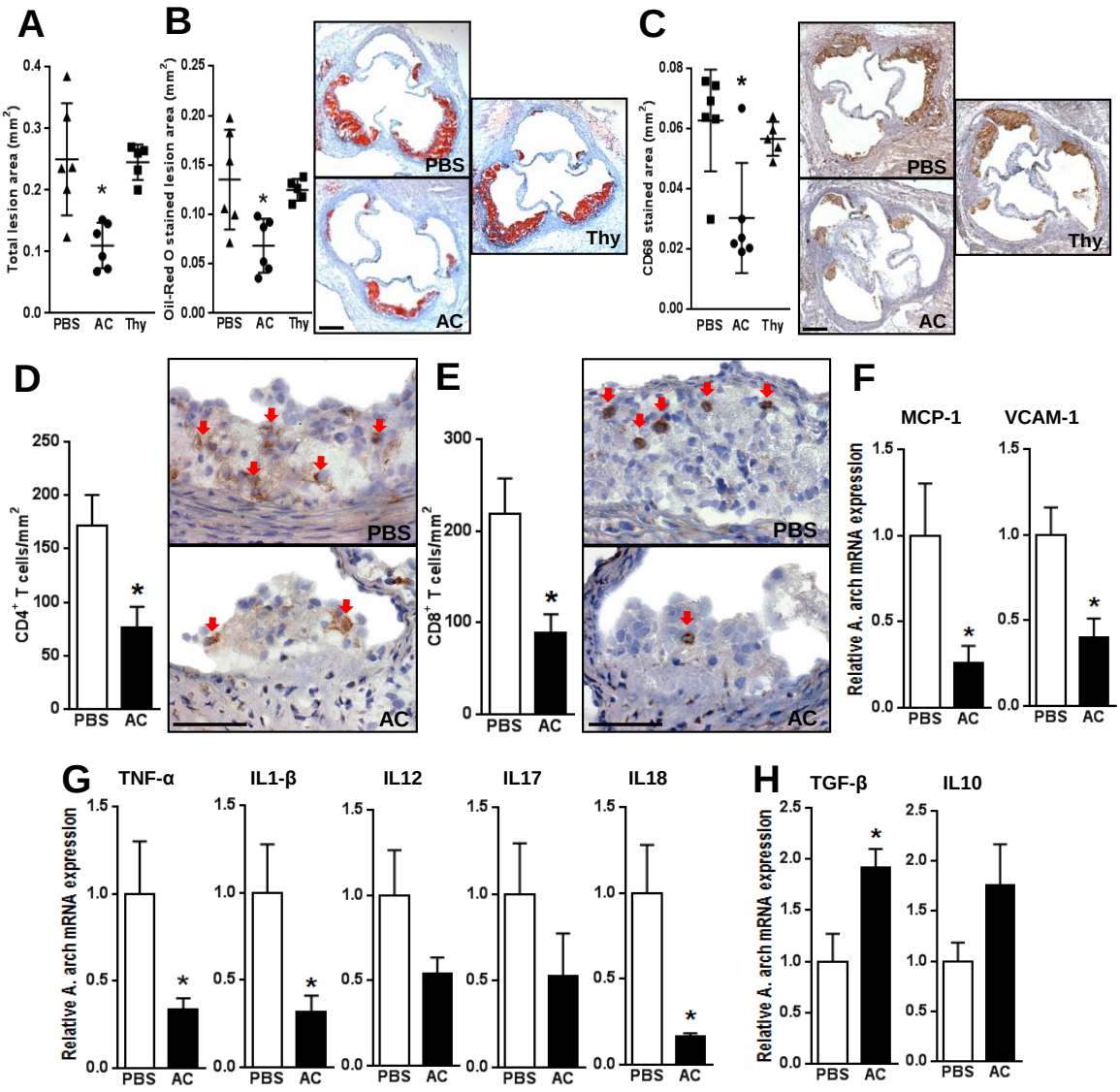

Fig 1- ACs transfer attenuated atherosclerosis development, proatherogenic leukocytes accumulation and local inflammation in atherosclerotic lesions.

Aortic sinus lesions were stained by Oil-red O for lipid content, showing (A) a reduction in total atherosclerotic lesion area and (B) lipid accumulation in ACs transferred group. Representative images showed total intimal lesion areas and ORO-stained lipid accumulation. Aortic sinus lesions were stained by anti-CD68, CD4 and CD8 Ab for macrophage, $CD4^+$ T cells and $CD8^+$ T cells accumulation in the lesions respectively. The graphs showing (C) reduction in macrophage accumulation in ACs transferred group, (D) reduction in $CD4^+$ T cells accumulation ACs transferred group and (E) reduction in $CD8^+$ T cells accumulation in ACs transferred group. Representative microimages showed $CD68^+$ macrophage, $CD4^+$ T cells and $CD8^+$ T cells accumulation. Real-time PCR analysis of arterial mRNA showed (F) reduction in mRNA expression of MCP-1 and VCAM-1 and (G) a decrease in expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and IL-18 in mice that transferred ACs compared with PBS. (H) Real-time PCR analysis of arterial mRNA also showed reduction in mRNA expression of anti-inflammatory cytokine TGF-β and IL10. Graphs represent mean±SEM, representative results of two experiments (AC: n=6, PBS: n=6 & Thy: n=6) *: $P<0.05$ compared to both controls, one-way ANOVA with Bonferroni post-test. Data represent mean±SEM, representative results of two individual experiments *: $P<0.05$ compared to PBS control, unpaired T-test.

Figure 2

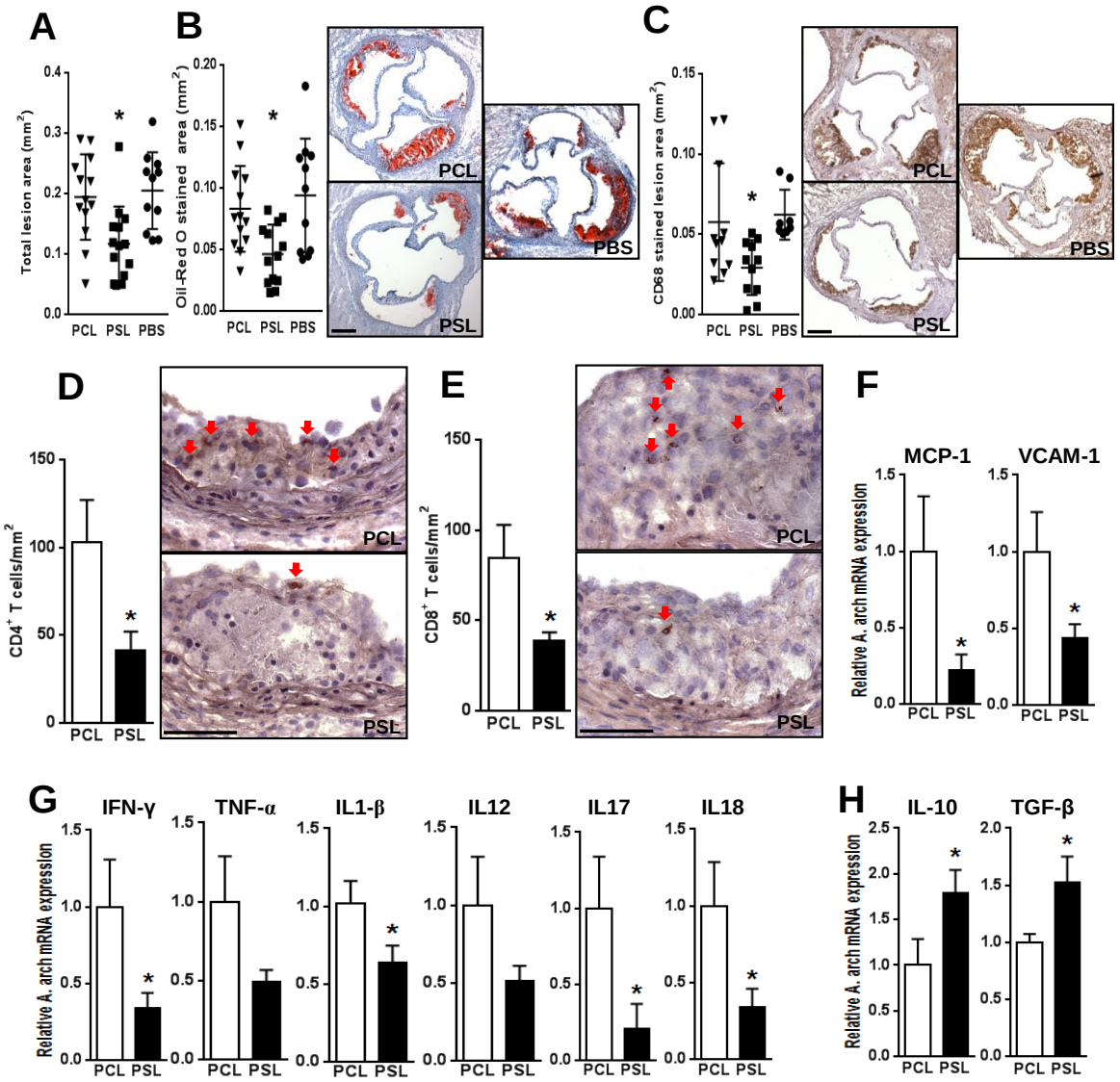

Fig 2- Phosphatidylserine liposomes treatment attenuates development of atherosclerotic lesions and reduces local inflammation.

Oil-red O staining of aortic sinus lesions for lipid content, showing (A) a reduction in total atherosclerotic lesion area and (B) lipid accumulation in PSL treated group. Representative images showed total intimal lesion areas and ORO-stained lipid accumulation. Immunostaining of aortic sinus lesions with anti-CD68, CD4 and CD8 Ab showing reduction in (C) macrophage, (D) CD4+ T cells and (E) CD8+ T cells accumulation in PSL treated group. Representative microimages showed CD68+ macrophage, CD4+ T cells and CD8+ T cells accumulation. Real-time PCR analysis of arterial mRNA showed (F) reduction in mRNA expression of MCP-1 and VCAM-1 and an increase in (G) expression of anti-inflammatory cytokines IL-10 and TGF-β. (H) Arterial expression of proinflammatory cytokines such as IFN-γ, IL-17 and IL-18 decreased in mice that treated with PSL compared with control. Graphs represent mean±SEM, representative results of three experiments (PSL: n=11, PCL: n=10 &PBS: n=11) *: P<0.05 compared to both controls, one-way ANOVA with Bonferroni post-test. Data represent mean±SEM, representative results of two individual experiments *: P<0.05 compared to PCL control, unpaired T-test.

Figure 3

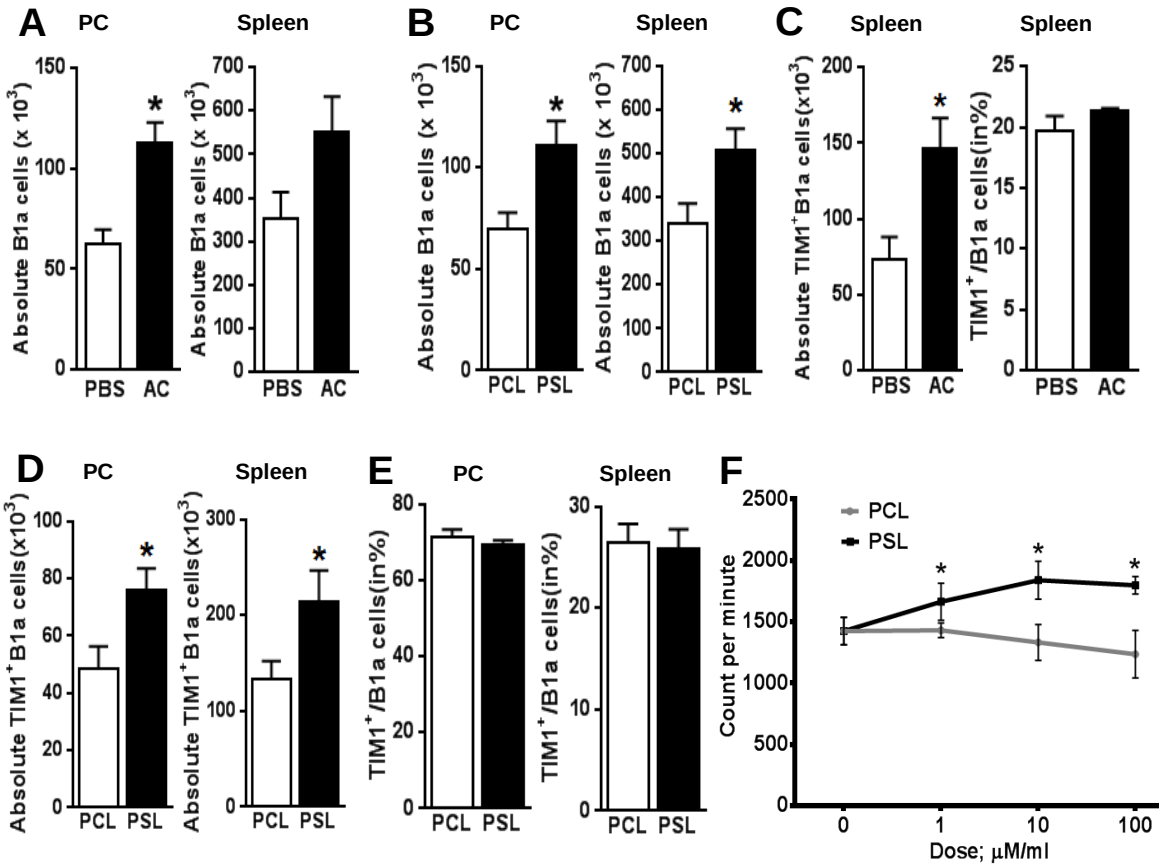

Figure 3- Liposomes containing phosphatidylserine and ACs treatment increased B1a cells expansion in peritoneal cavity and spleen.
FACS analysis of B1a cells in peritoneal cavity and spleen of ApoE-/- mice at the end point of 8WK HFD showed that (A) ACs were able to proliferate and expand B1a cells in spleen and peritoneal cavity and (B) PSL treatment also showed proliferation and expansion of B1a cells in spleen and peritoneal cavity. (C) B1a cells expressing TIM-1 were increased in spleen of ACs transferred group and in (D&E) spleen and peritoneal cavity of PSL treated mice without affecting the percentage of TIM1+ B1a cells in peritoneal cavity and spleen. In-vitro stimulation of peritoneal B1a cells by PSL revealed (F) a dose-response increase in B1a cell proliferation as assessed by 3H-thymidine incorporation. Data represent mean±SEM, representative results of two experiments for ACs study, three experiments for PSL study and two individual experiments for in-vitro experiments (PSL: n=11, PCL: n=10, AC: n=6, PBS: n=6) *: P<0.05 compared to control, unpaired T-test.

Figure 4

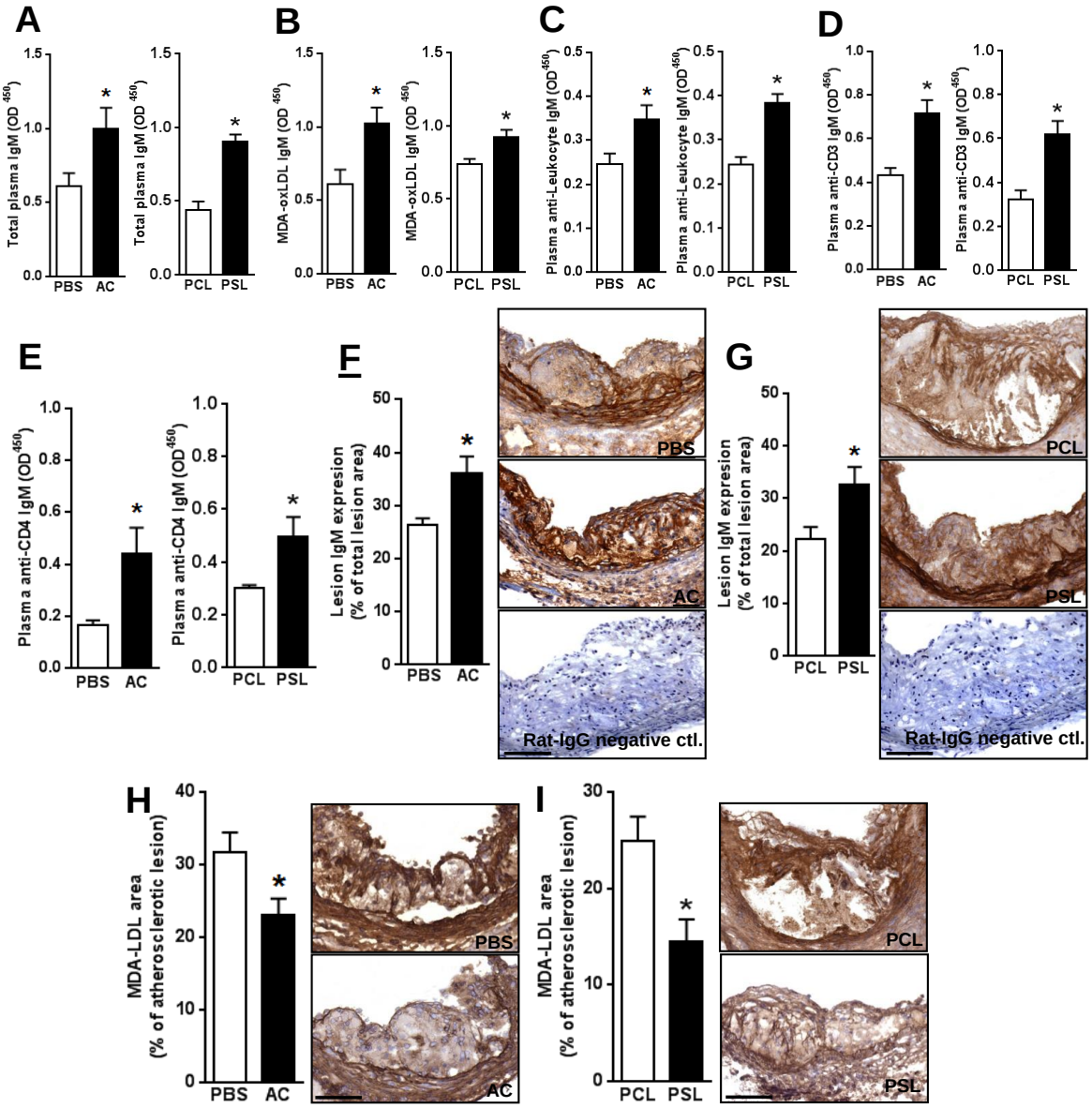

Figure 4- Treatment with PSL and ACs Increased plasma and lesions IgM
ELISA showed that PSL and ACs treatment increased (A) plasma total IgM and (B) MDA-oxLDL IgM antibodies. ELISA also showed that PSL and ACs treatment increased (C) plasma anti-leukocyte IgM, (D) anti-CD3 IgM and (E) anti-CD4 IgM antibodies. Immunohistochemical analysis of aortic sinus atherosclerotic lesions showed that increased total IgM deposits in (F) ACs transferred group and (G) PSL treated group compared with their own control. Representative microimages showed IgM deposits in the lesions. Immunostaining of aortic sinus lesions showed reduction of oxLDL accumulation in the lesions in (H) ACs and (I) PSL treated groups. Representative microimages showed oxLDL accumulation in the lesions. (PSL: n=11, PCL: n=10, AC: n=6, PBS: n=6) *: P<0.05 compared to control, unpaired T-test.

Figure 5

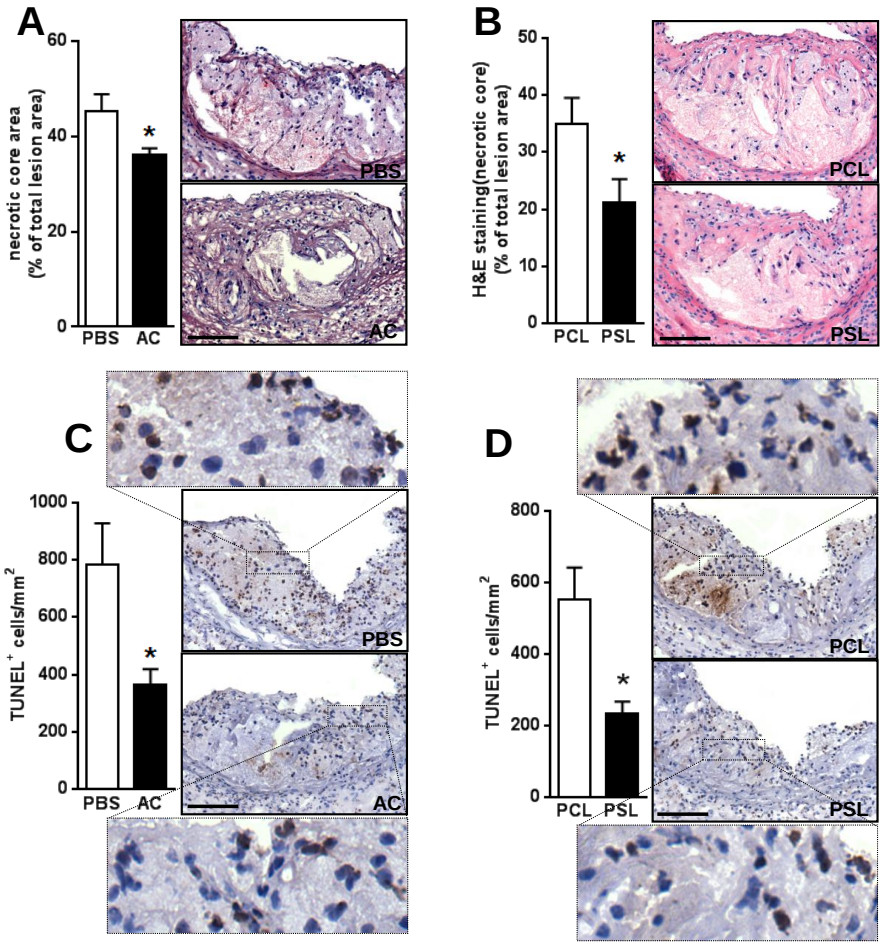

Figure 5- Mice Treated with PSL and ACs show reduced necrotic core and apoptotic cells in atherosclerotic lesions.
H&E stained aortic sinus lesions showed reduced necrotic cores of atherosclerotic lesions, identified as acellular areas in mice that received (A) ACs and (B) PSL. Apoptotic cells as TUNEL-positive cells in atherosclerotic lesions in (C) ACs and (D) PSL treated groups were reduced compared to their respective controls. Representative microimages showed necrotic core and apoptotic cells in atherosclerotic lesions. (PSL: n=11, PCL: n=10, AC: n=6, PBS: n=6) *: P<0.05 compared to control, unpaired T-test.

Figure 6

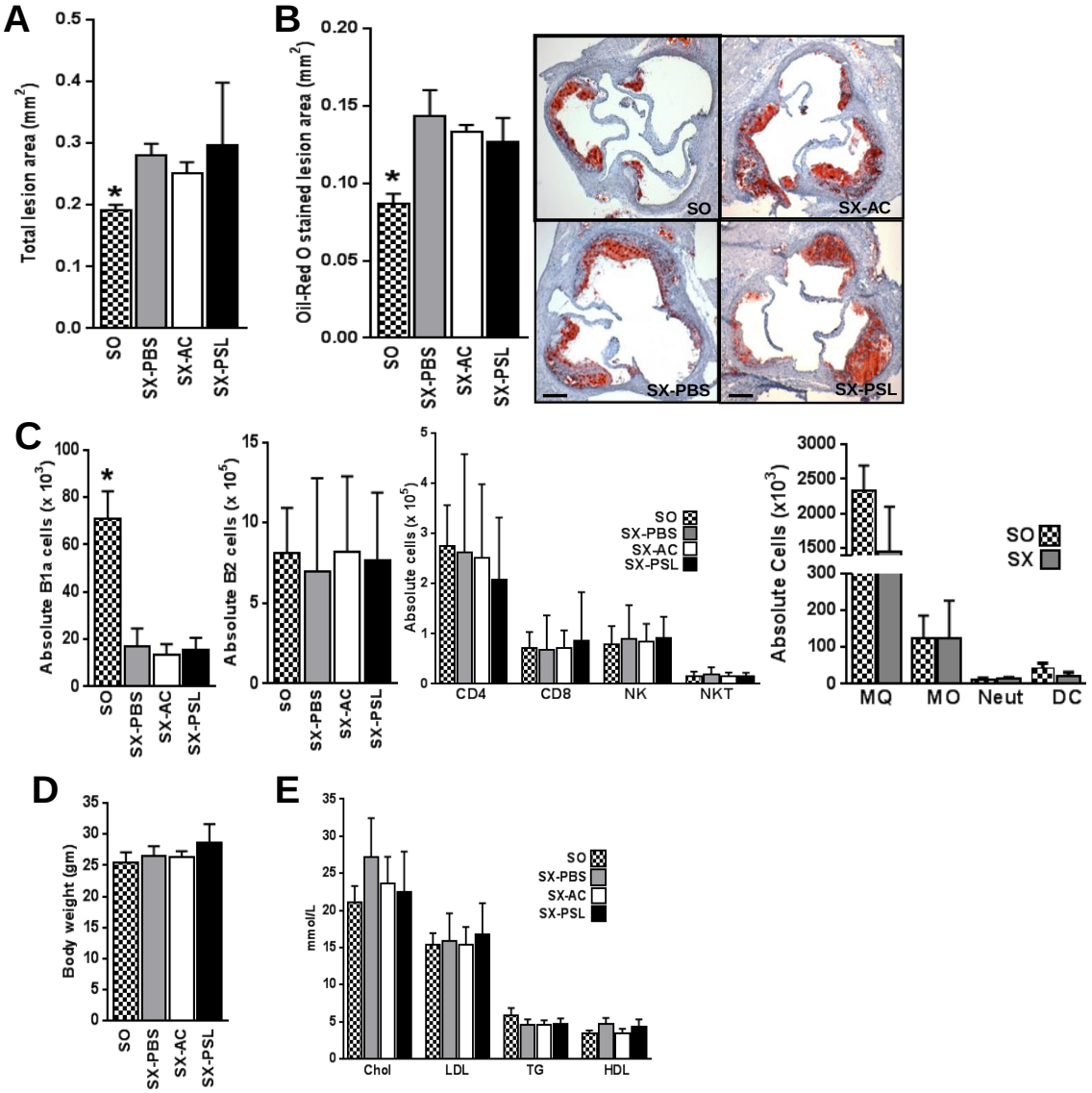

Figure 6- Figure 6- PSL and ACs treatment failed to protect against atherosclerosis development in absence of B1a cells.

Splenectomised ApoE-/- mice were received PSL and ACs as test and PBS as controls while fed a HFD for 8 weeks. Oil-red O stained for lipid content, showed no difference in (A) total atherosclerotic lesion area and (B) lipid accumulation in the splenectomised mice. Representative microimages showed total intimal lesion areas and ORO-stained lipid accumulation. FACS analysis showed (C) peritoneal B1a cells and non-B1a lymphocytes across the experimental groups after 8 weeks HFD and macrophages, monocytes, DC and neutrophils after 4 weeks HFD in splenectomised mice compared with sham operated mice. No difference in (D) body weight and (E) lipid profile was observed across the groups. Data represent mean±SEM, (SO; n=6, SX-PBS; n=7, SX-PSL; n=4, SX-AC; n=4) *: P<0.05, one-way ANOVA with Bonferroni post-test. Data represent mean±SEM, representative results of two individual experiments *: P<0.05 compared to IgG control, unpaired T-test.

Supplementary online data

Materials and Methods

Animals & Ethics

All experiment procedures approved by local animal ethics committee were carried out at the Precinct Animal Centre, Alfred Medical, Research, and Education Precinct (AMREP), Melbourne, Australia. ApoE$^{-/-}$ mice (6-8 week old male on C57BL/6 background) used in different experiments were maintained for 8 weeks under a 12 hour light/dark cycle with ad libitum sterile water and high fat diet (HFD) containing 21% fat and 0.15% cholesterol (Specialty Feeds, Glen Forrest, Western Australia). At the end of experiments, mice were culled using carbon dioxide (CO2) inhalation and tissues collected for analysis; spleen and peritoneal fluids for lymphocyte profile, aortic roots frozen in OCT embedding medium for histology and immunohistochemistry, aorta arches snap-frozen for mRNA expression and plasma for lipid and antibodies.

Generation of Apoptotic Cells

Thymus (6-8 week-old C57BL/6 mice culled by CO2 inhalation) was processed into single-cell suspension in preparation for radiation-induced apoptosis. Irradiated (6 Gy) thymocytes were incubated in complete 10% FCS DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 g/ml streptomycin, 20mM 2-mercaptoethanol for 6 hours at 37ºC under 10% CO2[1]. FACS analysis on cells stained with annexin V and propidium iodide (Invitrogen) showed >90% apoptotic (annexin V+ propidium iodide-) cells with ~2-3% post apoptotic (annexin V+ propidium iodide+) cells [2]. Apoptotic cells ($30x10^6$) were injected i.p. fortnightly starting from the beginning of 8 week HFD [2]. Viable thymocytes for injection were not subjected to irradiation.

Liposome preparation

Phosphatidylserine (PS; stearic acid at sn-1 and oleic acid at sn-2) and L-α-phosphatidylcholine (PC; palmitic acid at sn-1 and oleic acid at sn-2) were purchased from Avanti Polar Lipids (USA). PS liposomes (PSLs; PC and PS in molar ratio of 7:3) and PC liposomes (PCLs; PC only) were prepared as per manufacturer's instruction[3]. In brief, chloroform used as solvent in lipid preparation was evaporated using dry nitrogen stream, dried lipid films suspended in 1xPBS were subjected to 10 minute-sonication on ice to generate small unilamellar vesicle liposomes. Mice were treated alternate days with intraperitoneal injections of either 0.5mg/mouse PSL or 0.5 mg/mouse PCL during 8 week HFD[4].

Splenectomy

Peritoneal B1a cells were depleted by splenectomy as described[5]. Spleens of 6-8 week-old mice were removed surgically under aseptic conditions. An intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg) was given to induce anaesthesia. After confirming complete lack of reflexes, 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 haemorrhage in the abdominal cavity. Upon subcutaneous injection of Atipamezole-HCl (anaesthetic reversal 100mg/kg) and Carprofen (analgesic 5mg/kg), mice were placed in 37°C recovery chambers before they were returned to their cages. No post-splenectomy complication was observed.

In-vitro cell culture

Peritoneal B1a cells ($8x10^4$ cells) isolated from C57Bl/6 donor mice culled by CO2 inhalation[5] were cultured for 72 hours in the presence of either PSL or PCL in different concentrations. Culture condition was as follow:- 96 U-bottom plate was used to culture B1a cells in complete RPMI (RPMI + 1%PSG +10%FBS) supplemented with 2-mercaptoethanol (50μM), IL-4 (200 U/ml) and IL-5 (150U/ml) at 37ºC under 5% CO2. [3H]-thymidine was then added at a final concentration of 0.01mCi/ml (2 μCi/well; MP Biomedicals, Seven Hills, NSW, Australia) and incubated for a further 16 hours. Determination of [3H]-thymidine incorporation was performed in Packard Tri-Carb 1900TR liquid scintillation analyser (Packard Inc., Ramsey, MN, USA) and cell proliferation was presented as counts per minute[6].

Flow Cytometry

Lymphocytes from spleens and peritoneal cavity were analysed as described before[7, 8]. Different flurochrome-conjugated antibodies (BD Pharmingen, USA) purchased for FACS analysis were anti-CD19 (PE or FITC), anti-CD5 (APC), anti-CD11b (APC-Cy7), anti-TIM1 (PE), anti-IgD (PE), anti-IgM (PE-Cy7 or PerCP), anti-CD21 (APC), anti-CD23 (Pacific Blue), anti-CD24 (PerCP) and anti-B220 (AmCyan), anti-CD4 (Pacific Blue), anti-CD8a (PerCP), anti-TCR-b (FITC), and anti-NK1.1 (PE-Cy7) antibodies. Data acquired on FACS Canto II (BD Biosciences) were analysed using BD FACSDiva software (BD Biosciences). Peritoneal B1a B cells were defined as $CD5^+CD19^+CD1d^{low}CD43^{low}IgM^+$ (Supplementary Figure S2); other lymphocytes were defined as follows: B cell subsets such as FO ($B220^+IgM^{low}IgD^{high}CD24^+CD21^+$), MZ ($B220^+IgM^{high}IgD^{low}CD24^+CD21^-$), T1 ($B220^+IgM^{high}IgD^{low}CD24^-CD21^-$) and T2 ($B220^+IgM^{high}IgD^{high}CD24^+CD21^+$) B cells, CD4+ T cells (CD4+CD3+), CD8+ T cells (CD8+CD3+), NK cells (NK1.1+CD3-), NKT cells (NK1.1+CD3+), Macrophages (CD11b+F4/80+), Monocytes ($CD115^{high}CD11b^{high}$ Ly6C+), neutrophils ($CD115^-$ $CD11b^+Ly6G^+$) and dendritic cells ($MHCII^+CD11c^+33D1^+$).

Histological Lesion Analysis at Aortic Roots

Aortic sinuses dissected and embedded in OCT compound (Tissue-tek, Sakura Finetek and Torrance, CA) were kept at -80°C. Atherosclerotic lesion containing aortic sinus frozen sections (6 μm in thickness) were used to assess total intimal lesion area and lipid accumulation in Oil Red-O staining and to determine necrotic core areas in hematoxylin and eosin staining as described before[5, 9, 10].

Immunohistochemical Analysis at Aortic Roots

Frozen aortic sinus sections were subjected to immunohistochemical staining to determine different immune cells or proteins. Immune cells included macrophages (anti-CD68 antibody Serotec, Raleigh, NC), CD4 T cells (anti-CD4 antibody, BD Biosciences), CD8 T cells (anti-CD8 antibody, BD Biosciences) and proteins included immunoglobulin M (anti-IgM antibody, BD Pharmigen), oxLDL antigens (anti-MDA-oxLDL antibody, Abcam, UK) whilst apoptotic cells were identified by terminal dUTP nick end-labelling (TUNEL) as described before[5, 7, 8, 11].

Enzyme-Linked Immunosorbent Assay

Plasma total IgM and anti-oxLDL specific IgM levels were determined as described before[5, 7, 12, 13]. In a modified protocol to detect anti-CD3-binding and anti-CD4-binding IgM antibodies, recombinant CD3 and CD4 extracellular domain proteins (Life technology, USA) were used as coating antigen (50µl/well of 5µg/ml prepared in 1xPBS incubated for 18 hours at 4°C on 96-well flat-bottom plates[14]. Plasma IL5 level was determined according to manufacturer's instruction using IL5 ELISA kit (elisakit.com, Australia).

Plasma anti-leukocyte IgM detection using splenic leukocytes

A modified ELISA protocol was adapted from Lobo[14, 15]. Splenocytes ($2x10^6$ cells) from C57Bl/6 mice were activated with 10µg/ml lipopolysaccharide (Sigma-Aldrich) for 24 hours at 37°C in 5% CO2. After Fc blockage, plasma samples (diluted at 1:300 in 1% BSA) were added into activated cells and incubated for 2 hours at 37°C. Then, secondary HRP-conjugated anti-mouse IgM antibody was added into the wells and incubated for 1 hour at room temperature. TMB substrate was used to develop colour development and ELISA reader was used to detect the OD at 450 nm. Cells were washed three times at completion of each incubation step (addition of 1%BSA followed by spinning the cells at 300xg for 10 minutes). New 96 U-bottomed plates prior incubated with 1% BSA to prevent non-specific binding were used in each incubation time.

Arterial RNA extraction and mRNA Expression Analysis

Snap-frozen aortic arches were processed to extract total RNAs using RNeasy fibrous tissue mini kit (Qiagen) as described before[8, 10, 11]. Extracted total RNAs were used in determination of target gene expression using one-step QuantiFast SYBR Green RT-PCR kit (Qiagen) on 7500 Fast Real-Time PCR system (Applied Biosystem) as described before[8, 10, 11]. Housekeeping gene 18S (Applied Bioscience) was used together with gene of interest to determine target gene expression in the comparative cycle threshold (Ct) method to determine target-gene expression. The oligonucleotide sequences used were

TNF-α:	Sense (S), 5'-TATGGCCCAGACCCTCACA-3'
	Antisense (AS), 5'-TCCTCCACTTGGTGGTTTGC-3';
IFN-γ:	S, 5'-TCCTCAGACTCATAACCTCA GGAA-3'
	AS, 5'-GGGAGAGTCTCCTCATTTGTACCA-3';
IL-1β:	S, 5'-CCACCTCAATGGACAGAATATCAA-3'
	AS, 5'-GTCGTTGCTTGGTTCTCCTTGT -3';
TGF-β:	S, 5'-AGCCCTGGATACCAACTATTGC-3'
	AS, 5'-TCCAACCCAGGTCCTTCCTAA-3';
MCP-1:	S, 5'-CTCAGCCAGATGCAGTTAACG-3'
	AS, 5'-GGGTCAACTTCACATTCAAAGG-3';
VCAM-1:	S, 5'-AGAACCCAGACAGACAGTCC-3'
	AS, 5'-GGATCTTCAGGGAATGAGTAGAC-3,
IL-10:	S, 5'GAAGACAATAACTGCACCCA-3'
	AS, 5'-CAACCCAAGTAACCCTTAAAGTC-3';
IL-17:	S, 5'-TTCATCTGTGTCTCTAGTGCT-3'
	AS, 5'-AACGGTTGAGGTAGTCTGAG-3';
IL-18:	S, 5'GATCAAAGTGCAGTGAACC-3'
	AS, 5'-AACTCCATCTTGTTGTGTCC-3';

IL-12: S, 5'-CGTTTATGTTGTAGAGGTGGA-3'
AS, 5'-GTCATCTTCTTCAGGCGT-3'

Lipid Profiles

Plasma samples diluted in normal saline were sent for lipid profiling at Monash Pathology Laboratory where plasma concentration of different cholesterols and triglycerides were determined enzymatically using a cholesterol assay kit (Roche/Hitachi)[7].

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Supplementary Figures and Figure legends

Figure S1

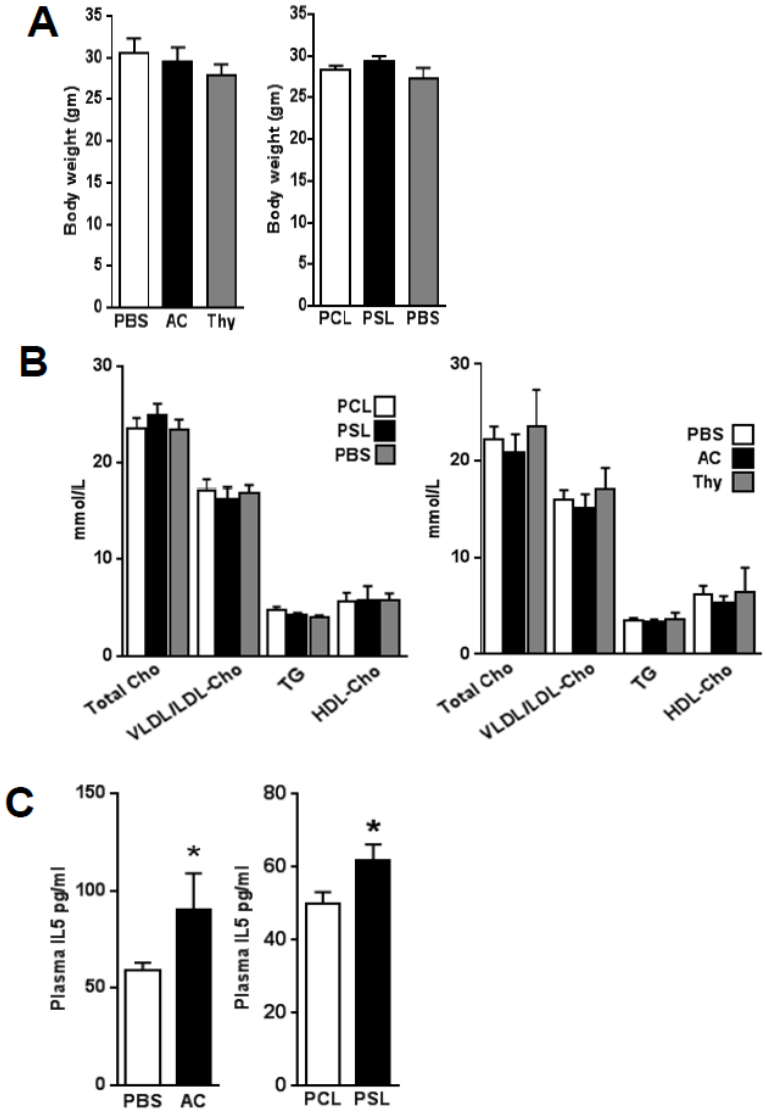

Fig S1. Body weight, lipid profile and plasma IL5 after PSL treatment or ACs transfer.
At the end point of 8 weeks HFD mice were culled and their body weight, plasma lipid profile and plasma IL5 level were determined. No difference in (A) body weight and (B) lipid profile was observed in both ACs and PSL studies. ELISA showed that (C) plasma IL5 was increased by PSL treatment and ACs transfer. Data represent mean±SEM (PSL: n=11, PCL: n=10, AC: n=6, PBS: n=6) *: $P<0.05$ compared to control (PBS or PCL).

Figure S2

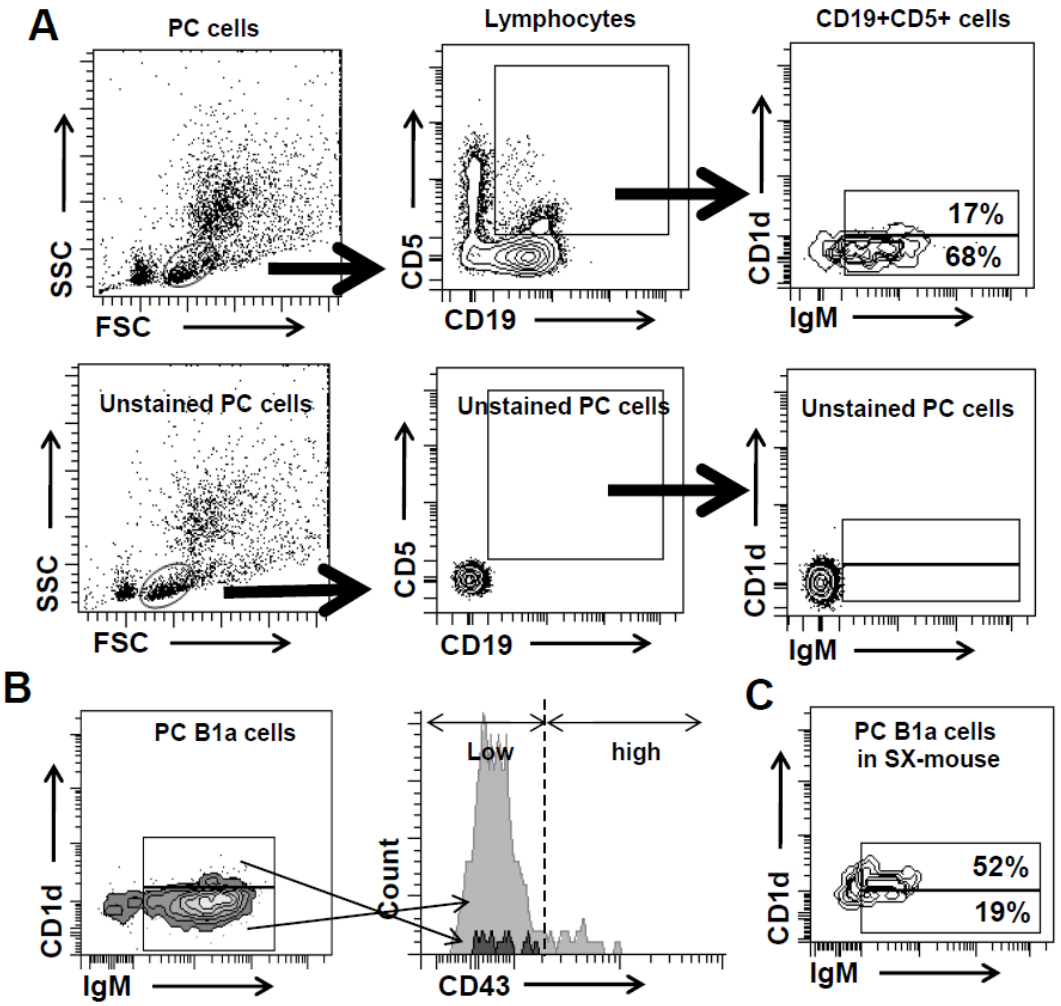

Fig S2. Peritoneal B1a B cell FACS analysis.

(A) Gating strategy to define peritoneal (PC) B1a cells. Upper panel - CD5+ CD19+ CD1d^{low}IgM+ B1a cells and lower panel – unstained PC lymphocytes for different fluorchorme-conjugated antibodies. (B) differential expression of CD43 in CD1+IgM+ and CD1d^{low}IgM+ subsets of peritoneal B1a cells. (C) significant reduction in CD1d^{low}IgM+ PC B1a cells in splenectomised mouse. All FACS dot plots/histogram were representative from multiple experiments done on different time. (n=10-20)

Figure S3

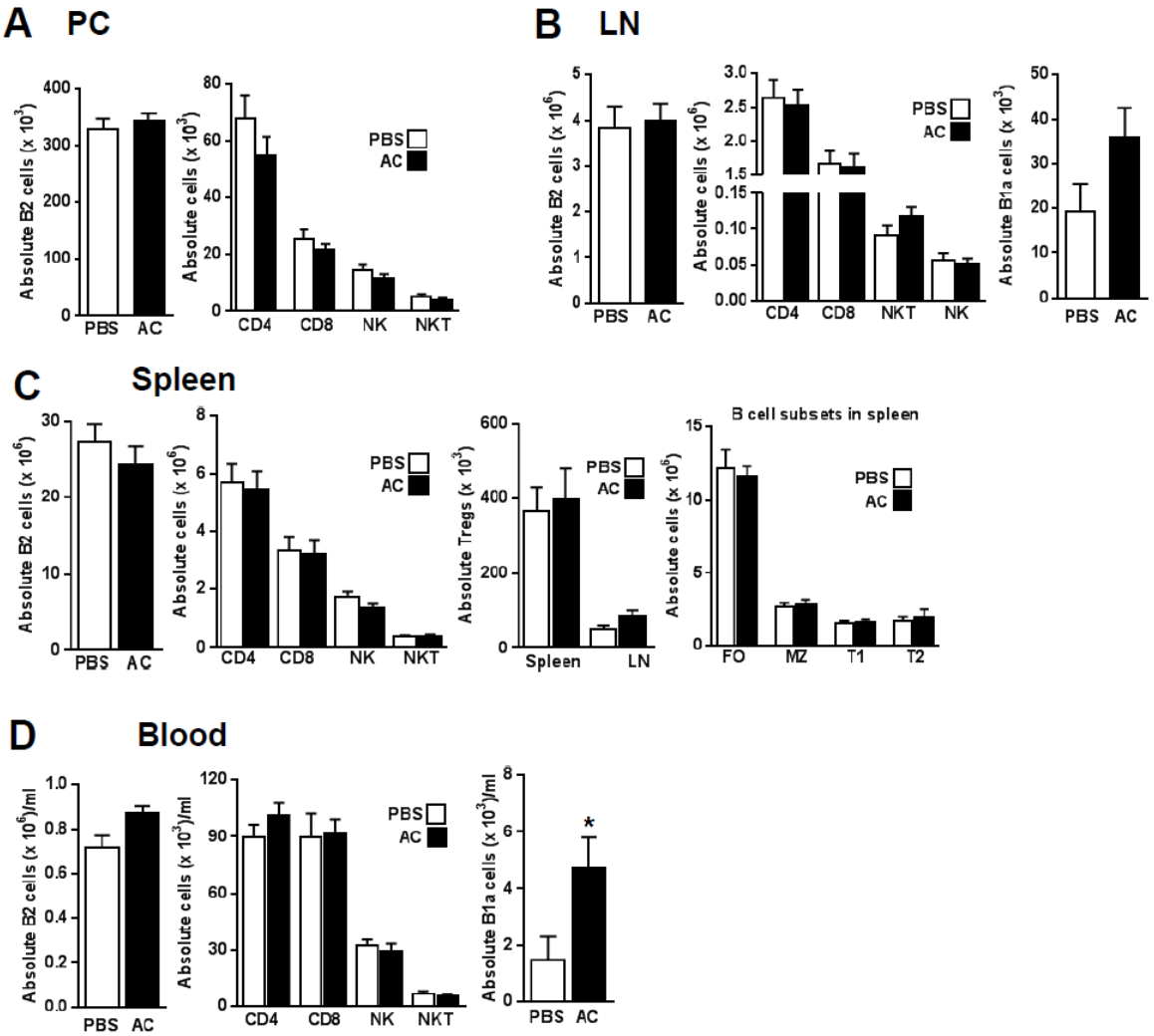

Fig S3. Lymphocyte population in peritoneal cavity, spleen, LN and blood of hyperlipidemic ApoE-/- mice treated with apoptotic cells (ACs).
FACS analysis shows B2 cells, $CD4^+$ and $CD8^+$ T cells, NKT cells, NK cells in (A) PC, (B) LN, (C) spleen and (D) blood and also B cell subsets such as FO, MZ, T1 and T2 B cells in (C) Spleen. Data represent mean±SEM (AC: n=6, PBS: n=6) *: $P<0.05$ compared to PBS.

Figure S4

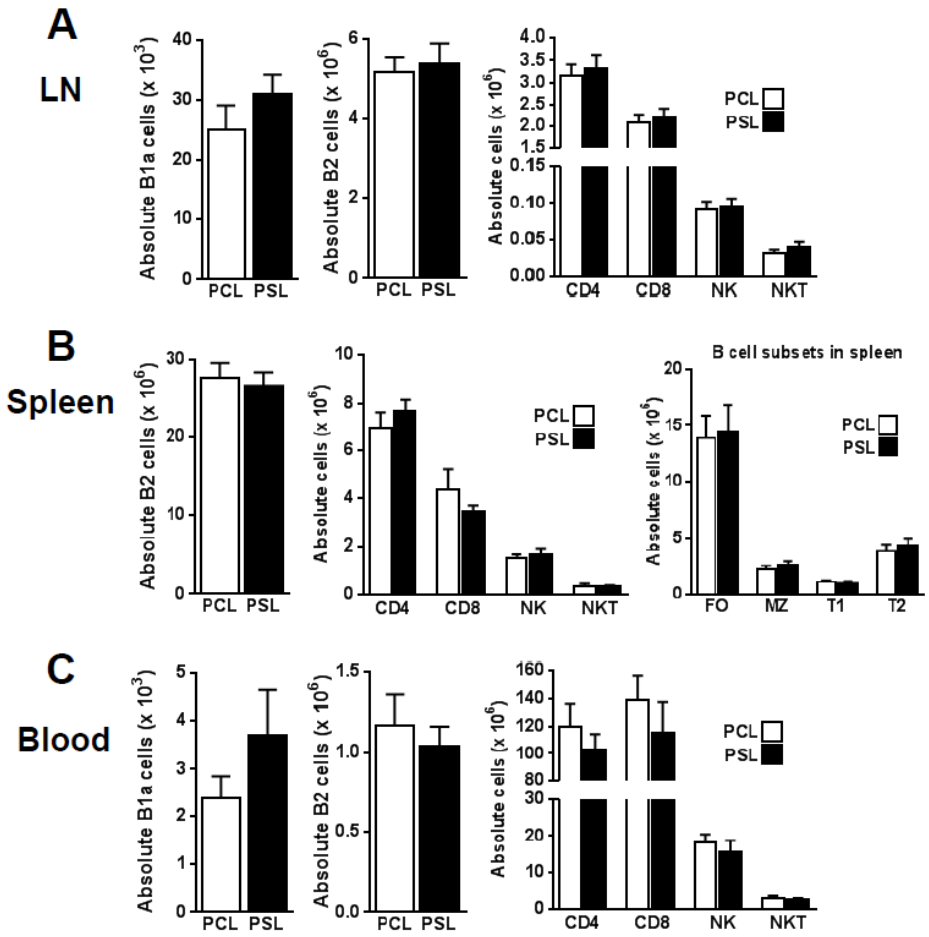

Fig S4. Lymphocyte population in LN, spleen and blood of hyperlipidemic ApoE-/- mice treated with Phosphatidylserine liposome (PSL).
FACS analysis shows B2 cells, $CD4^+$ and $CD8^+$ T cells, NKT cells, NK cells in (A) LN, (B) spleen and (C) blood and also B cell subsets such as FO, MZ, T1 and T2 B cells in (B) Spleen. Data represent mean±SEM (PSL: n=11, PCL: n=10).

Figure S5

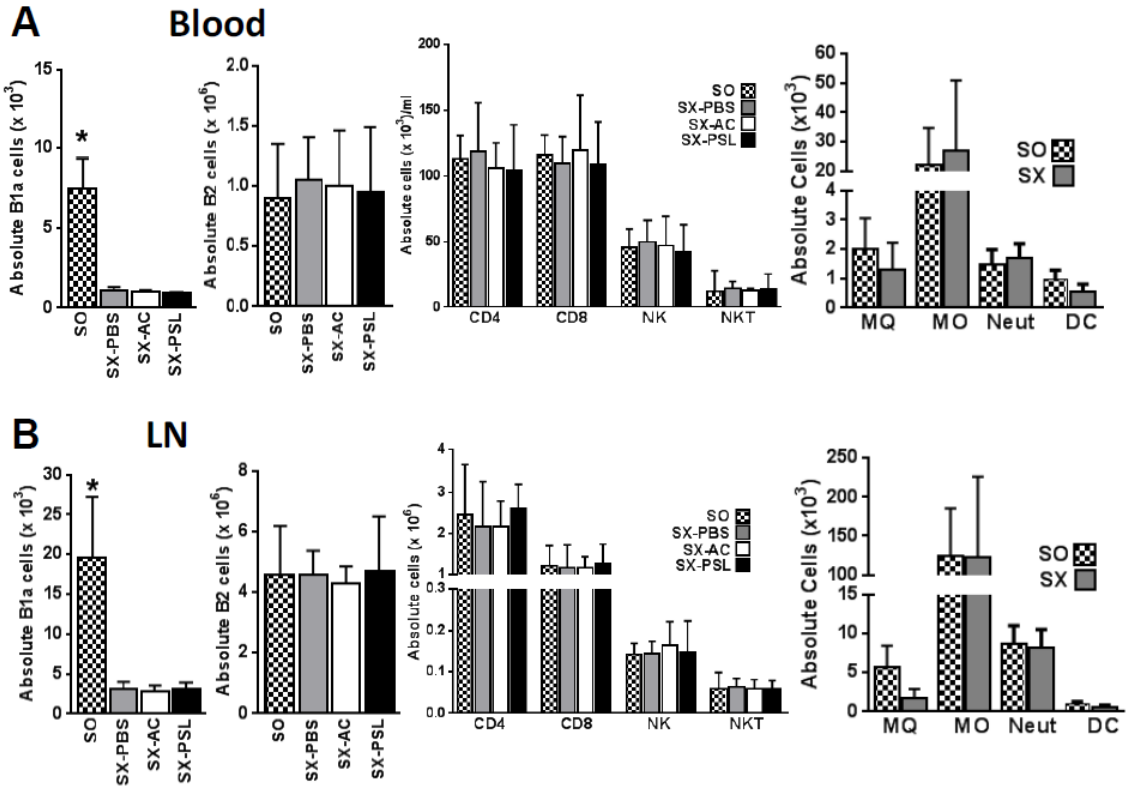

Figure S5- Leukocyte poppulation in blood and LN of splenectomised ApoE-/- mice

FACS analysis showed (A) Blood and (LN) B1a cells and non-B1a lymphocytes; B2 cells, CD4+ T cells, CD8+ T cells, NK cells, NKT cells, Macrophages (MQ), Monocytes (MO), neutrophils (Neut) and dendritic cells (DC) across the experimental groups after 8 weeks HFD and macrophages, monocytes, DC and neutrophils after 4 weeks HFD in splenectomised (SX) mice compared with sham operated (SO) mice. Data represent mean±SEM (SO; n=6, SX-PBS; n=7, SX-PSL; n=6, SX-AC; n=6) *: $P<0.05$ compared to SX groups.

Chapter 4. Anti-TIM-1 Monoclonal Antibody (RTM1-10) Ameliorates Atherosclerosis development and progression by Expansion of Atheroprotective B1a Cells

4.1 *Short Introduction*

B1a cells attenuate atherosclerosis by secreting natural IgM. Regulatory B cells expressing TIM-1 expanded through TIM-1 ligation by low affinity anti-T-cell immunoglobulin mucin domain-1 agonist monoclonal antibody (anti-TIM-1 mAb) induce tolerance. I examined the expansion of atheroprotective B1a cells using anti-TIM-1 mAb (RMT1-10) and its capacity to prevent and retard progression of established atherosclerosis. In prevention study, male ApoE$^{-/-}$ mice were treated with RMT1-10 while fed a HFD for 8 weeks to study its capacity to prevent atherosclerosis development, while in intervention study mice received a HFD for 6 weeks prior their treatment with RMT1-10 for another 8 weeks while fed a HFD to determine whether treatment attenuated already developed atherosclerosis. TIM-1^{+}IgM^{+} B1a cells and TIM-1^{+}IgM^{+}IL-10^{+} B1a cells were selectively expanded by RMT1-10 treatment. These effects reduced lesion size, markedly increased plasma and lesion IgM and decreased oxLDL in lesions. Lesion CD4^{+} and CD8^{+} T cells, macrophages and MCP-1, VCAM-1, proinflammatory cytokine expression, apoptotic cell numbers and necrotic cores were reduced. Splenectomy indicated that these effects were B1a cell-dependent. Treatment to HFD-fed ApoE-KO mice also increased TIM-1^{+}IgM^{+} B1a cells, TIM-1^{+}IgM^{+}IL-10^{+} B1a cells and IgM levels and greatly attenuated progression of established atherosclerosis. RMT1-10 treatment attenuates atherosclerosis development and progression by selectively expanding atheroprotective B1a cells. Antibody-based *in-vivo* expansion of B1a cells could be an attractive potential approach for treating atherosclerosis.

4.2 *Declaration for Thesis Chapter*

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 concept and design, Experimentation and handling, Data analysis, results interpretation, Preparation of manuscript	90

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
Peter Kanellakis	Experimentation	
Li Yi	Experimentation	5%
Christopher Tay	Experimentation	
Anh Cao	Experimentation	
Peter Tipping	Study concept, Study design, Results interpretation	
Alex Bobik	Study concept, Study design, Results interpretation,	
	Preparation of manuscript	
Ban-Hock Toh	Study concept, Study design, Results interpretation, Preparation of manuscript	
Tin Kyaw	Study design, Experimentation, results 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 13/02/15**
Main Supervisor's Signature	[redacted]	**Date 13/02/2015**

*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.

4.1 *Manuscript*

Anti-TIM-1 Monoclonal Antibody (RTM1-10) Ameliorates Atherosclerosis development and progression by Expansion of Atheroprotective B1a Cells

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TIM-1-mediated atheroprotective B1a expansion

*Equal contribution
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Background- Peritoneal B1a cells attenuate atherosclerosis by secreting natural IgM. Regulatory B cells expressing TIM-1 expanded through TIM-1 ligation by low affinity anti-T-cell immunoglobulin mucin domain-1 monoclonal antibody (anti-TIM-1 mAb) induce immune tolerance.

Objectives – We examined the expansion of peritoneal B1a cells using anti-TIM-1 mAb (RMT1-10) and their capacity to prevent and retard progression of established atherosclerosis.

Methods - Male Apolipoprotein E-knockout (ApoE-KO) mice were treated with RMT1-10 at the beginning of 8 week-high fat diet (HFD) to study its capacity to prevent atherosclerosis development. To determine whether RMT1-10 treatment attenuated developed atherosclerosis, male ApoE-KO mice were fed a HFD for 6 weeks, and then treated with RMT1-10 for another 8 weeks while fed a HFD.

Results- TIM-$1^+$$IgM^+$ B1a cells and TIM-$1^+$$IgM^+$$IL\text{-}10^+$ B1a cells were selectively expanded by RMT1-10. These effects reduced atherosclerotic lesion size, increased plasma IgM and lesion IgM deposits and decreased oxidatively modified Low Density Lipoprotein (oxLDL) in lesions. Lesion $CD4^+$ and $CD8^+$ T cells, macrophages and monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), proinflammatory cytokine expression, apoptotic cell numbers and necrotic cores were also reduced. Splenectomy (SX) that reduces Ba cells indicated that these effects are B1a cell-dependent. Of ApoE KO mice fed a HFD for 6 weeks and then treated with RMT1-10 also increased TIM-$1^+$$IgM^+$ B1a cells, TIM-$1^+$$IgM^+$$IL\text{-}10^+$ B1a cells and IgM levels and attenuated progression of established atherosclerosis.

Conclusions- RMT1-10 treatment attenuates atherosclerosis development and progression by selectively expanding atheroprotective B1a cells. Antibody-based *in-vivo* expansion of B1a cells could be an attractive approach for treating atherosclerosis.

Key Words: TIM-1; B1a cells; IgM; IL-10; RMT1-10; atherosclerosis;

Abbreviations and Acronyms

ApoE-KO = Apolipoprotein E-knockout
oxLDL = Oxidatively modified Low Density Lipoprotein
HFD = High Fat Diet
RMT1-10 = low affinity anti-TIM-1 monoclonal antibody
TIM-1 = T-cell immunoglobulin mucin domain 1
MCP-1 = monocyte chemoattractant protein-1
VCAM-1 = vascular cell adhesion molecule-1

In atherosclerosis, we have shown that peritoneal B1a cells are atheroprotective (1) and proposed that therapeutic expansion of atheroprotective B cells might be an approach to reduce atherosclerosis.(2) Their atheroprotective effect is associated with B1a-derived natural IgM which accumulates in atherosclerotic lesions to reduce apoptotic cell numbers and necrotic core size, thereby reducing the severity of inflammation in lesions; IgM plays a key role in recognizing phosphatidylserine on apoptotic cells, facilitating apoptotic cell removal by phagocytosis.(3) B1a cells including the $CD19^+CD5^+CD1d^+$ subset also produce IL-10.(4) B1a-derived IL-10 has been shown to suppress inflammatory disorders including arthritis, allergy, ulcerative colitis and experimental autoimmune encephalomyelitis by regulating $CD4^+$ Th1, Th2 and Th17 cell responses.(5-7) Thus B1a-derived IL-10 might also contribute to suppression of atherosclerosis as IL-10 is a potent anti-atherogenic cytokine.(8) Despite the importance of B1a cells in suppressing inflammatory disorders, its translation towards potential therapeutic strategies to control inflammatory disorders such as atherosclerosis has not hitherto been investigated.

TIM-1 is a member of the TIM family of cell surface phosphatidylserine receptors in human and in mice (9) that directly couples to phosphotryosine-dependent intracellular signalling pathways (10) and it provides a costimulatory signal for T cell activation.(10) However it has recently been found to be predominantly expressed on regulatory B cells, including the large majority of $CD5^+IgM^+$IL-10-expressing B cells.(11) Ligation of TIM-1 by a low affinity RMT1-10 promotes immune tolerance via IL-10-expressing B cells.(11) Treatment also increases $TIM\text{-}1^+$ B cell numbers and the percentage of $TIM\text{-}1^+$ B cells expressing IL-10 and IL-4,(11) preserving T-regs but inhibiting $CD4^+$ Th1 cell expansion.(12)

In the present study, we sought to investigate whether RTM1-10 treatment expands the B1a cell population and is effective in attenuating both development and progression of atherosclerosis. Here we show that a large population of B1a cells in the peritoneal cavity express TIM-1 and treatment with RTM1-10 markedly increases the number of peritoneal B1a cells expressing $TIM\text{-}1^+IgM^+$ and $TIM\text{-}1^+IgM^+IL\text{-}10^+$. The expansion of B1a cells attenuates both atherosclerosis development and progression of developed atherosclerosis, associated with increased plasma IgM and their deposits in lesions and decreased lesion oxLDL. This effect of RTM1-10 treatment on atherosclerosis is dependent on B1a cells because it is not seen in B1a cell-depleted splenectomised mice.

Materials and Methods

Animals & Ethics

All animal procedures were approved by Animal Ethics Committee of the Alfred Medical, Research, and Education Precinct (AMREP), Prahran, Melbourne, Australia. $ApoE^{-/-}$ on C57BL/6 back ground were bred and maintained at Precinct Animal Centre, AMREP.

Male $ApoE^{-/-}$ mice (6-8 week old) were fed a high-fat diet (HFD) containing 21% fat and 0.15% cholesterol (Specialty Feeds, Glen Forrest, Western Australia). At the end of experiments, mice were culled and spleen, lymph node (LN) and peritoneal fluids collected for differential cell analysis by flow cytometry; aortic roots frozen in OCT embedding medium, aorta arches snap-frozen and plasma kept in a -80°C freezer for subsequent analysis.

TIM-1 monoclonal antibody treatment
Rat anti-mouse TIM-1 IgG2a RMT1-10 (BioXcell, USA) was used to stimulate TIM-1 receptor and controlled with an isotype-matched control rat anti-mouse IgG2a Ab (Sigma).(12,13) In prevention study, ApoE-/- mice received either TIM-1 Ab or IgG control intera-peritoneally (0.2mg/mouse) (13,14) on alternate day while fed a HFD for 8 weeks. In intervention study, ApoE-/- mice were fed a HFD for 6 weeks to develop established atherosclerosis and then treated with the same regimen of either TIM-1 Ab or IgG control while they were fed a HFD for a further 8 weeks.

B1a cells depletion using Splenectomy
In splenectomy experiments, spleens of 6-8 week-old ApoE-/- mice were removed surgically under aseptic conditions to remove peritoneal B1a cells as previously described.(1,15,16) Briefly, under anesthesia with an intraperitoneal injection of ketamine (80 mg/kg) andxylazine (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 haemorrhage 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. SX-ApoE-/- mice received either TIM-1 Ab or IgG control (0.2mg/mouse) or PBS intera-peritoneally on alternate day while fed a HFD for 8 weeks. Sham-operation was performed according to the splenectomy procedure but without removing the spleen.(1)

Plasma Lipid Profiles
Blood was collected by cardiac puncture whilst the mice were under pentobarbitone sodium (80mg/kg i.p.) induced anaesthesia. Plasma was obtained after centrifugation and stored at 20°C. Plasma concentration of total cholesterol, high-density lipoprotein cholesterol, very-low-density lipoprotein/LDL (VLDL/LDL) cholesterol, and triglycerides) were determined enzymatically using a cholesterol assay kit (Roche/Hitachi) and automated chemistry analyser.(17)

Flow Cytometry
B lymphocytes and non-B-lymphocyte populations in spleen, LN and peritoneal cavity were analysed with fluorochrome conjugated antibodies (from BD Pharmingen, San Diego, CA unless otherwise stated) on a BD FACS-Canto II (BD Biosciences) as described.(1,17,18) For B cells, PE-conjugated anti-CD19, APC-conjugated anti-CD5, and APC-Cy7–conjugated anti-CD11b Abs were used. For non-B lymphocyte populations, Pacific Blue-conjugated anti-CD4, PerCP-conjugated anti-CD8a, APC-Cy7–conjugated anti-CD25, PE-conjugated anti-Foxp3 (eBioscience), FITC- conjugated anti-TCR-b, and PE-Cy7–conjugated anti-NK1.1 Abs were used. To stain for intracellular IL10, cells were stimulated for 5-6 hours with Cell Stimulation Cocktail plus Protein Transport Inhibitors (eBioscience, San Diego, CA). After blocking Fc receptors, surface markers were first stained with PE-conjugated anti-TIM1, APC-conjugated anti-CD5, FITC-conjugated anti-CD19 and PerCP-conjugated anti-IgM antibodies. Then fixed and permeablized cells were stained with Pacific Blue-conjugated anti-IL-10 antibody. Data acquired on FACS-Canto II (BD Biosciences) were analysed using BD FACS-Diva software (BD Biosciences).

Histological Lesion Analysis at Aortic Roots

Aortic root (proximal aorta) were dissected from mice and embedded in OCT compound (Tissue-tek, Sakura Finetek and Torrance, CA). Snap-frozen samples were kept at -80°C. Frozen sections (6 µm) were cut from the aortic sinus, the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off.(19) The aortic sinus was evaluated at this region because this part of the aorta is particularly susceptible to development of atherosclerosis in mice fed a HFD.(19) Atherosclerotic lesion containing aortic sinus frozen section were stained with oil red O and examined using light microscopy. Total intimal lesion areas and ORO-stained lipid deposition areas were 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 30mm intervals and averaged.(17) For analyses of necrotic core areas, aortic root atherosclerotic lesions were stained with haematoxylin and eosin (H&E) to identify acellular areas as necrotic cores.(1,20,21)

Immunohistochemical Analysis at Aortic Roots

CD68+ macrophage, CD4+ and CD8+ T cell accumulation, oxLDL antigen, IgM and IgG deposition and IL-1β, MCP-1 and VCAM-1 protein expression at aortic root atherosclerotic lesions were assessed by immunohistochemical analyses as described.(18,22) In brief, CD4+ and CD8+ T cells were manually counted under light microscope whilst other stained areas were quantified by Optimas software (Bedford Park). Mean values for positively stained areas and cell counts were calculated from three sections in the same way as described. Apoptotic cells identified by terminal dUTP nick end-labeling (TUNEL) (17,22) under light microscopy were expressed per lesion areas.

Enzyme-Linked Immunosorbent Assay

Plasma collected at the end of experiment were used to determine levels of total Ig, IgG and IgM levels by ELISA as described.(1,17) Anti-malondialdehyde (MDA)-oxidized (MDA)-LDL was used as coating antigen to determine oxLDL-specific IgG and IgM antibodies as described.(1,23)

Plasma anti-leukocyte IgM detection using splenic leukocytes

In a modified protocol, single-cell splenocyte suspensions were obtained from wild-type C57Bl/6 mice. Erythrocytes were removed using RBC lysing buffer (eBioscience, San Diego, CA). The cells were washed thrice with complete 10%FBS-RPMI and cell concentration adjusted to $2x10^6$ cells/0.2 ml per well. The cells were initially activated with 10µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 hours at 37°C in 5% CO2. Cells were incubated with FC blocking agent (Miltenyi Biotec, Germany) to prevent non-specific FC receptor-mediated antibody binding for 30 minutes before incubation with plasma samples (diluted at 1:300 in 1% BSA) at 37°C for 2 hours in plates pre-coated with 1% BSA. Then, secondary anti-mouse IgM Ab conjugated with HRP was added into the wells. The mix of cells with secondary anti-mouse IgM Ab were transferred into pre-coated plates with 1% BSA for 1 hour incubation and then addition of TMB substrate for colour development. Between each step cells were washed thrice with 1%BSA and the washing buffer removed by spinning the cells at 300xg for 10 minutes. ELISA to determine plasma

Plasma anti-lymphocyte IgM detection using recombinant protein

In a modified protocol, ELISAs were performed to determinate anti-CD3-binding and anti-CD4-binding IgM activity as follows: recombinant CD3 and CD4 extracellular domain proteins (Life technology, USA) were coated at 5µg/ml in 1xPBS (50µl/well) for 18 hours at 4°C on 96-well plates and subsequent steps were performed at room temperature. Wells were blocked with 1% BSA for 2 hours and sera to be tested were diluted at 1:300 in 1%BSA. Then, secondary anti-mouse IgM Ab conjugated with HRP was added into the wells, followed by addition of TMB substrate for colour development. The OD at 450 nm was read by ELISA reader and ELISA to determine plasma anti-CD3 and anti-CD4 IgM levels at end point was as described previously.(24)

Arterial RNA extraction and mRNA Expression Analysis

Total RNA from aortic arches was extracted using RNeasy fibrous tissue mini kit (Qiagen) to extract total RNA according to manufacturer's instruction and MultiRNA electrophoresis system (Shimadzu, Japan) was used to determine quantity and integrity of RNA. Single-step QuantiFast SYBR Green RT-PCR kit (Qiagen) on 7500 Fast Real-Time PCR system (Applied Biosystem) was used to measure mRNA expression in aorta arch. Target gene expression levels were analyzed using the comparative cycle threshold method with 18S rRNA primers (Applied Biosystems) and further details of method and primers were as described.(18,21,22)

Statistical Analyses

Results are expressed as means ± SEM. Comparisons between groups were carried out using Student t-test or Mann-Whitney U test, depending on whether the data was normally distributed, as assessed using the Kolmogorov-Smirnov test. For multiple comparisons, results were analysed using one-way ANOVA (after confirming normality of distribution) followed by Bonferroni post-test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Chronic RTM1-10 Treatment Expands B1a Cells

Previous studies using RTM1-10 treatment have been limited to short-term treatment.(11) We utilised a prolonged therapeutic strategy involving administration of RTM1-10 every alternate day for 8 weeks whilst ApoE-KO mice were fed a HFD. Chronic treatment with RTM1-10 doubled the number of peritoneal B1a cells ($P< 0.05$; Figure 1A-C) and whilst B1a cells in spleen tended to increase, this was not statistically significant (Figures 1C and S1A&B). TIM-1^+ B1a cells represented approximately half of peritoneal and spleen B1a cells (Figures 1A&B and S1A&B); these cells in peritoneal cavity increased nearly 3-fold following RTM1-10 treatment ($P< 0.05$; Figure 1D); smaller increases in the spleen were not significant (Figure 1D). Approximately 77%-90% of TIM-1^+ B1a cells expressed IgM (Figures 1A&B and S1A&B) and their numbers increased approximately 2-fold in the peritoneum and spleen ($P< 0.05$; Figure 1E). TIM-1^+ IL-10^+ B1a cells were a smaller population accounting for about half of TIM-1^+ B1a cells in the peritoneal cavity and spleen (Figures 1A&B and S1A&B). These were also increased, approximately 3-fold in the peritoneal cavity and spleen following treatment ($P< 0.05$; Figure 1F). TIM-1^+IgM$^+$IL-

following RTM1-10 treatment (P< 0.05; Figure 1G). In contrast to the effects on B1 total lymphocytes and B2 cell numbers in the peritoneal cavity as well as numbers o and $CD8^+$ T cells, NK and NKT cells were unaffected (Figure S2A&B).

RTM1-10 Treatment Increases IgM Plasma Levels and IgM Atherosclerotic Deposits

We next examined if RTM1-10 treatment elevated B1a-deriverd IgM levels. Consistent with the increase in B1a cell numbers, plasma levels of total IgM and MDA-oxLDL specific IgM were increased by 33% and 40% respectively by RTM1-10 treatment (P< 0.05; Figure 2A). In contrast plasma total Ig and IgG levels were unaffected, as were MDA-oxLDL-Ig and IgG levels (Data not shown). Furthermore plasma levels of anti-leukocyte, anti-CD3 and anti-CD4 specific IgM antibodies were also elevated by 78% and 100% respectively in RTM1-10 treated mice (P< 0.05; Figure 2B). As lesion IgM facilitates removal of apoptotic cells (26) and prevents accumulation of oxLDL in lesions,(1) we examined whether RTM1-10 treatment affected lesion IgM and oxLDL. RTM1-10 treatment increased lesion IgM levels by ~170% (P< 0.05; Figure 2C). MDA-oxLDL accumulation in lesions was reduced by nearly 30% (P< 0.05; Figure 2D).

RTM1-10 Treatment Reduces Atherosclerotic Lesions, Apoptotic Cells and Necrotic Core Size

We next investigated the effect of expanded B1a cells and increased IgM following RTM1-10 treatment on atherosclerosis development. Chronic RTM1-10 treatment of hyperlipidemic ApoE-KO mice attenuated atherosclerosis with 50% reduction in total lesion size. Lipid and macrophage accumulation were also markedly reduced, by 40% and 38% respectively (all P< 0.05; Figure 3 A&B). Because IgM facilitates removal of apoptotic cells,(27,28) we assessed the effects of RTM1-10 treatment on lesion apoptotic cell numbers. Treatment with RTM1-10 reduced apoptotic cell numbers in lesions by 30% (P< 0.05; Figure 3C). As apoptotic cell numbers are related to the size of the necrotic core,(29) we next assessed necrotic core size. Necrotic core in lesions of RTM1-10 treated mice were reduced by nearly 50% (P<0.05; Figure 3D). RTM1-10 treatment did not affect plasma lipids or body weights (Figure 3E-F).

RTM1-10 Treatment Reduces Arterial Inflammation

To determine whether RTM1-10 treatment reduced inflammation in developing lesions, we next assessed its effects on lesion T cell numbers and expression of proinflammatory proteins and cytokines. Immunohistochemistry was performed to assess effects on T cells as well as expression of MCP-1 and VCAM-1. RTM1-10 treatment reduced $CD4^+$ T cell numbers in lesions by 50% and $CD8^+$ T cell numbers by 35% (P< 0.05; Figure 4A&B). Also, expression of MCP-1 was reduced by nearly 40% in lesions (P< 0.05; Figure 4C) as was expression of VCAM-1, by 55% (P<0.05; Figure 4D); mRNA encoding MCP-1 and VCAM-1 was also reduced, by 43% and 40% respectively (P<0.05; Figure 4E). RTM1-10 treatment also reduced lesion expression of proinflammatory cytokines; mRNAs encoding TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17 and IL-18 were reduced, by 45%-65% (P<0.05; Figure 4F).

Anti-Atherosclerotic Effect of RTM1-10 Therapy is Dependent on B1a cells

Since TIM-1 can also be expressed on other immune cells, albeit at much lower levels,(11) we next assessed the effects of RTM1-10 on atherosclerosis

development in splenectomised ApoE-KO mice because B1a cells can be selectively depleted by splenectomy.(1,15) At the end of the study we confirmed the splenectomy-related depletion of B1a cells in the peritoneal cavity (Figure 5A). B1a cells in the peritoneal cavity were reduced by approximately 75%, averaging $1.45x10^4$ cells compared to $6.5x10^4$ cells in non-splenectomised mice ($P< 0.05$; Figure 5B), consistent with previous studies.(1,15) RTM1-10 treatment did not increase peritoneal B1a cells ($P>0.05$; Figure 5B) nor reduced atherosclerotic lesions in splenectomised ApoE-KO mice ($P<0.05$; Figure 5C&D).

Anti-TIM-1 Therapy Attenuates Progression of Developed Atherosclerosis

To determine therapeutic potential of RTM1-10 treatment on atherosclerosis, ApoE-KO mice were fed a HFD for 6 weeks after which they were treated with RTM1-10 for additional 6 weeks of HFD. RTM1-10 treatment selectively increased the number of B1a cells by 66% in the peritoneal cavity ($P<0.05$; Figure 6A) whilst the increase in the spleen did not reach statistical significance ($P>0.05$; Figure 6A). TIM-1^+ B1a cells represented approximately half of the B1a cell population and their number increased by nearly 2-fold in peritoneal cavity and by 55% in spleen ($P<0.05$; Figure 6B). TIM-1^+IgM$^+$ B1a cells increased approximately 3-fold in the peritoneal cavity and by 85% in spleen ($P<0.05$; Figure 6C). For TIM-1^+IL-10^+ B1a cells, approximately 3-fold increase was observed in the peritoneal cavity ($P<0.05$; Figure 6D), but an increase in spleen failed to reach statistical significance ($P>0.05$; Figure 6D). TIM-1^+IgM$^+$IL-10^+ B1a cells in the peritoneum and spleen were significantly increased ($P<0.05$; Figure 6E). Treatment also attenuated intimal lesion size, by 33% and lipid accumulation by 23% ($P<0.05$; Figure 6F); immunohistochemical comparisons indicated a 34% reduction in lesion macrophages ($P<0.05$; Figure 6G). IgM deposition in lesions was also increased by treatment, by 70% and accumulated MDA-oxLDL reduced by 46% ($P<0.05$; Figure S3A&B). Treatment reduced the number of apoptotic cells in lesions, by 32% ($P<0.05$) and necrotic cores by 21% ($P<0.05$; Figure S3C&D). Lesion MCP-1, VCAM-1 and IL-1β were also reduced, by 59%, 36% and 35% respectively ($P<0.05$; Figure S4A-C) as were lesion CD4+ and CD8+ T cells, by 36% and 49% respectively ($P<0.05$; Figure S4D-E).

Discussion

In a previous study we provided evidence of a protective role for B1a cells in the development of atherosclerosis.(1) However, at present there is no therapeutic strategy that harnesses the atheroprotective effects of B1a cells and adoptive transfers are not feasible due to limited availability of B1a cells. In this study we demonstrate that B1a cells express TIM-1 and that they may be therapeutically harnessed by their selective *in-vivo* expansion with RTM1-10 mAb to attenuate atherosclerosis. Treatment of mice with RMT1-10 induced sustained increase in B1a cells which attenuates not only atherosclerosis development but also progression of developed atherosclerosis. Our data demonstrate that RTM1-10 therapy may be used to attenuate both development and progression of atherosclerosis by selectively expanding the IgM$^+$ and IgM$^+$IL-10^+ B1a cells. Its effects on lesions *in-vivo* are critically dependent on B1a cells because their depletion by splenectomy abrogated the atheroprotective action of RTM1-10.

Unlike global depletion of the B cell population or the B2 cell population to attenuate atherosclerosis, we focused on expanding atheroprotective B1a cells by targeting TIM-1, to minimize perturbation of the immune system. TIM-1 is most abundantly expressed on B cells and TIM-1 ligation with RMT1-10 markedly expands IL-10

expressing spleen regulatory B cells.(11) As a substantial number of these B cells that express CD1d^{hi} CD5^{+} markers associated with regulatory B cells also express TIM-1,(11) we initially examined for TIM-1 expression by B1a cells and the extent to which these atheroprotective peritoneal B1a cells was affected by ligation with RMT1-10 in hyperlipidemic ApoE-KO mice, during atherosclerosis development. This population of CD1d^{hi} CD5^{+} regulatory B cells has also recently been found to be highly represented in peritoneal B1a cells.(30) Chronic RMT1-10 treatment doubled the number of B1a cells in the peritoneal cavity, increasing 3-fold the number of TIM-1^{+} B1a cells and within this group markedly increasing both TIM-1^{+}IgM^{+} and TIM-1^{+}IgM^{+}IL-10^{+} B1a cells in both the peritoneal cavity and spleen without altering their relative distribution. Effects were similar during treatment of already developed atherosclerosis in ApoE-KO mice. Our finding that TIM-1^{+}IgM^{+} B1a cells are much more abundant than either TIM-1^{+}IgM^{+}IL-10^{+} or TIM-1^{+}IL-10^{+} B1a cells, which are equal in number indicate that TIM-1 ligation with RMT1-10 also significantly expands TIM-1^{+}IgM^{+}IL-10^{+} B1a cells, the major B1a cell population. This expansion greatly increases plasma IgM levels and IgM accumulation in developing and developed atherosclerotic lesions whilst decreasing accumulation of oxidised LDL. IgM natural antibodies binding oxLDL protect against experimental atherosclerosis (1,31) and levels are inversely related to carotid artery atherosclerosis in humans.(32) RMT1-10 treatment appears to specifically target TIM-1^{+} B1a cells as no changes were observed in other lymphocyte populations including B2 lymphocytes; the latter is consistent with treatment not affecting plasma IgG or anti-MDA-LDL IgG levels.
The sustained selective expansion of TIM-1^{+}IgM^{+} and TIM-1^{+}IgM^{+}IL-10^{+} B1a cells in the peritoneal cavity and spleen by RMT1-10 suppressed atherosclerosis and reduced apoptotic cell numbers in lesions and necrotic core size; effects consistent with the increases in plasma and lesion IgM. Atherosclerotic mice not capable of producing IgM have greater numbers of apoptotic cells within atherosclerotic lesions.(1,31) These observations are consistent with a scavenger function for natural IgM produced by B1a cells in mopping up apoptotic debris.(33) Antigen-activated T cells are known to aggravate atherosclerosis.(34) IgM by binding ox-LDL can also modify the antigen environment, reducing the availability of such antigens to antigen presenting cells for processing and presentation to T cells.(33)
Naturally occurring anti-leukocyte IgM antibodies that are also secreted by B1a cells have been shown to modulate T cell activation and their migration.(24) Reduced CD4^{+} and CD8^{+} T cells in atherosclerotic lesion suggest that increased anti-leukocyte antibodies may have contributed to reduction of these atherogenic T cells (22,35) in atherosclerotic lesions. Anti-CD3 antibody is immunosuppressive and its therapeutic applications suggested in autoimmune diseases, tissue rejections and T cell carcinomas. Both development and progression of diabetes are effectively prevented by anti-CD3 antibody (36,37) and teplizumab (anti-CD3 antibody) improved patients with type I diabetes resulting from auto-destruction of beta cells by T cells.(38) We have shown in this study that RTM1-10 treatment not only expands B1a cells but also increases plasma levels of total and specific IgM antibodies (oxLDL, anti-leukocyte and anti-CD3).
In addition to suppressive effects of RTM1-10 treatment on T cells, macrophage accumulation in lesions was also reduced. This is associated with similar reductions in lesion MCP-1 and VCAM-1, which may contribute to the reduction in macrophage accumulation. MCP-1 is produced by vascular smooth muscle cells and

macrophages, is chemotactic for monocytes/macrophages and preventing its expression attenuates atherosclerosis development .(39) Similarly, VCAM-1 contributes to macrophage accumulation in developing lesions.(40) The reduction in MCP-1 and VCAM-1 expression is most likely the consequence of reduced T cell and macrophage activation; macrophage and T cell derived cytokines are reduced by RTM1-10 treatment and TNF-α, IL-6 and IL-1β are potent inducers of MCP-1 expression (41-43) whilst TNF-α and IL-1β are also known to induce VCAM-1 expression (44,45).

Our data clearly indicate that RMT1-10 treatment inhibits both atherosclerosis development and progression of already developed atherosclerotic lesions. Our studies also indicate that the expanded B1a cell subsets include TIM-1^{+}IgM^{+} and TIM-1^{+}IgM^{+}IL-10^{+} B1a cells and whilst many of the effects of the expanded B1a cell population can be attributed to their production of IgM, it is also possible that a component of their atheroprotective effects are mediated by IL-10. TIM-1^{+}IgM^{+}IL-10^{+} B1a cells in peritoneal cavity and spleen were also found to express CD1d (not shown) and are most likely similar, if not identical, to the recently described peritoneal and spleen B10 cells.(4,30) B10 cells (CD19^{+}CD5^{+}IgM^{hi}CD$1d^{+}$IL-10^{+}) are potent immunosuppressive cells capable of modulating Th1 CD4^{+} T cells and development of colitis (30) and other autoimmune inflammatory disorders.(46,47) In contrast to selective or global depletion of pathogenic immune cells, RTM1-10 therapy has the advantage of enhanced expansion of atheroprotective B1a cells and production of low affinity IgM antibodies without compromising the immune system. Further RTM1-10 therapy can also expect to expand other regulatory B cells (11) that may also contribute towards suppression of atherosclerosis.

Conclusion

In summary, we have demonstrated that targeting TIM-1 on B1a cells inhibits atherosclerosis development and progression by inducing expansion of two subpopulations of B1a cells, TIM-1^{+}IgM^{+} and TIM-1^{+}IgM^{+}IL-10^{+}; and increasing levels of IgM ,an important mechanisms of atherosclerosis suppression. Although the mechanism by which ligation of TIM-1 on B1a cells leads to their expansion remains to be clarified, our data indicate that expansion of B1a cells can be used as a promising therapeutic target to attenuate development and progression of atherosclerosis related vascular disorders.

Perspectives

Competency in medical knowledge: In addition to lipids that initiate and sustain atherosclerosis, immune involvement is fundamental for atherosclerosis development and progression and for the generation of unstable vulnerable plaques. B1a cells are present in relatively small numbers, but are potent immune regulators in inflammation and autoimmune diseases.
Translational Outlook 1: Since human B1 cells equivalent to mouse B1a cells have been identified, appropriate anti-TIM-1 antibody can be used to test its effect in properly designed clinical trials.
Translational Outlook 2: Further investigations are also warranted to understand the safety and efficacy of anti-human TIM-1 antibody in humans before its therapeutic approval.

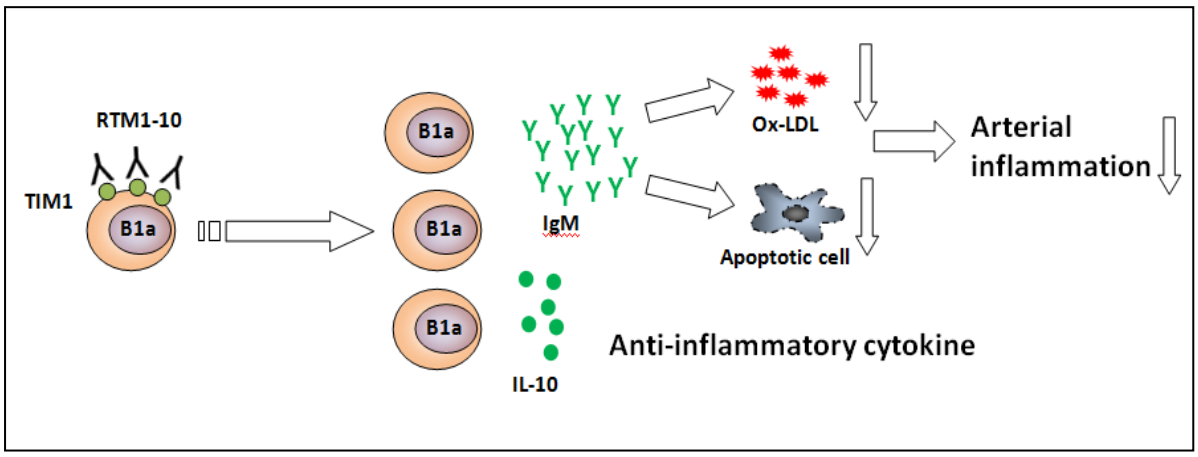

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Figure 1

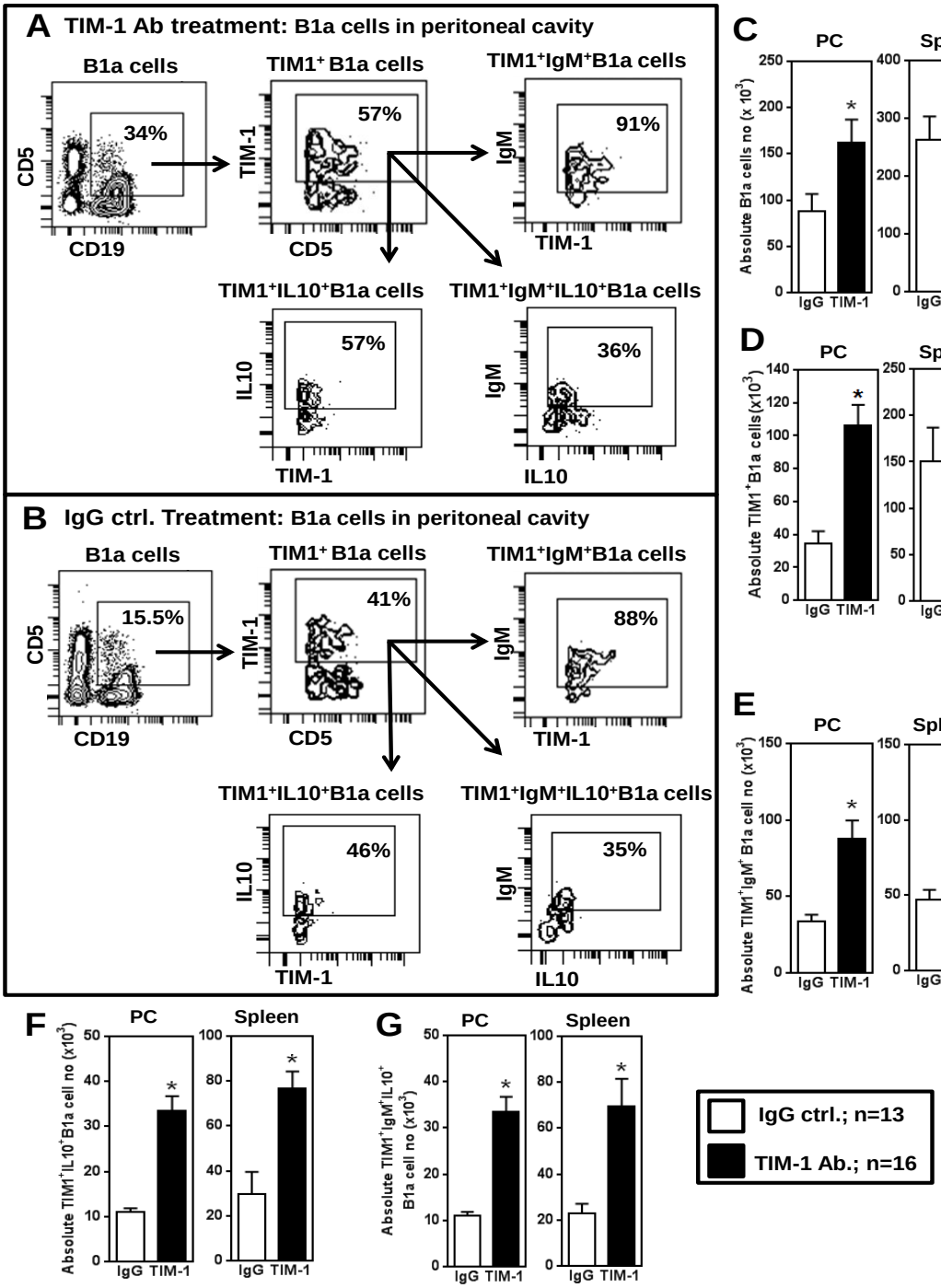


Figure 1- Peritoneal B1a cells assessed by flow cytometry shows B1a expansion in PC and spleen after RTM1-10 treatment.
Representative flow cytometry plots showed (A-B) expression of TIM-1, IgM and IL-10 on B1a cells in peritoneal cavity. FACS analysis showed an increase in (C) B1a cells, (D) TIM-1$^+$ B1a cells, (E) TIM-1$^+$IL-10$^+$ B1a cells, (F) TIM-1$^+$IgM$^+$IL-10$^+$ B1a cells and (G) TIM-1$^+$IgM$^+$IL-10$^+$ B1a cells in the spleen and PC in RTM1-10 treated group compared with IgG control group. Representative results of three individual experiments at different time points. *: P<0.05 compared to IgG control, unpaired T-test.

Figure 2

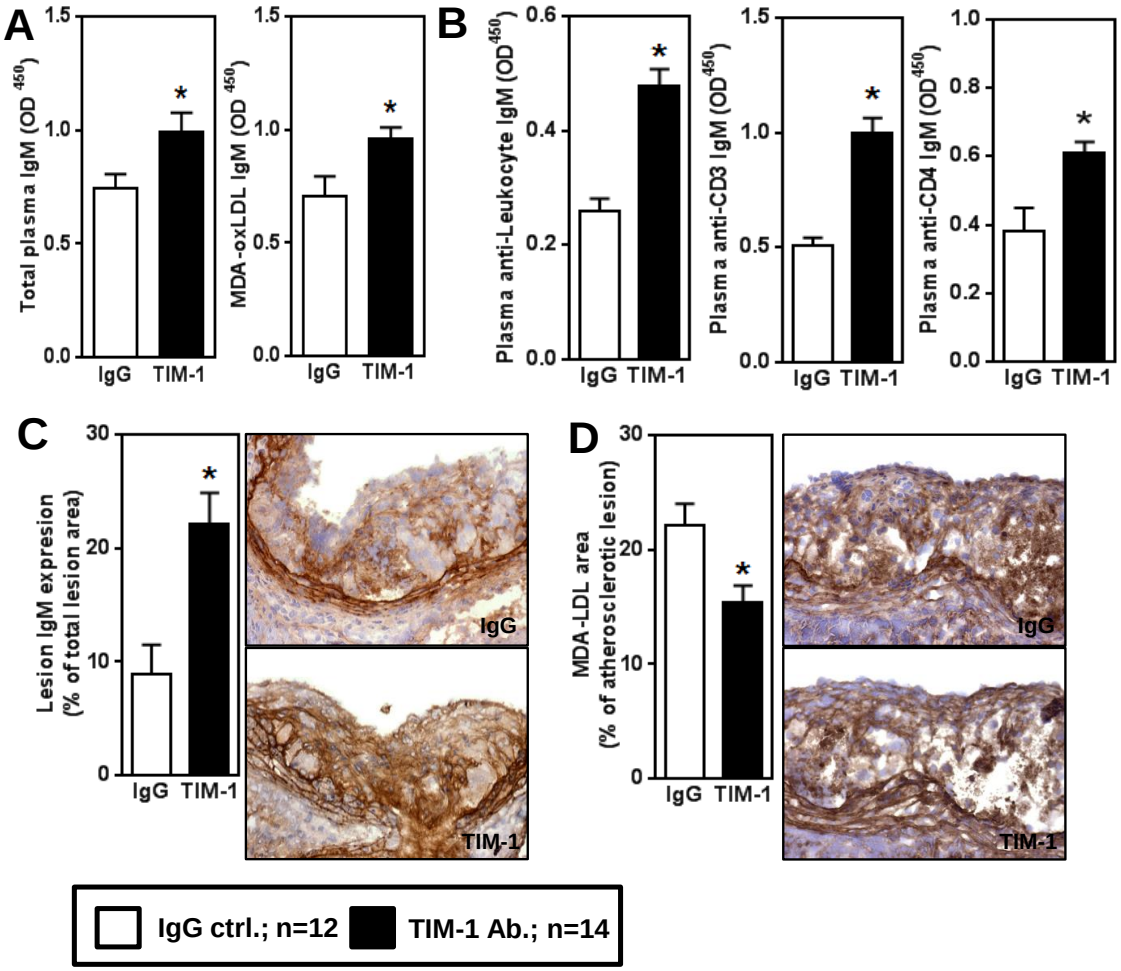


Figure 2- RTM1-10 treatment increases IgM level in plasma and atherosclerotic lesions in ApoE-KO mice fed a HFD.
ELISA showed that RTM1-10 treatment increased plasma IgM antibodies of (A) total and MDA-oxLDL and (B) anti-leukocyte, anti-CD3 and anti-CD4 antibodies. immunohistochemical analysis of aortic sinus atherosclerotic lesions showed (C) increased total IgM deposits and (D) reduced oxLDL accumulation in atherosclerotic lesions. *: P<0.05 compared to IgG control, unpaired T-test.

Figure 3

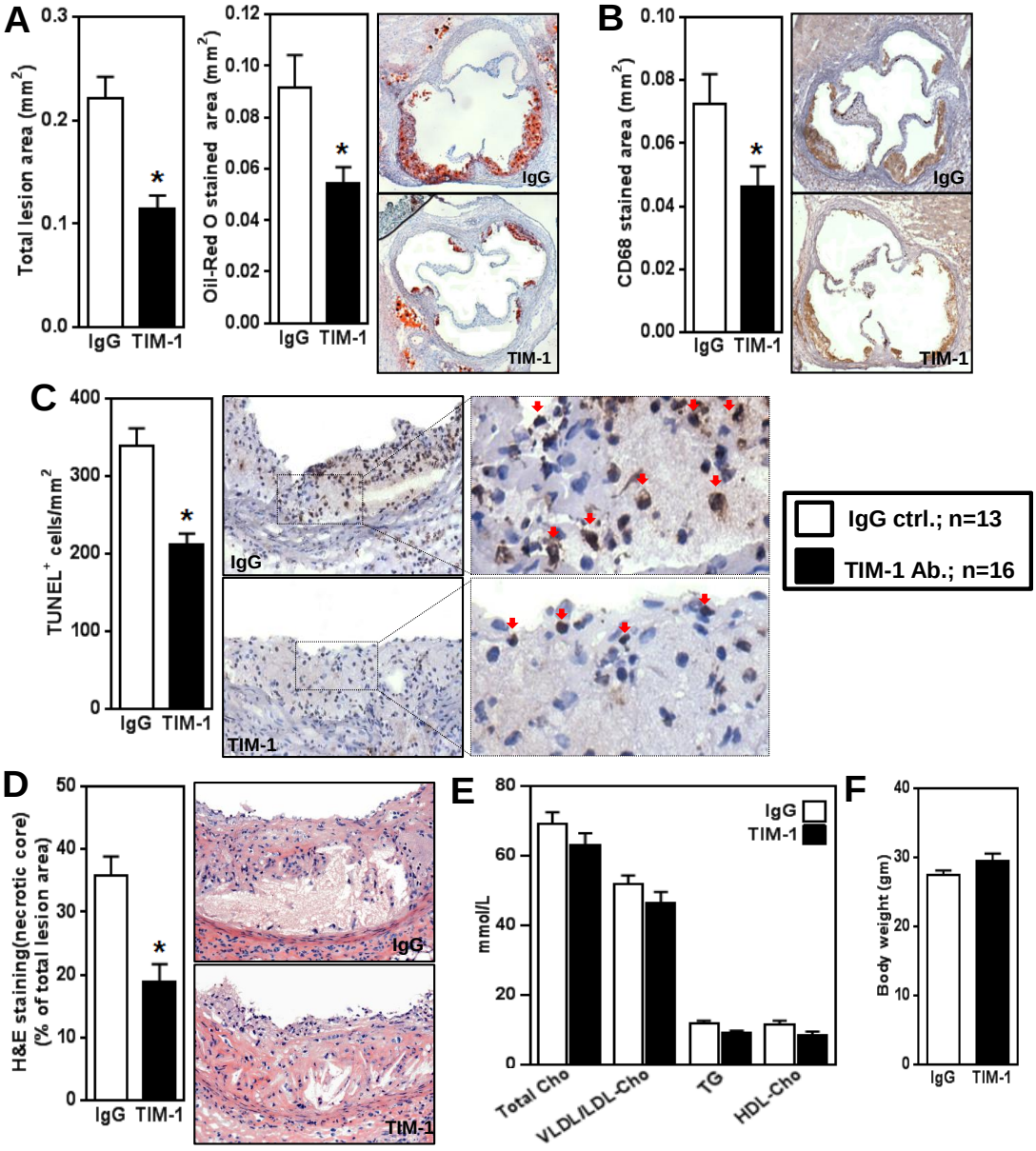


Figure 3- RTM1-10 reduces atherosclerotic lesion size, lipid and macrophage accumulation, necrotic cores and apoptotic cells.

Aortic sinus lesions stained by Oil-red O for lipid content, showed a significant reduction in (A) total intimal lesion area and lipid accumulation in RTM1-10 treated group. Aortic sinus lesions stained by anti-CD68 antibody for macrophage accumulation in lesions showed (B) significant reduction in macrophage accumulation in RTM1-10 treated group. (C) Apoptotic cells identified as TUNEL-positive cells and (D) necrotic cores of atherosclerotic lesions, identified as acellular areas in H&E stained atherosclerotic lesions were reduced in RTM1-10 treated group. No difference in (E) lipid profile and (F) body weight was observed. *: P<0.05 compared to IgG control, unpaired T-test.

Figure 4

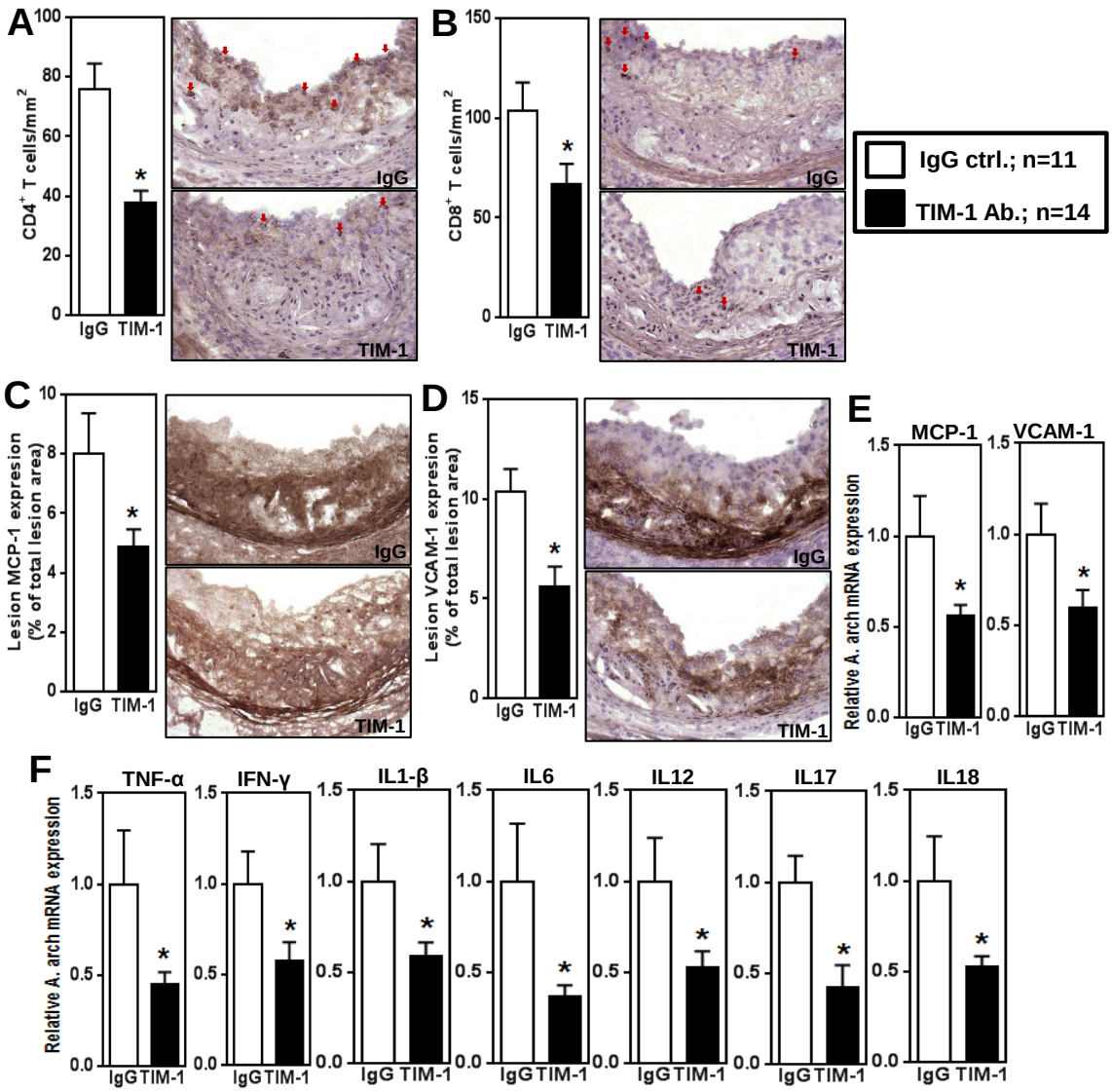


Figure 4- RTM1-10 Treatment reduces accumulation of $CD4^+$ and $CD8^+$ T cells and arterial inflammation.

Aortic sinus lesions stained with anti-CD4 Ab and anti-CD8 Ab showed reduced accumulation of (A) $CD4^+$ T cells and (B) $CD8^+$ T cells in RTM1-10 treated group. Immunohistochemical staining showed reduced expression of (C) MCP-1 and (D) VCAM-1 adhesion molecules in RTM1-10 treated mice. Real-time PCR analysis using arterial mRNA extracted from aorta arch showed significant reduction in mRNA expression of (E) adhesion molecules and (F) proinflammatory cytokines in RTM1-10 treated group. *: $P<0.05$ compared to IgG control, unpaired T-test.

Figure 5

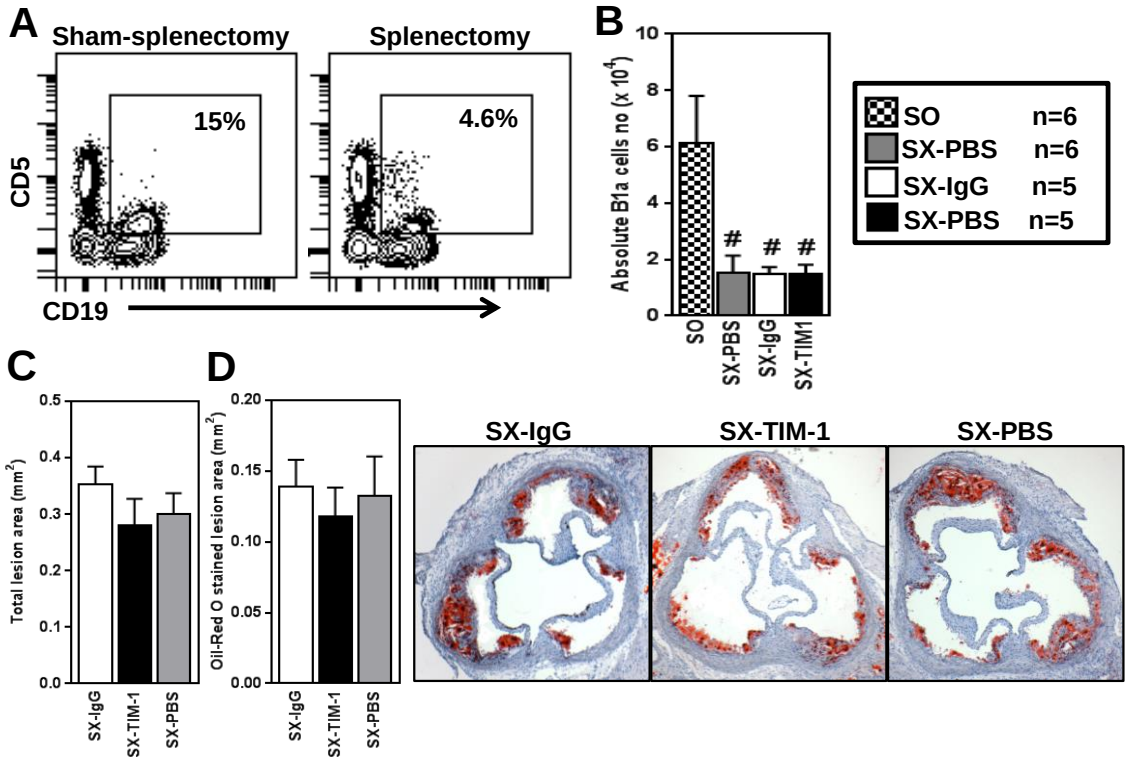


Figure 5- RTM1-10 treatment fails to protect against atherosclerosis development in the absence of peritoneal B1a cells.
Representative FACS plots show (A) a reduction of B1a cells in splenectomised mice. Splenectomised ApoE-KO mice received RTM1-10 while fed a HFD for 8 weeks. FACS analysis showed that (B) reduced peritoneal B1a cells in all splenectomised mice. Aortic sinus lesions stained by Oil-red O for lipid content, showed no difference in (C) total atherosclerotic lesion area and (D) lipid accumulation in splenectomised mice. #: P<0.05 compared to SO group, one-way ANOVA with Bonferroni post-test.

Figure 6

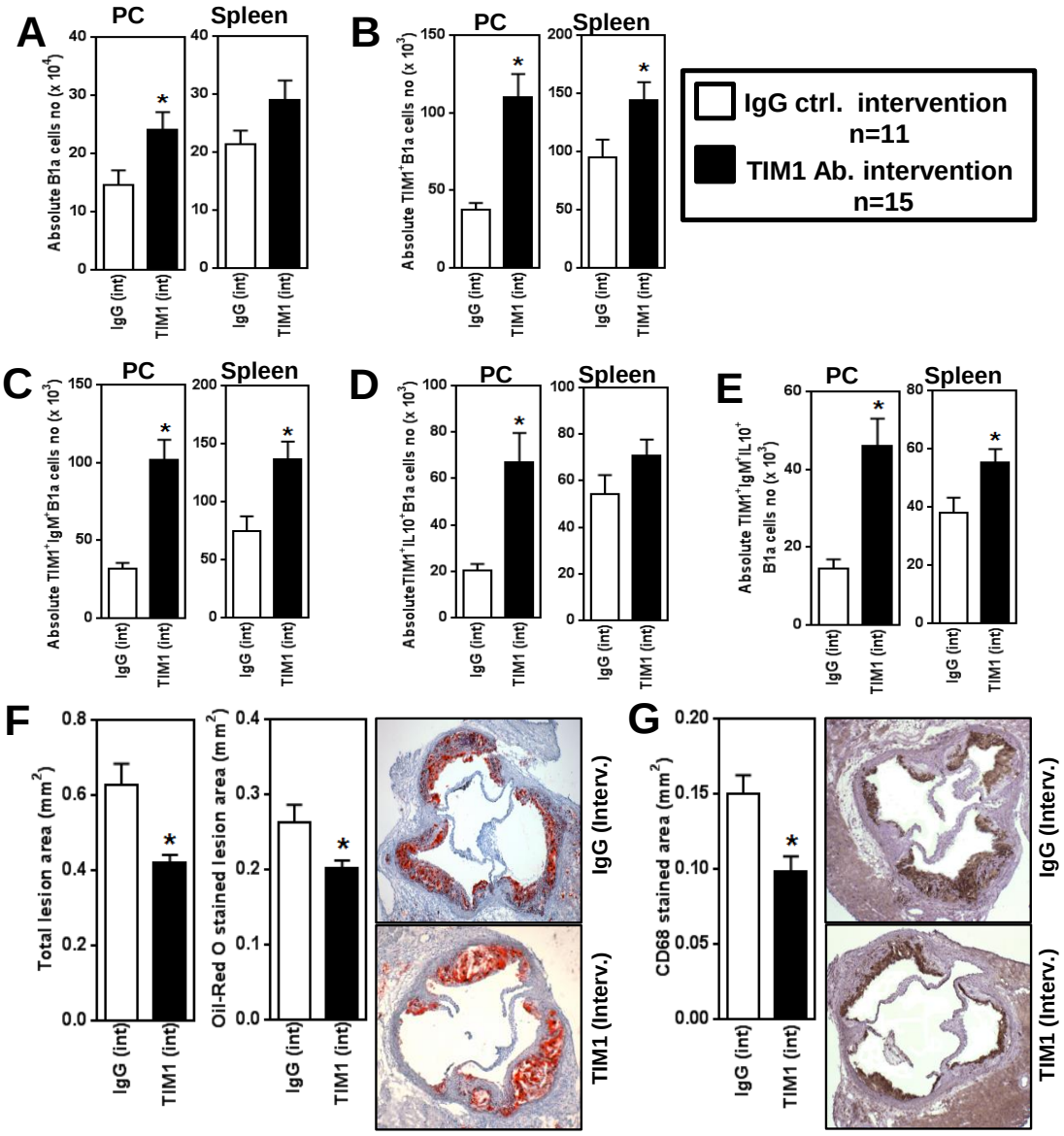


Figure 6- RTM1-10 reduces atherosclerotic lesions in established atherosclerosis. ApoE-KO mice were fed a HFD for 6 weeks before receiving 0.2mg RTM1-10 (Rat IgG2a) or 0.2 mg IgG (Rat IgG2a) intraperitoneally alternate days while fed a HFD for another 6 weeks. FACS analysis showed an increase in number of (A) B1a cells, (B) TIM-1$^+$ B1a cells, (C) TIM-1$^+$IgM$^+$ B1a cells, (D) TIM-1$^+$IL-10$^+$ B1a cells and (E) TIM-1$^+$IgM$^+$IL-10$^+$ B1a cells in the PC and spleen in treated group compared to control group. Oil-Red O-stained aortic sinus lesions showed significant reduction in (F) total intimal lesion area lipid accumulation in treated mice. Immunohistochemical analysis of aortic sinus lesions stained by anti-CD68 antibody for macrophage accumulation showed (G) a significant reduction in macrophage accumulation in RTM1-10 treated group. *: P<0.05 compared to IgG control, unpaired T-test.

Figure S1

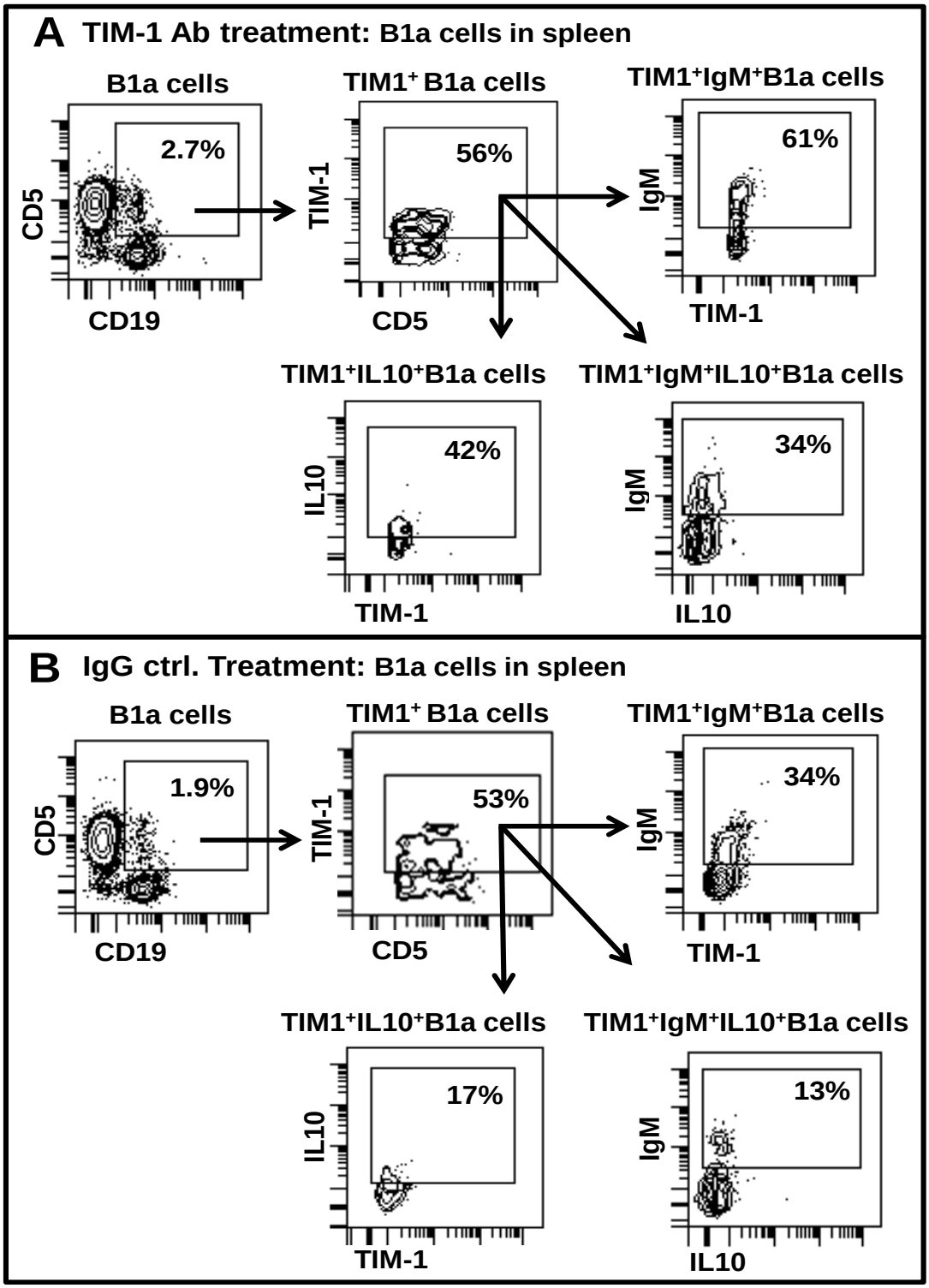


Figure S1- B1a cells and B1a cells subclasses expansion in spleen after TIM-1 mAb treatment

Compared to Figure1, representative flow cytometry plots show a comparable increased expression of TIM-1, IgM and IL10 on B1a B cells in spleen.

Figure S2

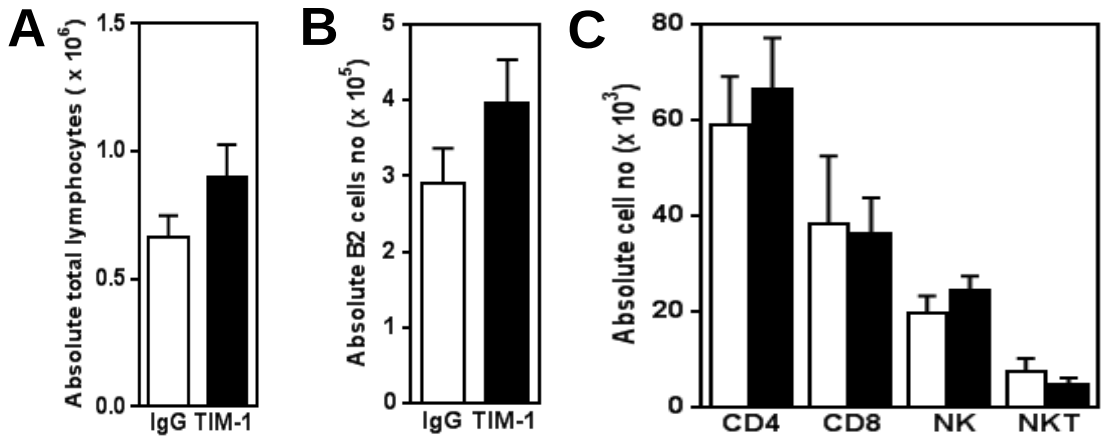


Figure S2- Non-B1a B cell population with TIM-1 mAb treatment.
FACS analysis showed no difference in number of (A) total lymphocytes, (B) B2 B cells, (C) CD4, CD8, NK and NKT cells in PC. Data represent mean±SEM. representative results of three individual experiments (IgG control n=13, Tim-1 mAb n=16). *: $P<0.05$ compared to IgG control, unpaired T-test.

Figure S3

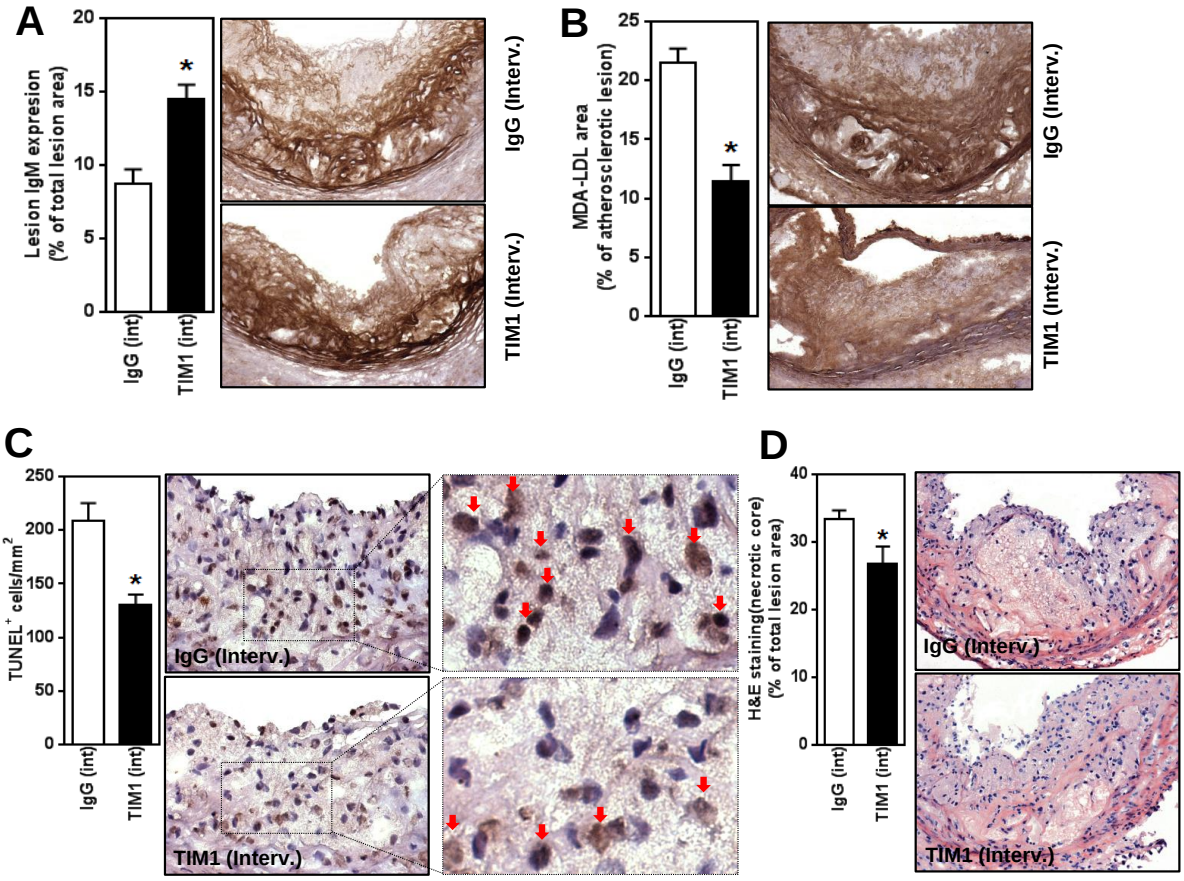


Figure S3- Intervention TIM-1 antibody treatment increases IgM deposits in atherosclerotic lesions in ApoE-/- mice while fed a HFD.
Immunohistochemical analysis of aortic sinus atherosclerotic lesions showed (A) increased IgM deposits and (B) reduced oxLDL accumulation in atherosclerotic lesions in TIM-1 mAb treated mice. In immunostaining of aortic sinus atherosclerotic lesions, (C) apoptotic cells as TUNEL-positive cells were reduced and (D) necrotic cores of atherosclerotic lesions identified as acellular areas in H&E stained of atherosclerotic lesions were reduced in anti-TIM-1 mAb treated group. Data represent mean±SEM. representative results of two individual experiments (IgG control n=11, Tim-1 mAb n=13). *: P<0.05 compared to IgG control, unpaired T-test.

Figure S4

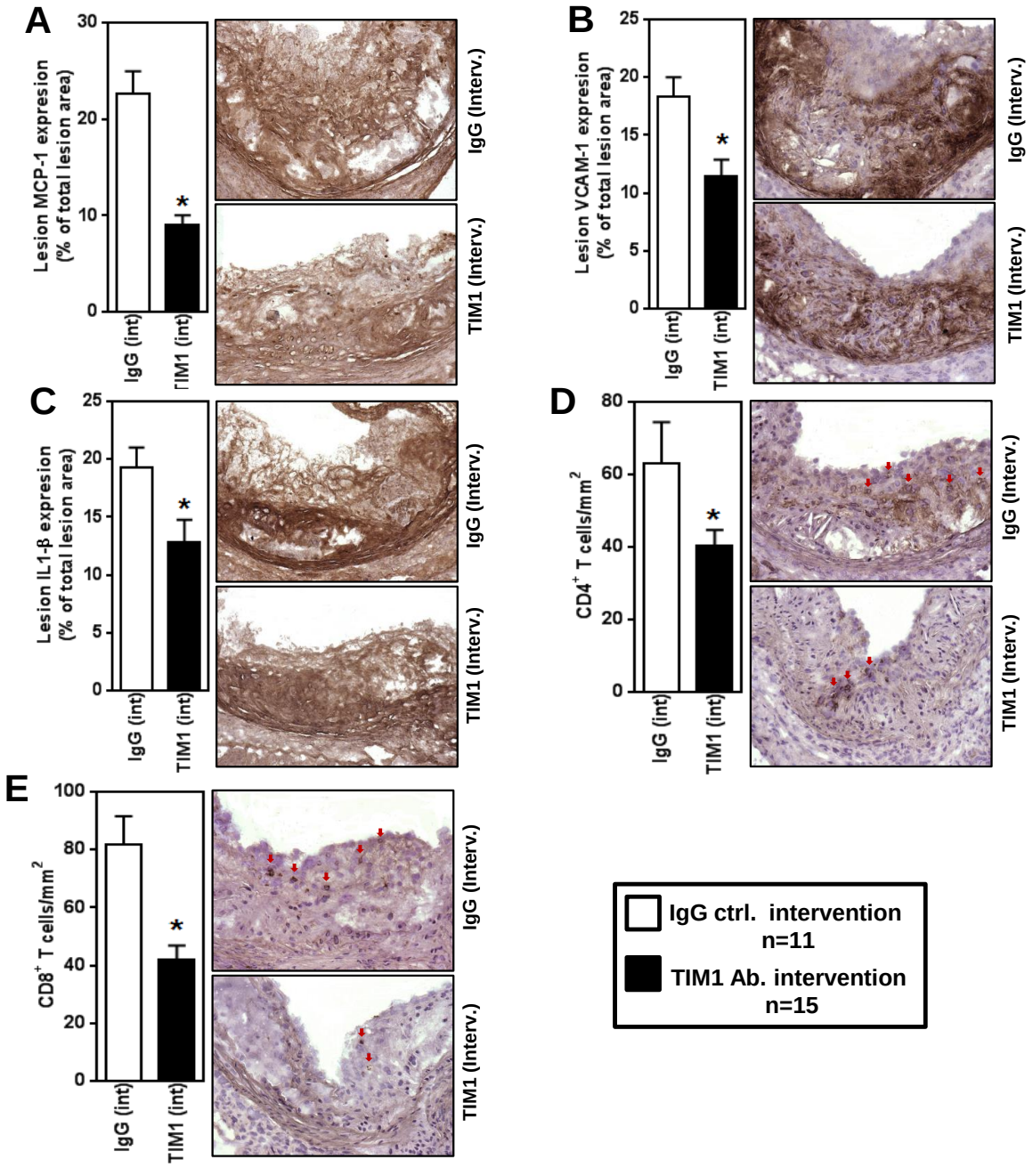


Figure S4- Intervention TIM-1 Ab. treatment reduces expression of VCAM-1 and MCP-1 and accumulation of IL1β, CD4 and CD8 T cells in atherosclerotic lesions.

Aortic sinus lesions stained by anti-MCP-1 Ab and anti-VCAM-1 Ab showed reduction expression (A-B) of both these molecules in anti-TIM-1 mAb treated mice. Aortic sinus lesions also stained with anti-IL1-β Ab, anti-CD4 Ab and anti-CD8 Ab showed reduced (C) IL1-β and (D) CD4 and (E) CD8 T cells in lesions in anti-TIM-1 mAb treated group. Data represent mean±SEM. representative results of two individual experiments (IgG control n=12, Tim-1 mAb n=15). *: P<0.05 compared to IgG control, unpaired T-test.

Chapter 5. Combined Discussion and Future Directions

5.1 Discussion

Both B cells and antibodies have been detected in all stages of experimental atherosclerosis [1, 2] and in of human atherosclerosis [3]. IgM antibodies can bind to oxidized LDL to clear oxLDL [4], suggesting that B cells are atheroprotective. $LDLR^{-/-}$ mice transplanted with bone marrow from B cell-deficient μMT mice showed increased atherosclerosis [5] while the adoptive transfer of unfractionated splenic B cells into splenectomised $ApoE^{-/-}$ mice markedly reduced atherosclerosis [6]. However, recent studies have established an atherogenic role for B cells using B cell-depleting anti-CD20 antibody [7, 8], which challenged the earlier studies. Our group also revealed an exacerbated atherosclerosis in both B cell-deficient $ApoE^{-/-}$ ($ApoE^{-/-}$μMT mice) and lymphocytes-deficient $ApoE^{-/-}$ mice ($ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice) when adoptively transferred with B2 B cells but not B1a B cells, thus identifying an atherogenic role for B2 B cell subset. In contrast, our group identified peritoneal B1a B cells as an atheroprotective IgM dependent B cell subset [9]. CD20 is a pan marker for B cells and CD20 mAb treatment treatment depleted both atherogenic B2 B cells and atheroprotective B1a B cells [7]. Short-term treatment with Rituximab also reduced atherosclerosis in rheumatoid arthritis patients [10, 11].

Over the years, studies revealed that Rituximab has proven well in safety terms with some controllable side effects [12], however some infection cases rarely occurred as a result of entire B cell population depletion [12]. CD20 is the only B cells marker that is expressed from the pre-B cell stage to the

mature B cell stage [13]. Recent studies have revealed that Rituximab may increase the chance of infections, such as hepatitis-B and *Pneumocystis* pneumonia [14]. Treatment with Rituximab wouldn't be the first choice for the patient at high risk of infection because Rituximab removes B1a B cells which are the first line of defence against infections. Studies have shown that statins potentially reduce the ability of Rituximab to deplete B cells effectively. As the majority of patients with atherosclerosis have hyperlipidaemia and they take statins, Rituximab may not be suitable for patients who are already on statins [15]. A therapeutic strategy that selectively depletes atherogenic B2 B cells or activates atheroprotective B1a B cells is highly demanded.

BAFF-R is essential for mediating BAFF induced survival signals of B2 B cell maturation [16] and binds exclusively to BAFF. We have recently examined the development of atherosclerosis in BAFF-R deficient ApoE$^{-/-}$ mice because B2 B cells but not B1a B cells are selectively depleted in BAFF-R deficient mice. We found that B2 B cells were significantly reduced while atheroprotective B1a B cells and non-B lymphocytes were unaffected and atherosclerotic lesions were significantly reduced in BAFF-R$^{-/-}$ApoE$^{-/-}$ mice compared with ApoE$^{-/-}$ control mice [17]. With this background, we blocked BAFF-R using anti-BAFF-R mAb to deplete atherogenic B2 B cells selectively with sparing of atheroprotective B1a B cells and the results revealed a therapeutic potential for BAFF-R mAb to treat atherosclerosis efficiently and not only reduced development of atherosclerosis lesions but ameliorated established atherosclerotic lesions in the ApoE$^{-/-}$ mice; these findings are in

accord with the report by Anton Rolink from whom we obtained the BAFF-R mAb [18, 19].

To explore a new therapeutic strategy in management of atherosclerosis apart from depletion of atherogenic lymphocytes we sought to activate atheroprotective B1a B cells. To address this I carried out experiments to supress the development of atherosclerosis or attenuate already established atherosclerosis. We have reported that B1a B cells are atheroprotective and their protection against atherosclerosis is dependent on IgM secretion [9]. Here I demonstrated that B1a B cell secretion of atheroprotective IgM is dependent on TLR4 activation in a MyD88 dependent manner. I found that both TLR4 and MyD88 deficient B1a B cells do not attenuate atherosclerosis or produce significant IgM, contrasting with suppressive effects of WT-B1a B cells and B1a B cells deficient in TLR2 or TLR9. IgM secreted by TLR4-activated B1a B cells bind not only to oxLDL and apoptotic cells but also to leukocytes, CD3 and CD4 and CD8 T lymphocytes. This study indicated that TLR4 is a major TLR receptor responsible for B1a B cell activation and IgM production. B1a B cells deficient in TLR4 are unable to attenuate atherosclerosis in splenectomised $ApoE^{-/-}$ mice. These cells do not increase IgM secretion following *in-vitro* activation by the TLR4 ligand LPS; neither do they produce IgM or MDA-oxLDL IgM in atherosclerotic mice *in-vivo*. While TLR4 activated peritoneal B1a B cells can also increase their expression of IL-10 [20], its contribution to atheroprotection may be due to IL-10 promotion of activated B cells to secrete IgM [21, 22]. In addition to neutralising apoptotic cells and oxLDL in atherosclerotic lesions [9, 23], this present study indicated

that IgM can also contribute to atheroprotection by interacting with lesion T cells and provide atheroprotection by binding to leucocytes, CD3 and CD4 T cells and reducing lesion proatherogenic CD4 and CD8 T lymphocytes [24, 25]. Studies by Lobo and colleagues have shown that increased IgM in inflammatory disorders inhibits immune cell activation and chemotaxis by interacting with CD3, CD4 CCR5 and CXCR4 expressing T cells, macrophages and dendritic cells [26] and inhibits cytokine production by activated splenocytes of prediabetic mice [27]. IgM can also inhibit $CD4^+$ T cell differentiation into Th1 and Th17 cells [28]. Further, removal of apoptotic cells by TLR4 activated B1a B cells may also contribute to attenuate inflammation mediated by post apoptotic necrotic cells [29-31]. The present study also indicated that B1a-derived IgM promotes the production of the anti-inflammatory cytokines TGF-β and IL-10 which is consistent with previous studies [32, 33]. These results revealed that TLR4- and MyD88-deficient B1a B cells failed to increase arterial mRNA expression of TGF-β and IL-10 in the absence of IgM antibodies highlights a healing role of B1a-derived IgM antibodies in addition to their atheroprotective action. Understanding the activation of B1a B cells through TLR4/MyD88 pathway may lead to novel strategies to augment B1a B cell IgM production to ameliorate atherosclerosis. In another series of studies I explored peritoneal B1a B cell activation using apoptotic cells (ACs) and the result highlighted a significant reduction in the development of atherosclerosis, an effect was that was mimicked by PS liposomes. B1a B cell activation with either apoptotic cells or PS liposomes induced B1a B cell expansion together with increased secretion of

polyreactive IgM antibodies, accounting for much of the immunosuppression by apoptotic cells and PS liposomes on atherosclerosis. Apoptotic cells can mediate immunosuppressive effects via multiple ligands on their cell surface. Annexin A1[34], DNA complex on their surface [35] and PS [36] highly expressed on ACs are the main immunosuppressive elements of ACs. My findings indicated a major role for PS expressed on ACs in attenuating development of atherosclerosis. TIM-1 as the main receptor for PS is highly expressed on regulatory B cells [37] and my results demonstrated a high expression of TIM-1 on B1a B cells, suggesting a role for their activation by PS in attenuating atherosclerosis. ACs and PSLs treatment failed to prevent atherosclerosis in the absence of B1a B cells in B1a-depleted splenectomised $ApoE^{-/-}$ mice. This direct activation resulted in B1a B cell expansion including the $TIM\text{-}1^{+}$B1a B cell population and their IgM production; suggesting that PS interacts with TIM-1 to initiate B1a B cell expansion and secretion of natural IgM. TIM-1 on B cells promotes their proliferation [38] mediated by T-independent antigens, whilst TIM-1 expressed by B1a B cells is likely responsible for the suppressive effects of PS on atherosclerosis. In my study, both ACs and PSLs treatment markedly increased polyreactive IgM antibodies, including anti-leukocyte, anti-CD3 and anti-CD4 IgM antibodies in plasma and atherosclerotic lesions. Natural anti-leukocyte as well as anti-CD3 and anti-CD4 IgM antibodies inhibit both T-cell activation and chemotaxis [39]. I found that ACs and PSLs treatment reduced accumulation of oxidised LDL (MDA-LDL), numbers of CD4+ and CD8+ T cells, macrophages and their secretion of TNF-α, IL-1β and MCP-1 [40] in the lesions; consistent with the

TLR4 study. These findings of reductions in lesion proinflammatory lymphocytes and cytokines are consistent with the IgM mediated reduction in oxLDL accumulation. Apart from immunosuppressive effect of IgM on proinflammatory responses of T cells and macrophages, IgM also promotes phagocytosis of apoptotic cells [29] and as a result reduction of post apoptotic necrotic cells in developing lesions. ACs and PSLs treatment also increased expression of anti-inflammatory cytokines IL-10 and TGF-β in lesions; consistent with previous studies showing that phagocytosis of apoptotic cells by macrophages markedly increases expression of both IL-10 [33] and TGF-β [32].

Towards finding a therapeutic strategy for atherosclerosis regard to B1a B cell activation by PS expressed on ACs, I demonstrated that treatment with RTM1-10 agonist mAb directed to TIM-1 attenuates not only atherosclerosis development but also progression of developed atherosclerosis by selectively expanding the IgM^+ and $IgM^+IL\text{-}10^+$ B1a B cells. Its effects on lesions are critically dependent on B1a B cells because their depletion by splenectomy abrogated the atheroprotective action of RTM1-10.

In both prevention and intervention studies, chronic RMT1-10 treatment doubled the number of B1a B cells in the peritoneal cavity, increasing 3-fold the number of $TIM\text{-}1^+$B1a B cells and within this group markedly increasing both $TIM\text{-}1^+IgM^+$ and $TIM\text{-}1^+IgM^+IL\text{-}10^+$ B1a B cells in both the peritoneal cavity and spleen without altering their relative distribution. My finding revealed an expansion of $TIM\text{-}1^+IgM^+$B1a, $TIM\text{-}1^+IL\text{-}10^+$B1a and $TIM\text{-}1^+IgM^+IL\text{-}10^+$B1a B cells as a result of TIM-1 ligation with RMT1-10 and this

expansion greatly increased IgM in plasma and IgM accumulation in developing and developed atherosclerotic lesions and as a result, accumulation of oxidised-LDL significantly decreased associated with reduced apoptotic cell numbers in the lesions and necrotic core size. RMT1-10 treatment specifically targeted TIM-1^{+}B1a B cells as no changes were observed in other lymphocyte populations including B2 lymphocytes; the latter is consistent with treatment not affecting plasma IgG or anti-MDA-LDL IgG levels. These observations are consistent with a scavenger function for natural IgM produced by B1a B cells in mopping up apoptotic debris [41].

Reduced CD4^{+} and CD8^{+} T cells in the lesions with increased anti-leukocyte antibodies, anti-CD4 and anti-CD8 mAb in plasma in RTM1-10 treatment mice is consistent with the results that we found in ACs and PSLs studies and studies by Lobo that showed that anti-leukocyte IgM antibodies secreted by B1a B cells modulate T cell activation and their migration [26]. Teplizumab (anti-CD3 antibody) is immunosuppressive medication with therapeutic effects on immune disorders such as diabetes type-I, tissue rejections and T cell carcinomas [42-44]. This study revealed that RTM1-10 treatment not only expands B1a B cells but also increases plasma levels of total and specific IgM antibodies (oxLDL, anti-leukocyte, anti-CD4 and anti-CD3). RTM1-10 mAb treatment reduced lesion MCP-1 and VCAM-1 and as a result macrophage accumulation in the lesions were significantly reduced [45, 46]; the reduction in MCP-1 and VCAM-1 expression is most likely the consequence of reduced T cell and macrophage activation. Macrophage and

T cell derived cytokines are reduced by RTM1-10 treatment and TNF-α, IL-6 and IL-1β are potent inducers of MCP-1 expression [47-49] whilst TNF-α and IL-1β are also known to induce VCAM-1 expression [50, 51].

My data clearly indicated that RMT1-10 treatment inhibits both atherosclerosis development and progression of already developed atherosclerotic lesions. These studies also indicated that the expanded B1a B cell subsets include TIM-1^+IgM$^+$ and TIM-1^+IgM$^+$IL-10^+ B1a B cells and whilst many of the effects of the expanded B1a B cell population can be attributed to their production of IgM, it is also possible that a component of their atheroprotective effects are mediated by IL-10. TIM-1^+IgM$^+$IL-10^+ B1a B cells in peritoneal cavity and spleen were also found to express CD1d (not shown) and are most likely similar to the recently described peritoneal and spleen B10 cells [52, 53]. B10 cells (CD19^+CD5^+IgM^{hi}CD$1d^+$IL-10^+) are potent immunosuppressive cells capable of modulating Th1 CD4^+ T cells and development of colitis [52] and other autoimmune inflammatory disorders. In contrast to selective or global depletion of pathogenic immune cells, RTM1-10 therapy has the advantage of enhanced expansion of atheroprotective B1a B cells and production of low affinity IgM antibodies without compromising the immune system. Further RTM1-10 therapy can also be expected to expand other regulatory B cells that may also contribute towards suppression of atherosclerosis.

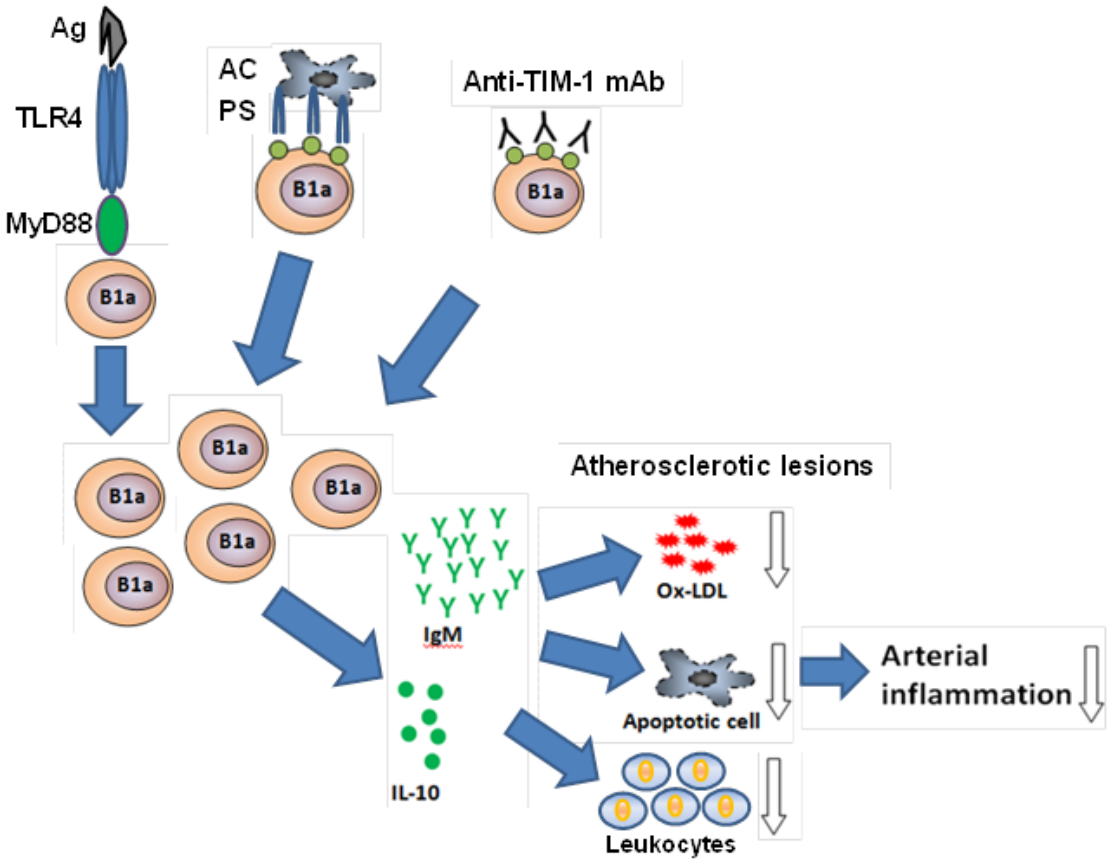
5.2 Conclusion

In summary, B1a B cells are atheroprotective and B2 B cells are atherogenic B cells with opposing roles in atherosclerosis. Therapeutic strategy toward proatherognic B2 B cell depletion or atheroprotective B1a B cell proliferation have potential to be exploited to threat atherosclerosis and attenuate its complications. Anti-BAFF-R mAb treatment in hyperlipidemic $ApoE^{-/-}$ mice not only prevented atherosclerosis development but also attenuated the progression of established atherosclerosis by selectively depletion of atherogenic B2 B cells and reduction of their proinflammatory cytokines while sparing atheroprotective B1a B cells. The combination of a lipid-lowering strategy, with anti-BAFFR antibody treatment has potential to even more effectively reduce progression of atherosclerosis and reduce the lethal complications of atherosclerosis-related myocardial infarction and stroke. On the other hand, I revealed that atheroprotective actions of B1a B cells via IgM are critically dependent on TLR4-and MyD88 activation. Activation of this TLR pathway may lead to novel strategies to augment B1a B cell IgM production to ameliorate atherosclerosis. B1a B cells can be specifically targeted by PS expressed on ACs to attenuate atherosclerosis through natural IgM production. Natural IgM secreted by PS stimulated-B1a B cells not only reduced inflammation by targeting leukocytes, T-cells and oxLDL, but also increased anti-inflammatory cytokines by apoptotic cells clearance and reduction of necrotic core development. Targeting TIM-1, a PS receptor using RTM1-10 mAb inhibited atherosclerosis development and progression by inducing expansion of two subpopulations of B1a B cells, $TIM\text{-}1^{+}IgM^{+}$ and

TIM-1^{+}IgM^{+}IL-10^{+}; and increasing levels of IgM, an important mechanisms of atherosclerosis suppression. These data indicated that B2 B cell depletion or B1a B cell expansion have potential to be exploited as a promising therapeutic target to attenuate development and progression of atherosclerosis related vascular disorders to reduce the morbidity and mortality of atherosclerosis associated with myocardial infarction and stroke. The suggestion with respect to B1a B cells is supported by the finding of B1a-like B cells in humans [54, 55]. Further studies are required to investigate the expression and function of TIM-1 on B1a-like B cells in humans and its relation to IgM and IL10 production.

Studies by Griffin et all. indicated that CD20^{+}CD27^{+}CD43^{+} B cells are the human counterpart of mouse B1a cells, especially CD11b^{-} population which spontaneously produce IgM [54, 55], but in contrast Covens and colleagues suggested that CD20^{+}CD27^{+}CD43^{+} B cells are preplasmablasts rather than belonging to B1 B cell lineage as they spontaneously secrete IgM, IgG, and IgA [56]. With these controversial findings, further studies are required to characterise human B1 B cells. My study suggest that new markers such as TIM-1 could provide a window to characterise human B1 B cells, isolate them and then examine their IgM and IL10 production level. As in my study TIM-1 Ab. treatment in mice reduced atherosclerotic lesions by B1a B cell activation through their natural IgM production, it is possible that TIM-1 antibody could have a therapeutic potential in humans with atherosclerotic lesions.

5.3 Summary


Ag
TLR4
MyD88
B1a
AC
PS
Anti-TIM-1 mAb
Atherosclerotic lesions
IgM
IL-10
Ox-LDL
Apoptotic cell
Leukocytes
Arterial inflammation

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