The role of natural IgM antibodies in atherosclerosis

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Submitted by

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Declaration

I hereby declare that this work was performed by myself under the supervision of Prof. Christoph J. Binder and has not been submitted to any other university. The review articles included were primarily written by myself and were corrected by Christoph Binder and co-authors. The published experimental work included in this thesis (Tsiantoulas et al., Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies. 2015, J Lipid Res 56: 440-448) was mainly performed by myself with contributions of co-authors. Moreover, the work for "Natural IgM antibodies regulate plasma IgE levels" (manuscript in preparation), which represents the second part of my thesis was also mainly done by myself with technical help from co-workers and collaborators.

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LIST OF ABBREVIATIONS

CVD cardiovascular disease

MP microparticles

OSE oxidation-specific epitope

LDL low density lipoprotein

OxLDL oxidized low density lipoprotein

Apoe apolipoprotein epsilon

Ldlr low density lipoprotein receptor

BCR B cell receptor

sIgM secreted immunoglobulin M

FO follicular

MZ marginal zone

T1 transitional stage 1

T2 transitional stage 2

Syk spleen tyrosine kinase

Btk Bruton’s tyrosine kinase

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Abstract in English

There is substantial evidence that humoral immunity and particularly natural IgM antibodies is a strong modulator of initiation and progression of atherosclerosis. Several epidemiological studies have shown that the risk for cardiovascular disease (CVD) is inversely associated with increased levels of IgM antibodies with specificity to oxidation-specific epitopes (OSE), which are lipid peroxidation products and are present on both oxidized low density lipoprotein and apoptotic cells. I investigated here whether microparticles (MP), which are generated upon membrane blebbing of activated and apoptotic cells and are increased in CVD patients, carry OSE and are recognized by OSE-specific IgM antibodies. I found that a large part of circulating MP express OSE on their surface and can be bound both *in vivo* and *in vitro* by OSE-specific IgM antibodies, particularly by those that are specific for malondialdehyde (MDA) epitopes. Moreover, I show that MP stimulation of monocytes results in IL-8 secretion, which is inhibited in presence of an MDA-specific natural IgM antibody compared to an isotype control suggesting that MDA bearing MP exhibit proinflammatory properties. In addition, I also found increased numbers of MDA^+ MP in the coronary compared to peripheral blood of acute myocardial infarction patients. These data identify a novel mechanism by which OSE-specific IgM antibodies protect from CVD. Because MP are a prototypic source of self-antigens, which have been shown to be critical regulators of B cell development, I hypothesized that natural IgM antibodies may also protect from atherosclerosis by modulating B cell responses. Here, I show that mice deficient in secreted IgM antibodies, which develop accelerated atherosclerosis, display enhanced BCR signaling impaired generation of $CD23^+$ B cells and robustly increased IgE antibody levels. Neutralization of IgE antibodies prevents the accelerated atherosclerosis in these mice. These data demonstrate that the protective role of natural IgM antibodies in atherosclerosis prominently includes the maintenance of B cell homeostasis, which is most likely based on their ability to bind self-antigens.

Abstract in German

Die humorale Immunität und vor allem natürliche IgM Antikörper spielen eine wesentliche Rolle in der Initiation und Progression von Atherosklerose. Mehrere epidemiologische Studien zeigten eine inverse Korrelation zwischen kardiovaskulären Krankheiten (KVK) und erhöhten IgM Antikörper-Spiegeln, welche sich gegen sogenannte oxidations-spezifische Epitope (OSE) richten. Diese Epitope entstehen durch Lipidperoxidation und befinden sich auf der Oberfläche von LDL (low-density lipoprotein) und apoptotischen Zellen. Diese Arbeit befasst sich mit der Fragestellung ob Mikropartikel (MP), die durch Membranabschnürung von aktivierten und apoptotischen Zellen entstehen und auch vermehrt in KVK Patienten zu finden sind, OSE exprimieren und durch OSE-spezifische IgM Antikörper erkannt werden. Ich konnte feststellen, dass ein Großteil der zirkulierenden MP OSE auf ihrer Oberfläche aufweisen und *in vivo* als auch *in vitro* von OSE-spezifischen IgM Antikörpern und vorallem durch jene, die Malondialdehyd (MDA)-Epitope erkennen, gebunden werden. Nach Stimulation mit Mikropartikeln wiesen Monozyten eine erhöhte Produktion von Interleukin-8 auf - ein Effekt, der durch Gegenwart von MDA-spezifischen IgM Antikörpern inhibiert werden konnte. Dies lässt die pro-inflammatorischen Eigenschaften von MDA-tragenden MP erkennen. Weiters konnte ich zeigen, dass die Zahl der MDA-tragenden MP im Koronarblut von Herzinfarktpatienten deutlich höher als im peripher gewonnenem Blut derselben Patienten ist. Dies weist auf einen neuen Mechanismus, durch welchen OSE-spezifische IgM Antikörper vor KVK schützen, hin. MP stellen prototypische Quellen von Antigenen dar, welche auch kritische Regulatoren der Entwicklung von B-Zellen sind. Basierend auf dieser Tatsache und der Erkenntnis, dass MP von natürlichen IgM Antikörpern gebunden werden, befasste ich mich weiters mit der Fragestellung ob die Regulation der B-Zellentwicklung Teil der Schutzfunktion von IgM Antikörpern in der Atherosklerose beinhaltet. So konnte ich zeigen, dass bei Mäusen, die keine IgM Antikörper sezernieren können und vermehrt atherosklerotische Läsionen bilden, B-Zell-Rezeptor Signale verstärkt, die Entstehung von $CD23^+$ B-Zellen beeinträchtigt, sowie IgE Antikörper stark erhöht sind. Durch Neutralisierung von IgE Antikörper wiederum konnte ich Fortschreiten der Atherosklerose in diesen Mäusen verhindern. Zusammenfassend zeigen meine Daten, dass die protektive Rolle von natürlichen IgM Antikörpern in Atherosklerose unter anderem auf die Erhaltung der B-Zellen Homöostase, welche wiederum auf dem Binden von Selbst-Antigenen basiert, zurückzuführen ist.

1. INTRODUCTION

1.1 Atherosclerosis, a chronic inflammatory disease

Atherosclerosis is a disease that affects mainly large and medium size arteries. It is characterized by the accumulation of cholesterol as well as different immune cells in the subendothelial space of the artery wall that leads to the formation of an atherosclerotic plaque (Glass & Witztum, 2001). Atherosclerotic plaque formation starts early in life and progresses for several decades silently. Rupture of an atherosclerotic plaque releases material that triggers thrombus formation leading to severe clinical manifestations, such as myocardial infarction and stroke, which both are leading causes of mortality and morbidity worldwide (Libby et al, 2013). Thrombus formation followed by myocardial infarction and stroke, can also occur in absence of plaque rupture, via a mechanism termed plaque erosion. These plaques differ in their characteristics with plaques that are prone to rupture. They contain few immune cells and lipids and are rich in smooth muscle cells and proteoglycans (Otsuka et al, 2014).

Hypercholesterolemia is major risk factor for atherosclerosis. The causative role of cholesterol in atherosclerotic plaque formation was originally demonstrated by Dr. Nikolai Anitschkow, a Russian pathologist who showed that cholesterol fed rabbits developed atherosclerosis. In 1950s, Dr. John Gofman identified two different cholesterol fractions - based on their density - the low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL) by subjecting rabbit hyperlipemic serum to ultracentrifugation. Gofman and colleagues later showed that rabbits, which were fed cholesterol and developed atherosclerosis had also elevated levels of the LDL fraction. Moreover, the authors found increased levels of LDL in cardiovascular disease (CVD) and diabetes patients (Gofman & Lindgren, 1950).

Since then, a large set of epidemiological data suggest that increased plasma levels of LDL are a major risk factor for CVD in humans, whereas increased levels of HDL associate with lower risk. The causative relationship of the latter with atheroprotection has been recently challenged as a number of pharmacological agents that increase HDL levels failed to confer clinical benefit (Rader & Tall, 2012).

LDL cholesterol lowering interventions, such as administration of statins, have a clear therapeutic benefit in the progression of atherosclerosis (Steinberg, 2002). Apart from hyperlipidemia, several lines of evidence - both experimental and

epidemiological - have shown that the immune system plays a key role in the initiation and progression of atherosclerosis. For example, patients suffering from systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are develop premature atherosclerosis and have an increased risk of ischemic heart disease, which cannot be explained by changes in total cholesterol levels or other traditional risk factors such as age, smoking or systolic blood pressure, pointing to a role for the immune system in the pathophysiology of atherosclerosis (Tsiantoulas et al, 2015b).

In the last two decades, an overwhelming amount of experimental studies mainly using the two best established murine models of atherosclerosis, the low density lipoprotein receptor deficient (*Ldlr* $^{-/-}$) and apolipoprotein E deficient (*Apoe*$^{-/-}$) mice, showed that both the innate and adaptive arms of the immune system contribute to the initiation and progression of atherosclerosis (Hansson & Hermansson, 2011). The initial steps of atherosclerotic plaque formation include activation of the endothelial cells and the subsequent accumulation of LDL cholesterol in the intima of the artery wall. In the subendothelial space of arteries, LDL undergoes oxidation and acquires novel pro-inflammatory properties such as a potential to activate endothelial cells. In response to this, monocytes are recruited and arrested in the activated endothelium via chemokines and adhesion molecules respectively, and migrate to the intima (Weber & Noels, 2011). Next, the newly recruited monocytes, which in the intima differentiate to macrophages, take up oxidized LDL (OxLDL) in order to prevent its accumulation. Uptake of OxLDL by macrophages, beyond their clearance capacity, results in cells that are characterized by a foamy appearance (known as foam cells) due to the formation of large lipid droplets in their cytoplasm. Foam cells eventually become apoptotic, and their accumulation has been shown to enhance lesion formation at later stages by promoting intimal inflammation (Tabas, 2010).

Although adaptive immunity (B and T lymphocytes) is not essential for atherosclerosis development (Dansky et al, 1997; Reardon et al, 2003), it clearly affects its progression. T lymphocytes are present in both mouse and human atherosclerotic lesions and consist of predominately $CD4^+$ T cells, though small numbers of $CD8^+$ T cells are found as well. T cell responses are both protective and proatherogenic and include several cell subsets. Th1 responses, which are characterized by IFNγ production, have been shown to promote atherosclerosis. On the other hand, the role of Th2 cells is not clearly established. Th2 responses are

characterized by IL-5, IL-10 and IL-13 production, which have been shown to play a protective role, as well as IL-4 production, for which the majority of the data suggest, a proatherogenic role. As all above mentioned cytokines are produced by other cells besides T cells, additional studies that include deletion of these cytokines in a T cell specific manner would shed more light on the role of Th2 cells (Ait-Oufella et al, 2014). Moreover, T regulatory cells, which are involved in the maintenance of self-tolerance and therefore prevention of autoimmunity, have been shown to play a protective role in atherosclerosis (Ait-Oufella et al, 2006). The exact underlying mechanism remains elusive but may involve suppression of effector Th1 responses as well as changes in the lipoprotein lipid profile (Ait-Oufella et al, 2014). Finally, the role of Th17 cells, which produce large amount of IL-17A cytokine, is still debatable as several studies, which include genetic ablation or exogenous administration of IL-17A, suggest either a protective or proatherogenic role (Taleb et al, 2014).

1.2 B cell development, subsets and their role in atherosclerosis

Even though they are found in very small numbers within atherosclerotic plaques (Galkina et al, 2006), B lymphocytes play a major role in atherosclerosis (Tsiantoulas et al, 2014; Tsiantoulas et al, 2015b). B cells are divided into two main subtypes, the B1 and B2 cells. B2 cells, which consist of marginal zone (MZ) and follicular (FO) B cells, have a half-life of only a few days. B1 cells develop from fetal liver and are characterized by their via self-renewal capacity. They are divided into B1a and B1b cells. B1 cells are primarily found in peritoneal and pleural cavities (Baumgarth, 2011).

The role of different B cell subsets in atherosclerosis is reviewed below in Tsiantoulas D, Sage AP, Mallat Z, Binder CJ (2015b) Targeting B cells in atherosclerosis: closing the gap from bench to bedside. *Arterioscler Thromb Vasc Biol* **35**(2)**:** 296-302 and Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ (2014) B cells and humoral immunity in atherosclerosis. *Circ Res* **114**(11)**:** 1743-1756.

ATVB in Focus
Modulation of Atherosclerotic Lesions by Circulating Cells: The Translational Spectrum

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Targeting B Cells in Atherosclerosis

Closing the Gap From Bench to Bedside

Dimitrios Tsiantoulas, Andrew P. Sage, Ziad Mallat, Christoph J. Binder

Abstract—Atherosclerotic plaque formation is strongly influenced by different arms of the immune system, including B lymphocytes. B cells are divided into 2 main families: the B1 and the B2 cells. B1 cells are atheroprotective mainly via the production of natural IgM antibodies that bind oxidized low-density lipoprotein and apoptotic cells. B2 cells, which include follicular and marginal zone B cells, are suggested to be proatherogenic. Antibody-mediated depletion of B cells has become a valuable treatment option for certain autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis that are also characterized by the development of premature atherosclerosis. Thus, B cells represent a novel interesting target for therapeutic modulation of the atherosclerotic disease process. Here, we discuss the effect of different of B-cell subsets in experimental atherosclerosis, their mechanism of action as well as potential ways to exploit these findings for the treatment of human disease. **(*Arterioscler Thromb Vasc Biol*. 2015;35:296-302. DOI: 10.1161/ATVBAHA.114.303569.)**

Key Words: antibodies ■ atherosclerosis ■ B lymphocytes ■ B-cell activating factor ■ belimumab ■ cardiovascular diseases ■ rituximab

Immunity and Atherosclerosis

Atherosclerosis is a multifactorial disease with multiple genetic and environmental risk factors and is characterized by the formation of a plaque in the artery wall. Plaque formation is initiated on trapping of low-density lipoproteins (LDL) in the intima where they undergo oxidation and acquire immunogenic properties. The oxidation of LDL results in the generation of many different immunogenic epitopes, termed oxidation-specific epitopes (OSEs), that are recognized by both innate and adaptive immune mechanisms. Monocytes that enter the intima differentiate to macrophages and take up oxidized LDL (oxLDL), which leads to their activation and results in the formation of foam cells. During this process, macrophages are stimulated by lipid-derived danger–associated molecular patterns such as oxidized phospholipids that promote cytokine secretion via scavenger receptor CD36 and Toll-like receptor signaling and cholesterol crystals, which activate the inflammasome followed by interleukin-1β production.[1,2] Plaque inflammation is further amplified and sustained as a result of recruitment/activation of the adaptive immune system and is an important and potentially central driving force in promoting vulnerable plaque features. Plaque rupture results in life-threatening manifestations, such as myocardial infarction and stroke. Surgery and reducing the risk of clotting are powerful end-stage solutions and lipid lowering is an effective preemptive treatment. However, significant risk remains and new strategies to target underlying causes of vulnerable plaque development and rupture are important future goals.[3] Although an adaptive immune system is not essential for atherosclerosis to develop,[4,5] many studies now demonstrate that it has a diverse range of important site-specific influences on plaque development and inflammation. (Auto)immune reactivity to a range of autoantigens, but most prominently modified LDL, is a mark of human cardiovascular disease and in experimental models plays a significant role in promoting atherosclerotic plaque progression. Atherosclerosis is a distinct case compared with typical autoimmune diseases because (1) the major autoantigen oxLDL is really a modified self-antigen or neo–self-antigen and (2) the oxLDL autoantigen, rather than playing a physiological function, is pathogenic and disease causing. There are also other autoantigens involved, such as heat shock protein 60,[6,7] and the impact of other autoimmune diseases in promoting atherosclerosis such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is well known.[8,9] The role of T cells and interferon-γ–secreting Th1 cells, in particular, as key drivers of plaque inflammation is well documented, and experimental approaches to dampen these responses by enhancing the activity of regulatory T cells are being tested. More recently, it was found that B cells could

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Nonstandard Abbreviations and Acronyms	
Apoe	apolipoprotein E
BAFF	B-cell activating factor
BAFFR	B-cell activating factor receptor
BCR	B-cell receptor
LDL	low-density lipoprotein
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor

also play both protective and pathogenic roles, and studies from animal models that have been reviewed extensively elsewhere[10,11] are beginning to dissect the different pathogenic and protective B-cell responses. Here, we will discuss these insights in light of translational aspects (Figures 1 and 2).

B-Cell Development, Subsets, and Functions

B cells are defined by their unique expression of surface (B-cell receptors [BCRs]) and secreted (antibody) immunoglobulin, produced from multigenic loci somatically rearranged during B-cell development, giving each B-cell clone a BCR with a different specificit .[12,13] Two major types of B cells, B1 and B2 cells, develop from hematopoietic stem cells. B1 cells develop from fetal liver hematopoietic stem cells and are subsequently maintained in the periphery via self-renewal, which is dependent on the spleen.[14–16] B2 cells have a half-life of only a few days and are continually replaced from bone marrow hematopoietic stem cells. Only B-cell clones encountering antigens, or in some cases in response to innate signals, become activated and persist. These differentiate into antibody-secreting plasma cells, or alternatively resting memory B cells, that respond more rapidly to subsequent antigen encounters. B1 cells are further divided into B1a, which express the CD5 on their surface and B1b cells. Both B1a and B1b cells primarily patrol peritoneal and pleural niches, and form a major (50%) proportion of peritoneal B cells in mice,[17] but only a minor population (<5%) in the spleen. B2 cells recirculate through the blood and lymphatics, encountering antigens in secondary lymphoid organs: the spleen, lymph nodes, and Peyer patches. Both B1- and B2-derived plasma cells are primarily found in the spleen and bone marrow,[17] suggesting the existence of common plasma cell niches allowing antibodies quick access to the blood. In cases of chronic inflammation such as atherosclerosis, tertiary lymphoid organs develop adjacent to diseased tissue, the arterial adventitia in the case of atherosclerosis, and may become major sites of adaptive immune activation.[18–20] It is likely that tertiary lymphoid organs accumulate B cells with relevant antigen specificit ,[21] or B-cell subsets that exhibit specific properties, for xample, circulating capacity.[22]

The workload of responding to different antigens is divided between different B-cell subsets. Responses are traditionally divided into T-cell dependent, those requiring helper T-cell signals (in addition to the antigen and antigen-specific B cell) and T-cell independent responses, with several subtypes of responses within each group now recognized.[23] B1 cells produce natural antibodies to common microbial epitopes and (neo)self-determinants such as OSEs independent of cognate T-cell help.[24] Multiple types of T-cell–independent responses are now recognized, including those to Toll-like receptor ligands such as bacterial polysaccharides. Marginal zone B cells, which differ from other B2 cells in only the final stages of their development, also contribute to innate antibody production. They can respond to multiple antigen types and their location in the marginal zone of the spleen provides them with the ability to respond

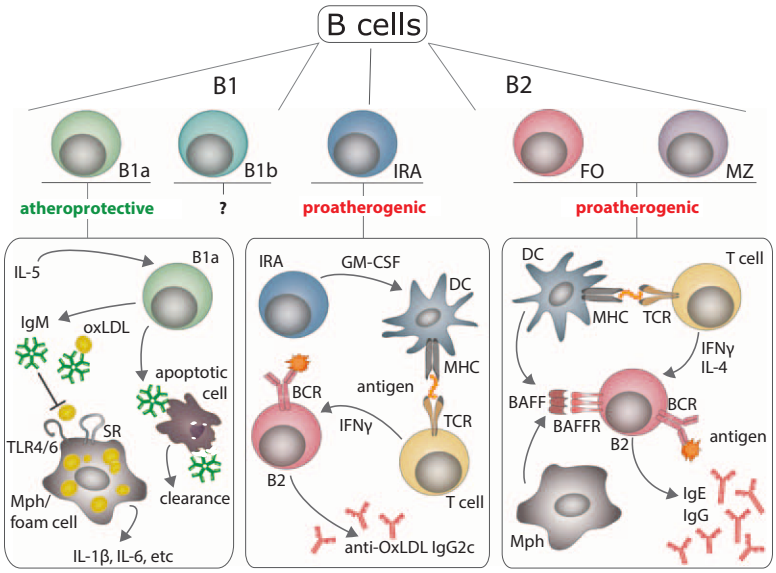

Figure 1. Role of different B-cell subsets in murine atherosclerosis. B cells consist of 2 major cell subsets, named B1 and B2 cells, which are characterized by different localization properties and activation requirements. B1 cells reside predominately in the peritoneum and are subdivided into B1a and B1b. B1a cells protect from atherosclerosis. Their atheroprotective properties depend mainly on the capacity to produce oxidized low-density lipoprotein (oxLDL)–specific natural IgM antibodies, which can be enhanced by interleukin (IL)-5 stimulation, that block oxLDL uptake and foam cell formation. In addition, natural IgM have also been shown to promote apoptotic cell clearance. The role of B1b in atherosclerosis remains still elusive. B2 cells are mainly found in the spleen and consist of marginal (MZ) and follicular (FO) B cells. B2 cells seem to be proatherogenic. Although the underlying mechanism is yet to be identified, this may include production of proatherogenic IgG and IgE antibodies. Innate response activator B cells (IRA), which are characterized by GM-CSF secretion, promote the expansion of IgG2c anti-oxLDL antibodies and aggravate atherosclerosis. BAFFR indicates B-cell activating factor receptor; BCR, B-cell receptor; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; MHC, major histocompatibility complex; Mph, macrophage; SR, scavenger receptor; TCR, T-cell receptor; and TLR, Toll-like receptor.

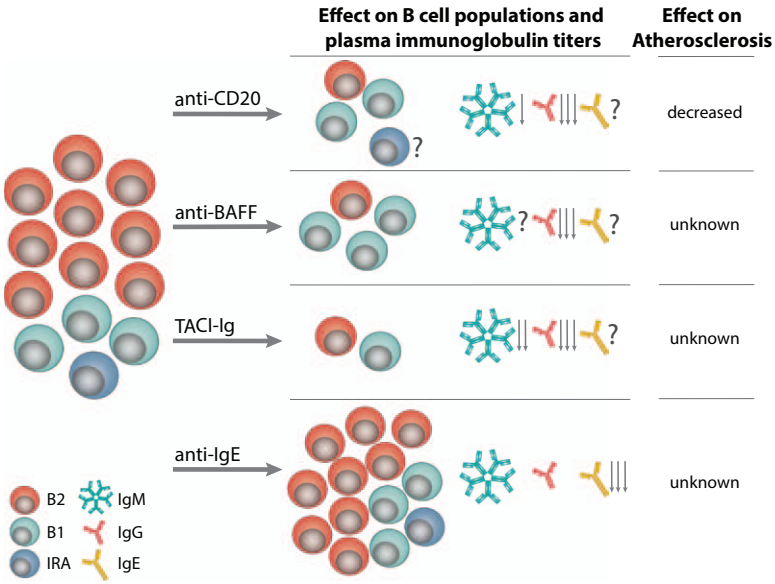

Figure 2. Biologicals that target B cells and their effect on murine atherosclerosis. Anti-CD20 antibodies cross-link the surface CD20 receptor on B cells, resulting in Fcγ-mediated depletion of predominantly B2 cells. Plasma IgM titers are moderately reduced, whereas IgG titers are strongly decreased after anti-CD20 treatment. Atherosclerosis-prone mice injected with anti-CD20 antibody develop reduced atherosclerotic plaque size. In contrast to B1 cells, B-cell activating factor (BAFF) signaling via the B-cell activating factor receptor (BAFFR) is essential for B2 cell survival. Treatment with a neutralizing antibody against BAFF leads to B2 cell apoptosis followed by strong reduction in plasma IgG titers. Combined neutralization of BAFF and a proliferation inducing ligand on treatment with the extracellular domain of the transmembrane activator and calcium modulator and cyclophilin ligand interactor receptor (TACI) fused to an Ig backbone (TACI-Ig) results in reduced numbers of B2 and to lesser extent of B1 cells. Moreover, TACI-Ig treatment inhibits plasma cell survival followed by reduction in total Ig plasma titers. The effect of both anti-BAFF and TACI-Ig treatment in atherosclerosis remains to be shown. Finally, IgE antibodies have been suggested to promote atherosclerotic plaque development and to correlate with cardiovascular disease risk. The effect of IgE antibody blockage via anti-IgE treatment in atherosclerosis is yet to be investigated. IRA indicates innate response activator B cells.

rapidly to blood-borne antigens.[25] Follicular B2 cells, which form the majority of recirculating mature B cells, respond to protein antigens presented to them complexed with immunoglobulin or complement and often immobilized on the surface of innate immune cells. These responses are T-cell dependent. On interaction with an antigen-specific T helper cell at the follicular T-cell zone border of secondary lymphoid organs, B2 cells migrate along with the T cell into the follicle and proliferate, forming germinal centers, where they undergo antibody isotype class switching, that is, from IgM to IgG, IgA or IgE, and affinity maturation through natural selection by competition for antigen and T-cell help.[26] In addition to antibody secretion, B cells can also be key sources of cytokines and chemokines. Production of granulocyte macrophage colony-stimulating factor by innate response activator B cells (IRA), a subset related to B1 cells, is important for dendritic cell activation,[27] and B-cell secretion of Ccl7 (monocyte chemotactic protein-3) is a key regulator of monocyte mobilization after acute myocardial infarction.[28]

B1 and B2 cells also display different survival properties. Mature B2-cell survival is dependent on B-cell activating factor receptor (BAFFR) signaling. BAFFR signaling prevents B2-cell apoptosis by binding the BAFF ligand. BAFF is mainly produced by stromal cells as well as by macrophages, monocytes, dendritic cells, and activated T cells[29,30] BAFF is recognized by 2 other receptors named transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) receptor and B-cell maturation antigen, both of which also bind a second ligand of the so-called BAFF system, named a proliferation inducing ligand, which has been shown to facilitate IgA class switching.[31] TACI receptor has been shown to mediate antibody class switching in mature B cells, whereas BCMA is essential for plasma cell survival. Disruption of BAFFR signaling, for example, by genetic deletion of BAFF or BAFFR, leads to mature B2-cell apoptosis, whereas B1-cell numbers remain unaltered. Interestingly, IRA B cells are also depleted in BAFFR-deficien mice, despite the fact that they are B1 cell derived.[32]

Our increased understanding of the diversity of B-cell functions has reignited research into B-cell regulation of atherosclerosis (Figure 1).

B Cells Are Modulators of Atherosclerosis

The role of B cells in murine atherosclerosis was first investigated by Caligiuri et al,[33] who showed that accelerated atherosclerosis on splenectomy was reversed by adoptive transfer of splenic B cells isolated from either wild-type or apolipoprotein E–deficient mice (*Apoe*$^{-/-}$). Notably, the latter had a stronger atheroprotective effect (below the sham operated mice) indicating that B cells acquire increased or even novel atheroprotective properties in hypercholesterolemic conditions. The results of this study were supported by Major et al[34] who performed B-cell–deficient (μMT) bone marrow transfer into lethally irradiated LDL receptor–deficient mice (*Ldlr*$^{-/-}$) that led to enhanced atherosclerotic plaque formation on atherogenic diet feeding. Collectively these data suggested an overall protective role of B cells in atherosclerosis. However, as described above, B cells are heterogeneous and consist of several cell subsets with different localization properties, activation requirements, survival characteristics, and immunoglobulin secretion profile Thus, different B-cell subsets may have different or even opposing roles in atherogenesis, and the understanding of this is critical for the optimal development of B-cell–targeting therapies.

We and others have investigated the effect of anti-CD20 antibody treatment in experimental atherosclerosis. Anti-CD20 treatment, which preferentially leads to B2-cell

depletion, whereas B1a cells remain nearly intact, reduced atherosclerosis in atherogenic diet–fed *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice.[35,36] In agreement with an effect that depends on B2-cell depletion, adoptive transfer of splenic B2 cells into lymphocyte-deficient Rag2$^{-/-}$γ-chain$^{-/-}$*Apoe*$^{-/-}$ or B-cell–deficient μMT/*Apoe*$^{-/-}$ mice aggravated atherosclerosis in 2 studies from 1 group, whereas another showed a protective effect[22,36] emphasizing the need for alternative models. Further evidence on the proatherogenic role of B2 cells came from studies on the role of BAFFR deletion in atherosclerosis-prone mice. BAFFR-deficient *Apoe*$^{-/-}$ mice as well as BAFFR-deficient *Ldlr*$^{-/-}$ bone marrow chimeric mice, which lack mature B2 cells, developed decreased atherosclerosis.[37,38] Similar data were obtained by Kyaw et al[39] who treated atherogenic diet–fed *Apoe*$^{-/-}$ mice with a blocking anti-BAFFR antibody. The mechanism by which B2-cell depletion protects mice from atherosclerosis is not entirely clear. Of note, anti-CD20 treatment failed to protect Western diet–fed *Apoe*$^{-/-}$ mice that were cotreated with a neutralizing antibody against interleukin-17 suggesting that Th17 responses may be involved in the protective mechanism of anti-CD20 treatment. Moreover, although in anti–CD20-treated mice the prototypic natural IgM antibody T15/E06 that binds oxLDL was largely unaffected, both total and anti–oxLDL-specific IgG titers were dramatically reduced.[35] This is particularly interesting, as previous epidemiological and experimental data point to proatherogenic role of IgG antibodies.[40] For example, IgG antibodies to $ApoB_{100}$ have been suggested to promote atherosclerosis in mice.[41] Alternatively, the proatherogenic role of B2 cells may be because of their capacity for IgE antibody production. IgE antibodies have been shown to be elevated in patients with coronary heart disease compared with healthy individuals[42] and to be a prognostic marker for myocardial infarction in the Helsinki Heart study.[43] Supporting experimental evidence on the proatherogenic role of IgE antibodies comes from Wang et al[42] who investigated the role of the high affinity receptor of IgE (FcεRI) in atherogenic diet–fed *Apoe*$^{-/-}$ mice. FcεRI-deficient *Apoe*$^{-/-}$ mice developed reduced atherosclerosis and plaque complexity. Whether these might constitute an underlying mechanism by which B2 cells promote atherosclerotic plaque formation remains to be shown.

In contrast to Kyaw et al,[36] Doran et al[22] found that adoptive transfer of splenic B2 cells from *ApoE*$^{-/-}$ mice reduced atherosclerosis in cholesterol-fed μMT/*Apoe*$^{-/-}$. Possible explanations for this discrepancy may include different cell ratio of follicular and marginal zone B cells or B2-cell purity of these cell preparations. In addition, Kyaw et al transferred 5×10^6, whereas Doran et al transferred 3×10^7 or 6×10^7 B2 cells into B-cell–deficient mice. Based on the fact that BCR interaction with self-antigens strongly controls the developmental fate and survival of B cells,[44] one may hypothesize that difference in the number of transferred B2 cells into B-cell–deficient mice affects the way the cells interact with the endogenous self-antigen pool. Thus, dependent on their numbers, transferred B cells may acquire distinct phenotypes and undergo distinct responses to those normally occurring in B-cell–sufficient mice. Notably, Doran et al suggest that local B-cell responses in the adventitia of affected arteries, at least after B-cell transfer, may be protective, whereas the localization of the pathogenic transferred B cells in 2 other studies was not defined. In conclusion, B2 cells seem to be proatherogenic although additional studies on the role of each B2-cell subset would provide more conclusive evidence on their role in atherosclerosis.

However, the data on B1a cells are more robust and suggest a strong atheroprotective role. Kyaw et al showed that splenectomy of *Apoe*$^{-/-}$ mice, which results in accelerated atherosclerosis, leads to ≈50% reduction of B1a cells in the peritoneum followed by a strong decrease in plasma IgM titers. Moreover, adoptive transfer of peritoneal B1a cells into splenectomized *Apoe*$^{-/-}$ recipients fed an atherogenic diet, reduced atherosclerosis even beyond the disease-accelerating effect resulting from splenectomy. This was dependent on the capacity of B1a cells to secrete natural IgM antibodies as there was no protective effect when the splenectomized *ApoE*$^{-/-}$ mice received B1a cells isolated from secreted IgM-deficient donor mice.[16] Natural IgM antibodies have been shown to be atheroprotective.[40] Lewis et al,[45] demonstrated that *sIgM*$^{-/-}$ crossed onto *Ldlr*$^{-/-}$ background develop strongly accelerated atherosclerosis when fed regular chow or atherogenic diet. The atheroprotective capacity of natural IgM may be to a large extent mediated by the IgM with specificity for OSEs. We have shown previously that a large part of B1-cell–derived natural IgM antibodies is directed against OSE, which are major antigenic determinants on the surface of apoptotic cells and on oxLDL. OSE-specific natural IgM have the potential to neutralize proinflammatory effects of oxLDL, inhibit foam cell formation, and promote clearance of apoptotic cells. A protective role for OSE-specific IgM is also supported by epidemiological data, which show that anti–oxLDL-specific IgM antibodies are inversely associated with cardiovascular disease adverse effects.[40] Thus, strategies that would promote the expansion of atheroprotective natural IgM antibodies may be beneficial in human atherosclerosis

The recently identified IRA B cells[32] also play a role in atherosclerosis. IRA B-cell–deficient *Ldlr*$^{-/-}$ mice, which were generated by reconstitution with granulocyte macrophage colony-stimulating factor and B-cell–deficient bone marrow, developed reduced atherosclerosis in the entire aorta. These mice had a strong reduction in interferon-γ–secreting $CD4^+$ T cells and anti-oxLDL IgG2c-specific antibodies.[27] Because IRA B cells are depleted in BAFFR-deficient mice,[32] this could be an alternative mechanism by which neutralization of BAFFR signaling protects from atherosclerosis.

A critical role of B cells in human atherosclerosis has been suggested by the finding that several critical genes involved in survival, proliferation, or activation status of B cells were identified as key drivers of CHD based on an integrated analysis of whole blood gene expression profiles from Framingham Heart Study participants and data from genome-wide association studies.[46] In line with this, it has been recently shown that increased numbers of a B-cell subset identified as $CD19^+CD86^+$ associate with increased risk for stroke but not with coronary artery disease.[47] Thus, developing or exploiting existing therapeutic approaches that modulate the survival or

activation status of B cells may provide a novel line of treatment in atherosclerosis (Figure 2).

Targeting B Cells in Atherosclerosis and Myocardial Infarction

B cells along with the antibodies they produce promote the pathology of several autoimmune disorders such as RA and SLE.[48] Interestingly, patients with both RA and SLE are characterized by increased risk of CVD complications, mainly ischemic heart disease, which is associated with the development of premature atherosclerosis.[49] Accelerated atherosclerosis in patients with SLE and RA seems to be independent of classical Framingham risk factors such as age, total cholesterol, high-density lipoprotein, and systolic blood pressure. This suggests that aggravated atherosclerosis in these patients may be a result of increased inflammation and altered immune responses, such as autoantibody production. For example, patients with SLE have been found to develop autoantibodies against ApoAI, which have been associated with acute coronary syndromes.[9]

The development of B-cell–targeting therapeutics for RA and SLE has gained a lot of attention in the past years. The first B-cell therapeutic agent that has been approved for clinical use in patients with RA is the anti-CD20 antibody (rituximab). Rituximab cross links the CD20 receptor present on all B cells, leading to Fcγ-mediated cell depletion[50] and consequently to decreased immunoglobulin/autoantibody titers. Another B-cell–depleting agent, a blocking antibody against BAFF (belimumab) has been approved by the Food and Drug Administration in 2011 for clinical use in patients with SLE, who have been shown to have increased plasma BAFF levels.[30] Belimumab, which is the first drug approved for SLE in 50 years, blocks soluble BAFF from binding to its receptor (BAFFR) resulting in apoptosis of mature B cells. Patients with SLE treated with belimumab show an improvement of clinical score,[51] which was associated with reduced B-cell numbers as well as reduced total immunoglobulins and autoantibody titers against dsDNA.[52] As mentioned above, anti–CD20-mediated depletion of B cells as well as BAFFR deficien y or treatment with an anti-BAFFR antibody has been found to reduce plaque burden in atherosclerosis-prone mice.

We have recently also shown that B-cell–derived CCL7 (MCP-3) drives monocyte mobilization leading to enhanced tissue injury in a mouse model of myocardial infarction. Treatment with an anti-CD20 or an anti-BAFF antibody, which leads to B-cell depletion and B-cell–derived CCL7 reduction, reduced infarct size and improved cardiac remodeling.[28] Thus, it can be speculated that rituximab- or belimumab-treated patients may also have a better outcome on myocardial infarction.

Besides anti-CD20 and anti-BAFF antibodies, additional B-cell–targeting agents are being developed that may have the potential to modulate atherosclerotic lesion formation as well. In line with this, a decoy form of the TACI receptor (TACI-immunoglobulin/Atacicept) has been tested in clinical phase II/III trial as treatment for patients with SLE. The results suggest a protective effect of Atacicept treatment in SLE at a high dose, although the recruitment of patients and treatment in this group was terminated prematurely because of 2 sudden deaths.[53] Combined neutralization of BAFF and APRIL on TACI-Ig treatment results in depletion of plasma cells and mature B cells as well as strong antibody level reduction in mice.[54,55] Although TACI-Ig could be considered as a therapeutic option in atherosclerosis, given its B-cell depleting properties, one should keep in mind that this treatment also strongly reduces IgM titers,[54] which have a protective effect in atherosclerosis.

Additional B-cell–modulating agents that are tested as treatment for patients with SLE and RA include anti-CD19 and anti-CD22 antibodies. CD19 is a B-cell–specific surface marker and is involved in the formation of the BCR complex as well as in its activation.[56] In contrast to CD20, a subset of plasma cells expresses CD19. Thus, targeting CD19 could also result in depletion of $CD19^+$ antibody-producing plasma cells and in more efficient plasma IgG reduction but, similar to TACI-Ig, anti-CD19 treatment may result in decrease of atheroprotective IgM titers as well. An antibody against CD19 named MDX1342 is in clinical trial as treatment of patients with RA.[57] CD22 is a transmembrane sialoglycoprotein and is expressed by the majority of mature B cells and is a negative modulator of BCR signaling. Epratuzumab is a humanized antibody (clinical phase III trial for patients with SLE) that binds CD22 induces its internalization and phosphorylation. Apart from the moderate B-cell–depleting capacity (mainly $CD27^-$ B cells), epratuzumab exhibits immunomodulatory properties such as inhibition of B-cell proliferation, in vitro.[57,58] In mice, CD22 deficien y results in strongly reduced marginal zone B cells,[59] thus investigation of the impact of CD22 deficien y could help to elucidate the role of different B2 cells in atherosclerosis. Finally, neutralizing IgE antibodies, for example, using omalizumab (an FDA-approved human anti-IgE antibody that neutralizes free IgE antibodies) may be an alternative more specific approach of limiting a B-cell–mediated proatherogenic mechanism in selective settings. Interestingly, IgE antibodies have been shown recently to be involved in the pathogenesis of SLE.[60]

All above-mentioned B-cell–depleting therapeutic approaches are also characterized by the risk of compromising immunity in general with an increased risk of infections and presumably cancer development as well as decreased responsiveness to vaccination. Moreover, different B-cell depletion strategies have also been found to result in different therapeutic effica y. For example, treatment of patients with SLE with rituximab showed no clinical benefit in 2 double-blind phase II/III clinical trials,[57] despite the fact that it is a efficient B-cell–depleting agent that should be beneficial in patients with SLE given the protective effect of belimumab. One may speculate that interfering with the BAFF-BAFFR signaling results in additional effects on top of B-cell depletion. For example, BAFF stimulation of human monocytes induces surface expression of TACI and promotes cell survival.[61] The effects of anti-CD20 treatment or the consequences of interfering with BAFF-BAFFR signaling on CVD in humans are not known, and only detailed understanding of the role of B cells and the BAFF system will help the identification of the best therapeutic option for CVD

Summary and Outlook

In addition to the use of genetic models resulting in B-cell deficiencies, the treatment of mouse models of atherosclerosis with B-cell–depleting agents has provided more information on the role of different B cells in plaque formation. For example, anti-CD20 treatment or blockage of the BAFFR signaling pathway that results in B2-cell depletion protects mice from atherosclerosis. It is particularly interesting that similar B-cell depletion strategies are approved as treatments in autoimmune diseases such as SLE and RA that are associated with increased risk of cardiovascular disease because of the development of accelerated atherosclerosis. Therefore, studies monitoring the effects of rituximab and belimumab treatment (and of other B-cell–targeting agents that are being developed) on CVD would be highly important for the understanding of the role of B cells in human atherogenesis and the potential of B-cell–targeting therapeutic strategies.

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Disclosures

None.

References

1. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*. 2010;10:36–46.
2. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol*. 2013;13:709–721.
3. Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. *Arterioscler Thromb Vasc Biol*. 2010;30:1282–1292.
4. Dansky HM, Charlton SA, Harper MM, Smith JD. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci USA*. 1997;94:4642–4646.
5. Reardon CA, Blachowicz L, Lukens J, Nissenbaum M, Getz GS. Genetic background selectively influences innominate artery atherosclerosis: immune system deficien y as a probe. *Arterioscler Thromb Vasc Biol*. 2003;23:1449–1454.
6. Harats D, Yacov N, Gilburd B, Shoenfeld Y, George J. Oral tolerance with heat shock protein 65 attenuates Mycobacterium tuberculosis-induced and high-fat-diet-driven atherosclerotic lesions. *J Am Coll Cardiol*. 2002;40:1333–1338.
7. Wick G, Jakic B, Buszko M, Wick MC, Grundtman C. The role of heat shock proteins in atherosclerosis. *Nat Rev Cardiol*. 2014;11:516–529.
8. Peters MJ, van Halm VP, Voskuyl AE, Smulders YM, Boers M, Lems WF, Visser M, Stehouwer CD, Dekker JM, Nijpels G, Heine R, Dijkmans BA, Nurmohamed MT. Does rheumatoid arthritis equal diabetes mellitus as an independent risk factor for cardiovascular disease? A prospective study. *Arthritis Rheum*. 2009;61:1571–1579.
9. Skaggs BJ, Hahn BH, McMahon M. Accelerated atherosclerosis in patients with SLE–mechanisms and management. *Nat Rev Rheumatol*. 2012;8:214–223.
10. Kyaw T, Tipping P, Toh BH, Bobik A. Current understanding of the role of B cell subsets and intimal and adventitial B cells in atherosclerosis. *Curr Opin Lipidol*. 2011;22:373–379.
11. Perry HM, Bender TP, McNamara CA. B cell subsets in atherosclerosis. *Front Immunol*. 2012;3:373.
12. Clark MR, Mandal M, Ochiai K, Singh H. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nat Rev Immunol*. 2014;14:69–80.
13. Schatz DG, Ji Y. Recombination centres and the orchestration of V(D)J recombination. *Nat Rev Immunol*. 2011;11:251–263.
14. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol*. 2011;11:34–46.
15. Wardemann H, Boehm T, Dear N, Carsetti R. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J Exp Med*. 2002;195:771–780.
16. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, Bobik A, Toh BH. B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res*. 2011;109:830–840.
17. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol*. 2012;42:120–129.
18. Houtkamp MA, de Boer OJ, van der Loos CM, van der Wal AC, Becker AE. Adventitial infiltrates associated with advanced atherosclerotic plaques: structural organization suggests generation of local humoral immune responses. *J Pathol*. 2001;193:263–269.
19. Mohanta SK, Yin C, Peng L, Srikakulapu P, Bontha V, Hu D, Weih F, Weber C, Gerdes N, Habenicht AJ. Artery tertiary lymphoid organs contribute to innate and adaptive immune responses in advanced mouse atherosclerosis. *Circ Res*. 2014;114:1772–1787.
20. Gräbner R, Lötzer K, Döpping S, et al. Lymphotoxin beta receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged ApoE-/- mice. *J Exp Med*. 2009;206:233–248.
21. Hamze M, Desmetz C, Berthe ML, Roger P, Boulle N, Brancherau P, Picard E, Guzman C, Tolza C, Guglielmi P. Characterization of resident B cells of vascular walls in human atherosclerotic patients. *J Immunol*. 2013;191:3006–3016.
22. Doran AC, Lipinski MJ, Oldham SN, et al. B-cell aortic homing and atheroprotection depend on Id3. *Circ Res*. 2012;110:e1–12.
23. Vinuesa CG, Chang PP. Innate B cell helpers reveal novel types of antibody responses. *Nat Immunol*. 2013;14:119–126.
24. Duan B, Morel L. Role of B-1a cells in autoimmunity. *Autoimmun Rev*. 2006;5:403–408.
25. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol*. 2013;13:118–132.
26. Zhang Y, Meyer-Hermann M, George LA, Figge MT, Khan M, Goodall M, Young SP, Reynolds A, Falciani F, Waisman A, Notley CA, Ehrenstein MR, Kosco-Vilbois M, Toellner KM. Germinal center B cells govern their own fate via antibody feedback. *J Exp Med*. 2013;210:457–464.
27. Hilgendorf I, Theurl I, Gerhardt LM, et al. Innate response activator B cells aggravate atherosclerosis by stimulating T helper-1 adaptive immunity. *Circulation*. 2014;129:1677–1687.
28. Zouggari Y, Ait-Oufella H, Bonnin P, et al. B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. *Nat Med*. 2013;19:1273–1280.
29. Mackay F, Schneider P. Cracking the BAFF code. *Nat Rev Immunol*. 2009;9:491–502.
30. Vincent FB, Morand EF, Schneider P, Mackay F. The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol*. 2014;10:365–373.
31. Castigli E, Scott S, Dedeoglu F, Bryce P, Jabara H, Bhan AK, Mizoguchi E, Geha RS. Impaired IgA class switching in APRIL-deficient mice. *Proc Natl Acad Sci USA*. 2004;101:3903–3908.
32. Rauch PJ, Chudnovskiy A, Robbins CS, et al. Innate response activator B cells protect against microbial sepsis. *Science*. 2012;335:597–601.
33. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest*. 2002;109:745–753.
34. Major AS, Fazio S, Linton MF. B-lymphocyte deficien y increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol*. 2002;22:1892–1898.
35. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vré E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF, Mallat Z. B cell depletion reduces the development of atherosclerosis in mice. *J Exp Med*. 2010;207:1579–1587.
36. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol*. 2010;185:4410–4419.
37. Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, Tipping P, Bobik A, Toh BH. Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS One*. 2012;7:e29371.

38. Sage AP, Tsiantoulas D, Baker L, Harrison J, Masters L, Murphy D, Loinard C, Binder CJ, Mallat Z. BAFF receptor deficien y reduces the development of atherosclerosis in mice–brief report. *Arterioscler Thromb Vasc Biol*. 2012;32:1573–1576.
39. Kyaw T, Cui P, Tay C, Kanellakis P, Hosseini H, Liu E, Rolink AG, Tipping P, Bobik A, Toh BH. BAFF receptor mAb treatment ameliorates development and progression of atherosclerosis in hyperlipidemic ApoE(-/-) mice. *PLoS One*. 2013;8:e60430.
40. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B cells and humoral immunity in atherosclerosis. *Circ Res*. 2014;114:1743–1756.
41. Hermansson A, Ketelhuth DF, Strodthoff D, Wurm M, Hansson EM, Nicoletti A, Paulsson-Berne G, Hansson GK. Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J Exp Med*. 2010;207:1081–1093.
42. Wang J, Cheng X, Xiang MX, et al. IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J Clin Invest*. 2011;121:3564–3577.
43. Kovanen PT, Mänttäri M, Palosuo T, Manninen V, Aho K. Prediction of myocardial infarction in dyslipidemic men by elevated levels of immunoglobulin classes A, E, and G, but not M. *Arch Intern Med*. 1998;158:1434–1439.
44. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol*. 2009;9:767–777.
45. Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO. Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation*. 2009;120:417–426.
46. Huan T, Zhang B, Wang Z, et al; Coronary ARteryDIsease Genome wide Replication and Meta-analysis (CARDIoGRAM) Consortium, International Consortium for Blood Pressure GWAS (ICBP). A systems biology framework identifies molecular underpinnings of coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2013;33:1427–1434.
47. Mantani PT, Ljungcrantz I, Andersson L, Alm R, Hedblad B, Björkbacka H, Nilsson J, Fredrikson GN. Circulating CD40+ and CD86+ B cell subsets demonstrate opposing associations with risk of stroke. *Arterioscler Thromb Vasc Biol*. 2014;34:211–218.
48. Browning JL. B cells move to centre stage: novel opportunities for autoimmune disease treatment. *Nat Rev Drug Discov*. 2006;5:564–576.
49. Roman MJ, Salmon JE. Cardiovascular manifestations of rheumatologic diseases. *Circulation*. 2007;116:2346–2355.
50. Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, Tedder TF. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med*. 2004;199:1659–1669.
51. Hahn BH. Belimumab for systemic lupus erythematosus. *N Engl J Med*. 2013;368:1528–1535.
52. Stohl W, Hiepe F, Latinis KM, Thomas M, Scheinberg MA, Clarke A, Aranow C, Wellborne FR, Abud-Mendoza C, Hough DR, Pineda L, Migone TS, Zhong ZJ, Freimuth WW, Chatham WW; BLISS-52 Study Group; BLISS-76 Study Group. Belimumab reduces autoantibodies, normalizes low complement levels, and reduces select B cell populations in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2012;64:2328–2337.
53. Isenberg D, Gordon C, Licu D, Copt S, Rossi CP, Wofsy D. Effica y and safety of atacicept for prevention of flares in patients with moderate-to-severe systemic lupus erythematosus (SLE): 52-week data (APRIL-SLE randomised trial). *Ann Rheum Dis*. 2014
54. Gross JA, Dillon SR, Mudri S, et al. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. impaired B cell maturation in mice lacking BLyS. *Immunity*. 2001;15:289–302.
55. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. 2004;199:91–98.
56. Mei HE, Schmidt S, Dörner T. Rationale of anti-CD19 immunotherapy: an option to target autoreactive plasma cells in autoimmunity. *Arthritis Res Ther*. 2012;14(suppl 5):S1.
57. Stohl W. Future prospects in biologic therapy for systemic lupus erythematosus. *Nat Rev Rheumatol*. 2013;9:705–720.
58. Jacobi AM, Goldenberg DM, Hiepe F, Radbruch A, Burmester GR, Dörner T. Differential effects of epratuzumab on peripheral blood B cells of patients with systemic lupus erythematosus versus normal controls. *Ann Rheum Dis*. 2008;67:450–457.
59. Samardzic T, Marinkovic D, Danzer CP, Gerlach J, Nitschke L, Wirth T. Reduction of marginal zone B cells in CD22-deficient mice. *Eur J Immunol*. 2002;32:561–567.
60. Dema B, Charles N, Pellefigues C, et al. Immunoglobulin E plays an immunoregulatory role in lupus. *J Exp Med*. 2014;211:2159–2168.
61. Chang SK, Arendt BK, Darce JR, Wu X, Jelinek DF. A role for BLyS in the activation of innate immune cells. *Blood*. 2006;108:2687–2694.

Significance

It is clear that some B-cell responses promote atherosclerosis, whereas others are protective. Natural antibody secretion from the B1a cell subset is a major protective pathway, but which types of B-cell responses or functions are most pathogenic is unclear. To study this in more detail is critical because (1) understanding the critical components specific to pathogenic B-cell responses will inform future therapeutic strategies against atherosclerosis; (2) B-cell responses are complex and it is important to understand which specific pathways and components are pathogenic rather than protective; (3) many autoimmune disease patients at high risk for cardiovascular disease are being treated by B-cell–targeting therapies; and (4) there are diverse opportunities to target B cells and many existing therapies used in autoimmune diseases and cancer could be translated for use in cardiovascular disease, and the previous successes of this mode of intervention bode well for future therapeutic developments.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Targeting B Cells in Atherosclerosis: Closing the Gap From Bench to Bedside

Dimitrios Tsiantoulas, Andrew P. Sage, Ziad Mallat and Christoph J. Binder

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1.3 Immunoglobulin M – the house keeper

IgM Abs comprise both natural IgM antibodies and adaptive IgM antibodies. In contrast to adaptive IgM, natural IgM antibodies are produced by B1 cells in a T cell independent manner. In uninfected mice more than 80% of the total IgM pool is derived from B1 cells (Baumgarth et al, 1999). Both epidemiological and experimental studies suggest that natural IgM exhibit atheroprotective properties (Tsiantoulas et al, 2014).

The role of natural IgM antibodies in atherosclerosis is reviewed below in Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ (2014) B cells and humoral immunity in atherosclerosis. *Circ Res* **114**(11)**:** 1743-1756.

This Review is in a thematic series on **The Immunopathogenesis of Arterial Diseases,** which includes the following articles:

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Danger Signaling in Atherosclerosis

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B Cells and Humoral Immunity in Atherosclerosis

Dimitrios Tsiantoulas, Cody J. Diehl, Joseph L. Witztum, Christoph J. Binder

Abstract: **Insights into the important contribution of inflammation and immune functions in the development and progression of atherosclerosis have greatly improved our understanding of this disease. Although the role of T cells has been extensively studied for decades, only recently has the role of B cells gained more attention. Recent studies have identified differential effects of different B-cell subsets and helped to clarify the still poorly understood mechanisms by which these act. B1 cells have been shown to prevent lesion formation, whereas B2 cells have been suggested to promote it. Natural IgM antibodies, mainly derived from B1 cells, have been shown to mediate atheroprotective effects, but the functional role of other immunoglobulin classes, particularly IgG, still remains elusive. In this review, we will focus on recent insights on the role of B cells and various immunoglobulin classes and how these may mediate their effects in atherosclerotic lesion formation. Moreover, we will highlight potential therapeutic approaches focusing on B-cell depletion that could be used to translate experimental evidence to human disease. (*Circ Res*. 2014;114:1743-1756.)**

Key Words: antibodies ■ atherosclerosis ■ B-lymphocytes ■ complement system proteins ■ immunity, humoral ■ immunoglobulin M

Atherosclerosis is a chronic inflammatory disease of large- and medium-sized arteries. It is characterized by the deposition and trapping of low-density lipoproteins (LDLs) in the artery wall, which then undergo a variety of changes, both enzymatic and nonenzymatic, that lead to a cascade of inflammatory responses followed by the recruitment of mostly macrophages and T cells.[1] The accumulation of LDL in the subendothelial space makes it susceptible to oxidation by various processes, and the resultant formation of oxidized LDL (OxLDL) has been suggested to be the key event in driving the subsequent inflammation that characterizes atherosclerosis.[2] Infiltrating macrophages take up OxLDL via scavenger receptors leading to the formation of lipid-laden foam cells, which are the hallmark cells of atherosclerotic lesions. With plaque progression, many foam cells undergo apoptosis resulting in the formation of an acellular necrotic core that is full of lipid gruel and cellular debris. Once the fibrous cap covering the necrotic core erodes or ruptures, allowing contact between the exposed material and the circulating coagulation system, thrombus formation is triggered, resulting in clinical events such as stroke and myocardial infarction,[3,4] which are leading causes of death globally.[5]

Inflammation is the response of the immune system to the presence of exogenous and endogenous antigens, and it is now widely accepted that immune mechanisms are involved in all phases of lesion development. However, knowledge

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Nonstandard Abbreviations and Acronyms

***Apoe*$^{-/-}$**	apolipoprotein E deficient
BAFF	B-cell–activating factor
BAFFR	B-cell–activating factor receptor
CFH	complement factor H
CHD	coronary heart disease
CuOx-LDL	copper-oxidized LDL
CVD	cardiovascular disease
Hsp60	heat shock protein 60
IFN-γ	interferon-γ
IL-10	interleukin-10
IRA	innate response activator
LDL	low-density lipoprotein
***Ldlr*$^{-/-}$**	low-density lipoprotein receptor–deficient
MDA-LDL	malondialdehyde-modified LDL
NAbs	natural antibodies
OSE	oxidation-specific epitope
OxLDL	oxidized LDL
OxPL	oxidized phospholipid
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism

of the antigens involved in initiating and sustaining the inflammatory response is only beginning to be understood. Although immune responses to infectious pathogens have been implicated in atherogenesis, evidence to support a primary role has been lacking, although such responses could have an important contributory role.[6] Among endogenous antigens, most studies have focused on heat shock protein 60 (Hsp60) and epitopes of OxLDL.[7–9] Hsp60, which shares high homology with mycobacterial Hsp65, has been shown to be expressed in endothelial cells in response to several proatherogenic triggers such as a high-cholesterol diet. This in turn results in the recall of a cross-reactive autoimmune response against endothelial Hsp60, but which originally developed against microbial Hsp65 following an infection. The resultant humoral and cellular response induces endothelial damage. Furthermore, oxidative modification of LDL results in the generation of a variety of oxidation-specifi epitopes (OSEs), such as the formation of adducts between apolipoprotein B and reactive oxidized lipid moieties, including phosphocholine-containing oxidized phospholipids (OxPLs), and their degradation products such as malondialdehyde and 4-hydroxynonenal. In addition, it is possible that immunogenic apolipoprotein B–derived peptides are also generated. Based on this, several model antigens are used to study these immune responses, including malondialdehyde-modified LDL (MDA-LDL) and copper-oxidized LDL (CuOx-LDL).[2,7,10] Although the exact contribution of these and potentially other antigens is not fully understood, it is now clear that both innate and adaptive immune responses are intimately involved in atherogenesis.

Innate immunity uses a variety of preformed, germline pattern recognition receptors to effect immune responses to pathogens and endogenous antigens. Because these pattern recognition receptors are limited in number, they recognize pathogen-associated molecular patterns on infectious agents and by analogy, danger-associated molecular patterns on endogenous antigens, which form common recognition motifs. These endogenous responses parallel antimicrobial responses and are necessary to sense tissue damage that needs to be resolved by the recruitment of phagocytes through sterile inflammation. In the case of atherogenesis, as noted above, heat shock proteins, cholesterol crystals, and OSEs seem to be important danger-associated molecular patterns that are recognized by innate pattern recognition receptors.[11] For example, OSEs, which are lipid peroxidation-derived modifications of proteins and lipids, represent conserved ligands for several arcs of innate immunity, including macrophage scavenger receptors, soluble innate proteins, and natural antibodies (NAbs). Notably, the same OSEs have been found on the surface of OxLDL and apoptotic cells, which both accumulate inside atherosclerotic lesions and provide a continuous trigger for sterile inflammatory responses [11]

Adaptive immunity also plays an important role in lesion development. Antibodies with specificity for plaque antigens (eg, OxLDL) and activated T cells are present in human lesions.[12,13] Important experimental evidence regarding the involvement of adaptive immunity came from studies with atherosclerosis-prone LDL receptor (*Ldlr*$^{-/-}$) or apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice that lack both functional T and B cells as a result of recombination activating gene deficien y or a severe combined immunodeficien y background.[14–16] These mice were found to exhibit significantly less lesion development indicating an important role for adaptive immunity. However, lesions still develop in these mice, and in the face of marked hypercholesterolemia, the immune-deficient mice develop similar lesions compared with those with intact adaptive immunity. Thus, although not a prerequisite for lesion development, adaptive immunity has an important modulatory role that can both enhance and retard lesion progression. There is now extensive evidence that Th1 responses and interferon-γ (IFN-γ) in particular are critical drivers of lesion formation. These effects are primarily mediated by $CD4^+$ T cells, but $CD8^+$ T cells seem to play a critical role as well.[9] Compelling evidence also demonstrates that the activity of proatherogenic T-cell subsets is tightly regulated by regulatory T cells, which act via multiple mechanisms and may directly alter plaque characteristics by the secretion of the fibrogenic cytokine transforming growth factor-β.[8] The roles of other T-cell subsets such as Th17 and Th2 are less consistent, and their effects may be best understood by the cytokines they secrete and how these function in relationship to IFN-γ–secreting Th1 cells. In contrast to the pronounced presence of T cells within lesions, only few B cells can be detected.[17] B cells participate in lymphocyte infiltrates of the adventitia surrounding affected arteries, which can be organized in tertiary lymphoid organs that have been suggested to regulate the inflammatory response of lesions.[18] Emerging data reviewed by Perry et al[19] and Kyaw et al[20] have recently identified an important role for B cells in atherogenesis. The potential mechanisms by which B cells may affect atherosclerosis include the production of

immunoglobulins and the subsequent formation of immune complexes with their cognate antigens. Antibodies with specificity for plaque antigens, such as OxLDL, have been detected in atherosclerotic lesions, and plasma antibody titers to OSEs and other antigens such as Hsp60 have been shown to correlate with several clinical manifestations of atherosclerosis.[12] Depending on the class of immunoglobulin, this can lead to the engagement of different Fc receptors resulting in cellular activation or inhibition. Moreover, antibodies may also provide an important function by neutralizing antigens and promoting their clearance. In addition, B cells mediate functions independent of antibody production, such as antigen presentation and the release of cytokines, including interleukin-10 (IL-10). In this review, we will discuss how different B-cell subsets may affect atherogenesis and particularly focus on the role of humoral immunity in mediating some of these effects.

B Cells Play a Role in Atherosclerosis

A recent network-driven integrative analyses of data from genome-wide association studies and whole blood gene expression profiles from Framingham Heart Study participants identified B-cell immune responses as causative in coronary heart disease (CHD).[21] Based on differential gene expression in whole blood extracts between healthy controls and patients with CHD, the authors identified modules enriched in genes involved in B-cell function, which were integrated with tissue-specific Bayesian networks and protein–protein interaction networks and led to the identification of B-cell–related genes as key drivers in CHD.

Evidence for a functional role of B cells in experimental atherosclerosis first came from studies by Caligiuri et al,[22] who demonstrated that splenectomized *Apoe*$^{-/-}$ mice develop aggravated atherosclerosis compared with sham-treated mice. This effect could be reversed by adoptive transfer of splenocytes. Interestingly, transfer of educated splenic B cells from older *Apoe*$^{-/-}$ donor mice not only rescued the proatherogenic effect, but also reduced lesion size below the size of sham-treated mice.[22] (It should be noted that transfer of T cells into splenectomized mice also rescued mice from enhanced atherosclerosis.) In fact, adoptive transfer of splenic B cells, but not T cells, into intact *Apoe*$^{-/-}$ mice also mediated moderate protection.[22] In line with this, Major et al[23] demonstrated that cholesterol-fed *Ldlr*$^{-/-}$ mice that were reconstituted with B-cell–deficient bone marrow from μ*MT* mice had increased plaque formation compared with mice reconstituted with wild-type bone marrow. Although these studies suggest an overall protective role of B cells, recent data have challenged this: 2 groups independently reported that anti-CD20–mediated B-cell depletion in atherosclerotic *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice results in a significant reduction of lesion size.[24,25] Thus, the general concept of B-cell involvement in atherosclerosis and the role of individual players still need to be elucidated.

The apparent discrepancy between the studies described above may be rooted in part in the differential roles of unique B-cell subsets (Table 1). Extensive studies of the peripheral blood, peritoneal cavity, spleen, and other lymphoid tissues have identified B1 and B2 cells as the 2 main B-cell subsets in mice based on their developmental origin. B2 cells, which represent the vast majority of B cells, are bone marrow derived and include follicular as well as marginal zone B cells. B1 cells, which can be further divided into B1a and B1b based on their surface marker expression, seem to be a developmentally and functionally distinct subset that is localized primarily in the peritoneal and pleural cavities.[26,27] Although B1a cells seem to be atheroprotective, the role of B2 cells in atherosclerosis is still debatable although the majority of studies suggest a proatherogenic role (Table 2 and see below). In addition, a distinct B1-cell subset termed innate response activator (IRA) that responds to lipopolysaccharide stimulation by secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) has recently been identified [33] It has been shown to play a vital role in protection against sepsis (Table 1). IRA B cells seem to be phenotypically distinct from other B1 cells because they also express the immature B-cell marker CD93. Moreover, in contrast to B1 cells, IRA B-cell survival is dependent on B-cell–activating factor receptor (BAFFR) signaling because these cells are depleted in BAFFR-deficient mice. Hilgendorf et al[32] showed that cholesterol-fed *Ldlr*$^{-/-}$ mice deficient in GM-CSF expressing B cells lack IRA B cells and develop reduced atherosclerosis. The authors propose that IRA B cells promote atherosclerosis by stimulating the expansion of mature dendritic cells that results in the generation

Table 1. Summary of Murine B-Cell Subsets and Their Surface Markers, Localization, and Frequencies in the Spleen

Murine B Cells	Cell Surface Markers	Localization	Frequency (of Splenic B Cells)
B1			
B1a	CD19^{+} B220^{low} IgM^{high} CD5^{+} CD43^{+} CD23^{-}	Spleen, peritoneum	≈2%
B1b	CD19^{+} B220^{low} IgM^{high} CD5^{-} CD43^{+} CD23^{-}	Spleen, peritoneum	<1%
IRA			
Innate response activator	IgM^{high} CD43^{+} CD23^{low} CD93^{+}	Spleen	<0.5%*
B2			
Marginal zone	CD19^{+} B220^{+} IgM^{high} CD21^{high} CD43^{-} CD23^{-}	Spleen	≈10%
Follicular	CD19^{+} B220^{+} IgM^{low} CD21^{+} CD43^{-} CD23^{+}	Spleen, circulation	≈80%
Bregs			
B regulatory	CD19^{+} B220^{+} IgM^{high} CD1d^{high} CD43^{-}	Spleen	≈1%

Bregs indicates regulatory B cells; and IRA, innate response activator.

*The frequency of IRA B cells increases to ≈4% in response to lipopolysaccharide injections.

Table 2. Summary of Experimental Studies With Respect to the Role of Different B-Cell Subsets in Atherosclerosis

Atherosclerosis Studies in Mice That Involve Different B-Cell Subsets	Effect on Atherosclerosis	B-Cell Subsets (Potentially) Affected					
		B1a	B1b	IRA	MZ	FO	Bregs
Adoptive CD19⁺ B-cell transfer (20×10^6) into splenectomized or sham-operated *Apoe*$^{-/-}$ mice[22]	↓	✓	✓	✓	✓	✓	✓
Reconstitution of *Ldlr*$^{-/-}$ mice with B-cell–deficient (μMT) bone marrow[23]	↑	✓	✓	✓	✓	✓	✓
CD20 antibody treatment of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice[24,25]	↓	(✓)	✓	?	✓	✓	✓
Adoptive transfer of splenic CD43⁻ B cells into lymphocyte-deficient *Rag2*$^{-/-}$*γ-chain*$^{-/-}$*Apoe*$^{-/-}$ (5×10^6 or 5×10^7) or B-cell–deficient μ*MT/Apoe*$^{-/-}$ (5×10^6) mice[25]	↑	…	…	…	✓	✓	✓
Adoptive transfer of splenic CD43⁻ B cells (3×10^7 or 6×10^7) into B-cell–deficient μ*MT/Apoe*$^{-/-}$ mice[28]	↓	…	…	…	✓	✓	✓
Adoptive transfer of wild-type but not *sIgM*$^{-/-}$ B1a cells (10^5 every 3 wk) into splenectomized atherosclerotic *Apoe*$^{-/-}$ mice[29]	↓	✓	…	…	…	…	…
BAFFR-deficient *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice[24,30]	↓	…	…	(✓)	✓	✓	?
Anti-BAFFR treatment of *Apoe*$^{-/-}$ mice[31]	↓	…	…	(✓)	✓	✓	?
Reconstitution of *Ldlr*$^{-/-}$ mice with 50% GM-CSF and 50% B-cell–deficient (μMT) bone marrow to achieve GM-CSF deficiency in B cells only[32]	↓	…	…	✓	…	…	…

Apoe$^{-/-}$ indicates apolipoprotein E deficient; BAFFR, B-cell–activating factor receptor; Bregs, regulatory B cells; FO, follicular; IRA, innate response activator; *Ldlr*$^{-/-}$, low-density lipoprotein receptor; and MZ, marginal zone.

of IFN-γ–secreting Th1 cells, accompanied by a switch from IgG1 to IgG2c antibodies against OxLDL. Moreover, these cells are increased in the spleen of patients with symptomatic compared with nonsymptomatic cardiovascular disease (CVD).[32] Finally, regulatory B cells with a distinct surface marker expression profile have been identified and are being discussed as modulators of autoimmunity, for example, by the secretion of IL-10 or direct interaction with pathogenic T cells.[34] The role of regulatory B cells in atherogenesis remains to be identified

In humans, different B-cell subsets have been described as well, but data regarding these are mostly derived from peripheral blood analysis. There is only limited knowledge regarding B cells that lack circulating capacities and substantial disagreement on the identity of such subsets.

B2 and B1 Cells: The Villain and the Good Guy?

A series of studies has pointed toward a differential role of B1 and B2 cells in atherogenesis (Table 2). For example, anti-CD20 treatment in vivo preferentially induces rapid death of B2 cells within a few hours on administration. Indeed, in a study by Ait-Oufella et al,[24] B2 cells were effectively depleted on CD20 antibody treatment, whereas B1a cells remained unchanged. These data were also confirmed by Kyaw et al,[25] who in addition showed that adoptive transfer of splenic B2 cells, but not B1 cells, into lymphocyte-deficient Rag2$^{-/-}$γ-chain$^{-/-}$ *Apoe*$^{-/-}$ or B-cell–deficient μMT/*Apoe*$^{-/-}$ mice aggravated atherosclerosis.[24,35] Moreover, disruption of the B-cell–activating factor (BAFF) signaling in atherosclerotic mouse models has further supported a proatherogenic function of B2 cells specifically. BAFF is expressed by monocytes, dendritic cells, and T cells and binds to 3 receptors predominately found on B cells, the BAFFR, transmembrane activator and calcium modulator and cyclophilin ligand interactor, and B-cell maturation antigen.[36] The interaction between BAFF and BAFFR is of crucial importance for the survival of B2 cells because mice deficient in either BAFF or BAFFR lack mature B2 cells but have near-normal B1 cell numbers.[37,38] In line with this, BAFFR-deficient *Apoe*$^{-/-}$ mice as well as BAFFR-deficient *Ldlr*$^{-/-}$ bone marrow chimeric mice display a nearly complete lack of mature B2 cells with fully preserved B1a cell numbers.[30,39] In both cases, atherosclerotic lesion formation was significantly reduced. In addition, in a recent study, Kyaw et al[31] showed that inhibition of the BAFFR using a blocking anti-BAFFR antibody in atherosclerotic *Apoe*$^{-/-}$ mice resulted in a selective depletion of B2 cells and decreased plaque formation. These data support a proatherogenic role for B cells and B2 cells in particular. The exact mechanisms by which B2 cells promote atherosclerosis remain elusive. Nevertheless, several possibilities have been suggested. For example, a link between anti-CD20–mediated B2-cell depletion and IL-17 upregulation has been proposed to mediate the atheroprotective effect of this treatment, because anti–IL-17 treatment abrogated the effect of anti-CD20.[24] In turn, increased Th17 cells were suggested to counterbalance the effects of proatherogenic IFN-γ secreting Th1 cells. On the contrary, the robust reduction of T-cell–dependent serum IgG antibodies as result of B-cell depletion (Figure 1) may have resulted in the removal of potentially pathogenic antibodies directed against plaque antigens. Furthermore, B-cell–mediated antigen presentation to pathogenic T cells would also be impaired after B-cell depletion. Interestingly, IRA cells, which secrete GM-CSF, were also shown to be depleted in BAFFR-deficient mice, and thus their depletion may also be responsible for the decreased atherogenesis in these mice.[33]

In contrast to the studies discussed above, McNamara and colleagues found that adoptive transfer of splenic B2 cells from *ApoE*$^{-/-}$ mice into cholesterol-fed μMT/*Apoe*$^{-/-}$ mice resulted in robust protection from atherosclerosis. In addition, they

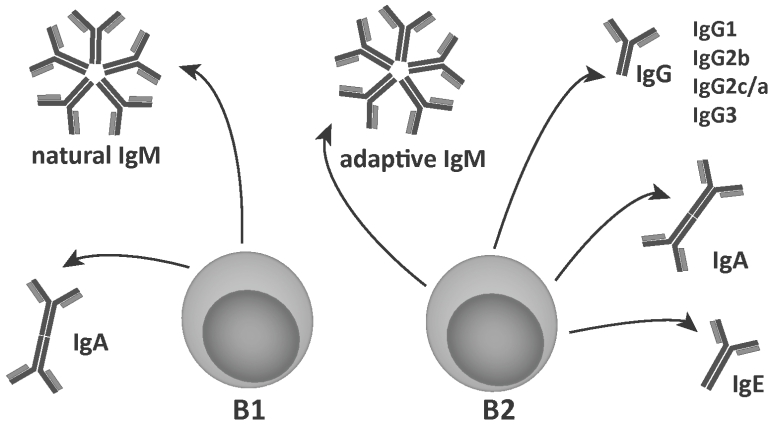

Figure 1. B1 and B2 cells have different immunoglobulin production profiles in mice. B cells are divided in 2 main subfamilies, the B1 and B2 cells. B1 cells produce germline-encoded natural IgM and IgA antibodies. B2 cells respond to T-cell help on antigen stimulation and produce adaptive IgM, followed by IgG (IgG1, IgG2a/c, IgG3), IgA, or IgE antibodies via a process termed class switching.

found that the effect was absent when B cells from *Apoe*$^{-/-}$ mice, which were also deficient in the transcription factor inhibitor of differentiation-3 (Id3), were transferred.[28] Of note, transferred *Id3*$^{-/-}$ B cells failed to home to aortic sites, which was accompanied by decreased chemokine receptor (CCR)6 expression on Id3-deficient B cells. A possible explanation for these contradictory data could be the different numbers and different genetic background (wild type versus *Apoe*$^{-/-}$) of transferred B2 cells, which may affect their fate and function in the recipient. Moreover, differences in the purity of transferred cells may also have contributed to these conflicting results. One may even speculate that the CD43^{-} B-cell preparations also contained potentially atheroprotective regulatory B cells and that an increased frequency of such cells could have contributed to the beneficial outcome in the study by McNamara and colleagues, for example, via IL-10 secretion.

Thus, at present, there is conflicting evidence concerning the role of B2 cells in atherogenesis. To clarify this seemingly complicated issue, it will be critical to dissect the functions of individual B2-cell subsets (marginal zone versus follicular) and to separate intrinsic biological properties of the B2 cells from effects mediated by the antibodies they secrete.

B1a cells on the contrary, which represent the much smaller subset of B cells, clearly protect from atherosclerosis in mice. It is currently not known whether B1b cells have the same atheroprotective potential as B1a cells. An intact spleen has been shown to be required for B1a cell maintenance, because splenectomized or genetically asplenic mice have significantly lower B1 cell numbers in the circulation and peritoneal cavities.[40] Moreover, it has been shown that the splenic microenvironment enhances the antibody production capacity of B1 cells.[41] Thus, splenectomy may result in the loss of this potentially atheroprotective B-cell subset. Indeed, Kyaw et al[29] showed that the peritoneal B1a cells of splenectomized *Apoe*$^{-/-}$ mice are reduced by 50%. Importantly, transfer of peritoneal B1a cells but not splenic B2 cells into splenectomized *Apoe*$^{-/-}$ mice resulted in an amelioration of the splenectomy-induced accelerated atherosclerosis.[29] Interestingly, in a long-term follow-up study on the cause of death of 740 World War II American servicemen who underwent splenectomy consequent to trauma, the risk of death attributable to ischemic heart disease was found to be 2-fold higher, suggesting a similar net protective effect of splenic cells in humans as well.[42] A human B1 cell equivalent has been recently described by Griffin et al[43] applying the criteria of spontaneous IgM secretion, efficient T-cell stimulation, and tonic intracellular signaling. Based on these criteria, they identified a small population of B1 cells present in umbilical cord and adult peripheral blood that express the unique phenotype of CD20^{+}CD27^{+}CD43^{+} CD70^{-}.[43] A recent study, however, has challenged this fact, as Covens et al[44] analyzed characteristics of this newly described B-cell population and found them to have a gene expression signature more similar to preplasmablasts than to murine B1 cells. Interestingly, this population of B cells was particularly enriched in umbilical cord blood and found to decline with age. It is tempting to speculate that such a decline correlates with a potential loss of protective capacities resulting in increased cardiovascular risk. Because the secretion of natural IgM antibodies is one of the major functions of B1 cells, it has been hypothesized that the atheroprotective effect of B1 cells depends on the secretion of IgM antibodies. Splenectomy of *Apoe*$^{-/-}$ mice is associated with decreased IgM levels, and patients subjected to splenectomy after trauma have been reported to have significantly lower serum IgM titers.[45] Of note, unlike transfer of wild-type B1a cells, B1a cells isolated from *sIgM*$^{-/-}$ donor mice, which express but do not secrete IgM, failed to attenuate the accelerated atherosclerosis in splenectomized mice, demonstrating that the IgM conferred the atheroprotection.[29] This is particularly interesting because we and others have previously characterized atheroprotective properties of specific B1 cell–der ved natural IgM antibodies.[46–50]

IgM: The House Keeper

Several studies in human subjects have shown that plasma levels of IgM antibodies to OSEs are inversely correlated with CVD. For example, levels of IgM antibodies to CuOx-LDL and MDA-LDL are inversely correlated with the carotid intima–media thickness or the risk of developing a >50% diameter stenosis in the coronary arteries.[51–53] Similarly, IgM titers to the OSE phosphocholine have been reported to be inversely correlated with the incidence of stroke[54] and heart attack[55] as well as with the CVD risk in patients with lupus.[56,57] Although these data are primarily correlative in nature, there is now increasing experimental evidence supporting a mechanistic role for IgM antibodies in protection against CVD, as reviewed below (Figure 2).

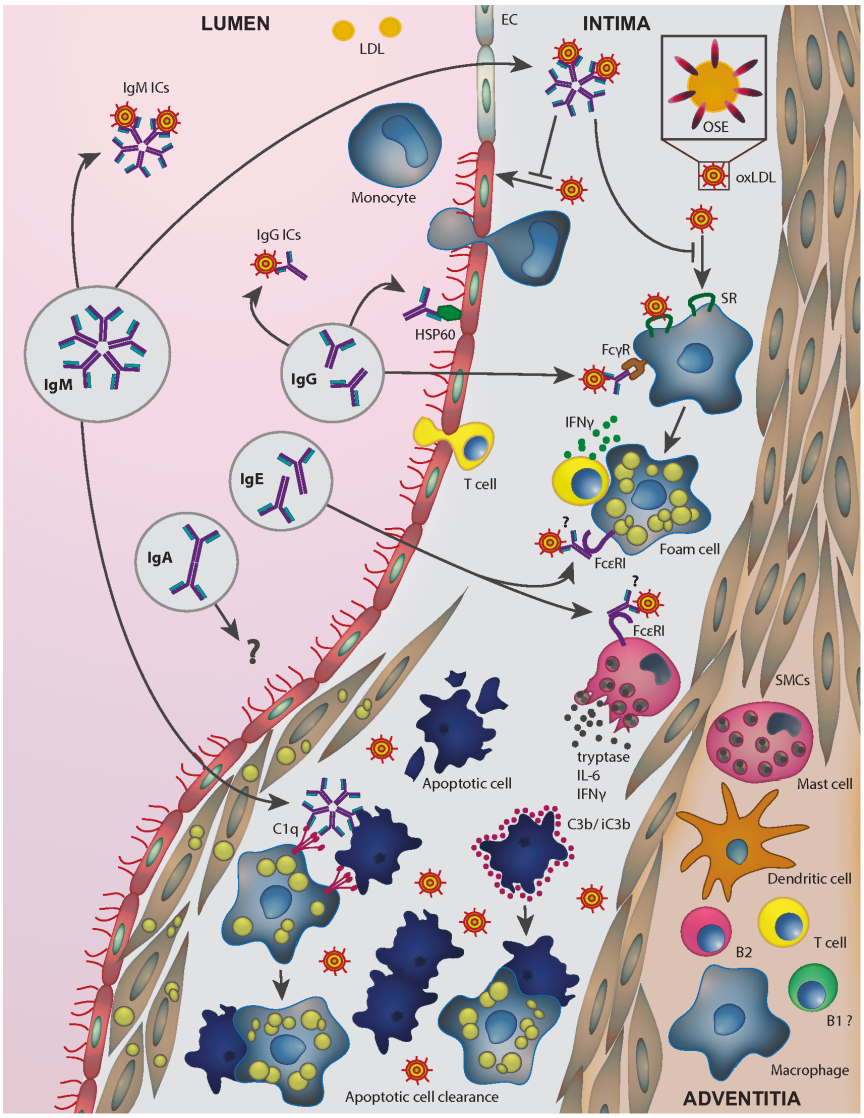

Figure 2. Role of immunoglobulins in lesion development. Different immunoglobulins are present in atherosclerotic plaques, which are directed against relevant antigens such heat shock proteins (HSPs) and oxidized low-density lipoproteins (OxLDLs). IgM antibodies may mediate atheroprotection by neutralizing the proinflammatory properties of OxLDL, inhibiting the uptake of OxLDL by macrophages, and by promoting apoptotic cell clearance. Their protective capacity may be largely dependent on their ability to recognize oxidation-specific epitopes (OSEs) present on both OxLDL and apoptotic cells. OxLDL-specific IgG antibodies could activate macrophages via Fcγ receptor engagement, thereby promoting atherogenesis, but may also exhibit protective neutralizing capacities. IgM and IgG immune complexes with LDL carrying OSEs are also found in the circulation and may promote clearance of proatherogenic LDL particles. HSP60/65-specific IgG recognize stressed endothelial cells and induce damage via antibody-dependent cellular cytotoxicity. IgE antibodies activate mast cells and macrophages via the engagement FcεRI resulting in plaque destabilization. Specificities of IgE for atherosclerosis antigens, for example, OxLDL, remain to be shown. The role of IgA antibodies in atherosclerosis is still elusive. Opsonization of apoptotic cells with C3b and iC3b enhances their uptake by macrophages. EC indicates endothelial cells; IC, immune complexes; IFN-γ interferon-γ; IL-6, interleukin-6; SMCs, smooth muscle cells; and SR, scavenger receptors.

IgM antibodies comprise both natural IgM antibodies and adaptive IgM antibodies. NAbs are primarily derived from B1 cells that spontaneously secrete T-cell–independent antibodies, whereas adaptive IgMs are secreted by B2 cells in a T-cell–dependent manner (Figure 1). In uninfected mice, 80% to 90% of the total IgM pool is derived from B1 cells.[58] Natural IgM antibodies are pre-existing germline-encoded products that do not require exogenous antigen stimulation for their generation.[59] Because of their specificity for microbial antigens, they play an important role in the first-line defense against infections with bacteria, viruses, and fungi. For example, *sIgM*$^{-/-}$ mice are particularly susceptible to bacterial peritonitis in a cecal ligation and puncture model.[60] Moreover, IgM antibodies are also necessary to mount an efficient protective response against influenza virus as well as to promote the recognition and clearance of fungi, such as *Pneumocystis murina*.[60–63] NAbs also have specificity for certain self antigens, and in addition to their antimicrobial properties, possess—so-called—housekeeping functions by regulating the safe disposal of apoptotic cells and (neo)self antigens, for example, altered self proteins. NAbs facilitate the removal of cellular debris,[64] because their repertoire includes specificities for highly conserved structures on apoptotic cells and other (neo)self antigens, such as OSEs that are found on apoptotic cells and on OxLDL.[65] We have shown that OSEs are a major target of natural IgM antibodies in mice and humans.[66] These studies originated in the characterization of a set of monoclonal IgM antibodies with specificity for epitopes of OxLDL that were cloned from the spleens of hypercholesterolemic *Apoe*$^{-/-}$ mice, which develop high anti-OxLDL autoantibody titers.[67] One clone that was studied in more detail is the anti-OxLDL IgM E06, which was shown to have fine specificity for the phosphocholine head group of OxPLs, but not the phosphocholine of native phospholipids.[68] Of relevance, E06 could bind to OxLDL and prevent its binding to CD36 and SR-B1 of macrophages and thus inhibit OxLDL uptake and prevent foam cell formation in vitro.[69] These and other data demonstrated that the phosphocholine of OxPL is a ligand for CD36 and mediated the uptake of OxLDL by macrophages.[11]

Subsequently, sequence analysis of the CDR3 region of E06 revealed a germline sequence, which was 100% identical in both the V_L and V_H chains to that of the prototypic B1

cell–derived NAb T15, which is an IgA. T15 has specificity for phosphocholine, which is also a prominent constituent of the capsular polysaccharide of *Streptococcus pneumonia* (but is not part of a phospholipid) and has been shown to provide optimal protection to mice from pneumococcal infections.[50,70,71] We first demonstrated an atheroprotective function of T15/E06 IgM in *Ldlr*$^{-/-}$ mice that were immunized with heat-killed pneumococcal extracts and fed an atherogenic diet for 16 weeks. This immunization resulted in high anti-OxLDL IgM titers attributable to a near monoclonal expansion of T15id^{+} IgM and concomitantly decreased lesion formation.[47] Plasma from these mice were able to block the uptake of OxLDL by macrophages effectively. Subsequently, Faria-Neto et al[49] confirmed the atheroprotective function of T15/E06 by showing that passive infusion of T15/E06 IgM antibodies reduced vein graft atherosclerosis in *Apoe*$^{-/-}$ mice. Moreover, several atheroprotective interventions are associated with an increase in T15/E06 IgM levels. For example, we could show that the atheroprotective immunization with homologous MDA-LDL resulted not only in the generation of high titers of antibodies against MDA-LDL, but also in the expansion of T15/E06 IgM. We found that this effect was dependent on the induction of IL-5, because MDA-LDL immunization of *Il5*$^{-/-}$ mice did not show an expansion of T15/E06 IgM. Moreover, IL-5–deficien *Ldlr*$^{-/-}$ bone marrow chimeras had significantly lower T15/E06 levels and developed accelerated atherosclerosis. The potential atheroprotective function of IL-5 and its role in modulating anti-OxLDL IgM levels was further confirmed in a human population, in which low serum IL-5 levels were associated with increased carotid intima–media thickness and low anti-OxLDL IgM levels.[72,73] Of note, in a genome-wide association study analysis of 15 596 patients with coronary artery disease and 34 992 controls, a single-nucleotide polymorphism (SNP) variant in the 3′ untranslated region of the *IL-5* gene was associated with increased risk of coronary artery disease.[74]

The mechanisms that underlie the protective properties of T15/E06 IgM are not entirely clear. As noted above, experiments performed in vitro have shown that T15/E06 prevents uptake of OxLDL by binding to the phosphocholine of OxPL, thereby inhibiting foam cell formation.[46,47] An additional mechanism by which T15/E06 IgM may limit plaque burden is by limiting the accumulation of apoptotic cells in developing lesions through the recognition of phosphocholine of OxPL formed on apoptotic cell surfaces.[65] Impaired efferocytosis has been linked to enhanced atherogenesis, and T15/E06 has the capacity to promote apoptotic cell clearance by macrophages in a C1q-dependent manner.[64,75,76] Finally, a key protective function is found in the ability of T15/E06 IgM to neutralize proinflamatory gene expression induced by OxPL present in OxLDL and the membranes of apoptotic cells.[11,48] For example, T15/E06 has been shown to inhibit IL-8 and adhesion molecule expression in endothelial cells stimulated with apoptotic cells or blebs and decreased monocyte adherence.[48,77] Moreover, T15/E06 was able to abrogate the recognition of POVPC (1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine) (an OxPL) by the macrophage scavenger receptor CD36,[69] which is also critically involved in the proinflammatory response of macrophages to OxPL by cooperating with Toll-like receptor 4 and 6.[78] Indeed, T15/E06 IgM has been found to prevent OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine)–induced IL-6 secretion by macrophages[79] and to block the ability of OxPL to decrease macrophage phagocytosis.[80] Many more natural IgMs with specificity for other OSEs exist, which represent a prominent fraction (20%–30%) of all natural IgMs in mice and humans.[66] For example, IgM with specificity for malondialdehyde epitopes, such as the natural IgM NA17, also bind apoptotic cells and enhance the in vivo clearance of injected apoptotic cells by peritoneal macrophages.[66] Malondialdehyde represents another important danger signal that is present in atherosclerotic lesions and promotes inflammatory cytokine expression in vivo.[66] Because of the prominent presence of malondialdehyde adducts in lesions, malondialdehyde-specific IgM may have a particularly important role in atheroprotection. Indeed, we have shown that the atheroprotective immunization of animal models of atherosclerosis with autologous MDA-LDL also leads to the induction of high titered IgM antibodies against MDA-LDL, which may in part be responsible for the protective effect of immunization.[72]

Because ~30% of natural IgM antibodies bind different OSEs, it can be expected that low IgM levels in general or total IgM deficien y would be associated with an increased propensity for lesion formation. Lewis et al[81] demonstrated the atheroprotective role of IgM antibodies using *sIgM*$^{-/-}$ mice, which cannot secrete IgM but possess surface-bound IgM antibodies and have the capacity to class switch and secrete all other immunoglobulin classes. When *sIgM*$^{-/-}$ mice were crossed onto *Ldlr*$^{-/-}$ mice, they develop dramatically accelerated atherosclerosis both on a low cholesterol diet and on an atherogenic diet. Considering the above described role for IgM in apoptotic cell clearance, the authors evaluated the apoptotic cell accumulation in the aortic root lesions of these mice. Although there was a trend toward increased numbers of apoptotic cells in the lesions of *sIgM*$^{-/-}$*Ldlr*$^{-/-}$ mice fed a low cholesterol diet, differences were not significant when stringent statistical tests were applied. Moreover, no differences in apoptotic cell accumulation were found in *sIgM*$^{-/-}$*Ldlr*$^{-/-}$ mice fed a high-cholesterol diet, despite differences in lesion size.[81] Similarly, adoptive transfer of B1a cells into splenectomized *Apoe*$^{-/-}$ mice, which rescued the aggravated atherosclerosis in these mice and increased lesional IgM deposition, did not significantly alter the apoptotic cell content compared with lesions from mice in which B1a cells from *sIgM*$^{-/-}$ mice were transferred.[29] These data suggest that promoting the clearance of apoptotic cells within the lesions alone does not fully explain the atheroprotective mechanisms of IgM, and additional mechanisms may be operative.

In this regard, immunoregulatory properties of IgM may be of particular importance. Natural IgMs possess a biased repertoire toward (neo)self antigens, and this interaction may be a critical factor for immune homeostasis. Mice deficient in sIgM display strong differences in their splenic B-cell populations with increased B1 and marginal zone cells and decreased follicular B cells, likely as a result of impaired B-cell receptor signaling. Moreover, IgG2a and IgG3 as well as IgA plasma levels are increased in *sIgM*$^{-/-}$ mice, which also show impaired IgG antibody responses at least to suboptimal doses

of a T-cell–dependent antigen.[82–85] Paradoxically, Lewis et al[81] reported decreased IgG2a/c titers against CuOx-LDL in atherosclerotic *sIgM*$^{-/-}$*Ldlr*$^{-/-}$ mice, which may reflect increased immune complex formation with OxLDL in the absence of circulating IgM. Interestingly, infusion of polyclonal IgM into *Apoe*$^{-/-}$ mice during the last 4 weeks of a 29-week atherogenic diet has been reported to delay lesion formation, whereas infusion of a monoclonal T15id^{+} IgM preparation failed to do so in this setting.[86] Atheroprotection in mice receiving polyclonal IgM was associated with a decreased frequency of CD4^{+} cells in the spleen. These data suggest that immunoregulatory functions of IgM, which may require the full repertoire of IgM specificities, provide an additional atheroprotective mechanism. In this regard, it is worthwhile to note that the repertoire (antibody specificities) of IgM antibodies differs between different B-cell subsets. For example, B1a cell–derived IgMs are more restricted to a germline-encoded repertoire, whereas the specificities of B1b cell–derived IgM can be more adaptive to antigen challenge.[27] Therefore, insights into the contribution of the polyclonal repertoire of natural IgM as well as the role of IgM with defined specificities, such as phosphocholine and malondialdehyde, will be critical for the understanding of the atheroprotective mechanism of IgM.

IgG: Old Friend or Foe?

IgG is the main immunoglobulin subtype in the circulation of humans. The IgG consists of 4 different subclasses both in humans (IgG1, IgG2, IgG3, and IgG4) and in mice (IgG1, IgG2a/c, IgG2b, and IgG3) that exhibit different affinity to Fcγ receptors as well as a different capacity to activate complement.[87,88] IgG antibodies are present in atherosclerotic lesions, and some of them have been shown to have specificity for OxLDL.[12] In addition, IgG antibodies with specificity for OxLDL and other plaque antigens have been documented in human plasma, and thus several epidemiological studies have measured their titers in association with CVD risk. Although many studies documented a positive correlation of anti-OxLDL IgG with manifestations of CVD, others have not.[9] In most cases, however, these correlations lose significance when multivariate analyses are performed with other risk factors of CVD. Another explanation for the inconsistency of these studies may also be found in the lack of reproducible antigens used to make such measurements. We have recently identified and characterized peptide mimotopes for malondialdehyde epitopes that can serve as a standardized antigen to assess antibodies specific to malondialdehyde epitopes.[89] Studies are currently ongoing to evaluate their use in large clinical studies. Hsp65-specific IgG titers have also been evaluated in relationship to CVD manifestations and found to be an independent risk factor for carotid intima–media thickness in one study,[90] but not in another.[91]

Despite all these caveats, IgG antibodies have often been suggested to be proatherogenic. However, there is little experimental evidence for this or even any functional role of IgG antibodies in atherosclerosis. Apart from the recent mouse studies, in which B-cell depletion was associated with decreased atherosclerosis and a profound reduction of both total and OxLDL-specific IgG antibodies and to a lesser extent IgM antibodies,[24,25,30,31,39] their role has been mostly implicated from immunization studies with either Hsp65 or models of OxLDL. For example, immunization of normocholesterolemic rabbits with Hsp65 induced arteritis even in the absence of elevated serum cholesterol levels and accelerated atherosclerosis on a cholesterol-enriched diet.[92] These effects were reproduced in studies using *Ldlr*$^{-/-}$ mice fed a regular chow diet, which developed high anti-Hsp65 IgG antibodies and increased atherosclerotic lesions.[93] A proatherogenic effect of anti-Hsp65 IgG was also demonstrated by George et al,[94] who showed that intraperitoneal injections of chow-fed *Ldlr*$^{-/-}$ mice with IgG preparations from Hsp65-immunized mice promoted fatty streak formation. These data suggest that at least in part Hsp65-specific IgGs h ve proatherogenic properties, for example, by damaging Hsp60-expressing endothelial cells[95] (Figure 2).

In contrast, immunization of *Ldlr*$^{-/-}$ Watanabe heritable hyperlipidemic rabbits with homologous MDA-LDL, which resulted in the induction of high IgG antibody titers against MDA-LDL, was shown to protect from atherosclerotic lesion formation compared with control PBS and keyhole limpet hemocyanin-immunized rabbits.[96] Similar data were obtained after immunization of hypercholesterolemic rabbits with homologous CuOx-LDL.[97] Moreover, immunization of either *Apoe*$^{-/-}$ or hypercholesterolemic *Ldlr*$^{-/-}$ mice with MDA-LDL resulted in the induction of robust IgG titers against MDA-LDL and decreased atherosclerosis.[72,98–100] In such immunization experiments, many complex immunoregulatory changes may be induced including both cellular and humoral effects, and the mechanisms by which immunization with OSEs protect from lesion formation remain to be defined. Clearly, more complex regulatory mechanisms may be involved beyond the simple induction of neutralizing antibodies. In support of this, immunization of *Ldlr*$^{-/-}$ mice with native LDL also resulted in atheroprotection in the absence of any measurable IgG titers to LDL antigens.[100] Nevertheless, there is considerable data to support a direct protective effect of OSE-specific antibodies. For example, passive transfer of a recombinant human IgG1 to MDA-LDL into *Apoe*$^{-/-}$ mice inhibited lesion formation.[101] Passive infusion of *Ldlr*$^{-/-}$ mice with the human recombinant Fab antibody IK17 against MDA-LDL[102] or adenoviral-mediated expression in vivo of an scFv version of IK17 both inhibited lesion formation, and in both of these cases, there was an enhanced capacity of plasma antibodies to inhibit OxLDL binding to macrophages.[102] Thus, considerable data demonstrate direct atheroprotective properties for malondialdehyde-specific antibodies. Importantly, to our knowledge; there is no information to date of adverse effects of OxLDL-specific IgG antibodies in vivo.

However, there are only limited insights into the mechanisms by which these anti-OxLDL IgGs act in vivo (Figure 2). Based on in vitro analysis of plasma from mice with high IgG titers to MDA-LDL, it seems that such IgGs have the capacity to block the binding of OxLDL to macrophages in vitro, but whether this occurs in vivo is unknown. On the contrary, Schiopu et al[101] reported that the recombinant human MDA-LDL–specific IgG1 that inhibited atherosclerosis actually increased uptake of OxLDL by macrophages in vitro. Of importance, the contributing role of Fc effector functions of these anti-OxLDL IgG antibodies is still elusive. Fcγ receptors,

which bind to the Fc portion of antibodies, are critical mediators of functions of IgG, and several studies in mouse models of atherosclerosis have addressed the global role of Fcγ receptors. Two categories of Fcγ receptors exist: the activating receptors that include the FcγRI, FcγRIIA, FcγRIII, and on the contrary, the inhibitory receptor FcγRIV. *Apoe*$^{-/-}$ mice lacking the Fc γ-chain (γ$^{-/-}$, which lack all activating receptors), thus only express the inhibitory receptor FcγRIIB, develop significantly less atherosclerosis when fed either regular chow or a high-cholesterol diet. Decreased lesion formation was associated with reduced numbers of lesional macrophages and T cells.[103] Consistent with a proatherogenic role of activating Fcγ receptors, another study also found strongly reduced lesion size in CD16 (FcγRIII)-deficient *Ldlr*$^{-/-}$ mice at both the aortic root and innominate artery, despite increased plasma total cholesterol. Interestingly, *Cd16*$^{-/-}$*Ldlr*$^{-/-}$ mice exhibited elevated IgG1 and IgG2c plasma titers against MDA-LDL and CuOx-LDL compared with *Ldlr*$^{-/-}$ control mice, whereas total plasma IgG levels were not different.[104] Although these data point to a proatherogenic role of activating Fcγ receptors, it is not clear whether these effects correspond directly to the engagement of Fc receptors by disease-specific IgGs, such as those to OSEs, or to a more global property of activating Fcγ receptors. It has been shown that human OxLDL-IgG immune complexes activate proinflammatory mitogen-activated protein kinase signaling in THP-1 human monocytes in an FcγRI (the human equivalent of murine FcγRI)-dependent manner.[105] In addition, indirect mechanisms may also have contributed to the atheroprotective effects in FcγR-deficient mice. For example, does the increase of OxLDL-specific IgGs mediate protection or is the relative activity of the inhibitory FcγRIIB increased in these mice? The latter point is of great interest because expression of FcγRIIB has been shown to protect mice from several autoantibody-mediated disease.[106] Indeed, *Apoe*$^{-/-}$ mice deficient in the inhibitory FcRγIIB receptor develop enhanced atherosclerosis, indicating a protective role for this immunomodulatory Fc receptor. Notably, these mice also had elevated IgG1 and IgG2c plasma titers against MDA-LDL and CuOx-LDL.[107]

In summary, it is clear that we are still far from a complete understanding of the biological roles and functions of OxLDL-specific IgGs in atherosclerosis. In addition to binding to OxLDL, these OSE antibodies also bind to apoptotic cells, although their impact on clearance or the proinflammatory properties of these cells is unknown. It still needs to be investigated whether their effects are mediated by FcγR and, if so, which cells expressing FcγR are involved. Furthermore, the roles of the different IgG isotypes, which have different affinities for Fcγ receptors, as well as different complement activating properties, need to be determined. These considerations add yet another layer of complexity to understanding their effector functions and net impact on atherogenesis.

IgE: The Underestimated Player

IgE antibodies have been extensively studied in allergy and asthma, where they are considered critical mediators of these pathologies. Little is known about their role in atherosclerosis, although a few epidemiological studies exist that suggest a contribution for this usually tightly controlled immunoglobulin. For example, Kovanen et al[108] found that high IgE levels are a prognostic factor for myocardial infarction and cardiac death in dyslipidemic men in the Helsinki Heart Study. This association was later confirmed by Wang et al,[109] who found that patients with CHD have elevated IgE levels compared with subjects without CHD. Moreover, in this study, IgE levels were positively correlated with the CVD severity, with patients with acute myocardial infarction having the highest serum IgE levels compared with patients with unstable angina pectoris and stable angina pectoris. The association with IgE was independent of sex, age, body mass index, hypertension, diabetes mellitus, or serum lipid profiles. This study and several others, including the one by Kovanen et al,[108] have also shown that IgE levels were directly associated with smoking. Therefore, IgE may be involved in the increased risk of atherosclerosis associated with smoking. Thus, epidemiological data suggest a proatherogenic role for IgE antibodies.

Although the role of IgE antibodies in experimental atherosclerosis has not been directly investigated, indirect evidence comes from studies in atherosclerosis-prone mice deficient in Fcε receptors. IgE antibodies mainly bind to the high-affinity IgE receptor (FcεRI) and to the low-affinity IgE receptor (FcεRII/CD23). Moreover, the IgE-binding protein galectin-3 has been shown to cross-link receptor-bound IgE and FcεRI.[110] A recent study demonstrated that *Apoe*$^{-/-}$ mice deficient in FcεRI develop significantly decreased atherosclerosis with reduced macrophage and apoptotic cell content. As a potential proatherogenic mechanism, the authors demonstrated that FcεRI on macrophages cooperates with Toll-like receptor 4 and on IgE binding led to cell activation, inflammatory cytokine secretion, and apoptosis (Figure 2). However, these effects were achieved with IgE concentrations that were >200-fold higher than the concentrations measured in atherosclerotic mice. Therefore, these effects may not entirely reflect an in vivo situation.[109]

Other FcεRI-mediated effects may also be operative because IgE antibodies are a major stimulus for mast cells, which have been implicated in atherosclerosis and in the destabilization of lesions in particular.[111] Studies in atherosclerotic *Ldlr*$^{-/-}$ mice have shown that mast cell deficien y results in reduced lesion formation and that this may be promoted by mast cell–derived IL-6 and IFN-γ.[112] Another study by Bot et al[113] also demonstrated a proatherogenic effect of mast cell activation in *Apoe*$^{-/-}$ mice and a role for mast cell degranulation in particular. In line with this, in vitro degranulation of mast cells by IgE treatment has been shown to promote OxLDL uptake by macrophages in coculture experiments.[114] Thus, mast cells may be a critical mediator of the proatherogenic effects of IgE binding to FcεRI (Figure 2). Interestingly, galectin-3 was found to be upregulated in atherosclerotic lesions of rabbits and humans. *Apoe*$^{-/-}$ mice deficient in galectin-3 or treated with a galectin-3 inhibitor were shown to develop significantly less atherosclerosis.[115,116] Although galectin-3 has many other potential functions, its proatherogenic effect may in part be mediated by its ability to bind and cross-link IgE antibodies. Critical insights into a direct role of IgE in atherosclerosis are still missing. Moreover, it will be important

to demonstrate whether such effects are dependent on certain antibody specificities for relevant antigens, for example, OxLDL, or whether they are antigen independent.[117,118]

IgA: Waiting to Go on Stage

IgA immunoglobulins have been selected to provide the first line of defense in mucosal areas (mucosal IgA). On the contrary, IgA antibodies are also found in the circulation. In humans, there are 2 classes of IgA, IgA1 and IgA2, whereas in mice, only 1 class exists. In addition, the human secreted IgA comprised monomeric IgA1 and IgA2, whereas in mice, the circulating IgA antibodies form dimers and oligomers.[119] There is little information about the role of IgA antibodies in CVD. Two studies by Muscari et al[120,121] found elevated IgA levels in patients with advanced vascular disease and myocardial infarction, respectively. Moreover, Kovanen et al[108] reported IgA levels to be correlated with myocardial infarction and cardiac death in dyslipidemic men after adjustment for CVD risk factors. However, there are no mechanistic studies available with respect to IgA antibodies in atherosclerosis. Recent insights on the effects of the gut microbiome on CVD,[122] however, could suggest a potentially important mechanism by which IgA antibodies could be modulated to impact atherogenesis.

Complement in Atherosclerosis

Besides its antimicrobial properties, complement is known to participate in the maintenance of immune homeostasis by sensing endogenous danger signals such as cellular debris and apoptotic cells. The complement cascade is composed of 3 pathways, the classical, the alternative, and the lectin pathway, which converge at the level of the C3 convertase resulting, if unopposed, in the generation of proinflammatory C5a and C5b leading to the formation of the lytic terminal complement complex. The classical pathway, which involves C1 and C4 upstream of the C3 convertase, is also strongly initiated by IgM and IgG antibodies bound to their cognate antigens (eg, microbes, apoptotic cells). Complement components have been described to be deposited in atherosclerotic lesions, and there are growing numbers of animal studies addressing the role of certain complement components. For a detailed review, see Speidl et al.[123] However, because of the fact that many complement components also play a key role in homeostasis, including the removal of apoptotic cells, the interpretation of these studies as indicators of immunoglobulin action is difficult. *Ldlr*$^{-/-}$ mice deficient in C3, the central component of all 3 pathways, have been shown to develop lesions of similar size in the abdominal and thoracic aorta. However, increased lipid and macrophage deposition and decreased collagen and smooth muscle cell content were found in the aortic root lesions of C3-deficient mice, suggesting an overall protective role for C3.[124] In addition, C3-deficient mice crossed on an *Apoe*$^{-/-}$*Ldlr*$^{-/-}$ background have been shown to develop 84% increased lesions in the aorta.[125] C3b and its degradation product iC3b have been shown to facilitate apoptotic cell uptake by macrophages in vitro. Similarly, the member of the classical pathway C1q, which also binds to apoptotic cells directly or via IgM,[123] has been found to be atheroprotective, because *Ldlr*$^{-/-}$ mice deficient in C1q develop significantly larger lesions compared with control mice.[81,126] In support of a proatherogenic role for complement activation, it has been shown that pharmacological inhibition of the C5a receptor CD88 in *Apoe*$^{-/-}$ mice results in decreased plaque size in the aortic root.[127] This finding is supported by epidemiological data showing a positive association of C5a levels with increased CVD risk independent of nonspecific inflammatory markers such as C-reactive protein or serum amyloid A.[128] Moreover, epidemiological studies have shown that C4 levels are associated with severe atherosclerosis.[120]

Thus, studies on the role of complement components do not allow any conclusion on immunoglobulin effector functions in atherosclerosis. In fact, homeostatic functions of complement may be of great relevance. This should also include regulators of complement activation, such as C4bp and complement factor H (CFH), which are present in atherosclerotic lesions and may also modulate immunoglobulin function.[129–131] CFH provides cofactor activity for factor I–mediated degradation of C3b into iC3b fragments, and deposition of iC3b on the surface of apoptotic cells has been shown to promote anti-inflammatory clearance mechanisms.[132] Our recent discovery that CFH binds malondialdehyde epitopes on cellular debris and inhibits their proinflammatory effects shines light on a potential function in atherosclerosis. In fact, we showed that the malondialdehyde-binding sites in CFH are localized to domains that are also hot spots of disease-associated mutations. For example, a common SNP rs1061170, which is highly associated

Table 3. Summary of B-Cell Depleting Compounds Already Approved or in Development for Treatment SLE and RA and Their Effects in Experimental Atherosclerosis (if Applicable)

Compound	Function	Effect on B Cells	Effect on Murine Atherosclerosis	Clinical Application	Reference
α-CD20	Cross-linking of CD20 receptor	Fcγ receptor–mediated B-cell depletion	Decreased atherosclerosis	RA	35
α-BAFF	Neutralization of circulating BAFF	B2 cell apoptosis	Unknown	SLE (Belimumab)	135
BAFFR-Fc	Neutralization of both circulating and membrane-bound BAFF	B2 cell apoptosis	Unknown	Unknown	136
α-BAFFR	BAFF receptor blockage	B2 cell apoptosis	Decreased atherosclerosis	Unknown	31
TACI-Fc	Neutralization of circulating and membrane-bound BAFF and APRIL	B2 cell and plasma cell apoptosis	Unknown	Phase 3 clinical trial for SLE (Atacicept)	137,138

APRIL indicates a proliferation inducing ligand; BAFFR, B-cell–activating factor receptor; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; and TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor.

with an increased risk for age-related macular degeneration, significantly impairs the binding of CFH to malondialdehyde. Although meta-analyses of clinical studies involving ≈48 000 individuals do not support a role for this specifi SNP in CVD,[133] it is tempting to speculate that the combination of several rare SNPs in the malondialdehyde-binding sites results in functional alterations of CFH binding to malondialdehyde. Such SNPs might not be detected in genetic association studies because of their low frequencies. In this regard, it will be interesting to evaluate the association of CFH binding to malondialdehyde with CVD in large clinical cohorts.

Translational Opportunities by Targeting B Cells and Their Effect in Antibody Production

Clearly, the involvement of immune mechanisms in atherosclerosis offers several novel therapeutic opportunities for targeting immune responses in atherosclerosis. The broad spectrum of such approaches is summarized in several reviews.[8,9,134] However, the advent and clinical application of several B-cell targeting compounds in autoimmune disease offer a hitherto unrecognized opportunity for novel therapeutic strategies in atherosclerosis (Table 3). This is of particular interest because diseases for which these compounds are mainly being used or developed, including rheumatoid arthritis and systemic lupus erythematosus (SLE), are also associated with a significantly increased risk of CVD. There are several B-cell–depleting compounds that are currently approved or in clinical trials. Foremost, the CD20-depleting antibody (Rituximab) results in B-cell elimination by cross-linking the CD20 receptor, which is expressed on all B cells.[35] It has been approved by the US Food and Drug Administration as a treatment for rheumatoid arthritis. Moreover, in 2011, the US Food and Drug Administration approved a neutralizing BAFF antibody (Belimumab) as treatment for SLE. The effects of these 2 treatments on CVD are not known; but as discussed, several animal studies demonstrated an atheroprotective effect of B-cell depletion by CD20[24] or inhibition of BAFFR signaling with an anti-BAFFR antibody.[31] In this regard, additional compounds that inhibit the BAFF–BAFFR signaling exist, such as the soluble form of BAFFR fused to an immunoglobulin backbone (BAFFR-Ig).[136] Similar to this, a decoy form of the transmembrane activator and calcium modulator and cyclophilin ligand interactor receptor (TACI-Ig) is currently in clinical phase III trial for SLE. TACI-Ig binds both BAFF and another ligand termed a proliferation inducing ligand[36] leading to the depletion of mature B2 as well as plasma cells with profound immunoglobulin reduction.[137,138] A careful accounting of the impact of these interventions on CVD should be done, which may reveal whether any of these B-cell depleting strategies modulate CVD risk or at least decrease the increased CVD risk associated with rheumatoid arthritis or SLE.

Concluding Remarks

Several studies in mouse models of atherosclerosis have established an important modulatory role for B cells in experimental atherosclerosis, and their effect is dependent on specific B-cell subsets. The contribution and effector functions of immunoglobulins and the different immunoglobulin classes in these effects still requires further investigation, but natural IgM antibodies, particularly those directed to OSEs, clearly mediate atheroprotection. There is still much to learn about the importance of B-cell immunity in human disease, but some genetic evidence exists which for the most part corroborates findings from mouse studies. Insights from patients with SLE or rheumatoid arthritis with increased CVD risk that are being treated with novel B-cell–depleting drugs may help establish this link in humans and could help identify novel therapeutic strategies for atherosclerosis.

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References

1. Tabas I, Williams KJ, Borén J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation*. 2007;116:1832–1844.
2. Steinberg D, Witztum JL. Oxidized low-density lipoprotein and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2010;30:2311–2316.
3. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell*. 2001;104:503–516.
4. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317–325.
5. Libby P, Lichtman AH, Hansson GK. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity*. 2013;38:1092–1104.
6. Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol*. 2011;12:204–212.
7. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol*. 2006;6:508–519.
8. Lahoute C, Herbin O, Mallat Z, Tedgui A. Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nat Rev Cardiol*. 2011;8:348–358.
9. Lichtman AH, Binder CJ, Tsimikas S, Witztum JL. Adaptive immunity in atherogenesis: new insights and therapeutic approaches. *J Clin Invest*. 2013;123:27–36.
10. Weismann D, Binder CJ. The innate immune response to products of phospholipid peroxidation. *Biochim Biophys Acta*. 2012;1818:2465–2475.
11. Miller YI, Choi SH, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, Boullier A, Gonen A, Diehl CJ, Que X, Montano E, Shaw PX, Tsimikas S, Binder CJ, Witztum JL. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res*. 2011;108:235–248.
12. Ylä-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler Thromb*. 1994;14:32–40.
13. Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci U S A*. 1995;92:3893–3897.
14. Dansky HM, Charlton SA, Harper MM, Smith JD. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci U S A*. 1997;94:4642–4646.

15. Daugherty A, Puré E, Delfel-Butteiger D, Chen S, Leferovich J, Roselaar SE, Rader DJ. The effects of total lymphocyte deficien y on the extent of atherosclerosis in apolipoprotein E-/- mice. *J Clin Invest*. 1997;100:1575–1580.
16. Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation*. 2000;102:2919–2922.
17. Zhou X, Hansson GK. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. *Scand J Immunol*. 1999;50:25–30.
18. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med*. 2006;203:1273–1282.
19. Perry HM, Bender TP, McNamara CA. B cell subsets in atherosclerosis. *Front Immunol*. 2012;3:373.
20. Kyaw T, Tipping P, Bobik A, Toh BH. Protective role of natural IgM-producing B1a cells in atherosclerosis. *Trends Cardiovasc Med*. 2012;22:48–53.
21. Huan T, Zhang B, Wang Z, et al; Coronary ARteryDIsease Genome wide Replication and Meta-analysis (CARDIoGRAM) Consortium, International Consortium for Blood Pressure GWAS (ICBP). A systems biology framework identifies molecular underpinnings of coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2013;33:1427–1434.
22. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest*. 2002;109:745–753.
23. Major AS, Fazio S, Linton MF. B-lymphocyte deficien y increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol*. 2002;22:1892–1898.
24. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vré E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF, Mallat Z. B cell depletion reduces the development of atherosclerosis in mice. *J Exp Med*. 2010;207:1579–1587.
25. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol*. 2010;185:4410–4419.
26. Montecino-Rodriguez E, Dorshkind K. New perspectives in B-1 B cell development and function. *Trends Immunol*. 2006;27:428–433.
27. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol*. 2011;11:34–46.
28. Doran AC, Lipinski MJ, Oldham SN, et al. B-cell aortic homing and atheroprotection depend on Id3. *Circ Res*. 2012;110:e1–e12.
29. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, Bobik A, Toh BH. B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res*. 2011;109:830–840.
30. Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, Tipping P, Bobik A, Toh BH. Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS One*. 2012;7:e29371.
31. Kyaw T, Cui P, Tay C, Kanellakis P, Hosseini H, Liu E, Rolink AG, Tipping P, Bobik A, Toh BH. BAFF receptor mAb treatment ameliorates development and progression of atherosclerosis in hyperlipidemic ApoE(-/-) mice. *PLoS One*. 2013;8:e60430.
32. Hilgendorf I, Theurl I, Gerhardt LM, et al. Innate response activator B cells aggravate atherosclerosis by stimulating T helper-1 adaptive immunity. *Circulation*. 2014;129:1677–1687.
33. Rauch PJ, Chudnovskiy A, Robbins CS, et al. Innate response activator B cells protect against microbial sepsis. *Science*. 2012;335:597–601.
34. Yang M, Rui K, Wang S, Lu L. Regulatory B cells in autoimmune diseases. *Cell Mol Immunol*. 2013;10:122–132.
35. Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, Tedder TF. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med*. 2004;199:1659–1669.
36. Mackay F, Schneider P. Cracking the BAFF code. *Nat Rev Immunol*. 2009;9:491–502.
37. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, Frew E, Scott ML. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science*. 2001;293:2111–2114.
38. Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M. TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *J Immunol*. 2004;173:2245–2252.
39. Sage AP, Tsiantoulas D, Baker L, Harrison J, Masters L, Murphy D, Loinard C, Binder CJ, Mallat Z. BAFF receptor deficien y reduces the development of atherosclerosis in mice–brief report. *Arterioscler Thromb Vasc Biol*. 2012;32:1573–1576.
40. Wardemann H, Boehm T, Dear N, Carsetti R. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J Exp Med*. 2002;195:771–780.
41. Holodick NE, Tumang JR, Rothstein TL. Immunoglobulin secretion by B1 cells: differential intensity and IRF4-dependence of spontaneous IgM secretion by peritoneal and splenic B1 cells. *Eur J Immunol*. 2010;40:3007–3016.
42. Robinette CD, Fraumeni JF Jr. Splenectomy and subsequent mortality in veterans of the 1939-45 war. *Lancet*. 1977;2:127–129.
43. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70-. *J Exp Med*. 2011;208:67–80.
44. Covens K, Verbinnen B, Geukens N, Meyts I, Schuit F, Van Lommel L, Jacquemin M, Bossuyt X. Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype. *Blood*. 2013;121:5176–5183.
45. Drew PA, Kiroff GK, Ferrante A, Cohen RC. Alterations in immunoglobulin synthesis by peripheral blood mononuclear cells from splenectomized patients with and without splenic regrowth. *J Immunol*. 1984;132:191–196.
46. Hörkkö S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Friedman P, Dennis EA, Curtiss LK, Palinski W, Witztum JL. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest*. 1999;103:117–128.
47. Binder CJ, Hörkkö S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med*. 2003;9:736–743.
48. Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammator . *J Exp Med*. 2004;200:1359–1370.
49. Faria-Neto JR, Chyu KY, Li X, Dimayuga PC, Ferreira C, Yano J, Cercek B, Shah PK. Passive immunization with monoclonal IgM antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein E-null mice. *Atherosclerosis*. 2006;189:83–90.
50. Shaw PX, Hörkkö S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, Witztum JL. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest*. 2000;105:1731–1740.
51. Karvonen J, Päivänsalo M, Kesäniemi YA, Hörkkö S. Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation*. 2003;108:2107–2112.
52. Tsimikas S, Brilakis ES, Lennon RJ, Miller ER, Witztum JL, McConnell JP, Kornman KS, Berger PB. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *J Lipid Res*. 2007;48:425–433.
53. Tsimikas S, Willeit P, Willeit J, Santer P, Mayr M, Xu Q, Mayr A, Witztum JL, Kiechl S. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol*. 2012;60:2218–2229.
54. Fiskesund R, Stegmayr B, Hallmans G, Vikström M, Weinehall L, de Faire U, Frostegård J. Low levels of antibodies against phosphorylcholine predict development of stroke in a population-based study from northern Sweden. *Stroke*. 2010;41:607–612.
55. Grönlund H, Hallmans G, Jansson JH, Boman K, Wikström M, de Faire U, Frostegård J. Low levels of IgM antibodies against phosphorylcholine predict development of acute myocardial infarction in a population-based cohort from northern Sweden. *Eur J Cardiovasc Prev Rehabil*. 2009;16:382–386.
56. Anania C, Gustafsson T, Hua X, Su J, Vikström M, de Faire U, Heimbürger M, Jogestrand T, Frostegård J. Increased prevalence of vulnerable atherosclerotic plaques and low levels of natural IgM antibodies against phosphorylcholine in patients with systemic lupus erythematosus. *Arthritis Res Ther*. 2010;12:R214.
57. Grönwall C, Akhter E, Oh C, Burlingame RW, Petri M, Silverman GJ. IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE. *Clin Immunol*. 2012;142:390–398.
58. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza

virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A*. 1999;96:2250–2255.
59. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol*. 2010;10:778–786.
60. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med*. 1998;188:2381–2386.
61. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med*. 2000;192:271–280.
62. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med*. 2008;205:3053–3064.
63. Rapaka RR, Ricks DM, Alcorn JF, Chen K, Khader SA, Zheng M, Plevy S, Bengtén E, Kolls JK. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina. *J Exp Med*. 2010;207:2907–2919.
64. Ogden CA, Kowalewski R, Peng Y, Montenegro V, Elkon KB. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. *Autoimmunity*. 2005;38:259–264.
65. Chang MK, Bergmark C, Laurila A, Hörkkö S, Han KH, Friedman P, Dennis EA, Witztum JL. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A*. 1999;96:6353–6358.
66. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Bäckhed F, Miller YI, Hörkkö S, Corr M, Witztum JL, Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest*. 2009;119:1335–1349.
67. Palinski W, Hörkkö S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, Witztum JL. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest*. 1996;98:800–814.
68. Friedman P, Horkko S, Steinberg D, Witztum JL, Dennis EA. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids. Importance of Schiff base formation and aldol condensation. *J Biol Chem*. 2002;277:7010–7020.
69. Boullier A, Gillotte KL, Hörkkö S, Green SR, Friedman P, Dennis EA, Witztum JL, Steinberg D, Quehenberger O. The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. *J Biol Chem*. 2000;275:9163–9169.
70. Masmoudi H, Mota-Santos T, Huetz F, Coutinho A, Cazenave PA. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. *Int Immunol*. 1990;2:515–520.
71. Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. *J Exp Med*. 1982;156:1177–1185.
72. Binder CJ, Hartvigsen K, Chang MK, Miller M, Broide D, Palinski W, Curtiss LK, Corr M, Witztum JL. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest*. 2004;114:427–437.
73. Sämpi M, Ukkola O, Päivänsalo M, Kesäniemi YA, Binder CJ, Hörkkö S. Plasma interleukin-5 levels are related to antibodies binding to oxidized low-density lipoprotein and to decreased subclinical atherosclerosis. *J Am Coll Cardiol*. 2008;52:1370–1378.
74. The IBC 50K CAD Consortium. Large-scale gene-centric analysis identifies novel variants for coronary artery disease. *PLoS Genet*. 2011;7:e1002260.
75. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol*. 2009;182:6031–6043.
76. Chen Y, Khanna S, Goodyear CS, Park YB, Raz E, Thiel S, Grönwall C, Vas J, Boyle DL, Corr M, Kono DH, Silverman GJ. Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol*. 2009;183:1346–1359.
77. Huber J, Vales A, Mitulovic G, Blumer M, Schmid R, Witztum JL, Binder BR, Leitinger N. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arterioscler Thromb Vasc Biol*. 2002;22:101–107.
78. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA, Lacy-Hulbert A, El Khoury J, Golenbock DT, Moore KJ. CD36 ligands promote sterile inflamm - tion through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol*. 2010;11:155–161.
79. Imai Y, Kuba K, Neely GG, et al. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*. 2008;133:235–249.
80. Thimmulappa RK, Gang X, Kim JH, Sussan TE, Witztum JL, Biswal S. Oxidized phospholipids impair pulmonary antibacterial defenses: evidence in mice exposed to cigarette smoke. *Biochem Biophys Res Commun*. 2012;426:253–259.
81. Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO. Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation*. 2009;120:417–426.
82. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol*. 1998;160:4776–4787.
83. Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A*. 1998;95:10089–10093.
84. Baker N, Ehrenstein MR. Cutting edge: selection of B lymphocyte subsets is regulated by natural IgM. *J Immunol*. 2002;169:6686–6690.
85. Notley CA, Baker N, Ehrenstein MR. Secreted IgM enhances B cell receptor signaling and promotes splenic but impairs peritoneal B cell survival. *J Immunol*. 2010;184:3386–3393.
86. Cesena FH, Dimayuga PC, Yano J, Zhao X, Kirzner J, Zhou J, Chan LF, Lio WM, Cercek B, Shah PK, Chyu KY. Immune-modulation by polyclonal IgM treatment reduces atherosclerosis in hypercholesterolemic apoE-/- mice. *Atherosclerosis*. 2012;220:59–65.
87. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol*. 2008;8:34–47.
88. Frank MM, Miletic VD, Jiang H. Immunoglobulin in the control of complement action. *Immunol Res*. 2000;22:137–146.
89. Amir S, Hartvigsen K, Gonen A, Leibundgut G, Que X, Jensen-Jarolim E, Wagner O, Tsimikas S, Witztum JL, Binder CJ. Peptide mimotopes of malondialdehyde epitopes for clinical applications in cardiovascular disease. *J Lipid Res*. 2012;53:1316–1326.
90. Xu Q, Willeit J, Marosi M, Kleindienst R, Oberhollenzer F, Kiechl S, Stulnig T, Luef G, Wick G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. *Lancet*. 1993;341:255–259.
91. Knoflach M, Kiechl S, Kind M, Said M, Sief R, Gisinger M, van der Zee R, Gaston H, Jarosch E, Willeit J, Wick G. Cardiovascular risk factors and atherosclerosis in young males: ARMY study (Atherosclerosis Risk-Factors in Male Youngsters). *Circulation*. 2003;108:1064–1069.
92. Xu Q, Dietrich H, Steiner HJ, Gown AM, Schoel B, Mikuz G, Kaufmann SH, Wick G. Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65. *Arterioscler Thromb*. 1992;12:789–799.
93. Afek A, George J, Gilburd B, Rauova L, Goldberg I, Kopolovic J, Harats D, Shoenfeld Y. Immunization of low-density lipoprotein receptor deficient (LDL-RD) mice with heat shock protein 65 (HSP-65) promotes early atherosclerosis. *J Autoimmun*. 2000;14:115–121.
94. George J, Afek A, Gilburd B, Shoenfeld Y, Harats D. Cellular and humoral immune responses to heat shock protein 65 are both involved in promoting fatty-streak formation in LDL-receptor deficient mice. *J Am Coll Cardiol*. 2001;38:900–905.
95. Schett G, Xu Q, Amberger A, Van der Zee R, Recheis H, Willeit J, Wick G. Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. *J Clin Invest*. 1995;96:2569–2577.
96. Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A*. 1995;92:821–825.
97. Ameli S, Hultgårdh-Nilsson A, Regnström J, Calara F, Yano J, Cercek B, Shah PK, Nilsson J. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol*. 1996;16:1074–1079.
98. George J, Afek A, Gilburd B, Levkovitz H, Shaish A, Goldberg I, Kopolovic Y, Wick G, Shoenfeld Y, Harats D. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. *Atherosclerosis*. 1998;138:147–152.
99. Zhou X, Caligiuri G, Hamsten A, Lefvert AK, Hansson GK. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2001;21:108–114.

100. Freigang S, Hörkkö S, Miller E, Witztum JL, Palinski W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. *Arterioscler Thromb Vasc Biol*. 1998;18:1972–1982.
101. Schiopu A, Bengtsson J, Söderberg I, Janciauskiene S, Lindgren S, Ares MP, Shah PK, Carlsson R, Nilsson J, Fredrikson GN. Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis. *Circulation*. 2004;110:2047–2052.
102. Tsimikas S, Miyanohara A, Hartvigsen K, et al. Human oxidation-specific antibodies reduce foam cell formation and atherosclerosis progression. *J Am Coll Cardiol*. 2011;58:1715–1727.
103. Hernández-Vargas P, Ortiz-Muñoz G, López-Franco O, Suzuki Y, Gallego-Delgado J, Sanjuán G, Lázaro A, López-Parra V, Ortega L, Egido J, Gómez-Guerrero C. Fcgamma receptor deficien y confers protection against atherosclerosis in apolipoprotein E knockout mice. *Circ Res*. 2006;99:1188–1196.
104. Kelly JA, Griffin ME, Fava RA, Wood SG, Bessette KA, Miller ER, Huber SA, Binder CJ, Witztum JL, Morganelli PM. Inhibition of arterial lesion progression in CD16-deficient mice: evidence for altered immunity and the role of IL-10. *Cardiovasc Res*. 2010;85:224–231.
105. Huang Y, Jaffa A, Koskinen S, Takei A, Lopes-Virella MF. Oxidized LDL-containing immune complexes induce Fc gamma receptor I-mediated mitogen-activated protein kinase activation in THP-1 macrophages. *Arterioscler Thromb Vasc Biol*. 1999;19:1600–1607.
106. Smith KG, Clatworthy MR. FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. *Nat Rev Immunol*. 2010;10:328–343.
107. Mendez-Fernandez YV, Stevenson BG, Diehl CJ, Braun NA, Wade NS, Covarrubias R, van Leuven S, Witztum JL, Major AS. The inhibitory FcγRIIb modulates the inflammatory response and influences atherosclerosis in male apoE(-/-) mice. *Atherosclerosis*. 2011;214:73–80.
108. Kovanen PT, Mänttäri M, Palosuo T, Manninen V, Aho K. Prediction of myocardial infarction in dyslipidemic men by elevated levels of immunoglobulin classes A, E, and G, but not M. *Arch Intern Med*. 1998;158:1434–1439.
109. Wang J, Cheng X, Xiang MX, et al. IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J Clin Invest*. 2011;121:3564–3577.
110. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol*. 2008;8:205–217.
111. Kovanen PT, Kaartinen M, Paavonen T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation*. 1995;92:1084–1088.
112. Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P, MacFarlane LA, Mallen-St Clair J, Shi GP. Mast cells promote atherosclerosis by releasing proinflammatory ytokines. *Nat Med*. 2007;13:719–724.
113. Bot I, de Jager SC, Zernecke A, Lindstedt KA, van Berkel TJ, Weber C, Biessen EA. Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation*. 2007;115:2516–2525.
114. Ma H, Kovanen PT. IgE-dependent generation of foam cells: an immune mechanism involving degranulation of sensitized mast cells with resultant uptake of LDL by macrophages. *Arterioscler Thromb Vasc Biol*. 1995;15:811–819.
115. Nachtigal M, Ghaffar A, Mayer EP. Galectin-3 gene inactivation reduces atherosclerotic lesions and adventitial inflammation in ApoE-deficient mice. *Am J Pathol*. 2008;172:247–255.
116. MacKinnon AC, Liu X, Hadoke PW, Miller MR, Newby DE, Sethi T. Inhibition of galectin-3 reduces atherosclerosis in apolipoprotein E-deficient mice. *Glycobiology*. 2013;23:654–663.
117. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, Krystal G. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity*. 2001;14:801–811.
118. Pandey V, Mihara S, Fensome-Green A, Bolsover S, Cockcroft S. Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosol Ca2+, degranulation, and membrane ruffling in the cultured rat basophilic leukemia-2H3 mast cell line. *J Immunol*. 2004;172:4048–4058.
119. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol*. 2008;8:421–434.
120. Muscari A, Bozzoli C, Gerratana C, Zaca' F, Rovinetti C, Zauli D, La Placa M, Puddu P. Association of serum IgA and C4 with severe atherosclerosis. *Atherosclerosis*. 1988;74:179–186.
121. Muscari A, Bozzoli C, Puddu GM, Rovinetti C, Vallar G, Renzi C, Scarani P, Molinaro N, Puddu P. Increased serum IgA levels in subjects with previous myocardial infarction or other major ischemic events. *Cardiology*. 1993;83:383–389.
122. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472:57–63.
123. Speidl WS, Kastl SP, Huber K, Wojta J. Complement in atherosclerosis: friend or foe? *J Thromb Haemost*. 2011;9:428–440.
124. Buono C, Come CE, Witztum JL, Maguire GF, Connelly PW, Carroll M, Lichtman AH. Influence of C3 deficien y on atherosclerosis. *Circulation*. 2002;105:3025–3031.
125. Persson L, Borén J, Robertson AK, Wallenius V, Hansson GK, Pekna M. Lack of complement factor C3, but not factor B, increases hyperlipidemia and atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice. *Arterioscler Thromb Vasc Biol*. 2004;24:1062–1067.
126. Bhatia VK, Yun S, Leung V, Grimsditch DC, Benson GM, Botto MB, Boyle JJ, Haskard DO. Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am J Pathol*. 2007;170:416–426.
127. Manthey HD, Thomas AC, Shiels IA, Zernecke A, Woodruff TM, Rolfe B, Taylor SM. Complement C5a inhibition reduces atherosclerosis in ApoE-/- mice. *FASEB J*. 2011;25:2447–2455.
128. Speidl WS, Exner M, Amighi J, Kastl SP, Zorn G, Maurer G, Wagner O, Huber K, Minar E, Wojta J, Schillinger M. Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *Eur Heart J*. 2005;26:2294–2299.
129. Oksjoki R, Kovanen PT, Mäyränpää MI, Laine P, Blom AM, Meri S, Pentikäinen MO. Complement regulation in human atherosclerotic coronary lesions. Immunohistochemical evidence that C4b-binding protein negatively regulates the classical complement pathway, and that C5b-9 is formed via the alternative complement pathway. *Atherosclerosis*. 2007;192:40–48.
130. Oksjoki R, Jarva H, Kovanen PT, Laine P, Meri S, Pentikäinen MO. Association between complement factor H and proteoglycans in early human coronary atherosclerotic lesions: implications for local regulation of complement activation. *Arterioscler Thromb Vasc Biol*. 2003;23:630–636.
131. Weismann D, Hartvigsen K, Lauer N, Bennett KL, Scholl HP, Charbel Issa P, Cano M, Brandstätter H, Tsimikas S, Skerka C, Superti-Furga G, Handa JT, Zipfel PF, Witztum JL, Binder CJ. Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. *Nature*. 2011;478:76–81.
132. Amarilyo G, Verbovetski I, Atallah M, Grau A, Wiser G, Gil O, Ben-Neriah Y, Mevorach D. iC3b-opsonized apoptotic cells mediate a distinct anti-inflammatory response and transcriptional NF-kappaB-dependent blockade. *Eur J Immunol*. 2010;40:699–709.
133. Sofat R, Casas JP, Kumari M, Talmud PJ, Ireland H, Kivimaki M, Marmot M, Hughes AD, Thom S, Ebrahim S, Whittaker JC, Smeeth L, Lawlor DA, Humphries SE, Hingorani AD. Genetic variation in complement factor H and risk of coronary heart disease: eight new studies and a meta-analysis of around 48,000 individuals. *Atherosclerosis*. 2010;213:184–190.
134. Amir S, Binder CJ. Experimental immunotherapeutic approaches for atherosclerosis. *Clin Immunol*. 2010;134:66–79.
135. Scholz JL, Crowley JE, Tomayko MM, Steinel N, O'Neill PJ, Quinn WJ 3rd, Goenka R, Miller JP, Cho YH, Long V, Ward C, Migone TS, Shlomchik MJ, Cancro MP. BLyS inhibition eliminates primary B cells but leaves natural and acquired humoral immunity intact. *Proc Natl Acad Sci U S A*. 2008;105:15517–15522.
136. Matsushita T, Fujimoto M, Hasegawa M, Matsushita Y, Komura K, Ogawa F, Watanabe R, Takehara K, Sato S. BAFF antagonist attenuates the development of skin fibrosis in tight-skin mice. *J Invest Dermatol*. 2007;127:2772–2780.
137. Gross JA, Dillon SR, Mudri S, et al. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. impaired B cell maturation in mice lacking BLyS. *Immunity*. 2001;15:289–302.
138. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. 2004;199:91–98.

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B Cells and Humoral Immunity in Atherosclerosis

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Collectively, these data suggest that in conditions of low or deficiency in IgM antibodies, there are additional distinct immunoregulatory mechanisms by which IgM antibodies confer atheroprotection and these may require the full or at least broader repertoire of IgM specificities.

1.4 Microparticles and their role in cardiovascular disease

Several pathologies - including atherosclerosis - in which accumulation of apoptotic cells is an important pathophysiological feature, are also characterized by increased numbers of circulating microparticles (MP). MP are defined as small anucleoid phospholipid vesicles (0.1-1 μm) that are released from activated or dying cells including platelets, red blood cells, endothelial cells and monocytes, and are present in the circulation (Rautou et al, 2011b). MP are formed when the membrane phospholipid asymmetry is lost resulting in phosphatidylserine (PS) exposure, although PS-negative MP have also been reported (Owens & Mackman, 2011). Elevated serum levels of MP have been shown to associate with increased risk of CVD, cancer and autoimmune disorders (Morel et al, 2011; Owens & Mackman, 2011; Rautou et al, 2011b). For example, levels of plasma MP are increased in patients with acute coronary syndrome (ACS) (Mallat et al, 2000) and individuals with high atherothrombotic risk, as well as in patients with venous thromboembolism (Morel et al, 2011).

MP can activate cells through specific interactions with cell surface receptors due to the high concentration of ligand molecules they carry on their surface, or they can directly transfer part of their content (e.g RNA and proteins) to recipient cells thereby modulating their function (Mause & Weber, 2010). For example, MP isolated from human atherosclerotic plaques have been shown to mediate monocyte adhesion on endothelial cells by direct ICAM-1 transfer to endothelial cells (Rautou et al, 2011a). On the other hand, monocyte-derived MP have been found to transfer IL-1β along with other components of the inflammasome such as NLRP3 and caspase-1 to endothelial cells, resulting in the expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (Wang et al, 2011b). In line with this, platelet derived MP have been shown to activate endothelial cells resulting in enhanced monocyte adhesion and to promote inflammation in arthritis by triggering cytokine responses from synovial fibroblasts in an IL-1 dependent manner (Boilard et al, 2010). Nevertheless, the pro-inflammatory moieties mediating effects of MP are far from fully

characterized. For example, also bioactive lipid moieties have been suggested to be involved (Barry et al, 1997).

MP have been also shown to modulate the function of adaptive immune cells such as B and T lymphocytes (Messer et al, 2009; Sheng et al, 2011), Notably, MP are elevated in several autoimmune conditions, which are associated with increased cardiovascular risk, such as RA and SLE (Sellam et al, 2009). Notably, MRL-lpr/lpr lupus prone mice develop more severe autoimmunity, in absence of secretory IgM antibodies (Boes et al, 2000). Moreover, mice lacking lactadherin, which is necessary for apoptotic cell clearance, have increased numbers of AnnexinV$^+$ MP in plasma and develop aggravated atherosclerosis (Ait-Oufella et al, 2007).

As mentioned above, MP are generated upon membrane blebbing of apoptotic cells, which also carry OSE on their surface. Thus, I hypothesized that OSE are present on the surface of MP as well. This is particularly interesting, as MP are found in the circulation, where they could be recognized by OSE-specific natural IgM antibodies. Thus, I hypothesized that an additional novel mechanism by which natural IgM antibodies protect from atherosclerosis is by limiting the proatherogenic effect of MP. Therefore, I investigated whether MP carry OSE and are recognized by natural IgM, and how this interaction affects MP effector functions. Moreover, as mentioned above self-antigens are critically involved in B cell development. Circulating MP represent a prototypic source of self-antigens. Thus the ability of natural IgM antibodies to bind cellular debris may also be important in regulation of B cell development. Indeed, it has been reported that deficiency of natural IgM antibodies results in altered BCR signaling (Notley et al, 2010), which as mentioned above is a critical regulator of B cell development. Therefore, I investigated whether natural IgM antibodies exhibit their atheroprotective properties also by modulating B cell homeostasis.

2. AIMS

1. To investigate whether microparticles carry OSE and are targeted by OSE specific natural IgM antibodies
2. To investigate the role of natural IgM antibodies in B cell receptor signaling and splenic B cell maturation
3. To identify the mechanisms by which natural IgM antibody deficiency aggravates atherosclerosis

3. RESULTS

3.1 Part 1: (Tsiantoulas et al, 2015a)

Title: Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies

3.1.1 A subset of circulating MP carries oxidation specific epitopes

It has been previously shown that apoptosis of EC, thymocytes, and Jurkat T-cells induced by various triggers, such as serum deprivation, staurosporine, dexamethasone, PMA, and UV-irradiation results in the formation of OSE on the surface of these cells (Amir et al, 2012; Chang et al, 1999; Chang et al, 2004; Chou et al, 2009; Tuominen et al, 2006). Moreover, *in vitro* Fe2+ and tert-butyl hydroperoxide-treated microvesicles of EC as well as microvesicles of THP-1 (human acute monocytic leukemia cell line) monocytes treated with unesterified cholesterol have been found to carry OSE (Huber et al, 2002; Liu et al, 2012). To test whether OSE are indeed present on MP in vivo, we isolated MP from plasma of healthy individuals by sequential centrifugation (Figure 1), and the presence of OSE on the surface of AnnexinV^{+} MP was determined by flow cytometry using previously characterized monoclonal anti-OxLDL IgM NAbs with specificity for PC (T15/E06) and MDA-type epitopes (LR04, NA17, and E014), respectively (Amir et al, 2012; Chou et al, 2009; Horkko et al, 1999; Palinski et al, 1996). These NAbs bind OxLDL and exhibit a selective specificity for certain OSE (Amir et al, 2012; Chou et al, 2009; Horkko et al, 1999; Palinski et al, 1996) (Figure 2).

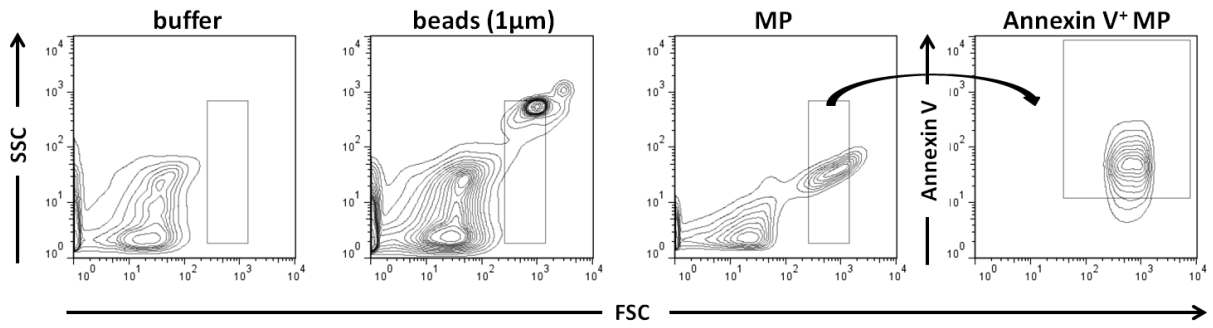

Figure 1. Flow-cytometric gating strategy for MP definition. The MP size gate was defined using monodisperse polystyrene reference beads with diameter of 1 µm and data acquired from buffer only to exclude noise. MP were stained with Annexin V and analyzed by flow cytometry. Representative flow cytometry plots are shown.

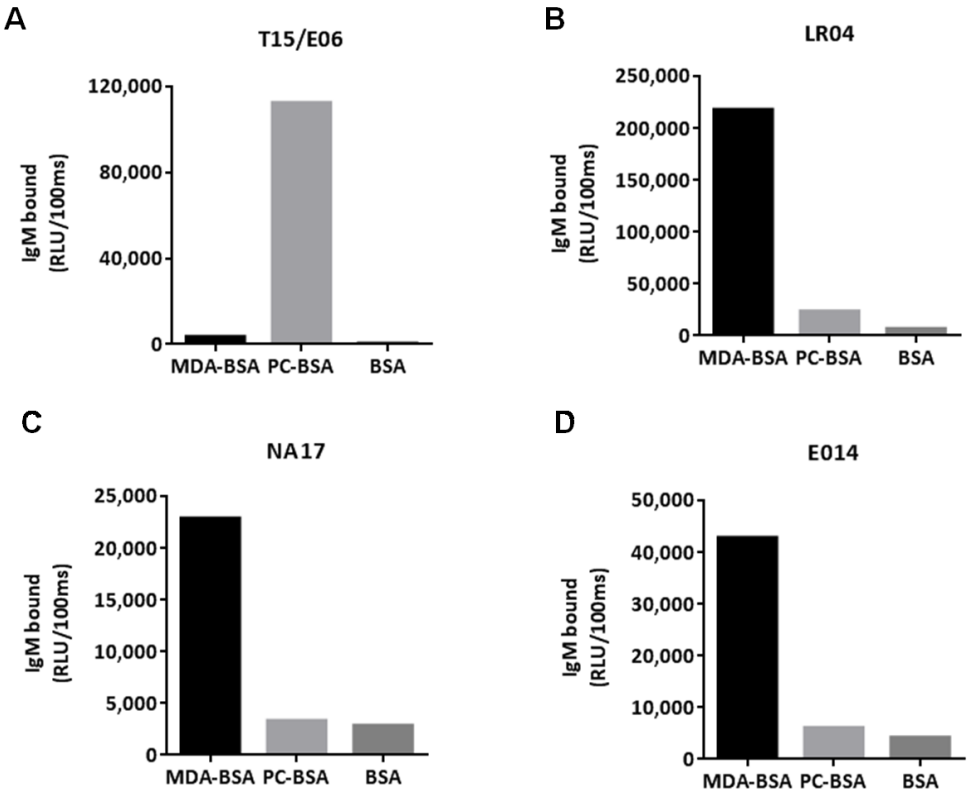

Figure 2. Binding properties of monoclonal IgM antibodies. The IgM NAb T15/E06 is specific for PC, whereas LR04, NA17, and E014 are directed against MDA-type epitopes. Binding of **(A)** T15/E06, **(B)** LR04, **(C)** NA17 and **(D)** E014 to either MDA-BSA or PC-BSA or native BSA was assessed by chemiluminescent ELISA. MDA-BSA contains different MDA-type epitopes including, advanced MAA-epitopes. Data are expressed as RLU/100ms.

A subset of circulating MP of all tested individuals was bound by OSE-specific IgM, while an isotype control antibody showed only minimal binding (Figure 3A). On average 26±2% of MP were bound by the OxPL-specific NAb T15/E06 (Figure 3B). Strikingly, 46±1% and 42±2% of MP showed reactivity with the MDA-specific antibodies LR04 and NA17, respectively (Chou et al, 2009; Palinski et al, 1996). In addition, the MDA-specific antibody E014 bound 32±1% of MP, suggesting specificity for a different type of MDA-adduct. A similar percentage of OSE-carrying MP was found in the plasma of a second cohort of healthy individuals (Figure 4).

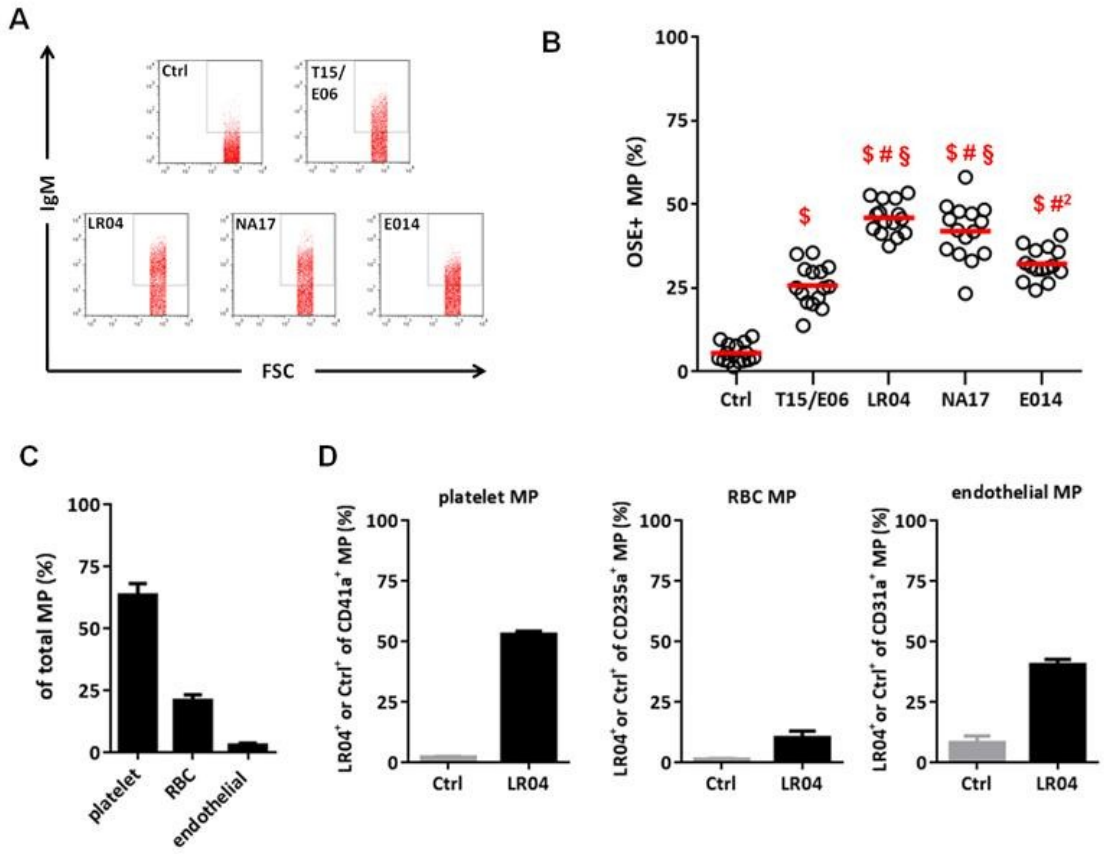

Figure 3. A subset of circulating MP carries OSE. (A,B) MP isolated from plasma of healthy volunteers were stained with Annexin V and OSE-specific IgM NAbs including T15/E06 (specific for PC), LR04, NA17 and E014 (specific for MDA-type epitopes) and analyzed by flow cytometry. **(A)** Representative flow cytometry plots of stained MP. **(B)** Quantification of the percentages of MP with positive staining for each antibody. Bonferroni's multiple comparison test; **$** P< 0.0001 compared to isotype control; **#** P< 0.0001 and **#²** P< 0.05 compared to T15/E06; and **§**P< 0.001 compared to E014. Black circles depict MP from individual donors identified by AnnexinV^{+} and size ≤1µm. **(C, D)** MP were stained with anti-CD41a (platelets), anti-CD235a (RBC) and anti-CD31 (EC) to identify their cellular origin and with LR04 to assess MDA^{+} MP within each cellular fraction. **(C)** Distribution of cellular origin and **(D)** percentages of LR04+ MP of MP of all donors. Data represent mean ±SEM.

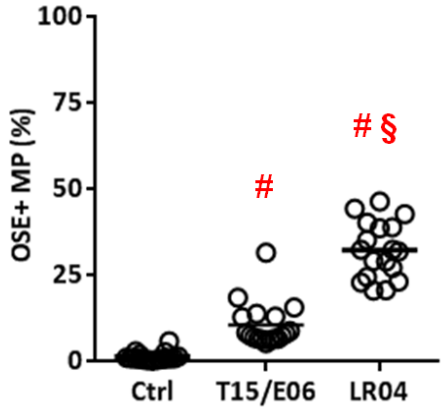

Figure 4. A subset of circulating MP carries OSE. Circulating plasma MP of 18 healthy volunteers were stained with biotinylated T15/E06 (specific for PC) and LR04 (specific for MDA/MAA) as well as an isotype control antibody and analyzed by flow cytometry. Shown are the percentages of MP with positive staining for each antibody. Bonferroni's multiple comparison test; (**#** P<0.001compared to isotype control; **§** P<0.001; compared to T15/E06). Circles depict MP from individual donors.

Moreover, co-staining of circulating MP with LR04 and T15/E06 antibodies revealed that PC epitopes are nearly exclusively present on MDA-carrying MP (Figure 5). To rule out that the presence of OSE was due to ex vivo oxidation, BHT was added to the plasma, in addition to EDTA, before isolating MP, as addition of BHT to plasma has been shown to prevent ex vivo oxidation of LDL (Jialal et al, 1995). Supplementation of plasma with BHT did not affect the presence of OSE (Figure 6).

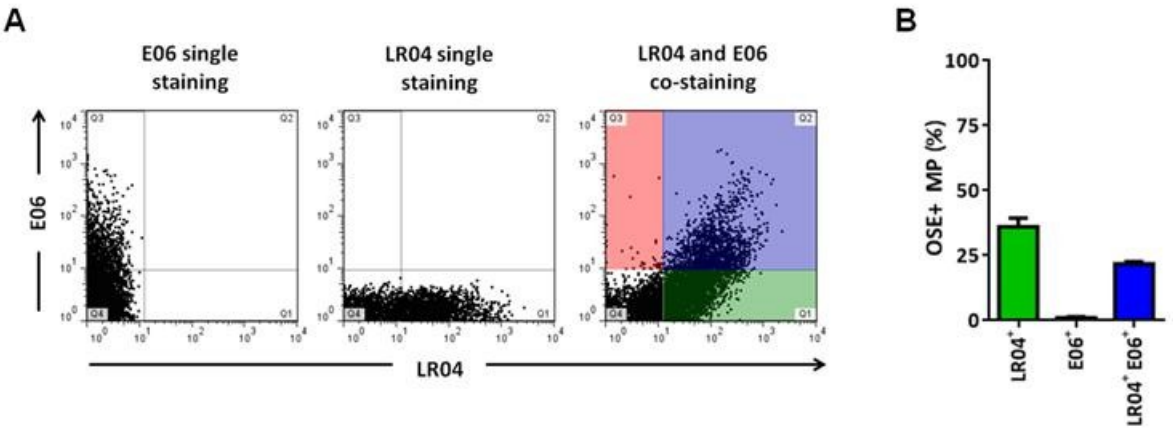

Figure 5. PC epitopes are nearly exclusively present on MP that carry MDA epitopes. Circulating plasma MP of healthy volunteers (n=3) were sequentially stained with T15/E06 (specific for PC), anti-mouse IgM FITC (II/41), LR04 (specific for MDA/MAA), anti-mouse IgM APC (II/41) and Annexin V and analyzed by flow cytometry. Data show dot plots of one representative experiment out of three **(A)** and the percentages of Annexin V^+ MP with positive staining for either LR04 or T15/E06 only or for both antibodies **(B)**.

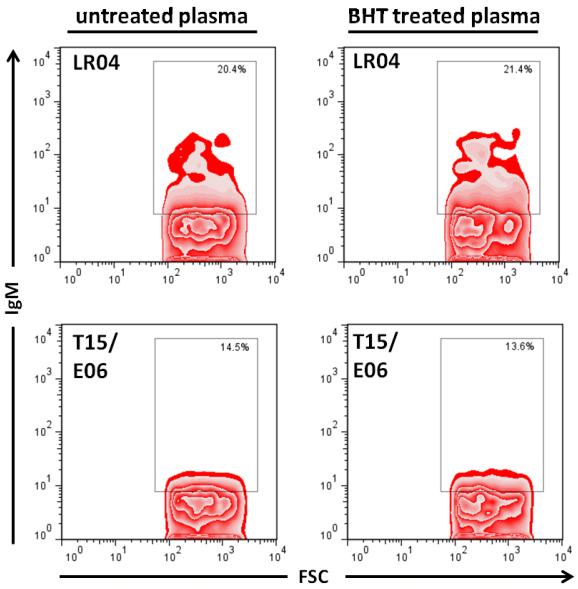

Figure 6. The presence of OSE on MP is not due to ex vivo oxidation. Circulating MP were isolated by sequential ultracentrifugation from plasma treated with BHT or left untreated. Subsequently, binding of LR04 and T15/E06 to MP was quantified by flow cytometry. Data show contour plots of a representative experiment.

3.1.2 MDA-epitopes are present on circulating MP irrespective of their cellular origin

As circulating MP originate from different parental cells, we investigated the cellular origin of the MP preparation, which contained 85±1% Annexin V^+ particles. MP origin was analyzed using antibodies specific for CD41a (platelets), CD235a (RBC), and CD31 (EC). Consistent with previous reports (Morel et al, 2011), a majority (64±5%) of MP were of platelet origin, while 21±2% were RBC-derived and 3±1% derived from EC (Figure 3C). MDA-carrying MP were present irrespective of the cellular origin of MP (Figure 3D). To further corroborate these results and to test whether MDA-epitopes can also be formed in vitro during the process of MP formation, we generated MP from platelets by Ca^{2+} ionophore stimulation. While the MDA-specific antibody LR04 did not bind to the surface of platelets, a large part of MP generated from these cells showed strong binding (Figure 7).

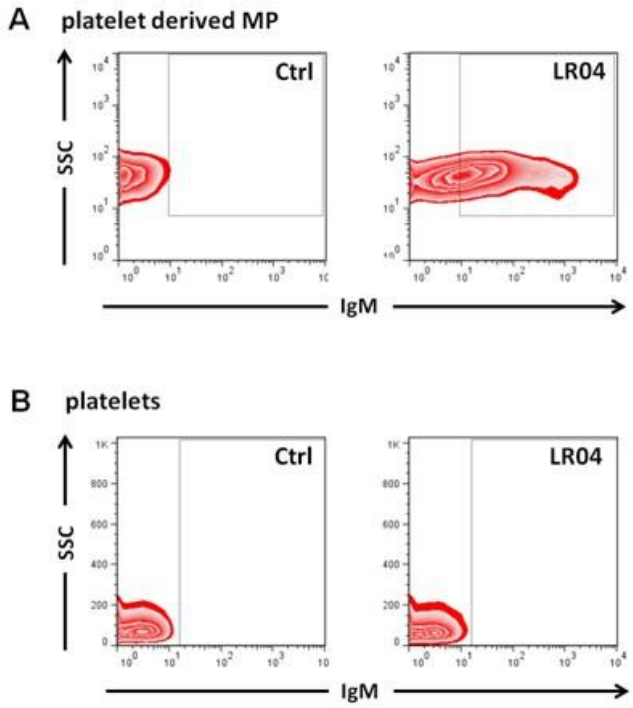

Figure 7. A subset of in vitro generated platelet-derived MP carry MDA epitopes. Platelet-derived MP were isolated from ionomycin-treated platelets, and binding of LR04 to MP and parental platelets was evaluated by flow cytometry. Shown are representative flow cytometry plots of **(A)** MP and **(B)** platelets stained with either a control antibody or LR04 as indicated. In contrast to parental cells, platelet-derived MP carry MDA epitopes.

3.1.3 Circulating microparticles carry surface bound IgM with specificity for MDA-LDL

Because OSE are a dominant target of IgM NAbs (Chou et al, 2009), we tested whether part of the isolated MP were already bound by IgM. Indeed, we found that 23±2% of circulating MP had endogenous IgM bound on their surface even after extensive washing (Figure 8A). Next, we eluted IgM from lysed MP and assessed the binding to MDA-modified LDL (MDA-LDL) and Cu^{2+}-oxidized LDL (CuOx-LDL), two model antigens for OSE, in comparison to circulating IgM in plasma of the same individuals. Normalization of antigen-specific IgM to total IgM revealed that IgM antibodies to MDA-LDL represented more than 80% of total IgM in MP-eluates, while plasma IgM to MDA-LDL was about 15% of total IgM (Figure 8B). The latter findings are consistent with our previous demonstration that MDA-LDL specific IgM represent ~15% of IgM NAbs in mice (Chou et al, 2009). The specificity of the MP-eluted IgM for MDA-LDL was tested in immunocompetition assays, in which neither soluble native LDL nor CuOx-LDL competed for the binding to coated MDA-LDL, while soluble MDA-LDL inhibited the binding by more than 75% (Figure 8C). Thus, MP-associated IgM are primarily composed of IgM with specificity for MDA-LDL. These data are consistent with the presence of OSE on a subset of circulating MP and support the notion that MDA-adducts are dominant.

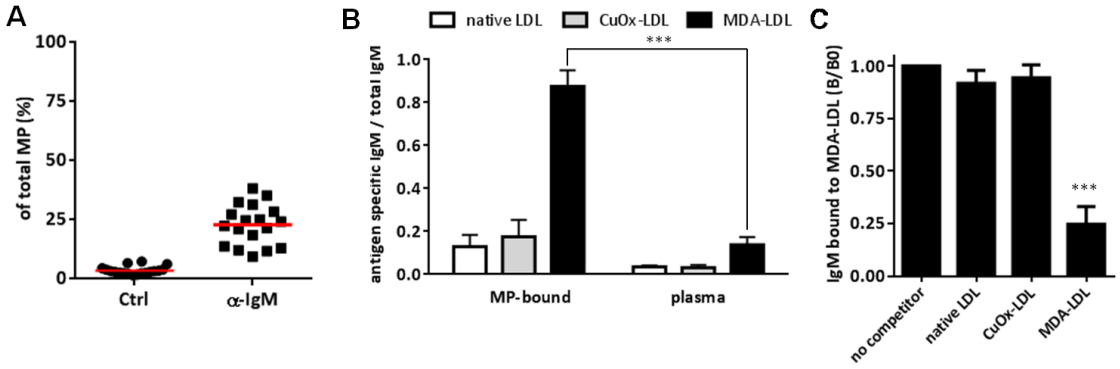

Figure 8. Circulating MP carry IgM that have specificity for MDA-LDL. (A) IgM antibodies are bound on the surface of circulating MP. MP isolated from healthy volunteers were stained with anti-human IgM and an isotype control antibody, and analyzed by flow cytometry. Symbols depict percentages of MP with bound IgM of individual donors. **(B)** IgM antibodies eluted from circulating MP are enriched for IgM with specificity for MDA-LDL compared to IgM in plasma. Binding of MP-eluted and plasma IgM to native LDL, CuOx-LDL, and MDA-LDL was measured by ELISA and the ratio of antigen-specific IgM/total IgM was calculated. Shown are the mean±SEM results of 4 samples. ***P< 0.0001; Bonferroni's multiple comparison test. **(C)** Competition immunoassay. Binding of MP-eluted IgM to coated MDA-LDL in the presence of soluble LDL, CuOx-LDL, and MDA-LDL. Data are expressed as a ratio of the binding in the presence of competitor divided by the binding in the absence of competitor (B/B_0). Shown are mean ±SEM data of 4 donors. ***P< 0.0001; Bonferroni's multiple comparison test.

3.1.4 MDA-adducts contribute to the proinflammatory effect of MP

We and others have previously shown that malondialdehyde-acetaldehyde (MAA)-modified BSA induces pro-inflammatory responses in various cells, including IL-8 secretion by THP-1 cells (Thiele et al, 2004; Weismann et al, 2011). To test whether MP induce chemokine expression via MDA-epitopes, we determined IL-8 secretion by THP-1 cells or primary human monocytes that were stimulated with platelet-derived MP in the presence of the MDA/MAA-specific IgM LR04 or an isotype control. A subset of platelet-derived MP was bound by LR04, but not an isotype control (Figure 9A). MP stimulation induced secretion of IL-8 by both THP-1 and primary human monocytes. Co-incubation with the MDA-specific antibody LR04, but not an isotype control, inhibited MP-induced IL-8 production by >25% and >90% in THP-1 cells and primary monocytes, respectively (Figure 9B, C). The more robust inhibition found in primary monocytes may be explained by previously observed differences between THP-1 cells and primary monocytes in their functional

responses and suggest that primary monocytes are more sensitive to MDA-dependent effects (Qin, 2012). In contrast, LR04 did not inhibit the IL-8 secretion induced with the specific TLR4 ligand KDO2-Lipid A stimulation (data not shown). Collectively, these data demonstrate the capacity of MDA-specific natural IgM antibodies to inhibit inflammatory effects of MP and indicate that the proinflammatory properties of MP are to a significant extent mediated by MDA/MAA-epitopes.

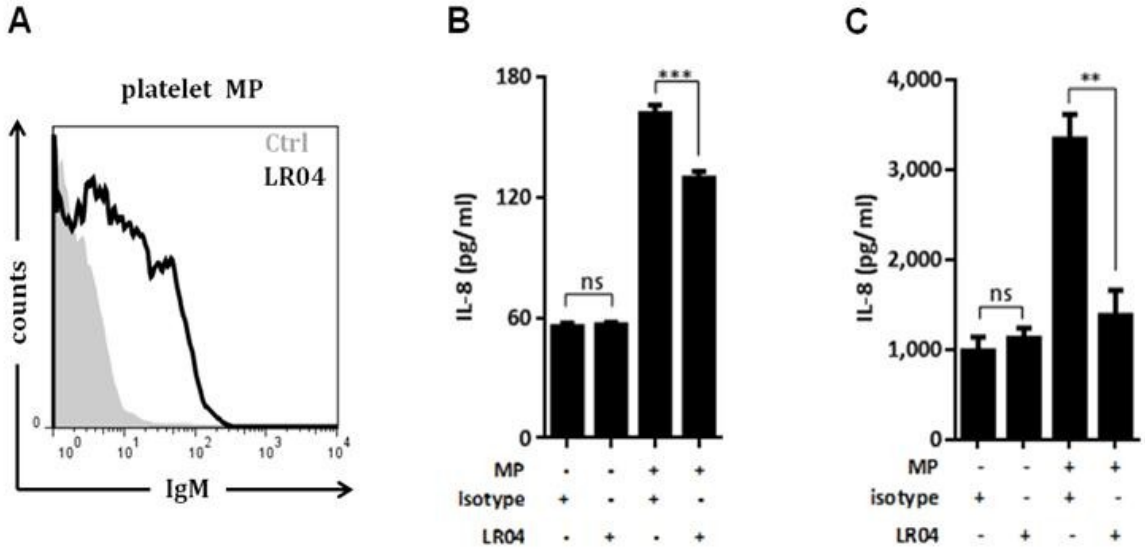

Figure 9. LR04, an MDA-specific IgM NAb, decreases the proinflammatory effect of platelet MP. (A) Platelet-derived MP stained with the MDA/MAA-specific LR04 or isotype antibody and analyzed by flow cytometry. **(B)** Stimulation of THP-1 human monocytes for 8 hours with in vitro generated platelet-derived MP resulted in IL-8 secretion, which was inhibited when MP were preincubated with LR04 compared to isotype control. Data are from one experiment representative of four in triplicate determinations. **(C)** Stimulation of primary human monocytes isolated from healthy donors (n=6) for 8 hours with in vitro generated platelet-derived MP resulted in IL-8 secretion, which was inhibited when MP were preincubated with LR04 but not an isotype control. Data are from two independent experiments. Data are presented as mean ±SEM. (ns; not significant, **P‹ 0.01, ***P‹ 0.001; Bonferroni's multiple comparison test or paired t test).

3.1.5 MDA-carrying MP are increased at the site of the coronary occlusion in ST-elevation myocardial infarction (STE-MI)

Several studies have reported that patients with ACS have increased levels of circulating MP, which could propagate the inflammatory responses of affected arteries. Therefore, we tested whether MP isolated from the peripheral and coronary circulations of patients with STE-MI also carry MDA-epitopes. Blood was obtained from the femoral artery sheath and from the site of occlusion of the affected coronary arteries of the same patients during primary percutaneous coronary intervention (pPCI) (Table 1). MP levels were significantly higher in the affected coronary arteries compared to the peripheral site of the same patient (Figure 10A). In comparing the source of isolated MP from the peripheral (88±2% Annexin V^+ particles) and coronary circulation (81±4% Annexin V^+ particles), the relative contribution of RBC-derived MP was similar between the two vascular sites (Figure 10B). In contrast, platelet-derived MP were decreased (Figure 10C), while EC-derived MP increased in the coronary compared to the peripheral blood (Figure 10D). Moreover, the frequency of monocyte/macrophage-derived MP identified by expression of CD14 was very low and not different between the two sites (periphery: 1.02% ±0.3 of total MP vs. coronary: 1.15% ±0.3 of total MP). Similar data were obtained after normalization for Annexin V^+ events (data not shown). Importantly, characterization of MP from both sites not only demonstrated higher numbers (Figure 10E), but also a higher percentage of MDA-carrying MP (Figure 10F) in the coronary circulation, despite similar levels of anti-MDA-LDL IgM at both sites (Figure 11). Moreover, the increased frequency of MDA-carrying MP was specifically found in platelet-derived MP (Figure 10G), but not red blood cell-derived MP (Figure 10H). Thus, the total number and frequency of MDA-carrying MP are elevated in the coronary circulation of STE-MI patients.

Number of patients		**14**
Age, years		54.9 ± 10.0
Female gender, n (%)		6 (42.9)
Diabetes, n (%)		2 (14.3)
History of hypertension, n (%)		5 (35.7)
Ever smoker, n (%)		10 (71.4)
Family history of CAD, n (%)		6 (42.9)
BMI > 25kg/m², n (%)		11 (78.6)
Troponin T max, ng/ml		3.9± 3.2
Culprit vessel, n (%)	LAD	4 (28.6)
	CX	3 (21.4)
	RCA	7 (50)
CAD, n (%)	1VD	8 (57.1)
	2VD	6 (42.9)
	3VD	0 (0)
CPKmax, U/l		2076.4 ± 1947.8
CRP, mg/dl		0.7 ± 0.8
Creatinine, mg/dl		1.1 ± 0.3
Total Cholesterol, mg/dl		220.0 ± 60.2
LDL, mg/dl		136.3 ± 59.7
HDL, mg/dl		39.7 ± 5.8
Triglycerides, mg/dl		185.9 ± 188.5

Table 1. STE-MI patient characteristics. Data are presented as mean ± SD. CPKmax, CRP, Creatinine, Cholesterol, LDL, HDL and Triglycerides were measured one day after pPCI. CAD, coronary artery disease; BMI, body mass index; LAD, left anterior descending artery; CX, left circumflex artery; RCA, right coronary artery; VD, vessel disease; CPKmax, peak value of serum creatine phosphokinase; CRP, C-reactive protein; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol.

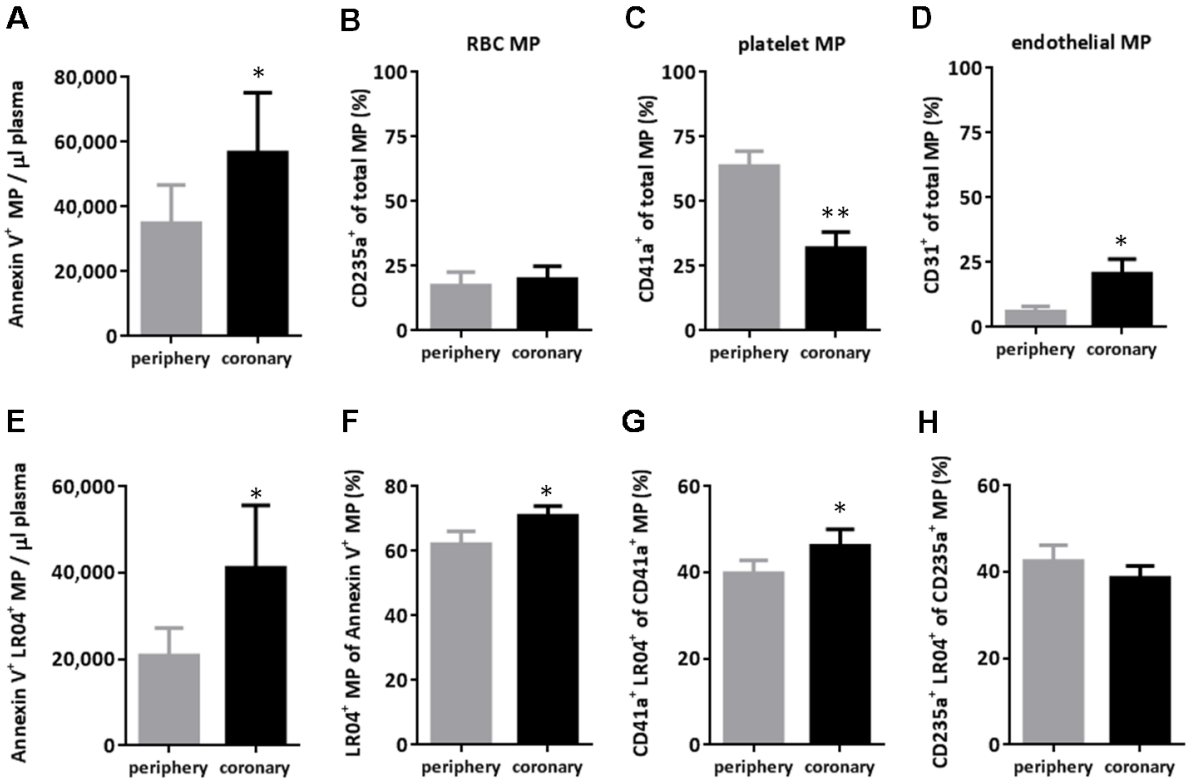

Figure 10. MDA-carrying MP are increased at the culprit lesion site in STE-MI. (A) Total numbers of MP were quantified by flow cytometry in plasma isolated from peripheral (femoral artery) and coronary blood samples of patients with STE-MI. (**B, C, D)** Cellular origin: Isolated MP were stained with anti-CD41a (platelet), anti-CD235a (RBC) and anti-CD31 (EC) to identify their cellular origin, and are presented as percentages of positive MP of total MP. **(E-H)** MDA-epitopes: Isolated MP were stained with Annexin V, anti-CD41a, or anti-CD235a and LR04, and analyzed by flow cytometry. Data show **(E)** total numbers of Annexin V^+ $LR04^+$ MP per µl plasma and **(F)** percentages of Annexin V^+ $LR04^+$ MP of total MP, as well as the percentages of $LR04^+$ MP of either **(G)** $CD41a^+$ platelet or **(H)** $CD235a^+$ RBC MP. All results are presented as mean ±SEM of 13-14 STE-MI patients, *P<0.05 and **P<0.01 (paired t test).

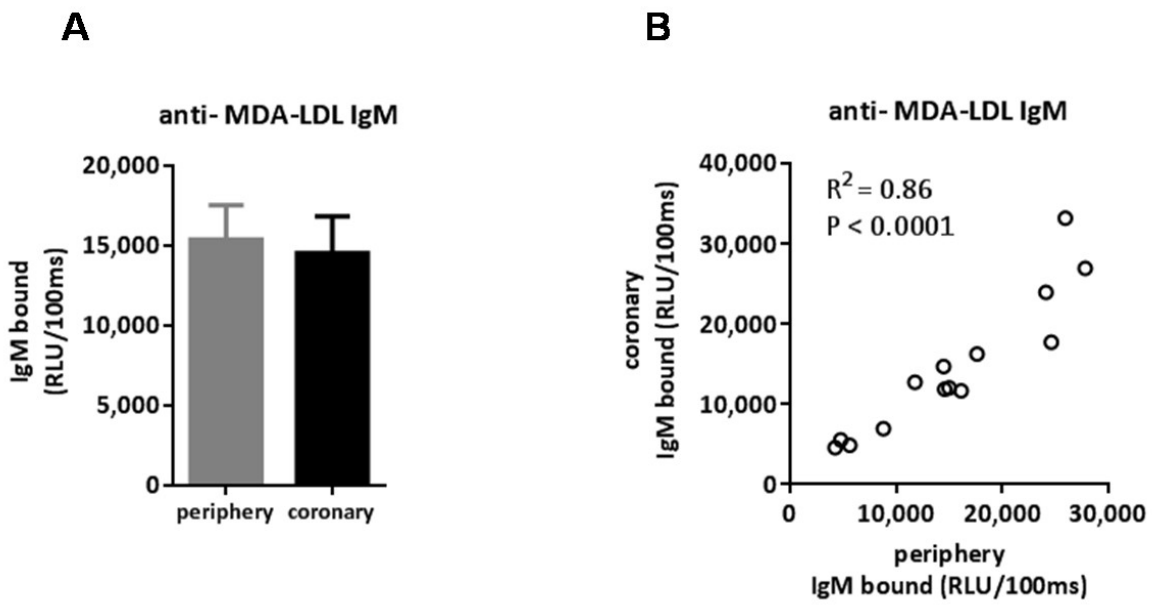

Figure 11. Similar IgM antibody titers specific for MDA-LDL in coronary and peripheral plasma of STE-MI patients. (A) Determination of IgM titers to MDA-LDL in diluted plasma isolated from peripheral and coronary blood of STE-MI patients by ELISA. Data are mean ± SEM of 14 AMI patients and expressed as RLU/100ms. **(B)** Correlation of IgM titers to MDA-LDL between peripheral and coronary circulation. Circles represent results of individual patients and correlation analysis was performed with Pearson's test; P<0.001.

3.2 Part 2: (Tsiantoulas et al in preparation)

Title: Natural IgM antibodies regulate plasma IgE levels

Levels of natural IgM antibodies with specificity for OSE have been shown to inversely correlate with cardiovascular disease risk (Tsiantoulas et al, 2014). Moreover, several experimental studies have shown that increased levels of certain OSE-specific IgM clones such as T15/E06, which binds OxLDL and apoptotic cells, confer an atheroprotective effect. The proposed mechanisms for the protective properties of OSE-specific natural IgM antibodies, which account for approximately 30% of total natural IgM, include blockage of scavenger receptor-mediated uptake of OxLDL by macrophages, promotion of apoptotic cell clearance (Tsiantoulas et al, 2014) and - as we show above - inhibition of the proinflammatory effect of MP. Consistent with such a protective function of natural IgM, it has been previously demonstrated that *sIgM*$^{-/-}$ mice deficient develop accelerated atherosclerosis. However, no significant difference in lesional apoptotic cell content between western diet fed *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice was observed (Lewis et al, 2009). This prompted us to hypothesize that different mechanisms exist that promote atherosclerosis in absence of polyclonal natural IgM pool. Indeed, natural IgM have been demonstrated to play a important role in the development of B cells (Baker & Ehrenstein, 2002; Boes et al, 1998; Ehrenstein et al, 1998; Notley et al, 2010), which – as reviewed above - are critical modulators of atherosclerosis development. Thus, we investigated whether altered B cell responses are responsible for the accelerated atherosclerosis in *sIgM*$^{-/-}$ mice.

3.2.1 Mature B cells display decreased surface B220 expression in *sIgM*$^{-/-}$ mice

B1 cells are characterized by reduced surface expression of the transmembrane protein tyrosine phosphatase B220 (Baumgarth, 2011), which is the biggest isoform of the CD45 receptor (CD45R) and has also been shown to be a negative regulator of BCR signaling (Rhee & Veillette, 2012; Shrivastava et al, 2004). CD45 E613R transgenic mice, which carry a point mutation (E613R) that abrogates the inhibitory effect of the CD45 receptor on BCR signaling develop increased B1 and reduced B2 cell numbers both in the spleen and the peritoneal cavity (Hermiston et al, 2005). These data suggest that CD45 is involved in the developmental fate of

B1 cells and B2 cells most likely by modulating BCR signaling. Because *sIgM*$^{-/-}$ mice have strongly increased B1 cell numbers both in the spleen and the peritoneal cavity (Boes et al, 1998), we investigated whether the lack of soluble natural IgM antibodies impacts the surface expression of B220 in splenic and peritoneal B cells (Figure 12). We found that in contrast to newly formed (NF) and transitional stage 1 (T1) B cells, B220 expression on the cell surface was significantly reduced in follicular and CD23^{+} transitional stage 2 (FO/T2), marginal zone (MZ) as well as CD21^{+}CD23^{-} splenic B cells in *sIgM*$^{-/-}$ compared to *sIgM*$^{+/+}$ mice (Figure 13A). In line with this, we also found reduced surface levels of B220 in bone marrow B cells that express a BCR (Figure 13B). Moreover, peritoneal B1a, B1b and CD23^{+} B2 cells were found to express decreased surface B220 in *sIgM*$^{-/-}$ mice (Figure 13C), which was completely restored to levels similar to wild type mice, upon intraperitoneal injection of 200µg of polyclonal IgM preparations every other day over a two week period (Figure 13D-F). These data identify soluble natural IgM antibodies as a novel regulator of surface B220 expression and suggest that soluble IgM antibodies may be modulators of BCR signaling.

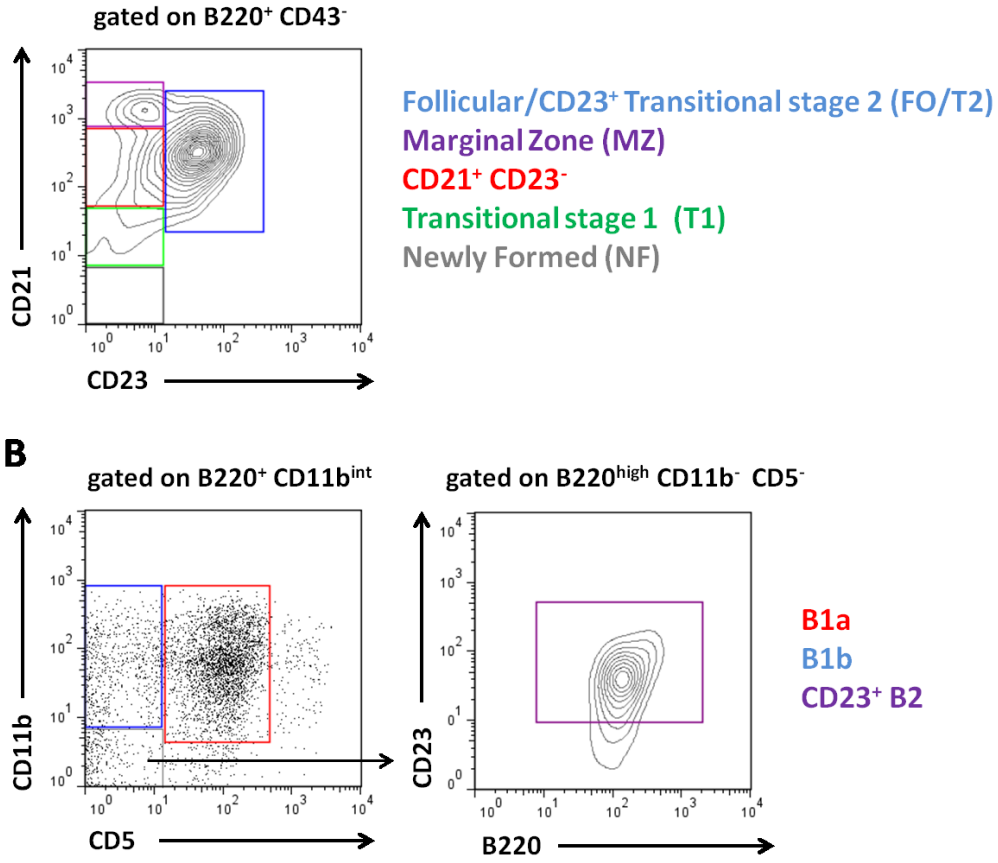

Figure 12. Gating strategy of mature splenic and peritoneal B cells. (A) Splenic B cell subsets. Follicular and $CD23^+$ transitional stage 2 B cells (FO/T2; blue) were defined as $B220^+CD21^+CD23^+CD43^-$, marginal zone (MZ; purple) B cells were defined as $B220^+CD21^{high}CD23^-CD43^-$, $CD21^+CD23^-$ B cells (were defined as $B220^+CD21^+CD23^-$ $CD43^-$; red), transitional stage 1 (T1; green) B cells were defined as $B220^+CD21^{low}CD23^-CD43^-$ and newly formed (NF; grey) B cells were defined as $B220^+CD21^-CD23^-CD43^-$. **(B)** Peritoneal B cell subsets. B1a cells (red) defined as $B220^+$ $CD11b^{intermediate}$ $CD5^+$, B1b (blue) defined as $B220^+$ $CD11b^{intermediate}$ $CD5^-$, and $CD23^+$ B2 cells (purple) defined as $B220^{high}CD11b^-$ $CD5^-$ $CD23^+$.

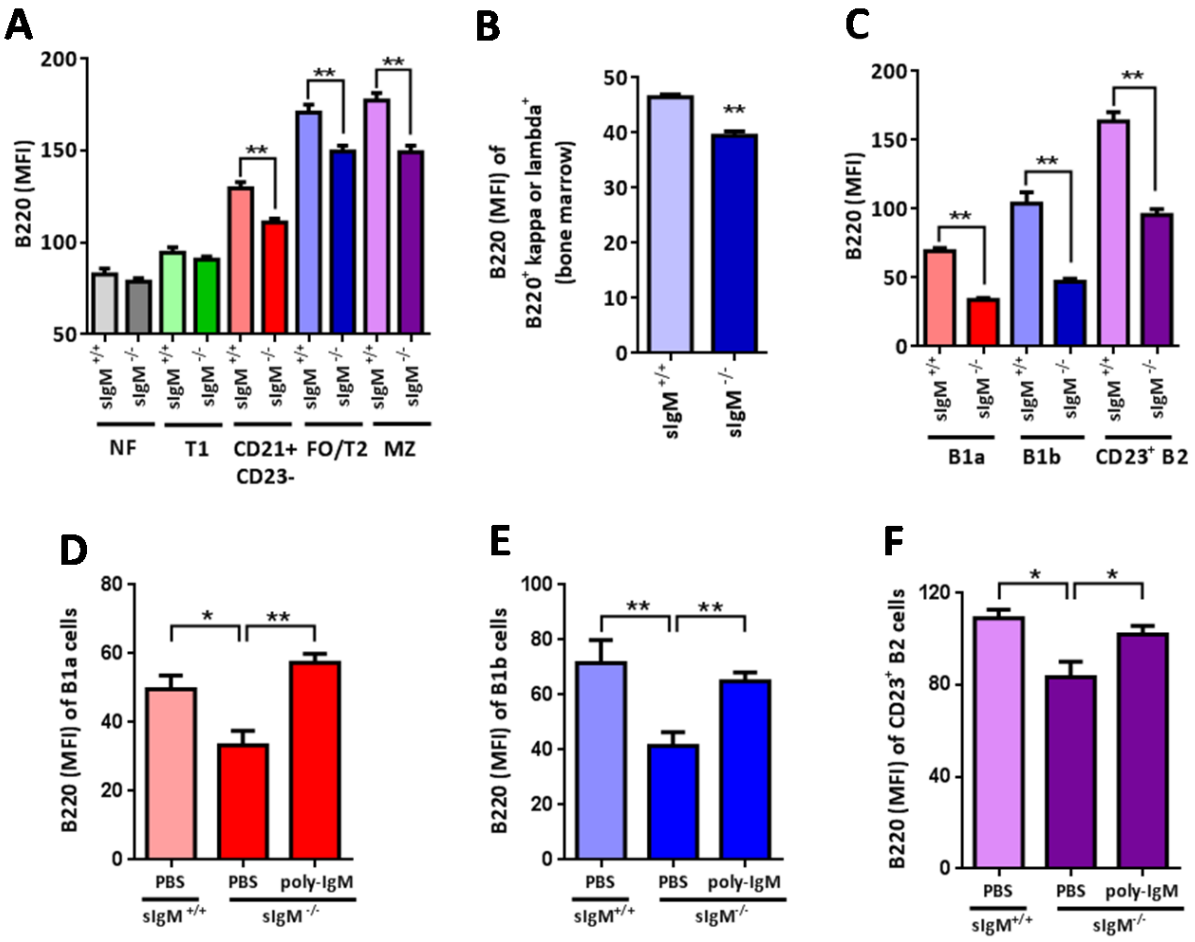

Figure 13. Mature splenic and peritoneal B cells display decreased surface B220 expression in *sIgM*$^{-/-}$ mice. (A) Quantification of B220 expression of splenic B cell subsets in *sIgM*$^{+/+}$ (light colored bars) and *sIgM*$^{-/-}$ (dark colored bars) mice, analyzed by flow cytometry. Bars represent the mean fluorescence intensity (MFI) of B220 expression in FO/T2 (blue), MZ (purple), CD21^{+}CD23^{-} B cells (red), T1 (green) and NF (grey) as defined in Figure 12A. **(B)** Quantification of B220 expression of bone marrow B cells in *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. Bars represent the MFI of B220 expression in B220^{+} B cells that also express either kappa or lambda light chains on their cell surface. **(C)** Quantification of B220 expression in peritoneal B cell subsets of *sIgM*$^{+/+}$ (light colored bars) and *sIgM*$^{-/-}$ (dark colored bars) mice analyzed by flow cytometry. Bars represent MFI of B220 expression in B1a cells (red), B1b (blue) and CD23^{+} B2 cells (purple) as defined in Figure 12B **(D, E, F)** Quantification of B220 expression in peritoneal B cell subsets of *sIgM*$^{+/+}$ (light colored bars) that were injected i.p. with DPBS and *sIgM*$^{-/-}$ (dark colored bars) mice that were injected with either DPBS or polyclonal IgM for every other day for two weeks. Bars represent the MFI of B220 expression in B1a cells (red), B1b (blue) and CD23^{+} B2 cells (purple) as defined in **(c)**. All results show mean ± SEM, (**A,C**: Values of one representative experiment of three independent experiments are shown with six mice per

group; Mann-Whitney test, **B**: Values of one representative experiment of two independent experiments are shown with six mice per group; Mann-Whitney test, **D,E,F**: Values of four to five mice per group are shown; One-Way ANOVA followed by Newman-Keuls test).*P<0.05, **P<0.01.

3.2.2 Soluble natural IgM antibodies decrease antigen mediated BCR signaling and reduce BCR editing.

We and others have previously shown that natural IgM antibodies recognize and promote the clearance of apoptotic cells (Chang et al, 2004; Chou et al, 2009; Ogden et al, 2005) resulting in prevention of autoimmune responses against self-antigens (Boes et al, 2000; Notley et al, 2011). Moreover, B cells that have robustly increased antigen-mediated BCR signaling, also display reduced B220 expression on their cell surface (Hojyo et al, 2014). Because we found reduced B220 cell surface expression in B cells of *sIgM*$^{-/-}$ mice, we were prompted to investigate the role of IgM antibodies in antigen mediated BCR signaling. Therefore, we isolated splenic B2 cells from *t11*µ*MT* mice (Klein et al, 1997) that exclusively express BCRs directed against the vesicular stomatitis virus (VSV) antigen, and stimulated them with UV-inactivated VSV antigens that were preincubated with either an anti-VSV specific IgM (that recognizes a different epitope than the BCR) or an isotype control antibody. To evaluate the strength of the VSV antigen mediated BCR signaling we quantified mRNA expression of the orphan nuclear hormone receptor Nur77 gene that has been shown to be rapidly upregulated upon BCR stimulation (Mittelstadt & DeFranco, 1993). The VSV antigen preincubated with the isotype control antibody stimulated Nur77 gene expression to the same extent as 10µg/ml of anti-IgM F(ab')$_2$. Interestingly, Nur77 expression was significantly reduced when the VSV antigen was bound by a monoclonal IgM VSV-specific antibody suggesting that soluble IgM interfere with the signaling triggered by BCR bound antigens (Figure 14A). Because the antigen-mediated BCR signaling strength determines evasion of immature B cells in the bone marrow from autoreactivity by influencing BCR editing (Clark et al, 2014), we examined the ratio of B cells expressing kappa to B cells expressing lambda light chains as a marker of BCR editing. We found a decreased ratio of kappa/lambda light chain expressing B cells in the bone marrow of *sIgM*$^{-/-}$ (Figure 14B) compared to *sIgM*$^{+/+}$ mice indicative of enhanced BCR editing. In line with this, splenic B cells showed also a decreased ratio of kappa/lambda light chain expressing B cells in

sIgM$^{-/-}$ mice (Figure 14C). Thus, our results suggest that soluble natural IgM antibodies modulate the interaction of self-antigens with B cells resulting in decreased BCR signaling and editing and thus influence the BCR repertoire present in peripheral mature B cells.

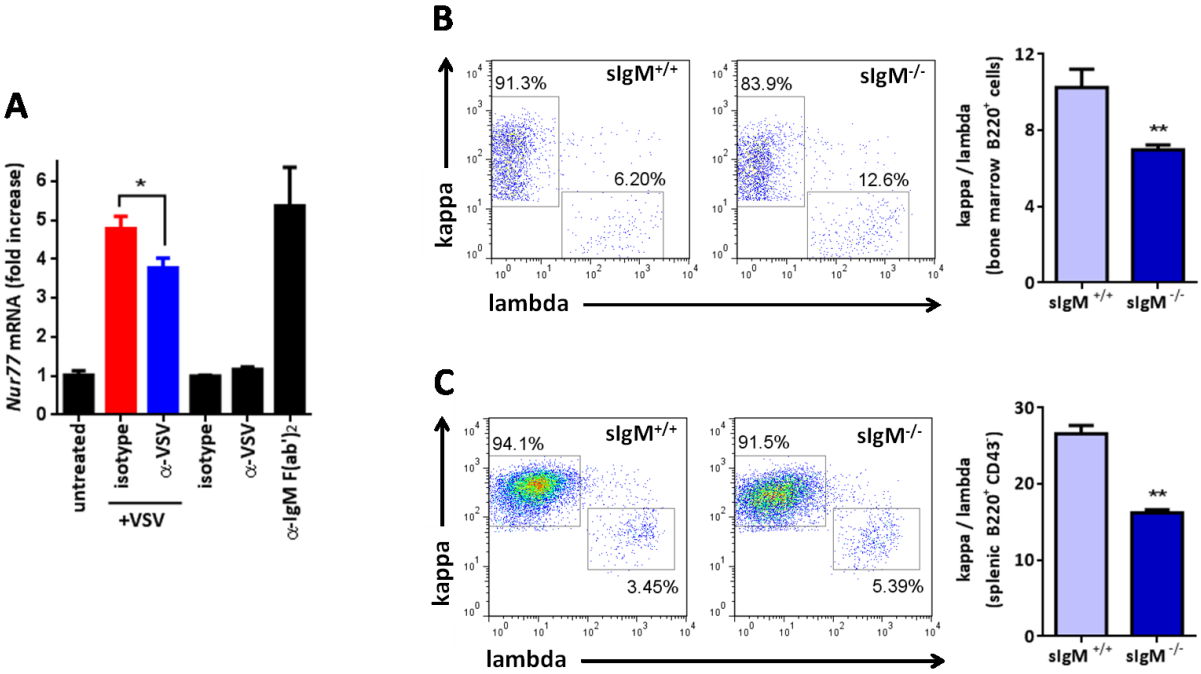

Figure 14. Natural IgM antibodies decrease the antigen mediated BCR signaling and reduce BCR editing in *sIgM*$^{-/-}$ mice. (A) Purified B2 (B220^{+} CD43^{-}) cells from *t11*µ*MT* mice were stimulated with either 10 µg/ml of anti-IgM F(ab')$_2$ or 465,000 PFU of inactivated vesicular stomatitis virus (VSV) for 30 minutes. VSV was preincubated with either 2 µg/ml of an anti-VSV (M4C11H2) or a control IgM antibody (MM-30). Nur77 mRNA was quantified by Real-time PCR. Data show one representative experiment of two independent experiments. **(B)** Ratio of kappa and lambda light chain expressing B220^{+} cells in bone marrow of *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. Representative flow cytometry plots show the percentage of either kappa or lambda light chain positive cells of B220^{+} B cells (within B220^{+} B cells that express kappa or lambda light chains), and bars represent the mean kappa/lambda light chain ratio of all mice. Data show one representative experiment of two independent experiments. **(C)** Ratio of kappa and lambda light chain expressing B2 cells in the spleen of *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. Representative flow cytometry plots show the percentage of either kappa or lambda light chain positive cells of B220^{+} CD43^{-} cells and bars represent the mean kappa/lambda light chain ratio of all mice. Data show one representative experiment of

three independent experiments with six mice per group. All results are represented as mean ± SEM, *P<0.05, **P<0.01 (**(A)** unpaired t test or **(B, C)** Mann-Whitney test).

3.2.3 Mature splenic B cells display increased BCR signaling in *sIgM*$^{-/-}$ mice *in vivo*.

Because the decreased kappa/lambda light chain ratio in bone marrow and splenic B cells (Figure 14B,C) of *sIgM*$^{-/-}$ mice indicates enhanced BCR editing, we hypothesized that B cells are exposed to strong BCR signaling in absence of soluble natural IgM antibodies. To investigate this, we quantified the phosphorylated spleen tyrosine kinase (pSyk) and Bruton's tyrosine kinase (pBtk) levels in splenic B cell subsets of *sIgM*$^{+/+}$ and *sIgM*$^{-/-}$ mice by flow cytometry. These kinases have been shown to be critically involved in antigen mediated BCR signaling and B cell maturation towards FO and MZ B cells (Cariappa et al, 2001; Cornall et al, 2000). Our results show that splenic NF and T1 B cells display similar pSyk and pBtk MFI in *sIgM*$^{+/+}$ and *sIgM*$^{-/-}$ mice. In contrast, both MZ and FO/T2 were found to have higher levels of both pSyk and pBtk. Similar results were obtained for CD21^{+}CD23^{-} splenic B cells (Figure 15A,B). To confirm our results, we quantified the expression of Nur77 in splenic B cell subsets of *Nur77-GFP/sIgM*$^{-/-}$ and *Nur77-GFP/sIgM*$^{+/+}$ reporter mice as a marker of BCR signaling intensity (Zikherman et al, 2012). In analogy to the levels of pBtk and pSyk we found higher Nur77 expression in MZ, FO/T2 and CD21^{+}CD23^{-} B cells of *sIgM*$^{-/-}$ mice (Figure 15C). Collectively, these data indicate that in absence of soluble natural IgM antibodies, mature splenic B cells subsets are exposed to higher BCR signaling in *sIgM*$^{-/-}$ mice.

3.2.4 Marginal zone B cells display stronger BCR signaling compared to follicular B cells *in vivo*.

Notably, we also found that MZ B cells display higher pSyk and pBtk levels (Figure 15A,B) in *wild type* mice. In line with this, we also show that Nurr77 expression of MZ B cells (light purple bar) is higher than that of FO/T2 (light blue bar) B cells in *Nur77-GFP* reporter mice (Figure 15C). Similar results were obtained when *Nur77-GFP* mice were housed in a different specific pathogen free animal facility (Figure 15D). Next, we gated on $B220^{+}CD93^{-}$ B cells in order to exclude all transitional B cells and compare the GFP MFI of MZ to FO B cells only. We obtained similar data showing that MZ have higher Nur77 expression compared to FO B cells (Figure 15E). This suggests that the MZ B cell compartment displays stronger BCR signaling compared to FO B cells.

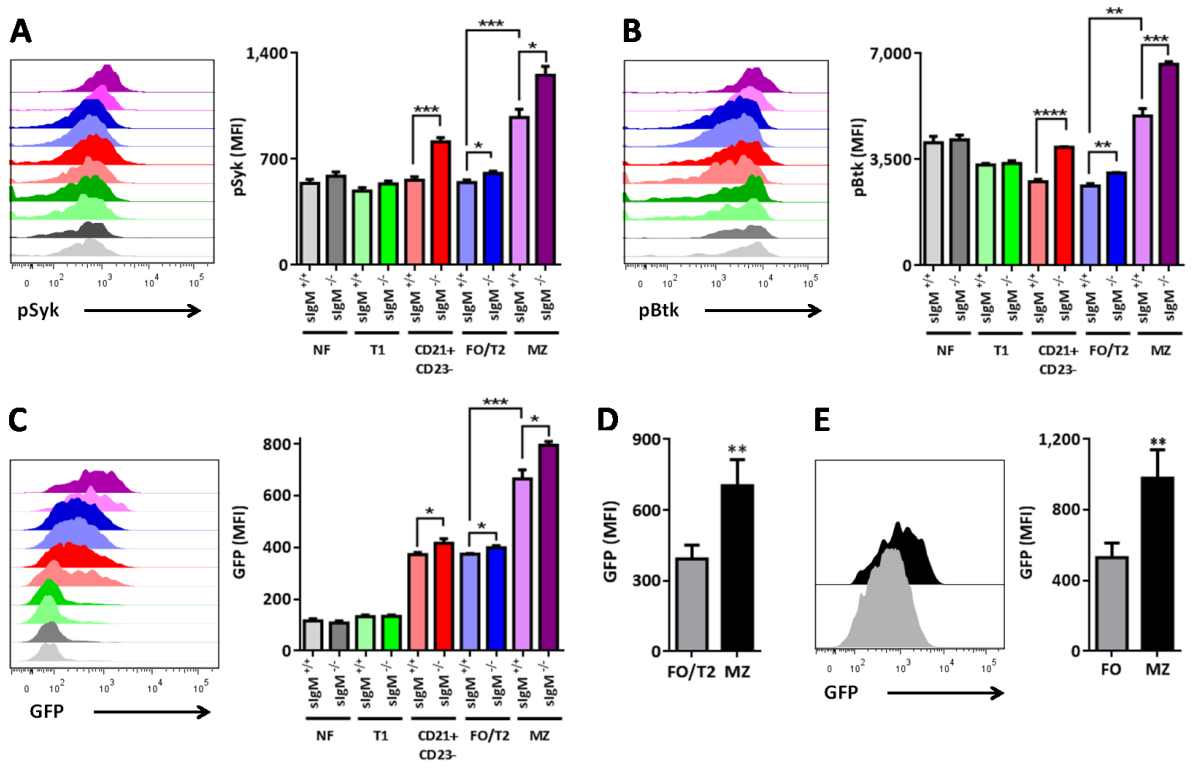

Figure 15. Increased B cell receptor signaling in *sIgM*$^{-/-}$ mice. Quantification of **(A)** phosphorylated spleen tyrosine kinase (pSyk) and **(B)** phosphorylated Bruton's tyrosine kinase (pBtk) in splenic B cell subsets of *sIgM*$^{+/+}$ (light colored bars) and *sIgM*$^{-/-}$ (dark colored bars) mice by flow cytometry. Shown are representative flow cytometry histograms for the MFI of pSyk and pBtk expression (left) and the mean MFI of pSyk and pBtk expression of all mice analyzed (bar graphs, right) in FO/T2 (blue), MZ (purple), $CD21^{+}CD23^{-}$ B cells (red), T1

(green) and NF (grey) as defined in Figure 12. **(C)** Quantification of Nurr77-GFP expression in splenic B cell subsets of *Nur77-GFP/sIgM*$^{+/+}$ (light colored) and *Nur77-GFP/sIgM*$^{-/-}$ (dark colored) mice analyzed by flow cytometry. Shown are representative flow cytometry histograms for the MFI of Nurr77-GFP expression (left) and the mean MFI of all mice analyzed (bar graphs, right) in FO/T2 (blue), MZ (purple), CD21^{+}CD23^{-} B cells (red), T1 (green) and NF (grey) as defined in Figure 12. **(D)** Shown are the mean MFI (bar graphs) of FO/T2 B cells (grey bar) and MZ B cells (black bar) as defined in Figure 12 of all *Nur77-GFP* mice analyzed, which were housed in a different animal facility. **(E)** Shown are representative flow cytometry histograms for the MFI of Nurr77-GFP expression (left) and the mean MFI of all mice analyzed (bar graphs, right) of B220^{high}CD21^{+}CD23^{+}CD93^{-} FO B cells (grey bar) and B220^{high}CD21^{high}CD23^{-}CD93^{-} MZ B cells (black bar) of *Nur77-GFP* mice. All results show mean ± SEM, (**A,B**: Values of one representative experiment of two independent experiments are shown with four mice per group; unpaired t test, **C**: Values of two independent experiments are shown with four to five mice per group; unpaired or paired t test, **D**: Values of one experiment are shown with five mice per group; paired t test, **E**: Values of two independent experiments are shown with five mice per group; paired t test.). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.2.5 Abnormal splenic B cell development results in reduced CD23^{+} B cells in *sIgM*$^{-/-}$ mice.

Newly formed splenic B cells further differentiate to transitional and then mature splenic B cells, which include FO and MZ B cells. Whether a transitional B cell will mature towards the FO or MZ zone B cell type is largely determined by the BCR signaling with the FO B cell differentiation to be promoted in conditions of strong BCR signaling (Loder et al, 1999; Pillai & Cariappa, 2009). Because our data show increased BCR signaling in the spleen of *sIgM*$^{-/-}$ mice, we were prompted to examine the splenic B cell differentiation in *sIgM*$^{-/-}$ mice. Therefore, we performed a detailed flow cytometry analysis of splenic B cell subsets in *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice. We found no difference in total viable or dying B cell numbers between *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice (Figure 16A-C). NF and T1 (Figure 17A) as well as total transitional B cell numbers (Figure 18B) were not different between *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice. Moreover and in agreement with a previous report (Baker & Ehrenstein, 2002), we also found increased MZ B cell numbers in young (12-14 week old) *sIgM*$^{-/-}$ mice (Figure 17A) but this was not the case for older (28 week old) mice (Figure 19A). Notably, we also found a robust increase in mature CD21^{+}CD23^{-} B cells in both

young and older *sIgM*$^{-/-}$ mice compared to *sIgM*$^{+/+}$ controls (Figure 17A and Figure 19C). Interestingly, our analysis revealed a strong decrease in CD23^{+} B cells, which consist of FO and CD23^{+} T2 B cells (FO/T2), in both young and older *sIgM*$^{-/-}$ mice (Figure 17A and Figure 19B). In line with this we found that the transitional stage 2 B cells, defined as B220^{+}CD93^{+}CD21^{+}, were shifted towards CD23^{-} cells in *sIgM*$^{-/-}$ mice (Figure 18C). Because, CD23^{+} B cells have circulating capacity, we examined their numbers in the peripheral blood as well as in the peritoneal cavity and we found that they were strongly reduced at both sites in *sIgM*$^{-/-}$ mice (Figure 17B,C). Collectively, these data show abnormal splenic B cell development, which results in impaired generation of CD23^{+} B cells in absence of soluble natural IgM antibodies.

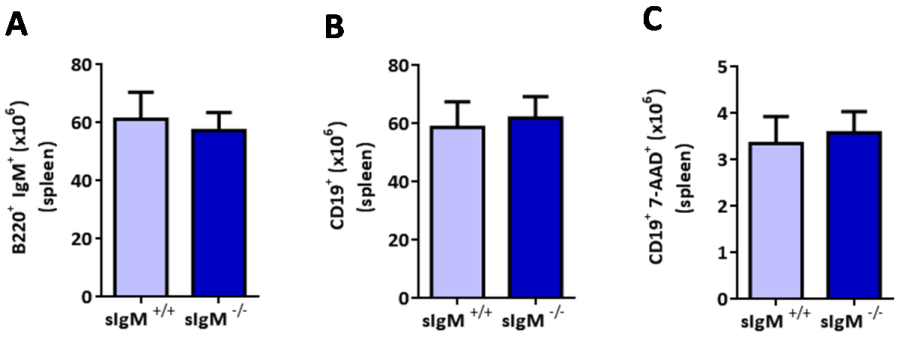

Figure 16. Total splenic B cell numbers are not different between *sIgM*$^{+/+}$ and *sIgM*$^{-/-}$ mice. Bars represent the absolute number of splenic B cells defined as **(A)** B220^{+} IgM^{+} or **(B)** CD19^{+} and the absolute numbers of **(C)** CD19^{+} 7-AAD^{+} dying B cells of *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. Data represent mean ± SEM values of one representative experiment of at least two independent experiments with six mice per group.

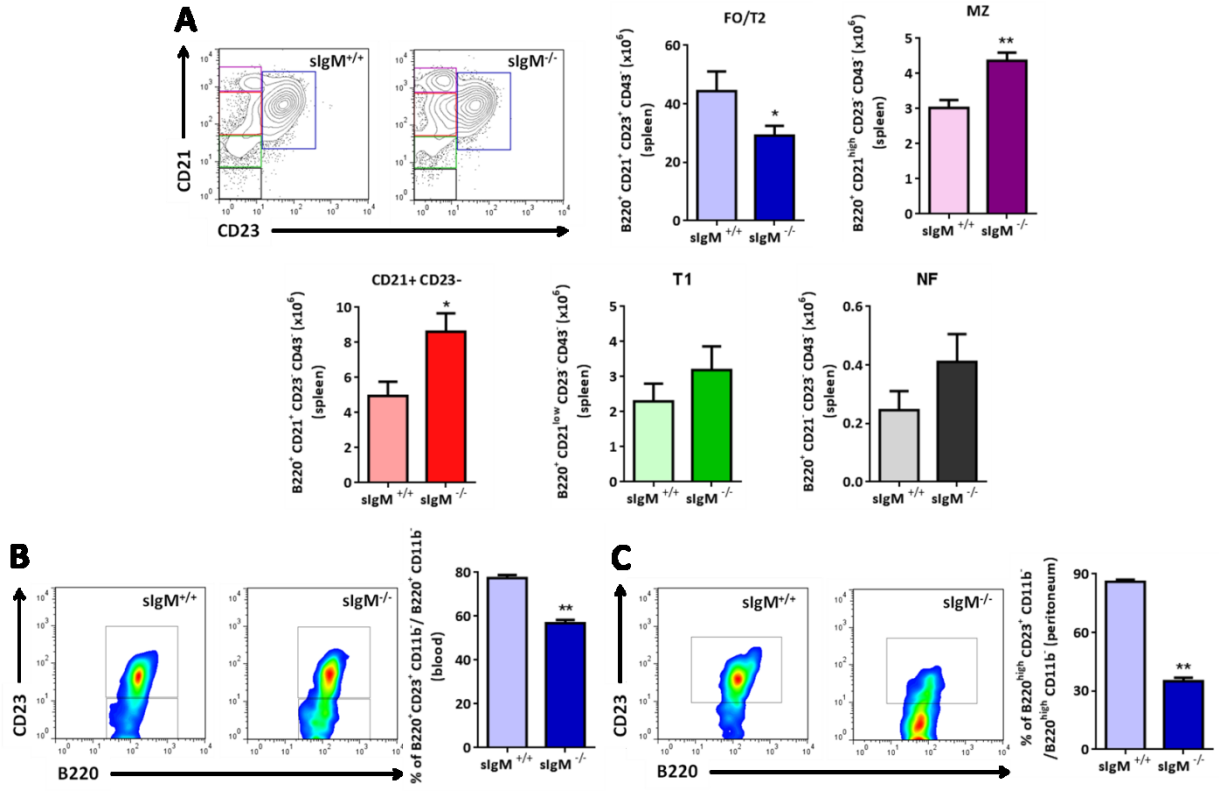

Figure 17. Abnormal splenic B cell development results in reduced CD23$^+$ B cells in *sIgM$^{-/-}$* mice. (A) Quantification of splenic B cell subsets in *sIgM$^{+/+}$* (light colored bars) and *sIgM$^{-/-}$* (dark colored bars) mice by flow cytometry. Flow cytometry plots show representative examples of different B cell populations identified by staining with antibodies against CD21 and CD23. Bar graphs represent absolute numbers of FO/T2 (blue), MZ (purple), CD21$^+$CD23$^-$ B cells (red), T1 (green) and NF (grey) cells as defined in Figure 12. Quantification of circulating **(B)** and peritoneal **(C)** CD23$^+$ B cells of *sIgM$^{+/+}$* (light blue bar) and *sIgM$^{-/-}$* (dark blue bar) mice by flow cytometry. Flow cytometry plots show representative examples CD23$^+$ B cells identified by staining with antibodies against B220 and CD23. Bar graphs represent percentages of CD23$^+$ B2 cells within the B2 population defined as B220$^+$ CD5$^-$ CD11b$^-$ of *sIgM$^{+/+}$* (light blue bar) and *sIgM$^{-/-}$* (dark blue bar) mice. Data shown are from one representative experiment of **(A,C)** at least three independent experiments and **(B)** two independent experiments . All results are represented as mean ± SEM of six mice per group, *P<0.05, **P<0.01 (Mann-Whitney test).

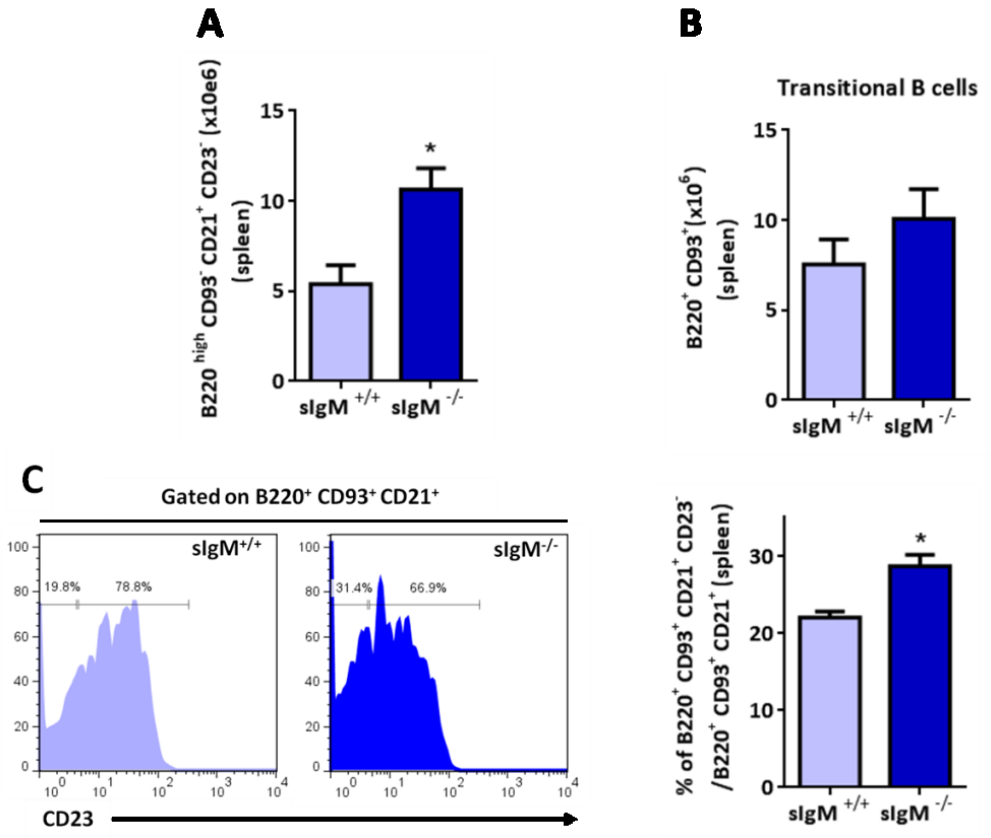

Figure 18. Transitional stage 2 B cells are shifted towards CD23$^-$ cells in the spleen of *sIgM*$^{-/-}$ mice. (A) Bars represent the absolute numbers of splenic B220^{high} CD93$^-$ CD21$^+$ CD23$^-$ B cells in *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice, analyzed by flow cytometry. **(B)** Bars represent the absolute numbers of splenic transitional B cells defined as B220$^+$ CD93$^+$ in *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. **(C)** Representative flow cytometry histograms and bar graphs showing the percentages of B220$^+$ CD93$^+$ CD21$^+$ CD23$^-$ within transitional stage 2 B cells defined as B220$^+$CD93$^+$CD21$^+$ in *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice, analyzed by flow cytometry. All results are represented as mean ± SEM of six mice per group. Data show one representative experiment of at least two independent experiments. *P<0.05 (Mann-Whitney test).

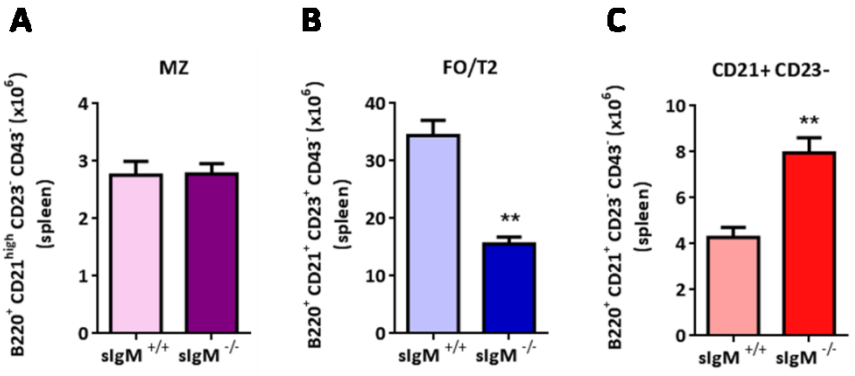

Figure 19. Marginal zone B cells are not increased in old *sIgM*$^{-/-}$ mice. Quantification of splenic B cell subsets in 28 week old *sIgM*$^{+/+}$ (light colored bars) and *sIgM*$^{-/-}$ (dark colored bars) mice by flow cytometry. Bars represent the absolute numbers of **(A)** MZ (purple), **(B)** FO/T2 (blue) and **(C)** CD21^{+}CD23^{-} B cells (red) as defined in Figure 12. All results are represented as mean ± SEM of six mice per group. Data show one of at least two independent experiments. **P<0.01 (Mann-Whitney test).

3.2.6 Natural IgM antibody deficiency results in increased serum IgE titers

CD23, which is the low affinity receptor for IgE (FcεRII) and expressed primarily in B cells, has been previously shown to be involved in regulation of plasma IgE levels (Cheng et al, 2010; Ford et al, 2006; Lewis et al, 2004; Yu et al, 1994). Because *sIgM*$^{-/-}$ mice have decreased CD23 expressing B cells in the spleen, periphery and peritoneal cavity (Figure 17A-C), we examined serum IgE levels in these mice. IgE levels were found to be >8 fold higher in both young (Figure 20A) and older (Figure 20B) *sIgM*$^{-/-}$ mice compared to *sIgM*$^{+/+}$ controls. In order to investigate whether the increased IgE levels were due to increased production or impaired clearance, we quantified the germline and mature IgE mRNA as well as the numbers of IgE^{+} B cells in the spleen of *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice. We found no difference in germline IgE mRNA between *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice (Figure 20C). Mature IgE mRNA was neither detectable in *sIgM*$^{+/+}$ nor in *sIgM*$^{-/-}$ splenocytes (Figure 20D). Moreover, our results show that *sIgM*$^{-/-}$ and *sIgM*$^{+/+}$ mice have equivalent numbers of IgE^{+} splenic B cells (Figure 20E, F). Thus, our data demonstrate for the first time that mice deficient in natural IgM antibodies have robustly increased IgE titers in their serum, most likely resulting from impaired clearance due to decreased CD23^{+} B cells.

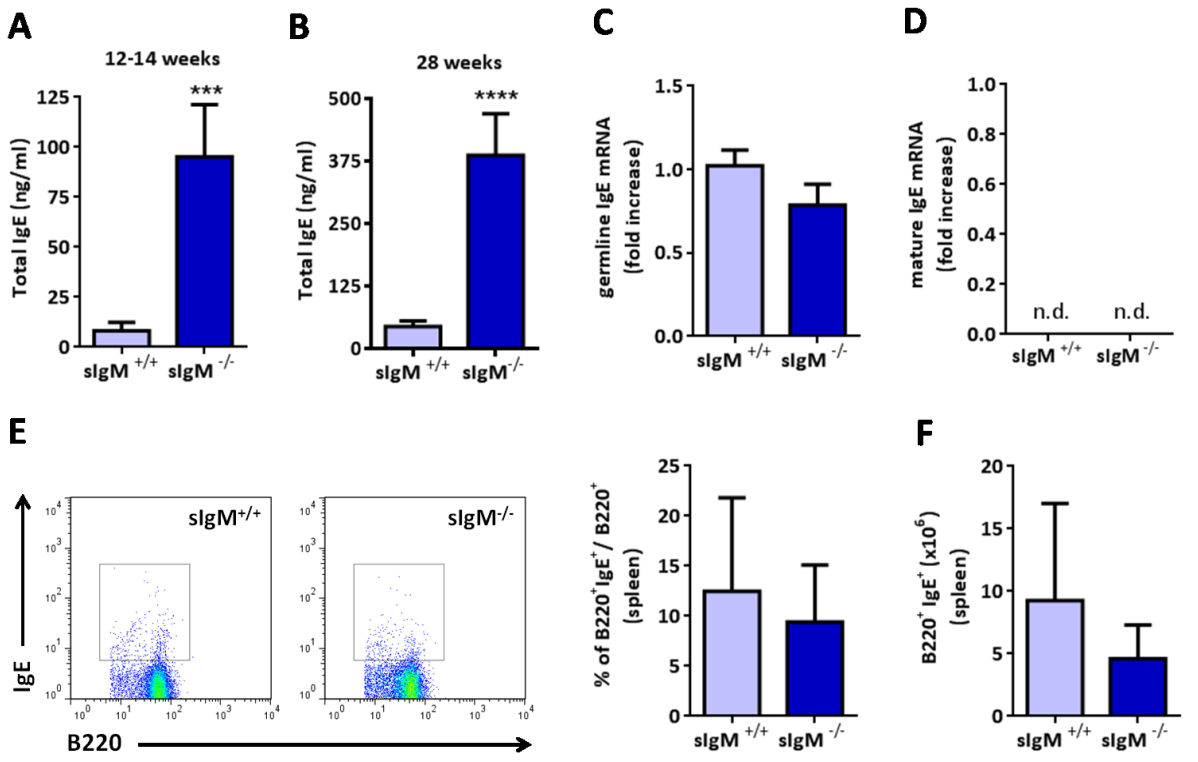

Figure 20. Increased plasma IgE titers in *sIgM*$^{-/-}$ mice. (A) Quantification of total plasma IgE antibody titers in **(A)** 12-14 week old (n=9-10 mice /group) and **(B)** 28 week old (n=10 mice /group) *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice by ELISA. Quantification of **(C)** germline IgE mRNA and **(D)** mature IgE mRNA in splenocytes of *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice by Real-time PCR (n.d.; not detectable). Quantification of IgE^{+} B cells. **(E)** Representative flow cytometry plots and bar graphs show the percentage of IgE^{+} B220^{+} B cells within total splenic B220^{+} B cells and **(F)** the splenic IgE^{+} B220^{+} B cell absolute number in the spleens of *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. All results are represented as mean ± SEM. **(C-F)** Data show one of at least two independent experiments with six mice per group. ***P<0.001, ****P<0.0001 (Mann-Whitney test).

3.2.7 IgE neutralization reverses the accelerated atherosclerosis in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice.

Wang et al have demonstrated that atherosclerosis-prone mice deficient in the high affinity receptor for IgE (FcεRI), develop smaller and less inflammatory atherosclerotic lesions (Wang et al, 2011a). Thus, we hypothesized that increased IgE levels may contribute to the accelerated atherosclerosis in *sIgM*$^{-/-}$ mice. Similar to *sIgM*$^{-/-}$ mice, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice fed a chow diet display reduced CD23^{+} B cells in the

spleen and the peritoneum (Figure 21A,B) , >9-fold increased serum IgE titers (Figure 21C) and no difference in IgE germline mRNA (Figure 21D). Mature IgE mRNA was not detectable in splenocytes isolated from *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ or *Ldlr*$^{-/-}$ mice. Moreover, we also found reduced soluble CD23 levels in the serum of *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice compared to *Ldlr*$^{-/-}$ controls (Figure 21E), which is in line with reduced generation of CD23^{+} B cells. In addition, atherogenic diet fed *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice that developed increased atherosclerosis despite similar serum cholesterol and triglyceride levels (Table 2; study 1), had also reduced splenic CD23^{+} B cells and increased serum IgE levels (Figure 22A-C).

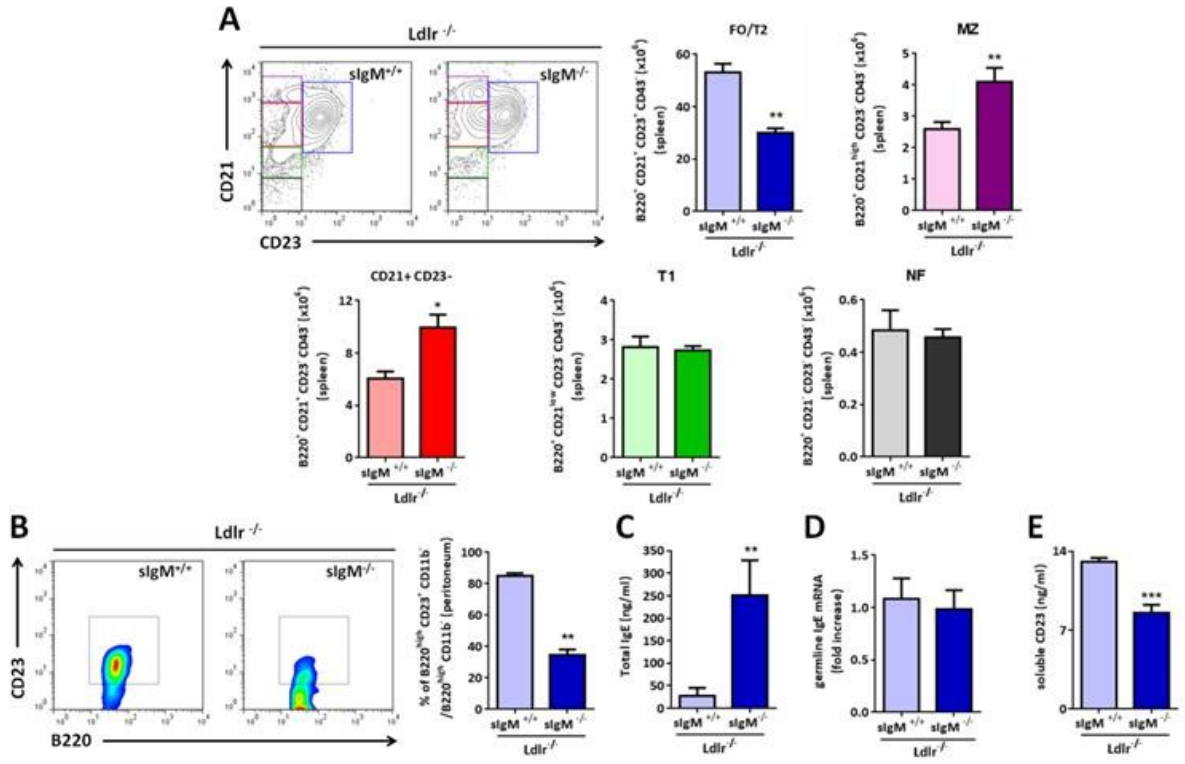

Figure 21. Reduced CD23^{+} B cells and increased plasma IgE titers in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice. **(A)** Quantification of splenic B cell subsets in *sIgM*$^{+/+}$ (light colored bars) and *sIgM*$^{-/-}$ (dark colored bars) mice by flow cytometry. Flow cytometry plots show representative examples of different B cell populations identified by staining with antibodies against CD21 and CD23. Bar graphs represent absolute numbers of FO/T2 (blue), MZ (purple), CD21^{+}CD23^{-} B cells (red), T1 (green) and NF (grey) cells as defined in Figure 12 of *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice. **(B)** Quantification of peritoneal CD23^{+} B cells of *sIgM*$^{+/+}$ (light blue bar) and *sIgM*$^{-/-}$ (dark blue bar) mice by flow cytometry. Flow cytometry plots show representative examples of CD23^{+} B cells identified by staining with antibodies against B220 and CD23. Bar graphs represent percentages of CD23^{+} B2 cells within the B2 population as

defined in Figure 12 of *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice. **(C)** Quantification of total plasma IgE antibody titers in *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) and *Ldlr*$^{-/-}$ *sIgM*$^{-/-}$ (dark blue bar) mice by ELISA. **(D)** Quantification of germline IgE mRNA by Real-time PCR in splenocytes of *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light colored bars) and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark colored bars) mice. Bars represent the fold increase compared to *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ mice. **(E)** Quantification of soluble CD23 in plasma of *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice by ELISA. All results are represented as mean ± SEM of six mice per group, *P<0.05, **P<0.01. ***P<0.001 (**(A,B,C)** Mann-Whitney test, **(E)** unpaired t test).

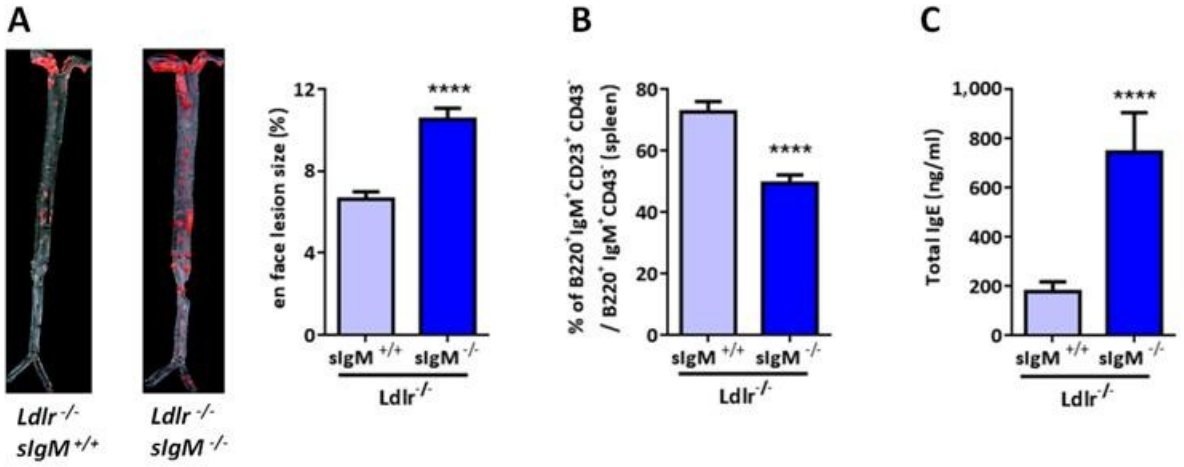

Figure 22. Reduced CD23^{+} B cells, increased plasma IgE titers and accelerated atherosclerosis in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice fed an atherogenic diet. (A) Representative photomicrographs and quantification of *en face* lesion size expressed as percentage of total area of Sudan IV stained aortas of female *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice that were fed an atherogenic diet for 16 weeks (n=13-15 mice/group). **(B)** Quantification of CD23^{+} B cells. Bars represent the percentage of splenic CD23^{+} B2 cells defined as B220^{+}IgM^{+}CD43^{-} CD23^{+} within B2 cells of *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ (light blue bar) and *Ldlr*$^{-/-}$ *sIgM*$^{-/-}$ (dark blue bar) mice analyzed by flow cytometry. **(C)** Quantification of total plasma IgE antibody titers in *Ldlr*$^{-/-}$ *sIgM*$^{+/+}$ (light blue bar) and *Ldlr*$^{-/-}$ *sIgM*$^{-/-}$ (dark blue bar) mice (n=13 mice/group) by ELISA. Results are represented as mean ± SEM, ****P<0.0001, (**(A)** unpaired t test or **(B, C)** Mann- Whitney test).

Study	experimental groups	final body weight (g)	total cholesterol (mg/dL)	triglycerides (mg/dL)
Study 1; 16 weeks on Western diet	$LDLR^{-/-}$ (n=11)	27 ±2.5	1762 ±355	767 ±310
	$LDLR^{-/-}$ $sIgM^{-/-}$ (n=15)	28 ±4.8	1770 ±236	757 ±277
Study 2; 6 weeks on Western diet	$LDLR^{-/-}$ / Ctrl Ab (n=14)	22.5 ±2.9	1391 ±258	691 ±185
	$LDLR^{-/-}$ $sIgM^{-/-}$ / Ctrl Ab (n=16)	22 ±2.9	1316 ±220	653 ±190
	$LDLR^{-/-}$ $sIgM^{-/-}$ / anti-IgE (n=15)	21.5 ±1.5	1298 ±263	652 ±200

Table 2. Whole body weight and serum or plasma total cholesterol and triglyceride quantification in *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice that were fed an atherogenic diet. Serum or plasma total cholesterol and triglycerides were measured under standardized conditions in an ISO 15189 accredited medical laboratory on Beckman Coulter AU5400 (Beckman Coulter) or Roche Cobas 8000 (Roche) instruments. All results are represented as mean ±SD.

To investigate whether increased IgE titers contribute to the accelerated atherosclerosis in mice lacking soluble natural IgM antibodies, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ or *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 6 weeks and at the same time treated with either a neutralizing anti-IgE antibody (R1E4) or a control antibody (Ctrl), respectively. Anti-IgE treatment resulted in complete neutralization of free IgE antibodies (Figure 23A) as well as >40% reduction of total serum IgE levels (Figure 23B), while it did not alter the reduced numbers of $CD23^+$ B cells in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (Figure 24A,B). Moreover, anti-IgE treatment did not change serum cholesterol and triglyceride levels (Table 2; study 2). While *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with the control antibody developed significantly increased atherosclerosis compared to *Ldlr*$^{-/-}$ mice in the aortic root, enhanced lesion formation in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice was fully reversed by anti-IgE treatment (Figure 23C). Because IgE antibodies robustly activate mast cells via the FcεRI and have been shown to promote atherosclerotic plaque formation (Bot et al, 2007; Sun et al, 2007), we examined the activation status of mast cells in the perivascular area of aortic root atherosclerotic lesions. We found that *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with the control Ab developed significantly increased frequency of activated mast cells compared to *Ldlr*$^{-/-}$ mice, while this effect was fully reversed by anti-IgE treatment (Figure 23D). Finally, because neutrophils, which have been shown to promote atherosclerosis (Doring et al, 2014), express the FcγRIV receptor (Jakus et al, 2008) that has been recently identified to bind IgE (Hirano et al, 2007; Mancardi et al, 2008) we were also prompted to examine neutrophil numbers in the

same perivascular area. We found that *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with the control Ab had significantly increased numbers of neutrophils compared to *Ldlr*$^{-/-}$ mice, and this was fully reversed by anti-IgE treatment (Figure 23E).

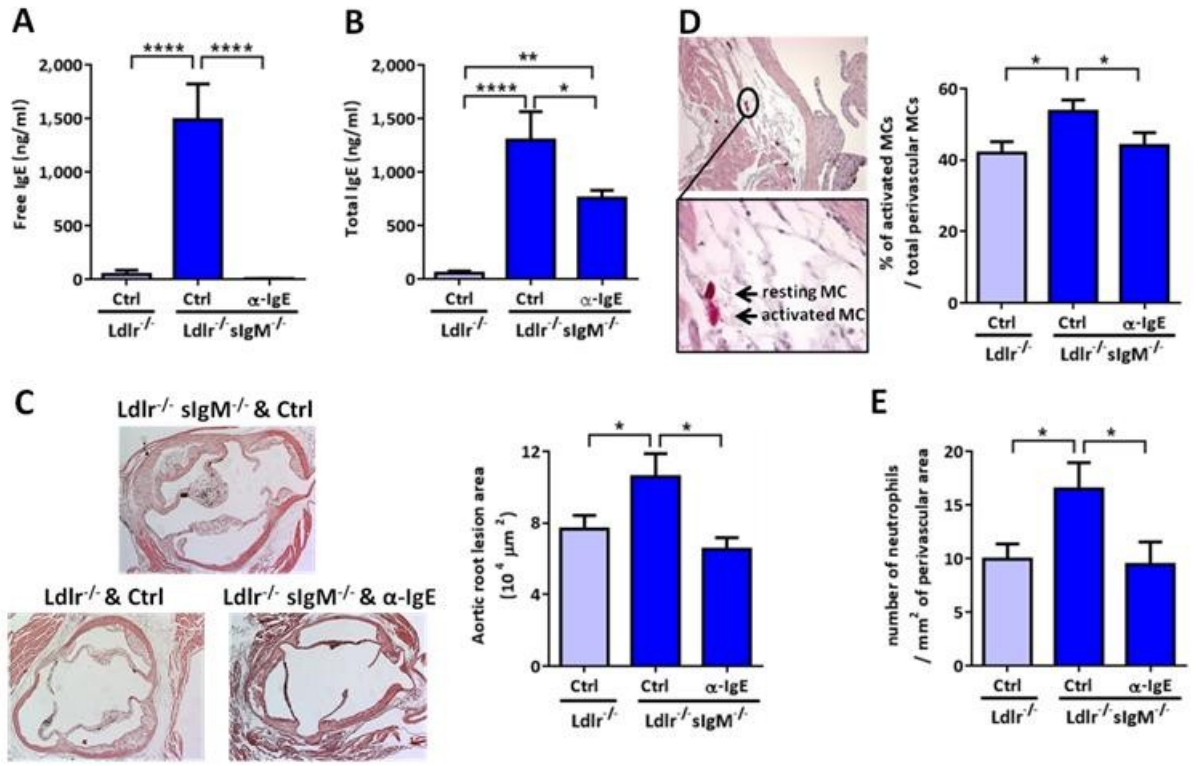

Figure 23. IgE neutralization reverses accelerated atherosclerosis in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice. Quantification of **(A)** free and **(B)** total plasma IgE antibody titers, **(C)** representative photomicrographs of H&E aortic root lesions (left) and bar graphs showing the intimal lesion size throughout the entire aortic origin expressed as µm^2/section in female *Ldlr*$^{-/-}$ (light blue bar) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bars) mice that were fed an atherogenic diet for 6 weeks. **(D)** Chloroacetate esterase staining of a resting and an activated mast cell (MC), (upper left; 10x, bottom left; 40x) and bar graphs (right) showing the percentage of activated mast cells in perivascular area of aortic root atherosclerotic lesions and **(E)** bar graphs showing the absolute numbers of neutrophils in the perivascular area of aortic root atherosclerotic lesions in female *Ldlr*$^{-/-}$ (light blue bar) or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bars) mice that were fed an atherogenic diet for 6 weeks. During the diet feeding, mice were injected intraperitoneally with 25µg diluted in 100µl DPBS (Sigma) of an anti-IgE neutralizing antibody (clone R1E4; kind gift of Dr. Daniel Conrad, Virginia, USA) or a control IgG (Ctrl; Jackson Immunoresearch Inc.) once every week, ((**A, B, C**) *Ldlr*$^{-/-}$ & Ctrl IgG; n=14, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ & Ctrl IgG; n=16, *Ldlr*$^{-/-}$ *sIgM*$^{-/-}$ & α-IgE; n=15, (**D,E**) *Ldlr*$^{-/-}$ & Ctrl IgG; n=13, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ & Ctrl IgG; n=15, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ & α-IgE; n=14). Data are represented as mean ± SEM, *P<0.05, **P<0.01, ****P<0.0001 (One-Way ANOVA test followed by (**A, B, C, D**) Newman-Keuls or (**D**) unpaired t test).

Similar data were obtained when *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 8 weeks and treated with a neutralizing anti-IgE or a control antibody as described above (data not shown). Similar to this study, anti-IgE treatment did not affect plasma cholesterol or triglyceride levels. Moreover, stimulation of bone marrow derived mast cells (BMMCs) with plasma collected from *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with the control antibody at the end of the diet feeding resulted in robust IL-6 secretion compared to stimulation with plasma from control antibody treated *Ldlr*$^{-/-}$ mice. Notably, stimulation with plasma of *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with the anti anti-IgE antibody, failed to stimulate IL-6 secretion by BMMCs (Figure 25). In summary, IgE neutralization reverses the accelerated atherosclerosis in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice at least in part by preventing mast cell activation and neutrophil recruitment.

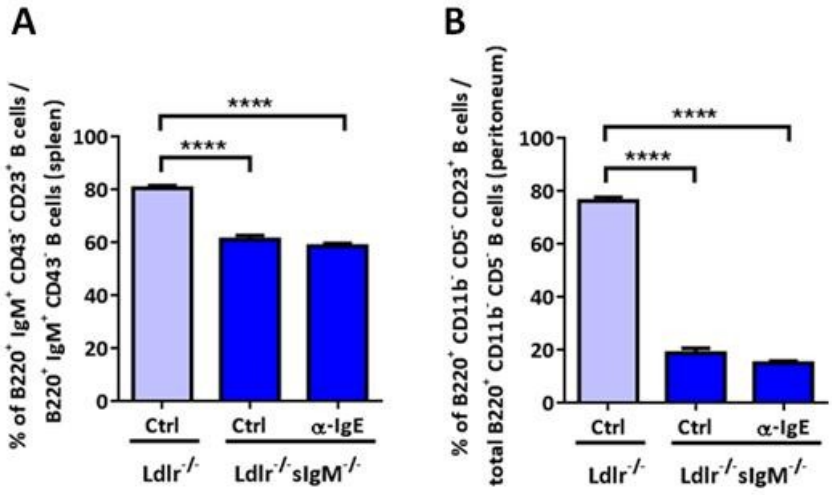

Figure 24. IgE neutralization does not alter the reduced CD23^{+} B2 cell population in *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice. Bars represent **(A)** the percentages of splenic CD23^{+} B2 cells defined as B220^{+}IgM^{+}CD43^{-}CD23^{+} within B2 cells, and **(B)** the percentages of peritoneal CD23^{+} B2 cells defined as B220^{+}CD11b^{-}CD5^{-}CD23^{+} within B2 cells in *Ldlr*$^{-/-}$ (light blue bar) and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bar) mice that were fed an atherogenic diet for 6 weeks. During the diet feeding mice were injected intraperitoneally with 25µg diluted in 100µl DPBS (Sigma) of an anti-IgE neutralizing antibody (clone R1E4; kind gift from Dr. Daniel Conrad, Virginia, USA) or a control IgG (Ctrl) (Jackson Immunoresearch Inc.) once every week (*Ldlr*$^{-/-}$ /Ctrl IgG; n=14, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ /Ctrl IgG; n=16, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ /α-IgE; n=15). Data are represented as mean ± SEM, ****P<0.0001 (One-Way ANOVA test followed by **(A)** Newman-Keuls test or **(B)** Dunn's test).

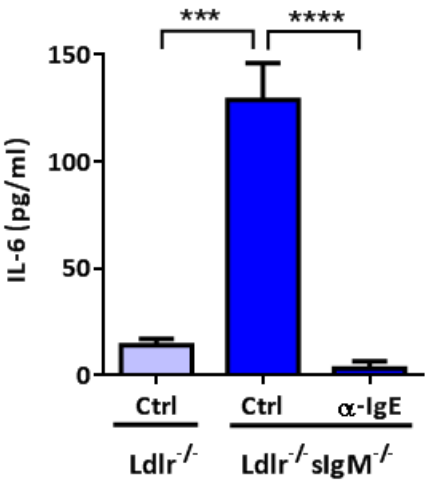

Figure 25. Plasma from atherogenic diet fed *Ldlr*$^{-/-}$ *sIgM*$^{-/-}$ mice triggers IL-6 secretion by bone marrow derived mast cells in an IgE dependent manner. *Ldlr*$^{-/-}$ (light blue bars) and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ (dark blue bars) mice were fed an atherogenic diet for 8 weeks and injected intraperitoneally with 25µg diluted in 100µl DPBS (Sigma) of an anti-IgE neutralizing antibody (clone R1E4; kind gift from Dr. Daniel Conrad, Virginia, USA) or a control IgG (Ctrl; Jackson Immunoresearch Inc.) once every week (*Ldlr*$^{-/-}$ /Ctrl IgG; n=8, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ /Ctrl IgG; n=9, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$/α-IgE; n=7).Quantification of IL-6 by ELISA in supernatants of bone marrow derived mast cells stimulated for 2 hours with pooled plasma from each group collected from the mice described above at the end of the diet feeding. Data are represented as mean ± SEM of quadruplicates. ***P<0.001, ****P<0.0001 (One-Way ANOVA test followed by Bonferroni's test).

4. DISCUSSION

In this study, we identified a subset of circulating MP that carries OSE and is increased at the site of coronary occlusion of patients with STE-MI. OSE+ MP are recognized by OxLDL-specific IgM antibodies, which have the capacity to decrease pro-inflammatory effects of MP. We further show that mice lacking secreted natural IgM antibodies have abnormal splenic B cell development resulting in impaired generation of CD23 expressing B cells and increased IgE plasma levels, which mediate the accelerated atherosclerotic lesion formation in these mice. Thus, natural IgM have the potential to protect from atherosclerosis both by neutralizing pro-inflammatory effects of stress-induced self-antigens as well as by regulating B cell homeostasis. The latter may be in part dependent on the ability of natural IgM to bind OSE on self-antigens.

4.1 Microparticles are recognized by OSE-specific natural IgM antibodies

There is substantial evidence from both epidemiological and experimental studies demonstrating that humoral immunity plays an important role in CVD (Tsiantoulas et al, 2014). OSE-specific IgM antibodies in particular have been shown to associate with lower CVD risk (Karvonen et al, 2003; Tsimikas et al, 2007). Moreover, high levels of OSE-specific IgM antibodies have been shown to predict a lower risk of cardiovascular events (Fiskesund et al, 2010; Gronlund et al, 2009). Specifically, a recent analysis of the Bruneck study, which prospectively followed 765 individuals for 15 years, demonstrated that high IgM titers to CuOx-LDL and MDA-LDL were associated with a significantly lower risk of adverse cardiovascular events (Tsimikas et al, 2012). Our findings that MP carry OSE and are recognized by the same antibodies that bind CuOx-LDL and MDA-LDL suggest a novel atheroprotective mechanism for OxLDL-specific IgM.

Indeed, patients with ACS were found to have increased circulating MP of EC origin (Mallat et al, 2000). Moreover, in a recent study of 45 AMI patients, the number of MP in the blood collected distal to the coronary lesions with an aspiration catheter of the culprit coronary artery was also found to be increased before pPCI compared to MP in the blood collected from the femoral artery (Min et al, 2013). We now report that STE-MI patients have increased numbers of EC-derived MP at the coronary occlusion site, suggesting that MP are generated most likely from injured and dying

cells at this site. In turn, these MP may contribute to pro-inflammatory and pro-coagulatory responses during atherothrombosis. It has been recently demonstrated by immunological and mass spectroscopy techniques the presence of OSE, including a variety of OxPL as well as MDA-type epitopes, on embolized material from vulnerable plaques trapped on distal protection devices during PCI procedures (Ravandi et al, 2014). MDA-modified proteins induce the secretion of pro-inflammatory cytokines, such as IL-8, in monocytes and macrophages (Shanmugam et al, 2008; Weismann et al, 2011). Because MP also carry MDA-epitopes, they may propagate inflammation locally by triggering inflammatory responses via the same mechanisms. In turn, these can be inhibited by MDA/MAA-specific IgM antibodies. Additional biological activities may also be mediated by other OSE present on MP, including PC of OxPL, which we found to be nearly exclusively present on MDA-carrying MP. Notably, PC epitopes have been found to mediate EC activation when present on apoptotic cells or blebs (Chang et al, 2004; Huber et al, 2002). It has been previously shown that bioactive lipid-bearing platelet-derived MP can activate platelets and EC (Barry et al, 1997). One may hypothesize that MDA-modified lipids or proteins on the surface of MP contribute to these effects. Interestingly, activation of platelets has also been shown to generate MDA and induce MDA-modifications in LDL (Fogelman et al, 1980; Hammarstrom & Falardeau, 1977). Whether OSE-specific IgM could also interfere with the pro-coagulatory potential of MP and subsequent thrombus formation remains to be shown.

4.2 Deficiency in natural IgM antibodies accelerated atherosclerosis but does not result in apoptotic cell accumulation in atherosclerotic lesions

In support of an atheroprotective role of IgM, deficiency of natural IgM antibodies has been shown to result in accelerated atherosclerosis in mice (Lewis et al, 2009). On the other hand, pneumococcal vaccination, which results in a robust increase of the PC specific T15/E06 natural IgM antibody levels or infusion of T15/E06 antibody in intact atherogenic diet fed *Apoe*$^{-/-}$, reduces atherosclerotic lesion size. OSE-specific natural IgM antibodies, such as E06 and NA17, have been shown to bind apoptotic cells and inhibit their proinflammatory properties *in vitro* (Chang et al, 2004) and promote their uptake by macrophages *in vivo* (Chou et al, 2009), respectively. Indeed, accumulation of apoptotic cells in atherosclerotic lesions has

been shown to enhance atherosclerosis (Tabas, 2010; Tabas et al, 2009). Together these data suggest that OSE-specific natural IgM antibodies confer atheroprotection also via promoting apoptotic cell clearance. Surprisingly, however, deficiency in secreted IgM antibodies did not lead to increased accumulation of apoptotic cells in atherosclerotic lesions (Lewis et al, 2009). This suggests that there are additional mechanisms by which natural IgM protect from atherosclerosis that require the presence of the full or at least a broader repertoire.

4.3 Soluble natural IgM antibodies decrease BCR signaling and editing, and modulate B cell maturation

Natural IgM antibodies have been shown to regulate B cell responses (Baker & Ehrenstein, 2002; Boes et al, 1998; Boes et al, 2000; Ehrenstein et al, 1998; Notley et al, 2010), which are strong modulators of atherosclerotic lesion development (Tsiantoulas et al, 2014; Tsiantoulas et al, 2015b). Thus, we investigated whether alterations of B cell responses could be responsible for the accelerated atherosclerosis in mice lacking secreted IgM.

In line with previous reports (Baker & Ehrenstein, 2002; Nguyen et al, 2015), we found that natural IgM antibody deficiency results in increased numbers of splenic MZ and $CD21^{+}CD23^{-}$ B cells and decreased numbers of FO B cells, which altogether point to an abnormal splenic B cell development in *sIgM*$^{-/-}$ mice. A major determinant of the developmental fate of splenic B cells is the self-antigen mediated BCR signaling strength (Pillai & Cariappa, 2009). We report here that both MZ, $CD21^{+}CD23^{-}$ and FO B cells display increased BCR signaling as judged by increased pBtk, pSyk and Nur77 expression. Additional evidence that supports further the increased BCR signaling in natural IgM antibody deficiency comes from our finding that bone marrow B cells of *sIgM*$^{-/-}$ mice display reduced kappa/lambda light chain ratio compared to *sIgM*$^{+/+}$ controls. These data are indicative of enhanced BCR editing - presumably in order to avoid autoreactivity - most likely due to exposure to very strong self-antigen mediated BCR signaling. This finding is particularly interesting as products of editing at the lambda locus may trigger autoimmune responses (Doyle et al, 2006). Notably, decreased kappa/lambda light chain ratio has been reported for some autoimmune diseases, such as juvenile idiopathic arthritis (Low et al, 2007). Moreover, kappa/lambda light chain ratio was also decreased in splenic *sIgM*$^{-/-}$ B cells compared to *sIgM*$^{+/+}$ controls. This also suggests that the

splenic BCR repertoire in *sIgM*$^{-/-}$ mice is more prone to autoreactivity, which goes along with 1) stronger BCR signaling in splenic *sIgM*$^{-/-}$ B cells and 2) the increased autoantibody levels specific for anti-double stranded DNA and histones in *sIgM*$^{-/-}$ mice, crossed onto a lupus-prone mouse model (lpr) background (Boes et al, 2000).

In line with these data, we also report that self-antigen mediated BCR signaling is decreased in presence of an antigen specific soluble IgM antibody, *in vitro*. An obvious mechanism by which soluble IgM antibodies could regulate antigen-mediated BCR signaling would be by modulating the exposure to self-antigens for example by competing for the binding to BCR or by promoting the clearance of certain self-antigens. We here show that in the presence of a soluble VSV-specific IgM antibody, which recognizes a different epitope on the VSV antigen than does the BCR (membrane bound IgM), VSV-mediated BCR signaling is reduced in VSV-specific BCR transgenic B cells.

The data mentioned above are in contrast to a previous report, which has shown that CD43^{-} splenic B cells of *sIgM*$^{-/-}$ mice display reduced intracellular basal phosphorylation of extracellular signal kinase (pErk) compared to B cells of *sIgM*$^{+/+}$ mice (Notley et al, 2010), suggesting that natural IgM deficiency decreases BCR signaling in splenic B cell subsets. In our hands, pErk levels were not different in MZ or in FO B cells, but there was a strong tendency for CD21^{+}CD23^{-} cells to have increased basal pErk in *sIgM*$^{-/-}$ mice (data not shown). In addition, it is important to note that the authors of that study found also increased frequencies of dying B cells in the spleen of *sIgM*$^{-/-}$ mice compared to *sIgM*$^{+/+}$ controls, which their inclusion in this analysis may account for the decreased pErk signaling in *sIgM*$^{-/-}$ mice.

An important observation made in our studies was the finding that BCR signaling was significantly stronger in MZ compared to FO B cells of wild type mice *in vivo*. These data suggest that MZ B cell differentiation is favored in conditions of enhanced BCR signaling, whereas FO B cells preferentially develop upon weaker BCR signaling. In line with our findings, it has been reported that BCR transgenic B cells specific for the self-Thy-1/CD90 glycoprotein differentiate towards MZ or FO B cells in presence or absence of the Thy-1 antigen, respectively (Wen et al, 2005). Moreover, B cell differentiation towards a MZ like phenotype is positively associated with increasing numbers of surface BCR copies, which also suggests that enhanced antigen mediated BCR signaling favors MZ B cell development (Heltemes & Manser, 2002).

On the other hand, other studies have pointed to an inverse association between BCR signaling strength and FO B cell differentiation (Pillai & Cariappa, 2009). For example, mice lacking Btk display impaired B cell proliferation upon anti-IgM $F(ab')_2$ stimulation, which is indicative of impaired BCR signaling, and have robustly reduced FO B cells (Khan et al, 1995; Loder et al, 1999). Similar results were obtained with mice deficient in the Btk interacting partner PLC-γ2 (Wen et al, 2003). Based on our findings an alternative interpretation of these data, would be that by increasing the strength of BCR signaling, MZ cells are deleted due to exposure to very strong signaling, whereas FO B cells may be still within the BCR signaling strength range that allows their survival. Moreover, because in both Btk and PLC-γ2 deficient mice the reduction in FO B cells is not accompanied by a concomitant increased differentiation towards MZ B cells one could suggest that deficiency in Btk or PLC-γ2 may impact cell survival in a cell intrinsic manner, rather than B cell differentiation *per se*.

Moreover, as mentioned above we found increased numbers of splenic mature $CD21^{+}CD23^{-}$ B2 cells that also display stronger BCR signaling in *sIgM*$^{-/-}$ compared to *sIgM*$^{+/+}$ mice. The exact role of these cells is not known. A possible function could be that the $CD21^{+}CD23^{-}$ B2 cell population functions - in addition to T2 B cells - as a source or an intermediate step for future FO and MZ B cells. Based on our data, these cells display basal BCR signaling similar to FO B cells in wild type mice, which would suggest that they are more prone to differentiate to FO B cells. Based on this, it is very tempting to hypothesize that in *sIgM*$^{-/-}$ mice $CD21^{+}CD23^{-}$ B2 cells accumulate at this stage due to increased BCR signaling and perhaps under these conditions are more prone to differentiate to MZ B cells.

In line with the altered BCR signaling in *sIgM*$^{-/-}$ mice, we also show that bone marrow, mature splenic and peritoneal B cells in *sIgM*$^{-/-}$ mice display reduced surface expression of the protein tyrosine phosphatase B220. These data indicate that increased BCR signaling associates with decreased B220 surface expression. This is in line with a recent report showing that ZIP10 deficient B cells, which display increased antigen mediated BCR signaling have also decreased B220 surface expression (Hojyo et al, 2014). Moreover, we show here that intraperitoneal infusion of polyclonal IgM into *sIgM*$^{-/-}$ mice normalized the decreased B220 expression on the surface of all peritoneal B cells. Little is known on how B220 expression is regulated on the surface of B cells. Interestingly, B1 cells which their differentiation is favored

upon strong BCR signaling (Casola et al, 2004) and are the main producers of natural IgM antibodies display lower levels of B220 than B2 cells (Baumgarth, 2011). Thus, our findings also identify soluble IgM as regulator of B220 expression on the cell surface of B cells, which is likely a result of increased BCR signaling.

4.4 *sIgM*$^{-/-}$ mice display robustly increased IgE titers and are characterized by decreased CD23^{+} B cells

The decrease in FO B cells, which is the only mature B cell subset expressing the CD23 surface marker, was accompanied by strongly reduced frequencies of CD23 expressing B cells in both periphery and peritoneum. CD23, which is the low affinity receptor for IgE (FcεRII) has been implicated in the clearance of plasma IgE antibody levels (Cheng et al, 2010). Consistent with this, our data show that the increase in plasma IgE antibody titers is not due to preferential class switch to IgE, as neither IgE mRNA nor the numbers of IgE expressing splenic B cells are altered in *sIgM*$^{-/-}$ mice compared to wild type controls. Notably, *sIgM*$^{-/-}$ mice can class switch and produce all Ab classes similarly to wild type mice ((Boes et al, 1998) and data not shown). Thus, our data suggest that CD23 expression levels do not influence IgE production but rather regulate IgE levels most likely by promoting their clearance.

IgE antibodies have been extensively studied in allergy and asthma, where they are considered critical mediators of these pathologies. Little is known about their role in atherosclerosis, though a few epidemiological studies exist that suggest a contribution for this usually tightly controlled immunoglobulin. For example, Kovanen et al found that high IgE levels are a prognostic factor for MI and cardiac death in dyslipidemic men in the Helsinki Heart Study (Kovanen et al, 1998). This association was later confirmed by Wang et al, who found that patients suffering from CHD have elevated IgE levels compared to non-CHD subjects (Wang et al, 2011a). Collectively, epidemiological data mentioned above suggest a proatherogenic role for IgE antibodies.

While the role of IgE Abs in experimental atherosclerosis has not been directly investigated, indirect evidence comes from studies in atherosclerosis-prone mice deficient in FcεRI receptor. As discussed above it has been demonstrated that *Apoe*$^{-/-}$ mice deficient in FcεRI develop significantly decreased atherosclerosis with reduced macrophage and apoptotic cell content in their lesions (Wang et al, 2011a). We here show for the first time that IgE neutralization confers an anti-atherogenic effect *in*

vivo as the accelerated atherosclerosis in atherogenic diet fed *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice treated with an IgE neutralizing antibody was restored to similar levels with *Ldlr*$^{-/-}$ mice, which were treated with a control antibody.

It is important to note that plasma IgE levels in wild type C57BL/6 mice are particularly low (approximately 50ng/ml) compared to all other immunoglobulin subclasses. Interestingly, high fat diet consumption for 12 weeks - starting at the age of 10 weeks - resulted in a significant increase of plasma IgE titers in *Apoe*$^{-/-}$ mice (Wang et al, 2011a). Thus it is tempting to propose that at least in conditions of advanced atherosclerosis IgE neutralization could confer atheroprotective effect also in natural IgM competent mice.

IgE Abs binding to FcεRI results in robust stimulation of mast cells, which have been implicated in atherosclerosis and in the destabilization of lesions in particular (Kovanen et al, 1995). Another study by Bot et al also demonstrated a proatherogenic effect of mast cell activation in *Apoe*$^{-/-}$ mice and a role for mast cell degranulation in particular (Bot et al, 2007). Studies in atherosclerotic *Ldlr*$^{-/-}$ mice have shown that mast cell deficiency results in reduced lesion formation, and that this may be promoted by mast cell-derived IL-6 and IFNγ (Sun et al, 2007). We found that *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ have increased frequencies of activated mast cells in the perivascular area of aortic root atherosclerotic lesions due to the increased IgE antibody levels in these mice. In addition, BMMC stimulation with plasma collected from control antibody treated western diet fed *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice resulted in IL-6 secretion by BMMCs, whereas plasma from α-IgE treated western diet fed *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice failed to do so. Moreover, it will be important to demonstrate whether the proatherogenic effects of IgE are dependent on certain antibody specificities for relevant antigens, e.g. OxLDL, or whether they are antigen independent (Kalesnikoff et al, 2001; Pandey et al, 2004).

The pro-atherogenic role of reduced natural IgM titers is also supported by Kyaw et al, who has demonstrated that splenectomized *Apoe*$^{-/-}$ mice have 50% reduced B1a peritoneal cells along with 50% reduced IgM plasma antibody titers and accelerated atherosclerosis (Kyaw et al, 2011). Interestingly, injection of wild type B1a cells – in contrast to *sIgM*$^{-/-}$ B1a cells - to splenectomized recipients restores the plasma IgM levels and rescues the accelerated atherosclerosis. Despite the normalization of plasma and lesional IgM antibodies in splenectomized mice that received wild type B1a cells, there was no difference in the apoptotic cell content in

atherosclerotic lesions compared to *sIgM*$^{-/-}$ B1a cell injected animals, further hinting at distinct pro-atherogenic mechanisms when the polyclonal IgM pool is absent or strongly reduced. Notably, splenectomy in humans, which also causes reduction in IgM titers (Drew et al, 1984) has been reported to result in elevated plasma IgE titers (Balsalobre & Carbonell-Tatay, 1991; Balsalobre et al, 1993; Chelazzi et al, 1985), though the underlying mechanism remains still elusive. Additional evidence that links the levels of plasma IgM and IgE antibodies comes from patients with selective IgM deficiency. These patients, who frequently develop asthma and allergic disorders, have often elevated IgE titers, while B cell numbers as well as other immunoglobulin subclasses - such as IgG - remain usually unchanged. (De la Concha et al, 1982; Goldstein et al, 2008; Ideura et al, 2008; Louis & Gupta, 2014; Yamasaki, 1992).

4.5 Translational aspects in targeting B cells and humoral immunity

B cells along with the antibodies they produce promote the pathology of several autoimmune disorders such as SLE (Browning, 2006). Notably, IgE antibodies have been recently shown to be involved in the pathogenesis of SLE (Dema et al, 2014). Interestingly, SLE patients are characterized by increased risk of CVD complications, mainly ischemic heart disease, which is associated with the development of premature atherosclerosis (Roman & Salmon, 2007). Accelerated atherosclerosis in SLE patients seems to be independent of classical Framingham risk factors such as age, total cholesterol, HDL and systolic blood pressure. This suggests that aggravated atherosclerosis in these patients may be a result of increased inflammation and altered immune responses. Therefore, depleting B cells may represent an alternative therapeutic approach in atherosclerosis to limit their pro-atherogenic effects, such as IgE antibody production. The development of B cell targeting therapeutics for RA and SLE has gained a lot of attention in the last years. The first B cell therapeutic agent that has been approved for clinical use in RA patients is the α-CD20 antibody (Rituximab). Rituximab cross links the CD20 receptor present on all B cells, leading to Fcγ mediated cell depletion and consequently to decreased immunoglobulin/autoantibody titers (Uchida et al, 2004). Another B cell depleting agent, a blocking antibody against BAFF (Belimumab) has been approved by the FDA in 2011 for clinical use in SLE patients, who have been shown to have increased plasma BAFF levels (Vincent et al, 2014). Belimumab, which is the first drug approved for SLE in 50 years, blocks soluble BAFF from binding to its receptor

(BAFFR) resulting in apoptosis of mature B cells. SLE patients treated with Belimumab show an improvement of clinical score (Hahn, 2013).

All above mentioned B cell depleting therapeutic approaches are also characterized by the risk of compromising immunity in general with an increased risk of infections and presumably cancer development as well as decreased responsiveness to vaccination. Thus, more selective interventions that target B cell responses in atherosclerosis would be of great interest. Neutralizing IgE antibodies for example by using Omalizumab (an FDA approved human anti-IgE antibody that neutralizes free IgE antibodies) may provide a novel therapeutic approach against the development of atherosclerosis while maintaining the homeostasis of the immune system.

4.6 Summary and Conclusions

In summary, natural IgM antibodies seem to confer atheroprotection via distinct mechanisms. When total IgM levels and presumably the polyclonal repertoire are in physiological levels, an increase in OSE-specific natural IgM seems to be beneficial in atherosclerosis. The exact mechanism by which OSE-specific natural IgM protect from cardiovascular disease remains still elusive but there is evidence that mechanisms such as apoptotic cell clearance may be involved. We have identified here a subset of circulating MP that carry OSE and are also recognized by natural IgM antibodies. We also show that MP induced inflammation is decreased in presence of an MDA specific natural IgM antibody. These findings reveal a novel mechanism by which OSE-specific IgM could exhibit their atheroprotective properties. Understanding further the functional properties of the interaction between MP and IgM antibodies may help to identify novel diagnostic and therapeutic approaches for CVD.

On the other hand, when the entire IgM repertoire is absent, this results in impaired generation of CD23 expressing B cells, as a result of abnormal splenic B cell development most likely due to enhanced BCR signalling. It would be particularly interesting to investigate whether and how BCR signalling is affected in atherogenic conditions. In addition, these changes in B cell development result in robustly increased IgE titers, which mediate the accelerated atherosclerosis in *sIgM*$^{-/-}$ mice. These data also call for further studies with respect to the role of IgE antibodies in atherosclerosis, particularly if α-IgE therapies could be beneficial in conventional

atherosclerosis, which may be associated with a similar phenotype at advanced stages of disease due to increased consumption of free IgM.

In conclusion, natural IgM antibodies play a role both in “war and peace”. On one hand, they provide the first line of defense against exogenous antigens that are present on invading pathogens, and confer protection against endogenous sterile inflammation (e.g atherosclerosis). On the other hand, in healthy conditions they comprise a fundamental and critical regulator of immune homeostasis. These functions of natural IgM antibodies are largely mediated by their capacity to recognize highly conserved structures such as OSE, which are present on OxLDL, apoptotic cells and MP.

5. MATERIALS AND METHODS

5.1 Collection of blood samples and isolation of circulating MP

Blood samples were obtained from the antecubital vein of healthy individuals of two independent cohorts: a) 18 healthy individuals (10 female, 8 male) with a mean age of 28.4 and 28.7 years, and b) 15 healthy individuals, 10 male and 5 female, with mean age 32.1 and 32.8 years, respectively. Moreover, blood samples were collected from a 6 French femoral sheath (herein after referred to as periphery) and from the coronary aspiration catheter of 14 patients during primary percutaneous intervention for ST-segment elevation acute myocardial infarction (STE-MI), as described (Distelmaier et al, 2009). Detailed patient characteristics are shown in Table 1. Patients received 250 mg aspirin and were treated with unfractionated heparin to achieve an activated clotting time ≥300s. A total of 10 ml blood was aspirated with a Pronto® V3 thrombus extraction catheter or the Medtronic Export Aspiration Catheter at the site of thrombotic occlusion prior to any intervention. Because of a 2 ml 0.9% sodium chloride flush of the aspiration catheter prior to thrombectomy all analyses were normalized for hematocrite.

Blood samples were collected into K2EDTA containing collection tubes (Vacutainer® tubes, Becton Dickinson). Samples were immediately centrifuged at room temperature for 30 minutes at 2,000g to obtain platelet-poor plasma. In some experiments, plasma was additionally treated with 40 µmol of butylated hydroxytoluene (BHT). The resulting MP containing plasma was carefully removed without disturbing the cell pellet. An aliquot of the plasma sample was stored at -20°C and the rest was transferred to autoclaved tubes and centrifuged at 21,000g for 30 minutes at 4°C to pellet MP. In some experiments, plasma was centrifuged for 2 minutes at 13,000g to remove remnant platelets and aggregates prior to the ultracentrifugation step. After centrifugation, plasma was harvested and pelleted MP were resuspended and washed with Dulbecco's Phosphate Buffered Saline (DPBS; Sigma Aldrich) at 18,000 for 30 minutes. This washing step was repeated at least three times. Finally, MP were resuspended in DPBS and stored at -20°C and/or used for further experiments. All studies were conducted after patients' or healthy individuals' written informed consent under the approval of the Ethics Committee of the Medical University of Vienna (EK-N: 303/2005 and 2051/2013).

5.2 Inclusion criteria of STE-MI patients

Patients in the setting of STE-MI were included if all of the following criteria applied: 1) chest pain at the time of coronary angiography associated with ST-segment elevation in 2 or more contiguous leads, 2) Thrombolysis In Myocardial Infarction 0–1 flow in a native culprit coronary artery, and an intraluminal filling defect suggestive of thrombus within 50 mm of the respective coronary ostium, 3) Absence of complete heart block, and 4) no thrombolytic therapy/or treatment with glycoprotein IIbIIIa antagonists.

5.3 Flow cytometric analysis of MP

To assess the presence of PS, isolated MP were stained with Annexin V conjugated to Phycoerythrin (PE) (eBiosciences) in calcium containing buffer (eBiosciences). To determine the presence of OSE on their surface, isolated MP were stained with 2 µg/ml of biotinylated or 40 µg/ml of unconjugated antibodies in filtered (0.1µm) PBS containing 0.5% fatty acid free BSA (PAA Laboratories) of the following IgM primary antibodies: anti-keyhole limpet hemocyanin (anti-KLH; MM-30; Becton Dickinson) as isotype control; T15/E06 specific for oxidized phosphocholine-containing phospholipids; and NA-17, E014, and LR04, which are directed against MDA, including advanced malondialdehyde-acetaldehyde (MAA)-adducts (Amir et al, 2012; Chou et al, 2009; Horkko et al, 1999; Palinski et al, 1996). The samples were incubated with the antibodies for 30 minutes at 4°C. Then MP were pelleted at 18,000g for 15 minutes. Supernatants were removed and avidin conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson) or anti-mouse IgM conjugated to allophycocyanin (APC) (II/41; eBiosciences) was added at a final concentration of 0.5µg/ml or 1µg/ml respectively, and incubated for 20 minutes in darkness at 4°C. To identify MP that carry both MDA and PC epitopes, MP were sequentially stained with T15/E06, then anti-mouse IgM FITC at 2.5µg/ml (II/41; Becton Dickinson), followed by LR04 and then anti-mouse IgM APC. To determine surface bound human IgM, MP were stained with an anti-human IgM-PE (MHM 88; Becton Dickinson) or with the corresponding isotype control antibody (MOPC-21; Becton Dickinson) and incubated for 15 minutes in darkness at 4°C.

To identify the cellular origin, MP were stained with anti-human CD235a conjugated to R-Phycoerythrin (PE) (HIR 2), anti-human CD41a-FITC (HIP 8), anti-human CD14-PercP Cy5.5 (61D3) or biotinylated anti-human CD31 (WM59) all

bought from eBiosciences, and incubated for 30 minutes in darkness at 4°C. After incubation with antibodies, MP were pelleted at 18,000g for 15 minutes and the samples were then incubated with 0.5µg/ml streptavidin-PercP (Biolegend) for 20 minutes in darkness at 4°C (Becton Dickinson).

To quantify circulating MP in the peripheral and coronary blood of AMI patients, diluted plasma samples were stained with Annexin V-PE. All samples were acquired on a FACS Calibur (Becton Dickinson) for 30 seconds at low speed. Forward and sideward scatter (FSC, SSC) were set at logarithmic gain. MP were identified as Annexin V positive events with size ≤1µm, using monodisperse polystyrene microparticle size standards with a mean size of 1µm as reference. MP-free buffer was acquired in order to exclude false events due to noise (figure 1). Samples were analyzed on a FACS Calibur (Becton Dickinson). All acquired row data were analyzed with Flow Jo software (Treestar).

5.4 In vitro generation of platelet derived MP

Purified platelets were obtained by apheresis from the Department of Transfusion Medicine of the Medical University of Vienna and incubated with 10µM of ionomycin in PBS containing 1mM $CaCl_2$, for 30 minutes at 37°C. After adding 5mM EDTA to stop the reaction, cells and debris were pelleted by sequential centrifugation at 2,000g and 3,900g for 10 minutes, respectively. After centrifugation, the MP containing supernatant was carefully removed and MP were pelleted and washed with DPBS and centrifuged at 18,000g for 30 minutes. This washing step was repeated at least three times. Finally, MP were resuspended in DPBS+0.01% BHT and stored at -20°C for further experiments. The MP total protein amount was estimated using the BCA protein assay (Pierce).

5.5 Flow cytometric analysis of platelets

Purified platelets were stained with 10µg/ml of LR04 or an isotype control antibody (MM-30; Biolegend) for 30 minutes at 4°C. After washing in filtered PBS+0.5% BSA, cells were pelleted at 500g and incubated with 0.25µg/ml of anti-IgM APC (II/41; eBiosciences) for 20 minutes in darkness at 4°C. Cells were washed again and samples were acquired on a FACS Calibur (Becton Dickinson) and data were analyzed using Flow Jo software (Treestar).

5.6 Elution of MP-associated IgM

Isolated and extensively washed circulating MP were lysed in RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS (pH=7.6) supplemented with a complete protease inhibitor cocktail (Roche Applied Science). After overnight incubation at 4°C, the MP lysates were transferred to microdialysis cups with a molecular weight cut-off of 10 kDa (Micro DispoDialyzers, Harvard Apparatus) and extensively dialyzed against PBS containing 0.27mM EDTA.

5.7 Antigen binding of monoclonal IgM antibodies

The specificity of T15/E06, LR04, NA17 and E014 is extensively documented in the literature.(Amir et al, 2012; Chou et al, 2009; Horkko et al, 1999; Palinski et al, 1996) To confirm these specificities with the aliquots used, we performed direct binding studies to native BSA, PC-BSA or MDA-BSA (which contains different types of MDA-adducts) using chemiluminescent ELISA as described (Chou et al, 2009).

5.8 Antibody measurements and competition assay

Total IgM and IgM antibodies to native LDL, CuOx-LDL, MDA-LDL were measured as described previously.(Chou et al, 2009) In brief, 5µg/ml of native LDL, CuOx-LDL and MDA-LDL diluted in PBS containing 0.27 mM EDTA and 0.02% sodium azide (PBS/EDTA) were coated to 96-well white round-bottomed MicroFluor microtiter plates (ThermoLabsystems) and incubated overnight at 4°C. Coated plates were then blocked with Tris-buffered saline containing 1% BSA (TBS/BSA) for 1 h at room temperature. After washing 3 times with PBS/EDTA, 50 µl of diluted plasma (1:10,000) or MP-lysates (1:20) in TBS/BSA were added and incubated overnight at 4°C. Plates were washed again 3 times with PBS/EDTA and binding of the IgM antibodies was detected with an alkaline phosphatase-conjugated goat anti-human IgM (A3437; Sigma Aldrich) diluted 1:30,000 in TBS/BSA. Following an incubation time of 1 h at room temperature, the wells were washed 3 times as described above, followed by a single rinse with distilled water. Then, 25 µl of a 30% LumiPhos Plus solution in dH_20 (Lumigen Inc) was added. After 90 min, the light emissions were measured on a WALLAC VIKTOR II luminometer (Perkin Elmer) and expressed as

relative light units (RLU) per 100ms. For the determination of total IgM, plates were coated with 5µg/ml of the polyclonal goat anti-human IgM antibody (I2386; Sigma Aldrich) and IgM in plasma (1:10,000) or MP-eluates (1:20) diluted in TBS/BSA was measured as above. The ratio of antigen specific IgM per total IgM was calculated for each sample.

To quantify the IgM antibodies against MDA-LDL in the peripheral and coronary blood of the acute myocardial infarction patients, plasma was diluted (1:800) in TBS/BSA and binding to coated MDA-LDL was measured as described. Obtained data were normalized to hematocrite values in the periphery and coronary blood, respectively.

To determine the specificity of the MP-bound IgM, an ELISA-based immunocompetition assay using native LDL, Cu-OxLDL or MDA-LDL as competitors was performed. MP lysates (1:50) were diluted in TBS/BSA and mixed 1:1 with 50µg/ml of native LDL, Cu-OxLDL or MDA-LDL and binding to coated MDA-LDL was measured as described above.

5.9 Isolation of human primary monocytes

Blood was obtained from the antecubital vein of healthy individuals (n=6) as described above. Peripheral monocytes were isolated using Ficoll-Paque (Sigma) followed by positive selection using CD14 Microbeads (Miltenyi) according to the manufacturer's instructions. The purity of the different preparations was evaluated by flow cytometry using anti-human CD14/PercP Cy5.5 (61D3). Samples were analyzed with a FACS Calibur Becton Dickinson), and data were analyzed using the Flow Jo software (Treestar). The purity of all preparations was found to be >96%.

5.10 In vitro MP stimulation and neutralization assay

To characterize the pro-inflammatory ability of MP in vitro, $5x10^4$ THP-1 or primary human monocytes (after resting for 36 hours in RPMI + 10% FBS) were stimulated with 200 µg/ml of in vitro generated platelet-derived MP for 8 hours at 37^oC in RPMI media containing 50µg/ml BSA. In parallel experiments, MP were co-cultured with 25 or 50µg/ml of either LR04 or control antibody (MM-30; Biolegend). At the end of the 8 hour stimulation, cell free supernatants were collected by pelleting the cells in a 96 well V bottom plate at 400g for 3 minutes. IL-8 protein levels were quantified in the supernatant by ELISA using a commercial kit (Becton Dickinson).

5.11 Mice, treatments and diets

Ldlr$^{-/-}$ mice (on C57BL/6 background) and *sIgM*$^{-/-}$ (on 129 background) mice were bought from The Jackson Laboratories (USA). *SIgM*$^{-/-}$ mice were backcrossed onto C57BL/6 background for at least 10 generations. *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice were generated by intercrossing *Ldlr*$^{-/-}$ and *sIgM*$^{-/-}$ mice. *t11*µ*MT* mice (Klein et al, 1997) (on C57BL/6 background) were kindly provided by Dr. Andreas Bergthaler (CeMM, Research Center for Molecular Medicine, Vienna, Austria). *Nur77-GFP* reporter mice (on C57BL/6 background) were kindly provided by Dr. Norbert Gerdes (LMU, Munich, Germany). *Nur77-GFP/sIgM*$^{-/-}$ mice were generated by intercrossing *Nur77-GFP* and *sIgM*$^{-/-}$ mice. All mice were bred in our in-house breeding facility.

All mice included in atherosclerosis studies were matched for age and whole body weight. For the atherosclerosis study 1, female *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ were fed an atherogenic diet (0.2% cholesterol, 21% fat; E15721-347 bought from Ssniff, Germany) for 16 weeks starting at the age of 12 weeks. For the atherosclerosis study 2, female *Ldlr*$^{-/-}$ or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ were fed an atherogenic diet for 6 weeks, starting at the age of 13-18 weeks. During the diet feeding period, the mice were injected intraperitoneally once every week, with 25µg diluted in 100µl DPBS (Sigma) of either an α-IgE neutralizing antibody (clone R1E4; kind gift by Dr. Daniel Conrad, Virginia, USA) or a control IgG (Jackson Immunoresearch Inc.). All experimental studies were approved by the Animal Ethics Committee of the Medical University of Vienna (Austria) BMWF-66.009/0157-II/3b/2013.

5.12 Plasma or serum cholesterol and triglyceride quantification

Fresh blood was collected from the vena cava at the time of sacrifice in MiniCollect Gold cap TUBE or MiniCollect K3EDTA TUBE (both from Greiner Bio-One). Blood was centrifuged at 1000g for 30 minutes at room temperature. Serum or plasma total cholesterol and triglycerides were measured under standardized conditions in an ISO 15189 accredited medical laboratory on Beckman Coulter AU5400 (Beckman Coulter) or Roche Cobas 8000 (Roche) instruments using the Beckman Coulter OSR6516 and OSR60118 or Roche CHOL2 and TRIGL reagents, respectively.

5.13 Quantification of atherosclerotic lesion size

Atherosclerotic lesion size was evaluated by computer assisted image analysis using Adobe Photoshop Elements 6.0 and ImageJ software as described previously (Binder et al, 2004; Binder et al, 2003; Cardilo-Reis et al, 2012). Briefly, lesion size in the aortic arch, descending thoracic and abdominal aorta was quantified in Sudan IV stained *en face* preparations of the entire aorta and is expressed as percentage of the whole aortic surface area. Lesion size in the aortic root was quantified in Hematoxylin and Eosin (H&E) stained cross sections (n=9/mouse) with 50µm distance that were collected starting with the appearance of all 3 valve leaflets.

5.14 Mast cell and neutrophil staining

Mast cells and neutrophils were visualized in the perivascular area of cross sections (1-2/mouse) by staining with a naphthol AS-D chloroacetate esterase staining kit (Sigma) and counted manually. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. Neutrophils were identified as round cells with a characteristic lobular nucleus and pink granular cytoplasm. Perivascular size was measured using a Leica image analysis system (Leica Ltd, UK). Morphometric analyses were performed by a blinded independent operator.

5.15 Bone marrow derived mast cell culture and stimulation

Bone marrow derived mast cells (BMMCs) were grown by culturing bone marrow cells at a density of $0.25x10^6$ cells in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from PAA) and murine Interleukin-3 containing supernatant (supernatant from WEHI cells overexpressing murine Interleukin-3) for 4 weeks. BMMCs ($5x10^5$) were incubated with plasma (1:12 dilution in medium) from either *Ldlr*$^{-/-}$ or *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice (that were fed an atherogenic diet and were injected intraperitoneally once every week, with 25µg diluted in 100µl DPBS (Sigma) of either an α-IgE neutralizing antibody (clone R1E4; kind gift by Dr. Daniel Conrad, Virginia, USA) or a control IgG

(Jackson Immunoresearch Inc.)) for 2 hours at 37°C, after which supernatant was collected. IL-6 was determined by ELISA according to the manufacturer's protocol (BD Biosciences).

5.16 Total and free IgE, and soluble CD23 quantification by ELISA

Total and free IgE serum titers were quantified by ELISA with the Mouse IgE ELISA MAX kit (Biolegend). To determine free IgE serum levels, plates were coated with the R1E4 antibody (described in mice treatments and diets section of the methods) at 5 μg/mL. Serum soluble CD23 was determined by ELISA (protocol and reagents were kindly provided by Dr. Daniel Conrad, Virginia, USA). Briefly, 96-well white round-bottomed MicroFluor microtiter plates (Thermo Lab systems) plates were coated with 10 μg/mL of 2H10 mAb overnight and then washed 3 times with PBS/EDTA and blocked with Tris-buffered saline containing 1% BSA (TBS/BSA) for 1 h at room temperature. After washing the plates as before, diluted murine plasma and recombinant CD23 were added in TBS/BSA to the wells and incubated for 1 hour at room temperature. Plates were washed and bound soluble CD23 was detected with a rabbit anti-CD23 antibody. Following an incubation time for 1 hour at room temperature and a washing step as before, an anti-rabbit IgG conjugated to alkaline phosphatase (Sigma; A3687) was added for 1 hour at room temperature. Wells were washed again as before and rinsed once with distilled water, and 25 μl of a 30% LumiPhos Plus solution in dH_20 (Lumigen Inc) was added. After 75 min the light emission was measured with a Synergy 2 luminometer (BIO-TEK) and expressed as RLU per 100ms.

5.17 Total RNA extraction, cDNA synthesis and Real-time PCR analysis

Total RNA was extracted with the peqGold total RNA kit (Peqlab) and cDNA was synthesized using the High capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative Real-time PCR analysis was performed with the KAPA SYBR green FAST BioRad icycler kit (Peqlab). For the germline and mature IgE mRNA quantification, CD19 was used as reference gene and the data are expressed as fold change over *sIgM*$^{+/+}$ or *Ldlr*$^{-/-}$*sIgM*$^{+/+}$ mice. Cyclin B or 36B4 mRNA was used as reference for the Nur77 mRNA quantification, and the data are expressed as fold change over unstimulated cells.

Primers:
germline IgE forward: 5-TGGGCATGAATTAATGGTTACTAGAG-3 (Pongratz et al, 2006),
germline IgE reverse: 5-TGGCCAGACTGTTCTTATTCGAA-3 (Pongratz et al, 2006),
mature IgE forward: 5-TCAAGGAACCTCAGTCACCGTCTC-3 (Pongratz et al, 2006),
mature IgE reverse: 5-TTACAGGGCTTCAAGGGGTAGAGC-3 (Pongratz et al, 2006),
CD19 forward: 5-AAGAGGGAGGCAATGTTGTG-3,
CD19 reverse: 5-AAAAGCCACCAGAGAAACCA-3,
36B4 forward: 5-AGGGCGACCTGGAAGTTCC-3 (Lam et al, 2013),
36B4 reverse: 5-CCCACAATGAAGCATTTTGGA-3 (Lam et al, 2013),
Cyclin B forward: 5-CAGCAAGTTCCATCGTGTCATCAAGG-3,
Cyclin B reverse: 5-GGAAGCGCTCACCATAGATGCTC-3,
Nur77 forward: 5-GGCATGGTGAAGGAAGTTGT-3 (Papac-Milicevic et al, 2012),
Nur77 reverse: 5-TCGATCAGTGATGAGGACCA-3 (Papac-Milicevic et al, 2012)

5.18 Flow cytometry

Peritoneal cells were collected upon peritoneal lavage with HBSS (Gibco) media containing 2% FBS. To isolate circulating leukocytes, fresh blood collected from the vena cava was diluted in DPBS (Sigma) containing 2% dextran sulfate (Sigma) at approximately 1:1 v/v ratio and incubated at 37°C for at least 30-40 minutes. The upper phase that includes leukocytes was carefully collected without disturbing the lower phase that includes the erythrocytes. Bone marrow cells were isolated from the tibia and the femur bones on cell strainers with 100 μm diameter (BD Biosciences), and erythrocytes were lysed upon incubation with erythrocyte lysis buffer (MORPHISTO). Isolated spleens were mechanically dissociated in single cell suspensions using cell strainers with 100 μm diameter (BD Biosciences), and erythrocytes were lysed as above. Cells were added in a 96 well V-bottom plate (Thermo Scientific) and incubated for 20 min at 4°C, with 2.5 μg/ml of a blocking anti-CD16/32 antibody (clone 93; eBiosciences) diluted in DPBS (Sigma) containing 10% FBS (FACS buffer). After two washing steps with FACS buffer (393 g for 3 minutes at 4°C), cells were stained with different combinations (as defined in figure legends) of the following antibodies: anti-B220 PercP-Cy5.5 (clone RA3-6B2; eBiosciences),

anti-CD23 FITC, anti-CD23 eFluor450 (clone B3B4; eBiosciences), anti-CD43 PE (clone S7; BD Biosciences), anti-IgM APC (clone II/41; eBiosciences), anti-CD21 biotinylated (clone 7E9; Biolegend), anti-CD93 PE (clone AA4.1; eBiosciences), anti-CD19 PE (clone 1D3; BD Biosciences), anti-kappa FITC (clone 187.1; BD Biosciences), anti-lambda biotinylated (clone RML-42; Biolegend), anti-IgE FITC (clone RME-1; Biolegend), anti-IgE PE (clone 23G3; eBiosciences), anti-CD11b APC (clone M1/70; eBiosciences), anti-CD5 (clone 53-7.3; eBiosciences), streptavidin APC or streptavidin eFluor 450 (eBiosciences).

To determine the amount of the phosphorylated kinases pBtk and pSyk, cells were first fixed and permeabilized with fixation and permeabilzation solution (Miltenyi) for 30 minutes at 4°C and then stained intracellularly in permeabilzation buffer (Miltenyi) with the following antibodies: pBTK/ITK (Y551/Y511) APC (clone M4G3LN; eBiosciences), pSYK (Y348) APC (clone moch1ct, eBiosciences). Finally, to identify dead cells staining with 7-AAD viability solution (eBiosciences) was performed where indicated. Data were acquired on a FACS Calibur (Becton Dickinson) or LSR Fortessa (Becton Dickinson) and were analyzed using Flow Jo software 7.6 (Treestar).

5.19 Polyclonal IgM treatment

Female *sIgM*$^{-/-}$ mice (n=5) were injected intraperitoneally six times, every two days for two weeks with 200 µg/mouse of polyclonal IgM (Rockland) diluted in 100µl DPBS and compared to *sIgM*$^{-/-}$ (n=5) and *sIgM*$^{+/+}$ (n=4) mice that were injected with DPBS only. All mice were sacrificed and analyzed on day 14.

5.20 Antigen-specific stimulation of *t11*µ*MT* B cells

Isolated spleens from *t11*µ*MT* mice were mechanically dissociated in single cell suspensions as described above and then untouched B2 cells were isolated with the B cell isolation kit (Miltenyi) according to the manufacturerer's instructions. Purified *t11*µ*MT* B cells ($3x10^5$) were stimulated in RPMI (supplemented with 10% FBS, 1% penicillin/streptomycin, 1% NaPy, 0.1% 2-mercaptoethanol and 1µg/ml insulin (Sigma)) for 30 minutes at 37°C with 465,000 PFU of UV-inactivated vesicular stomatitis virus (VSV) Indiana. VSV particles were preincubated for 30 minutes at 4°C with either 2µg/ml of anti-VSV IgM antibody (produced in supernatants of the M4C11H2 hybridoma, (Fehr et al, 1997)) or a control IgM antibody (clone MM-30;

Biolegend). As positive control, cells were stimulated with 10 µg/ml of anti-mouse IgM $F(ab')_2$ (Jackson Immunoresearch Inc.).Then cells were pelleted in a 96 well V bottom plate (Thermo Scientific), total RNA was extracted and Nur77 mRNA quantified by Real-time PCR.

5.21 Statistical Analyses

Statistical analyses were performed using Graph Pad Prism 5 for Windows (Graph Pad Software). Experimental groups were compared using Student's unpaired or paired t test or Mann-Whitney test as indicated. To analyze multiple group data, either One-Way or Two-Way ANOVA tests followed by Bonferroni's Multiple Comparison or Newman-Keuls or Dunn's or unpaired t test were performed as appropriate. For correlation analysis, the Pearson's test was used. Data are presented as mean ±SEM or as mean ±SD where indicated. A P value of <0.05 was considered significant.

6. REFERENCES

IBC 50K CAD Consortium Ait-Oufella H, Kinugawa K, Zoll J, Simon T, Boddaert J, Heeneman S, Blanc-Brude O, Barateau V, Potteaux S, Merval R, Esposito B, Teissier E, Daemen MJ, Leseche G, Boulanger C, Tedgui A, Mallat Z (2007) Lactadherin deficiency leads to apoptotic cell accumulation and accelerated atherosclerosis in mice. *Circulation* **115**(16)**:** 2168-2177

Ait-Oufella H, Sage AP, Mallat Z, Tedgui A (2014) Adaptive (T and B cells) immunity and control by dendritic cells in atherosclerosis. *Circ Res* **114**(10)**:** 1640-1660

Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z (2006) Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med* **12**(2)**:** 178-180

Amir S, Hartvigsen K, Gonen A, Leibundgut G, Que X, Jensen-Jarolim E, Wagner O, Tsimikas S, Witztum JL, Binder CJ (2012) Peptide mimotopes of malondialdehyde epitopes for clinical applications in cardiovascular disease. *J Lipid Res* **53**(7)**:** 1316-1326

Baker N, Ehrenstein MR (2002) Cutting edge: selection of B lymphocyte subsets is regulated by natural IgM. *J Immunol* **169**(12)**:** 6686-6690

Balsalobre B, Carbonell-Tatay F (1991) Total serum IgE concentration in splenectomized patients after abdominal trauma. *Rev Esp Fisiol* **47**(1)**:** 43-44

Balsalobre B, Enguidanos MJ, Hernandez-Godoy J, Planelles D, Mir A (1993) Changes in total serum IgE concentrations after splenectomy. *J Investig Allergol Clin Immunol* **3**(5)**:** 268-270

Barry OP, Pratico D, Lawson JA, FitzGerald GA (1997) Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* **99**(9)**:** 2118-2127

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

Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA (1999) Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* **96**(5)**:** 2250-2255

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

Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ (2003) Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med* **9**(6)**:** 736-743

Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J (1998) Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* **160**(10)**:** 4776-4787

Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J (2000) Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci U S A* **97**(3)**:** 1184-1189

Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J, Lee DM (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science* **327**(5965)**:** 580-583

Bot I, de Jager SC, Zernecke A, Lindstedt KA, van Berkel TJ, Weber C, Biessen EA (2007) Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115**(19)**:** 2516-2525

Browning JL (2006) B cells move to centre stage: novel opportunities for autoimmune disease treatment. *Nat Rev Drug Discov* **5**(7)**:** 564-576

Cardilo-Reis L, Gruber S, Schreier SM, Drechsler M, Papac-Milicevic N, Weber C, Wagner O, Stangl H, Soehnlein O, Binder CJ (2012) Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype. *EMBO Mol Med* **4**(10)**:** 1072-1086

Cariappa A, Tang M, Parng C, Nebelitskiy E, Carroll M, Georgopoulos K, Pillai S (2001) The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. *Immunity* **14**(5)**:** 603-615

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

Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, Dennis EA, Witztum JL (1999) Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A* **96**(11)**:** 6353-6358

Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL (2004) Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *J Exp Med* **200**(11)**:** 1359-1370

Chelazzi G, Pinotti G, Nicora C, Rossi D, Senaldi G (1985) Increased total serum IgE concentration in patients who have undergone splenectomy after trauma. *J Clin Pathol* **38**(11)**:** 1309-1310

Cheng LE, Wang ZE, Locksley RM (2010) Murine B cells regulate serum IgE levels in a CD23-dependent manner. *J Immunol* **185**(9)**:** 5040-5047

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

Clark MR, Mandal M, Ochiai K, Singh H (2014) Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nat Rev Immunol* **14**(2)**:** 69-80

Cornall RJ, Cheng AM, Pawson T, Goodnow CC (2000) Role of Syk in B-cell development and antigen-receptor signaling. *Proc Natl Acad Sci U S A* **97**(4)**:** 1713-1718

Dansky HM, Charlton SA, Harper MM, Smith JD (1997) T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci U S A* **94**(9)**:** 4642-4646

De la Concha EG, Garcia-Rodriguez MC, Zabay JM, Laso MT, Alonso F, Bootello A, Fontan G (1982) Functional assessment of T and B lymphocytes in patients with selective IgM deficiency. *Clin Exp Immunol* **49**(3)**:** 670-676

Dema B, Charles N, Pellefigues C, Ricks TK, Suzuki R, Jiang C, Scheffel J, Hasni S, Hoffman V, Jablonski M, Sacre K, Gobert D, Papo T, Daugas E, Crampton S, Bolland S, Rivera J (2014) Immunoglobulin E plays an immunoregulatory role in lupus. *J Exp Med*

Distelmaier K, Adlbrecht C, Jakowitsch J, Winkler S, Dunkler D, Gerner C, Wagner O, Lang IM, Kubicek M (2009) Local complement activation triggers neutrophil recruitment to the site of thrombus formation in acute myocardial infarction. *Thromb Haemost* **102**(3)**:** 564-572

Doring Y, Drechsler M, Soehnlein O, Weber C (2014) Neutrophils in Atherosclerosis: From Mice to Man. *Arterioscler Thromb Vasc Biol*

Doyle CM, Han J, Weigert MG, Prak ET (2006) Consequences of receptor editing at the lambda locus: multireactivity and light chain secretion. *Proc Natl Acad Sci U S A* **103**(30)**:** 11264-11269

Drew PA, Kiroff GK, Ferrante A, Cohen RC (1984) Alterations in immunoglobulin synthesis by peripheral blood mononuclear cells from splenectomized patients with and without splenic regrowth. *J Immunol* **132**(1)**:** 191-196

Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS (1998) Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A* **95**(17)**:** 10089-10093

Fehr T, Bachmann MF, Bucher E, Kalinke U, Di Padova FE, Lang AB, Hengartner H, Zinkernagel RM (1997) Role of repetitive antigen patterns for induction of antibodies against antibodies. *J Exp Med* **185**(10)**:** 1785-1792

Fiskesund R, Stegmayr B, Hallmans G, Vikstrom M, Weinehall L, de Faire U, Frostegard J (2010) Low levels of antibodies against phosphorylcholine predict development of stroke in a population-based study from northern Sweden. *Stroke* **41**(4)**:** 607-612

Fogelman AM, Shechter I, Seager J, Hokom M, Child JS, Edwards PA (1980) Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc Natl Acad Sci U S A* **77**(4)**:** 2214-2218

Ford JW, Kilmon MA, Haas KM, Shelburne AE, Chan-Li Y, Conrad DH (2006) In vivo murine CD23 destabilization enhances CD23 shedding and IgE synthesis. *Cell Immunol* **243**(2)**:** 107-117

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

Glass CK, Witztum JL (2001) Atherosclerosis. the road ahead. *Cell* **104**(4)**:** 503-516

Gofman JW, Lindgren F (1950) The role of lipids and lipoproteins in atherosclerosis. *Science* **111**(2877)**:** 166-171

Goldstein MF, Goldstein AL, Dunsky EH, Dvorin DJ, Belecanech GA, Shamir K (2008) Pediatric selective IgM immunodeficiency. *Clin Dev Immunol* **2008:** 624850

Gronlund H, Hallmans G, Jansson JH, Boman K, Wikstrom M, de Faire U, Frostegard J (2009) Low levels of IgM antibodies against phosphorylcholine predict development of acute myocardial infarction in a population-based cohort from northern Sweden. *Eur J Cardiovasc Prev Rehabil* **16**(3)**:** 382-386

Hahn BH (2013) Belimumab for systemic lupus erythematosus. *N Engl J Med* **368**(16)**:** 1528-1535

Hammarstrom S, Falardeau P (1977) Resolution of prostaglandin endoperoxide synthase and thromboxane synthase of human platelets. *Proc Natl Acad Sci U S A* **74**(9)**:** 3691-3695

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

Heltemes LM, Manser T (2002) Level of B cell antigen receptor surface expression influences both positive and negative selection of B cells during primary development. *J Immunol* **169**(3)**:** 1283-1292

Hermiston ML, Tan AL, Gupta VA, Majeti R, Weiss A (2005) The juxtamembrane wedge negatively regulates CD45 function in B cells. *Immunity* **23**(6)**:** 635-647

Hirano M, Davis RS, Fine WD, Nakamura S, Shimizu K, Yagi H, Kato K, Stephan RP, Cooper MD (2007) IgEb immune complexes activate macrophages through FcgammaRIV binding. *Nat Immunol* **8**(7)**:** 762-771

Hojyo S, Miyai T, Fujishiro H, Kawamura M, Yasuda T, Hijikata A, Bin BH, Irie T, Tanaka J, Atsumi T, Murakami M, Nakayama M, Ohara O, Himeno S, Yoshida H, Koseki H, Ikawa T, Mishima K, Fukada T (2014) Zinc transporter SLC39A10/ZIP10 controls humoral immunity by modulating B-cell receptor signal strength. *Proc Natl Acad Sci U S A* **111**(32)**:** 11786-11791

Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Friedman P, Dennis EA, Curtiss LK, Palinski W, Witztum JL (1999) Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest* **103**(1)**:** 117-128

Huber J, Vales A, Mitulovic G, Blumer M, Schmid R, Witztum JL, Binder BR, Leitinger N (2002) Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arterioscler Thromb Vasc Biol* **22**(1)**:** 101-107

Ideura G, Agematsu K, Komatsu Y, Hatayama O, Yasuo M, Tsushima K, Hanaoka M, Koizumi T, Fujimoto K, Kubo K (2008) Selective IgM deficiency accompanied with IgG4 deficiency, dermal complications and a bronchial polyp. *Allergol Int* **57**(1)**:** 99-105

Jakus Z, Nemeth T, Verbeek JS, Mocsai A (2008) Critical but overlapping role of FcgammaRIII and FcgammaRIV in activation of murine neutrophils by immobilized immune complexes. *J Immunol* **180**(1)**:** 618-629

Jialal I, Fuller CJ, Huet BA (1995) The effect of alpha-tocopherol supplementation on LDL oxidation. A dose-response study. *Arterioscler Thromb Vasc Biol* **15**(2)**:** 190-198

Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, Krystal G (2001) Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* **14**(6)**:** 801-811

Karvonen J, Paivansalo M, Kesaniemi YA, Horkko S (2003) Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation* **108**(17)**:** 2107-2112

Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, Davidson L, Muller S, Kantor AB, Herzenberg LA, et al. (1995) Defective B cell development and function in Btk-deficient mice. *Immunity* **3**(3)**:** 283-299

Klein MA, Frigg R, Flechsig E, Raeber AJ, Kalinke U, Bluethmann H, Bootz F, Suter M, Zinkernagel RM, Aguzzi A (1997) A crucial role for B cells in neuroinvasive scrapie. *Nature* **390**(6661)**:** 687-690

Kovanen PT, Kaartinen M, Paavonen T (1995) Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* **92**(5)**:** 1084-1088

Kovanen PT, Manttari M, Palosuo T, Manninen V, Aho K (1998) Prediction of myocardial infarction in dyslipidemic men by elevated levels of immunoglobulin classes A, E, and G, but not M. *Arch Intern Med* **158**(13)**:** 1434-1439

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

Lam MT, Cho H, Lesch HP, Gosselin D, Heinz S, Tanaka-Oishi Y, Benner C, Kaikkonen MU, Kim AS, Kosaka M, Lee CY, Watt A, Grossman TR, Rosenfeld MG, Evans RM, Glass CK (2013) Rev-Erbs repress macrophage gene expression by inhibiting enhancer-directed transcription. *Nature* **498**(7455)**:** 511-515

Lewis G, Rapsomaniki E, Bouriez T, Crockford T, Ferry H, Rigby R, Vyse T, Lambe T, Cornall R (2004) Hyper IgE in New Zealand black mice due to a dominant-negative CD23 mutation. *Immunogenetics* **56**(8)**:** 564-571

Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO (2009) Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* **120**(5)**:** 417-426

Libby P, Lichtman AH, Hansson GK (2013) Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* **38**(6)**:** 1092-1104

Liu ML, Scalia R, Mehta JL, Williams KJ (2012) Cholesterol-induced membrane microvesicles as novel carriers of damage-associated molecular patterns: mechanisms of formation, action, and detoxification. *Arterioscler Thromb Vasc Biol* **32**(9)**:** 2113-2121

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

Louis AG, Gupta S (2014) Primary selective IgM deficiency: an ignored immunodeficiency. *Clin Rev Allergy Immunol* **46**(2)**:** 104-111

Low JM, Chauhan AK, Moore TL (2007) Abnormal kappa:lambda light chain ratio in circulating immune complexes as a marker for B cell activity in juvenile idiopathic arthritis. *Scand J Immunol* **65**(1)**:** 76-83

Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A (2000) Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* **101**(8)**:** 841-843

Mancardi DA, Iannascoli B, Hoos S, England P, Daeron M, Bruhns P (2008) FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation. *J Clin Invest* **118**(11)**:** 3738-3750

Mause SF, Weber C (2010) Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res* **107**(9)**:** 1047-1057

Messer L, Alsaleh G, Freyssinet JM, Zobairi F, Leray I, Gottenberg JE, Sibilia J, Toti-Orfanoudakis F, Wachsmann D (2009) Microparticle-induced release of B-lymphocyte regulators by rheumatoid synoviocytes. *Arthritis Res Ther* **11**(2)**:** R40

Min PK, Kim JY, Chung KH, Lee BK, Cho M, Lee DL, Hong SY, Choi EY, Yoon YW, Hong BK, Rim SJ, Kwon HM (2013) Local increase in microparticles from the aspirate of culprit coronary arteries in patients with ST-segment elevation myocardial infarction. *Atherosclerosis* **227**(2)**:** 323-328

Mittelstadt PR, DeFranco AL (1993) Induction of early response genes by cross-linking membrane Ig on B lymphocytes. *J Immunol* **150**(11)**:** 4822-4832

Morel O, Morel N, Jesel L, Freyssinet JM, Toti F (2011) Microparticles: a critical component in the nexus between inflammation, immunity, and thrombosis. *Semin Immunopathol* **33**(5)**:** 469-486

Nguyen TT, Elsner RA, Baumgarth N (2015) Natural IgM Prevents Autoimmunity by Enforcing B Cell Central Tolerance Induction. *J Immunol*

Notley CA, Baker N, Ehrenstein MR (2010) Secreted IgM enhances B cell receptor signaling and promotes splenic but impairs peritoneal B cell survival. *J Immunol* **184**(7)**:** 3386-3393

Notley CA, Brown MA, Wright GP, Ehrenstein MR (2011) Natural IgM is required for suppression of inflammatory arthritis by apoptotic cells. *J Immunol* **186**(8)**:** 4967-4972

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

Otsuka F, Joner M, Prati F, Virmani R, Narula J (2014) Clinical classification of plaque morphology in coronary disease. *Nat Rev Cardiol* **11**(7)**:** 379-389

Owens AP, 3rd, Mackman N (2011) Microparticles in hemostasis and thrombosis. *Circ Res* **108**(10)**:** 1284-1297

Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, Witztum JL (1996) Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest* **98**(3)**:** 800-814

Pandey V, Mihara S, Fensome-Green A, Bolsover S, Cockcroft S (2004) Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosol Ca2+, degranulation, and membrane ruffling in the cultured rat basophilic leukemia-2H3 mast cell line. *J Immunol* **172**(7)**:** 4048-4058

Papac-Milicevic N, Breuss JM, Zaujec J, Ryban L, Plyushch T, Wagner GA, Fenzl S, Dremsek P, Cabaravdic M, Steiner M, Glass CK, Binder CJ, Uhrin P, Binder BR (2012) The interferon stimulated gene 12 inactivates vasculoprotective functions of NR4A nuclear receptors. *Circ Res* **110**(8)**:** e50-63

Pillai S, Cariappa A (2009) The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* **9**(11)**:** 767-777

Pongratz G, McAlees JW, Conrad DH, Erbe RS, Haas KM, Sanders VM (2006) The level of IgE produced by a B cell is regulated by norepinephrine in a p38 MAPK- and CD23-dependent manner. *J Immunol* **177**(5)**:** 2926-2938

Qin Z (2012) The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis* **221**(1)**:** 2-11

Rader DJ, Tall AR (2012) The not-so-simple HDL story: Is it time to revise the HDL cholesterol hypothesis? *Nat Med* **18**(9)**:** 1344-1346

Rautou PE, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, Nalbone G, Castier Y, Leseche G, Lehoux S, Tedgui A, Boulanger CM (2011a) Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. *Circ Res* **108**(3)**:** 335-343

Rautou PE, Vion AC, Amabile N, Chironi G, Simon A, Tedgui A, Boulanger CM (2011b) Microparticles, vascular function, and atherothrombosis. *Circ Res* **109**(5)**:** 593-606

Ravandi A, Leibundgut G, Hung MY, Patel M, Hutchins PM, Murphy RC, Prasad A, Mahmud E, Miller YI, Dennis EA, Witztum JL, Tsimikas S (2014) Release and capture of bioactive oxidized phospholipids and oxidized cholesteryl esters during percutaneous coronary and peripheral arterial interventions in humans. *J Am Coll Cardiol* **63**(19)**:** 1961-1971

Reardon CA, Blachowicz L, Lukens J, Nissenbaum M, Getz GS (2003) Genetic background selectively influences innominate artery atherosclerosis: immune system deficiency as a probe. *Arterioscler Thromb Vasc Biol* **23**(8)**:** 1449-1454

Rhee I, Veillette A (2012) Protein tyrosine phosphatases in lymphocyte activation and autoimmunity. *Nat Immunol* **13**(5)**:** 439-447

Roman MJ, Salmon JE (2007) Cardiovascular manifestations of rheumatologic diseases. *Circulation* **116**(20)**:** 2346-2355

Sellam J, Proulle V, Jungel A, Ittah M, Miceli Richard C, Gottenberg JE, Toti F, Benessiano J, Gay S, Freyssinet JM, Mariette X (2009) Increased levels of circulating microparticles in primary Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. *Arthritis Res Ther* **11**(5)**:** R156

Shanmugam N, Figarola JL, Li Y, Swiderski PM, Rahbar S, Natarajan R (2008) Proinflammatory effects of advanced lipoxidation end products in monocytes. *Diabetes* **57**(4)**:** 879-888

Sheng H, Hassanali S, Nugent C, Wen L, Hamilton-Williams E, Dias P, Dai YD (2011) Insulinoma-Released Exosomes or Microparticles Are Immunostimulatory and Can Activate Autoreactive T Cells Spontaneously Developed in Nonobese Diabetes Mice. *J Immunol*

Shrivastava P, Katagiri T, Ogimoto M, Mizuno K, Yakura H (2004) Dynamic regulation of Src-family kinases by CD45 in B cells. *Blood* **103**(4)**:** 1425-1432

Steinberg D (2002) Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat Med* **8**(11)**:** 1211-1217

Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P, MacFarlane LA, Mallen-St Clair J, Shi GP (2007) Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat Med* **13**(6)**:** 719-724

Tabas I (2010) Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* **10**(1)**:** 36-46

Tabas I, Seimon T, Timmins J, Li G, Lim W (2009) Macrophage apoptosis in advanced atherosclerosis. *Ann N Y Acad Sci* **1173 Suppl 1:** E40-45

Taleb S, Tedgui A, Mallat Z (2014) IL-17 and Th17 Cells in Atherosclerosis: Subtle and Contextual Roles. *Arterioscler Thromb Vasc Biol*

Thiele GM, Duryee MJ, Willis MS, Sorrell MF, Freeman TL, Tuma DJ, Klassen LW (2004) Malondialdehyde-acetaldehyde (MAA) modified proteins induce pro-inflammatory and pro-fibrotic responses by liver endothelial cells. *Comp Hepatol* **3 Suppl 1:** S25

Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ (2014) B cells and humoral immunity in atherosclerosis. *Circ Res* **114**(11)**:** 1743-1756

Tsiantoulas D, Perkmann T, Afonyushkin T, Mangold A, Prohaska TA, Papac-Milicevic N, Millischer V, Bartel C, Horkko S, Boulanger CM, Tsimikas S, Fischer MB, Witztum JL, Lang IM, Binder CJ (2015a) Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies. *J Lipid Res* **56**(2)**:** 440-448

Tsiantoulas D, Sage AP, Mallat Z, Binder CJ (2015b) Targeting B cells in atherosclerosis: closing the gap from bench to bedside. *Arterioscler Thromb Vasc Biol* **35**(2)**:** 296-302

Tsimikas S, Brilakis ES, Lennon RJ, Miller ER, Witztum JL, McConnell JP, Kornman KS, Berger PB (2007) Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *J Lipid Res* **48**(2)**:** 425-433

Tsimikas S, Willeit P, Willeit J, Santer P, Mayr M, Xu Q, Mayr A, Witztum JL, Kiechl S (2012) Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol* **60**(21)**:** 2218-2229

Tuominen A, Miller YI, Hansen LF, Kesaniemi YA, Witztum JL, Horkko S (2006) A natural antibody to oxidized cardiolipin binds to oxidized low-density lipoprotein, apoptotic cells, and atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* **26**(9)**:** 2096-2102

Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, Tedder TF (2004) The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* **199**(12)**:** 1659-1669

Vincent FB, Morand EF, Schneider P, Mackay F (2014) The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol* **10**(6)**:** 365-373

Wang J, Cheng X, Xiang MX, Alanne-Kinnunen M, Wang JA, Chen H, He A, Sun X, Lin Y, Tang TT, Tu X, Sjoberg S, Sukhova GK, Liao YH, Conrad DH, Yu L, Kawakami T, Kovanen PT, Libby P, Shi GP (2011a) IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J Clin Invest* **121**(9)**:** 3564-3577

Wang JG, Williams JC, Davis BK, Jacobson K, Doerschuk CM, Ting JP, Mackman N (2011b) Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner. *Blood* **118**(8)**:** 2366-2374

Weber C, Noels H (2011) Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med* **17**(11)**:** 1410-1422

Weismann D, Hartvigsen K, Lauer N, Bennett KL, Scholl HP, Charbel Issa P, Cano M, Brandstatter H, Tsimikas S, Skerka C, Superti-Furga G, Handa JT, Zipfel PF, Witztum JL, Binder CJ (2011) Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. *Nature* **478**(7367)**:** 76-81

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

Wen R, Chen Y, Xue L, Schuman J, Yang S, Morris SW, Wang D (2003) Phospholipase Cgamma2 provides survival signals via Bcl2 and A1 in different subpopulations of B cells. *J Biol Chem* **278**(44)**:** 43654-43662

Yamasaki T (1992) Selective IgM deficiency: functional assessment of peripheral blood lymphocytes in vitro. *Intern Med* **31**(7)**:** 866-870

Yu P, Kosco-Vilbois M, Richards M, Kohler G, Lamers MC (1994) Negative feedback regulation of IgE synthesis by murine CD23. *Nature* **369**(6483)**:** 753-756

Zikherman J, Parameswaran R, Weiss A (2012) Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature* **489**(7414)**:** 160-164

Curriculum Vitae

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Education History:

1995 – 2001	2nd High School of Ampelokipoi, Thessaloniki, Greece
2001 – 2007	Molecular Biology and Genetics School, Democritus University of Thrace, **Diploma university degree with grade "very good" awarded on 13.11.2007**
2006 – 2007	Diploma thesis, B' Dept. of Neurology, Aristotle University of Thessaloniki
2008 –	PhD student at the Medical University of Vienna, Prof. Christoph Binder, Laboratory of Immunity and Atherosclerosis, CeMM Research Center for Molecular Medicine, Dept. of Laboratory Medicine

Publications (2009-):

1. Tsiantoulas D, Perkmann T, Afonyushkin T, Mangold A, Prohaska TA, Papac-Milicevic N, Millischer V, Bartel C, Horkko S, Boulanger CM, Tsimikas S, Fischer MB, Witztum JL, Lang IM, Binder CJ (2015) Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies. *J Lipid Res* **56:** 440-448
2. Tsiantoulas D, Sage AP, Mallat Z, Binder CJ (2015) Targeting B cells in atherosclerosis: closing the gap from bench to bedside. *Arterioscler Thromb Vasc Biol* **35:** 296-302
3. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ (2014a) B cells and humoral immunity in atherosclerosis. *Circ Res* **114:** 1743-1756
4. Gonen A, Hansen LF, Turner WW, Montano EN, Que X, Rafia A, Chou MY, Wiesner P, Tsiantoulas D, Corr M, VanNieuwenhze MS, Tsimikas S, Binder CJ, Witztum JL, Hartvigsen K (2014) Atheroprotective immunization with malondialdehyde-modified LDL is hapten specific and dependent on advanced MDA adducts: implications for development of an atheroprotective vaccine. *J Lipid Res* **55:** 2137-2155
5. Zouggari Y, Ait-Oufella H, Bonnin P, Simon T, Sage AP, Guerin C, Vilar J, Caligiuri G, Tsiantoulas D, Laurans L, Dumeau E, Kotti S, Bruneval P, Charo IF, Binder CJ, Danchin N, Tedgui A, Tedder TF, Silvestre JS, Mallat Z (2013) B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. *Nat Med* **19:** 1273-1280
6. Tsiantoulas D, Gruber S, Binder CJ (2012) B-1 cell immunoglobulin directed against oxidation-specific epitopes. *Front Immunol* **3:** 415
7. Sage AP, Tsiantoulas D, Baker L, Harrison J, Masters L, Murphy D, Loinard C, Binder CJ, Mallat Z (2012) BAFF receptor deficiency reduces the development of atherosclerosis in mice--brief report. *Arterioscler Thromb Vasc Biol* **32:** 1573-1576
8. Tsakiri A, Tsiantoulas D, Frederiksen J, Svane IM (2010) Increased immunopotency of monocyte derived dendritic cells from patients with optic neuritis is inhibited in vitro by simvastatin. *Exp Neurol* **221:** 320-328
9. Irony-Tur-Sinai M, Grigoriadis N, Tsiantoulas D, Touloumi O, Abramsky O, Brenner T (2009) Immunomodulation of EAE by alpha-fetoprotein involves elevation of immune cell apoptosis markers and the transcription factor FoxP3. *J Neurol Sci* **279:** 80-87

Book Chapter

Natural Antibodies and Atherosclerosis; Dimitrios Tsiantoulas & Christoph J. Binder; pp. 289-304 in Inflammation and Atherosclerosis; Springer 2012; Editors G.Wick & C. Grundtmann

Oral-Poster presentations:

1. Journal of Internal Medicine (JIM) symposium, 2014 (Stockholm, Sweden): Deficiency in natural IgM antibodies leads to increased IgE plasma titers resulting in aggravated atherosclerosis, (oral presentation)
2. Gordon's Research Conference for Atherosclerosis, 2013 (Stowe, USA): Deficiency in natural IgM antibodies leads to increased IgE plasma titers resulting in aggravated atherosclerosis, (poster presentation)
3. Austrian Atherosclerosis Society meeting, 2013 (St. Gilgen, Austria): Natural IgM antibodies protect from atherosclerosis by regulating the B cell development, (oral presentation)
4. 17th International Vascular Biology Meeting, 2012 (Wiesbaden, Germany): Natural IgM antibodies protect from atherosclerosis by binding circulating microparticles resulting in neutralization of their proinflammatory activities and regulation of splenic B cell maturation, (oral presentation)
5. Austrian Atherosclerosis Society meeting, 2012 (St. Gilgen, Austria): Natural IgM antibodies suppress the pro-inflammatory effect of circulating microparticles in atherosclerosis, (oral presentation)
6. Inflammation and Cardiovascular disease Summer school, 2011 (Obergurgl, Austria): How do the Natural IgM Antibodies protect from Atherosclerosis? (oral presentation)
7. Atherosclerosis, Thrombosis and Vascular Biology 2011 meeting, (Chicago, USA): Natural IgM antibodies suppress the proinflammatory effect of apoptotic blebs in atherosclerosis, (poster presentation)
8. 8th B cell Forum, 2010 (Dresden, Germany): Characterization of B-cell populations and atherosclerosis in serum IgM-deficient Low-Density Lipoprotein knock-out mice, (poster presentation)
9. 21th Congress of the European Committee - 10th Annual meeting of the American Committee for Treatment and Research in Multiple Sclerosis, 2005 (Thessaloniki, Greece): Opposite expression of cytotoxic T lymphocyte antigen-4 and forkhead box P3 mRNA in multiple sclerosis patients during relapses, (poster presentation)

Awards:

Travel award of the European Vascular Biology Organisation (EVB) for the 17th International Vascular Biology Meeting, 2012 (Wiesbaden, Germany)