

# Humoral immunity in atherosclerosis and myocardial infarction: from B cells to antibodies

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Received 2 June 2021; revised 30 July 2021; editorial decision 15 August 2021; accepted 24 August 2021; online publish-ahead-of-print 27 August 2021

## Abstract

Immune mechanisms are critically involved in the pathogenesis of atherosclerosis and its clinical manifestations. Associations of specific antibody levels and defined B-cell subsets with cardiovascular disease activity in humans as well as mounting evidence from preclinical models demonstrate a role of B cells and humoral immunity in atherosclerotic cardiovascular disease. These include all aspects of B-cell immunity, the generation of antigen-specific antibodies, antigen presentation and co-stimulation of T cells, as well as production of cytokines. Through their impact on adaptive and innate immune responses and the regulation of many other immune cells, B cells mediate both protective and detrimental effects in cardiovascular disease. Several antigens derived from (oxidized) lipoproteins, the vascular wall and classical autoantigens have been identified. The unique antibody responses they trigger and their relationship with atherosclerotic cardiovascular disease are reviewed. In particular, we focus on the different effector functions of specific IgM, IgG, and IgE antibodies and the cellular responses they trigger and highlight potential strategies to target B-cell functions for therapy.

## Keywords

B cells • Antibodies • Immunoglobulins • Atherosclerosis • Myocardial infarction

This article is part of the Spotlight Issue on Cardiovascular Immunology.

## 1. Introduction

Ischaemic heart disease and stroke constitute a major part of the burden of cardiovascular diseases and are the main clinical manifestations of atherosclerosis, a chronic inflammatory process affecting large and medium-sized arteries.<sup>1</sup> Intimal accumulation and retention of low-density lipoprotein (LDL) in the artery wall renders it susceptible to various modifications including oxidation, leading to the generation of structurally altered LDL particles that are taken up by monocyte-derived macrophages forming foam cells. Oxidation of LDL causes the formation of different types of oxidation-specific epitopes (OSEs) that constitute a class of danger-associated molecular patterns (DAMPs) targeted by several components of innate immunity.<sup>2</sup> OSEs trigger pro-inflammatory activation of endothelial cells and macrophages, allowing them to sense intracellular cholesterol crystals and activate the NLRP3 inflammasome, triggering IL-1 $\beta$  production.<sup>3</sup> In addition to modified LDL, accumulation of dying cells derived from foam cells undergoing cell death contributes

to plaque inflammation. Indeed, impaired clearance of lesional apoptotic cells represents an important cause of chronic inflammation.<sup>4</sup> Moreover, dying cells carry the same pro-inflammatory OSEs, thereby further sustaining a vicious cycle of inflammation.

It is now evident that this process of vascular inflammation is modulated by adaptive immunity, which plays a critical role in plaque progression. T cells represent a major portion of plaque-infiltrating leukocytes and analyses showing the presence of oligoclonal T-cell receptor repertoires indicate restriction for specific antigens.<sup>5–7</sup> Preclinical studies with atherosclerosis-prone mice lacking adaptive immune cells have established a net negative effect of adaptive immunity in atherogenesis, as these mice developed less atherosclerosis particularly when plasma cholesterol levels were not exceedingly high.<sup>8</sup> Multiple studies dissecting the contribution of different T-cell subsets demonstrated functional roles for CD8<sup>+</sup> as well as CD4<sup>+</sup> T cells and IFN $\gamma$ -secreting T helper 1 (Th1) cells as major drivers of lesion progression. In turn, these responses are tightly regulated by regulatory T (T<sub>reg</sub>) cells. These important aspects have

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been reviewed extensively.<sup>5</sup> B cells, representing the other main lymphocyte population, are also major modulators of atherogenesis.<sup>8–10</sup> Powerful evidence of their contribution to atherosclerotic cardiovascular disease (ACVD) comes from the integration of GWAS and transcriptomic data that identified B-cell functions as key drivers of coronary heart disease.<sup>11</sup> Epidemiological data reporting increased cardiovascular risk with autoimmune diseases, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) that have a major B-cell component further support a role for B-cell immunity in ACVD.<sup>12</sup>

The pathophysiological processes and remodelling of the atherosclerotic lesions can eventually culminate in the rupture or erosion of the plaque and subsequent occlusive atherothrombosis, which results in clinical events, such as acute myocardial infarction (MI). This leads to ischaemic injury and cardiomyocyte death, as well as the release of pro-inflammatory factors that cause the activation and infiltration of immune cells.<sup>13</sup> In addition to the recruitment of innate immune cells, such as monocytes and neutrophils, there is a growing appreciation of the potential contribution of lymphocytes to the modulation of ischaemic myocardial injury.<sup>14</sup> Importantly, ischaemic cell death following prolonged vascular obstruction or cellular damage upon ischaemic-reperfusion (I/R) injury after restoration of blood flow often leads to substantial fibrosis and cardiac remodelling that can eventually result in heart failure, which may also be partially driven by deregulated inflammatory responses.<sup>15</sup>

B cells play important roles in adaptive as well as innate immunity. Their main functions are the generation of humoral immunity in the form of antigen-specific antibodies, antigen presentation, and co-stimulation of T cells as well as production of cytokines, allowing the regulation of other immune cells. Several B-cell subsets with different functional properties exist in mice and humans. B cells can broadly be divided into innate-like B1 cells, which are made up of B1a and B1b cells, and B2 cells, which can differentiate into marginal zone (MZ) cells and follicular (FO) B cells<sup>16</sup> (Figure 1). While the differential contribution of these different B-cell subsets in atherosclerosis has been extensively addressed,<sup>8–10,17</sup> recent studies provided critical novel insights into their regulation and role of antibody-mediated effector functions in ischaemic cardiovascular disease, which will be the focus of this review.

## 2. B cell functions

### 2.1. B-cell receptor and signalling

The expression of B-cell receptors (BCRs) allows B lymphocytes to recognize a vast range of antigens. Somatic recombination and hypermutation during B-cell development yield a highly varied BCR repertoire that is estimated to consist of more than  $10^{11}$  distinct specificities.<sup>18</sup> Whether the BCR repertoire is altered in the context of ACVD and how it modulates disease is unknown.

BCR engagement and crosslinking results in proliferation, actin remodelling, receptor-mediated endocytosis, antigen processing, and the induction of B-cell effector functions. Given the central role of BCR signalling in regulating B-cell development, differentiation, tolerance, and activity, the exquisite fine-tuning of the BCR signalling threshold by co-receptors is essential to ensure an efficient B-cell response while preventing autoimmunity. For example, the co-receptor complex made up of CD19, CD21, and CD81 greatly enhances BCR signalling upon crosslinking, while inhibitory receptors, such as FcγRIIb, Siglec-G, and CD22 dampen BCR signalling. There is also crosstalk with other pathways operating in B cells, such as toll-like receptor (TLR) or B-cell-activating factor receptor (BAFFR)-signalling.<sup>19</sup> It is currently not understood if and how the

atherosclerotic environment, including dyslipidaemia and the presence of pro-inflammatory DAMPs, could affect BCR signalling.

Immunoglobulins represent the secreted form of BCRs and consist of a linked pair of identical heavy and identical light chains, which contribute to the antigen-binding site that is defined by complementarity-determining regions (CDRs) of the variable (V) regions. The less variable constant (C) regions of heavy chains ( $C_H$ ) determine the isotype and effector function of the secreted immunoglobulin, which can be either IgM, IgG, IgA, IgE or IgD, with the latter not typically occurring as a secreted form. Light chain constant ( $C_L$ ) regions are made of  $\lambda$  or  $\kappa$  segments, although their functional distinction is unknown.<sup>18</sup>

### 2.2. Humoral response

Immunoglobulins exert much of their function via the terminal part of their  $C_H$  domains called Fc region, which differ between antibody isotypes. Antibodies can mediate several effector functions depending on their isotype and the antigen they bind, including direct neutralization, complement-dependent clearance, opsonization and Fc-mediated uptake, cellular activation, induction of lytic cell death, and immune regulation.<sup>20</sup>

Fc receptors, which often consist of an intracellular  $\gamma$ -signalling domain and an Fc-binding  $\alpha$ -chain with differing affinities for different isotype subclasses, are expressed in various combinations on cells and typically determine the effects of antibodies on effector cells they bind.<sup>20</sup>

Various antibody effector functions have been described in ACVD depending on the isotype and antigens that are recognized (Figure 2).

#### 2.2.1 IgM

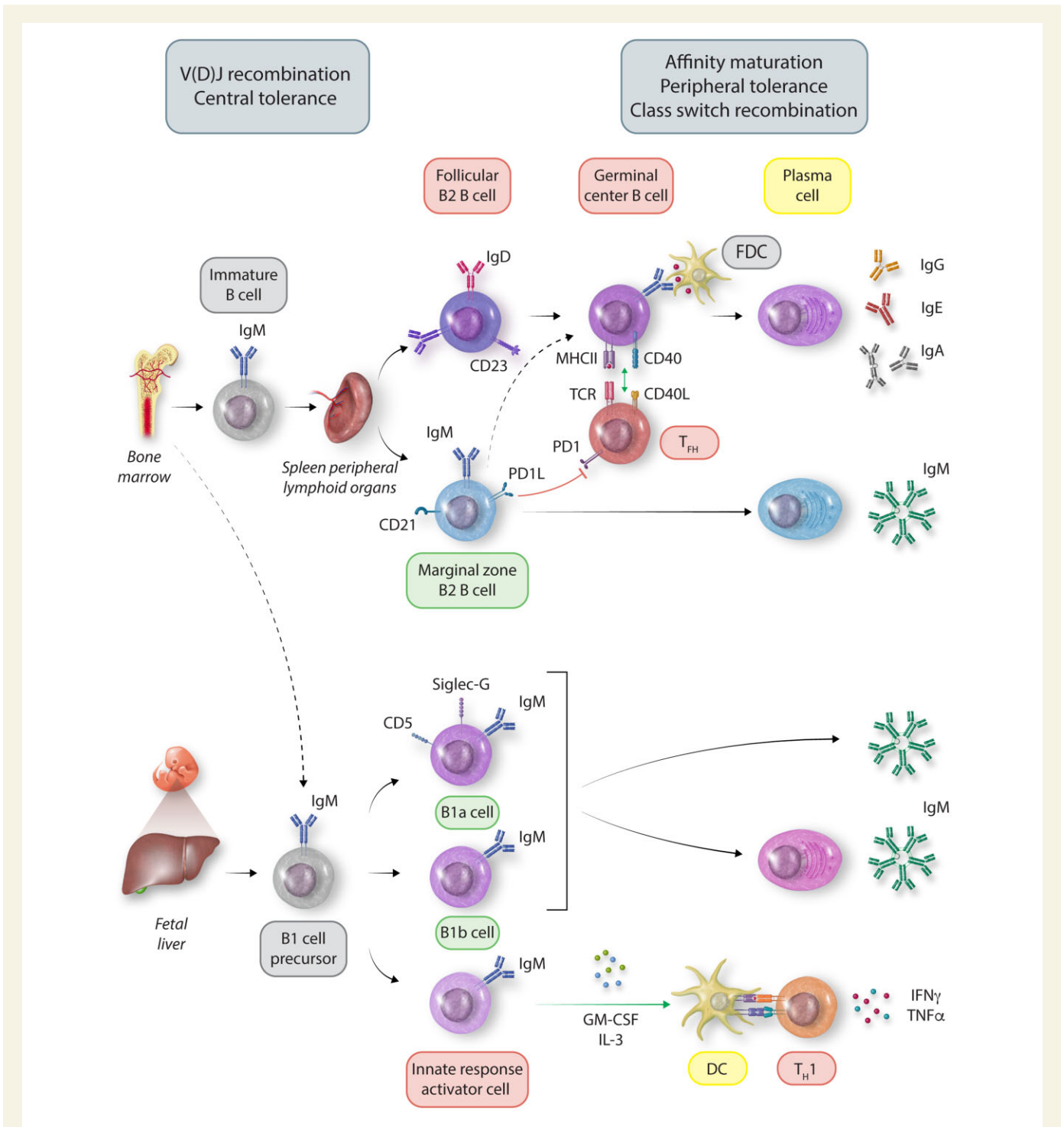
IgM antibodies usually form the first-line humoral response that occurs before affinity maturation. Therefore, secreted IgM (sIgM) are often of low affinity. Nonetheless, their usually pentameric form endows IgM antibodies with high avidity particularly to multivalent antigens, such as pathogen-associated molecular patterns (PAMPs) and DAMPs often composed of repetitive motifs or repetitive adducts, as in OSE. The recognition of such motifs appears to be essential, as they represent the most prevalent targets of so-called natural antibodies (NAbs).<sup>21</sup> These are germline-encoded antibodies that recognize highly conserved exogenous and endogenous (neo-)antigens and are constitutively produced by B1 cells, usually in the absence of foreign antigen exposure. Most IgM is derived from innate-like B1 or marginal zone B cells rather than follicular B cells.<sup>22</sup>

Most IgM antibodies are unable to diffuse outside the bloodstream, although they can be transferred to mucosal surfaces via polymeric immunoglobulin receptor (pIgR) that also binds IgA. The  $Fc\alpha/\mu R$  binds IgA and IgM and allows receptor-mediated endocytosis. The  $Fc\mu R$ , also known as Toso or Fas apoptotic inhibitory molecule 3 (FAIM3), was only recently identified as a receptor for IgM and likely plays a role in regulating B-cell activity and differentiation.<sup>22</sup>

IgM can act as a potent neutralizing antibody. Moreover, IgM strongly binds C1q, allowing it to activate the classic complement cascade and to facilitate target cell lysis or opsonization and clearance, which also occurs by direct IgM surface binding. Interestingly, IgM may also play a role in the regulation of BCR signalling and B-cell differentiation, as mice lacking sIgM show altered B-cell subset distributions.<sup>22,23</sup>

#### 2.2.2 IgG

IgG is the most prevalent antibody in the circulation. Several subclasses exist in mice (IgG1, IgG2a/b, IgG2c, and IgG3) and humans



**Figure 1** Diversity and development of B cells that may modulate ASCVD. Bone marrow-derived B-cell progenitors undergo BCR somatic V(D)J recombination and induction of central tolerance in the bone marrow, then differentiate to follicular (FO) or marginal zone (MZ) B2 cells in the spleen. FO B cells can undergo germinal center (GC) reactions upon interaction with T follicular helper ( $T_{FH}$ ) cells and follicular dendritic cells (FDC) in GCs, where affinity maturation, peripheral tolerance induction, and often class switch recombination yield highly specific antibodies. Differentiation to antibody-secreting plasma cells can occur. MZ B cells often secrete IgM but can also class switch and enter GC reactions. Moreover, MZ B cells can regulate  $T_{FH}$  cell activity. In contrast to B2 cells, B1 cells generally derive from foetal progenitor cells and self-renew but can in some conditions differentiate from bone marrow-derived progenitors. B1a and B1b cells can secrete natural antibodies (NAbs), typically of IgM isotype, or differentiate to usually IgM+ plasma cells. B1-derived innate response activator (IRA) B cells secrete GM-CSF and IL-3, activating DCs and thereby inducing Th1 responses. Cell types with proposed proatherogenic functions are shown in red, atheroprotective in green, controversial/mixed in yellow, unknown in grey.

(IgG1-4), and there is additional heterogeneity in their effector functions. Most IgGs have high antigen-neutralizing capacity. Some subtypes can act as strong opsonins, either directly or by recruitment of complement, potentially inducing complement-dependent cytotoxicity (CDC) or phagocytic engulfment. Moreover, some IgGs can induce antibody-dependent cellular cytotoxicity (ADCC) via NK cells.<sup>18</sup> The latter functions may be particularly relevant for the activity of autoantibodies targeting stressed endothelial cells in the context of ACVD.<sup>24,25</sup>

Fc $\gamma$ Rs can be subdivided into activating and inhibitory receptors. In mice, the activating receptors are Fc $\gamma$ R1b, Fc $\gamma$ R1c, and Fc $\gamma$ R1d, while in humans, they are Fc $\gamma$ R1, Fc $\gamma$ R1a, Fc $\gamma$ R1c, Fc $\gamma$ R1d, and Fc $\gamma$ R1e. Activating Fc $\gamma$ Rs typically contain immunoreceptor tyrosine activating motifs (ITAMs) that crosstalk with other signalling pathways, while the sole inhibitory receptor in mice and humans, Fc $\gamma$ R1b, carries inhibitory motifs that counteract activating signals. While most cells express combinations of different receptors, B cells express only the inhibitory Fc $\gamma$ R1b. Most Fc $\gamma$ Rs bind different IgG subclasses with different affinities and often require multiple IgGs bound by antigen to induce efficient activation. In addition to IgG, Fc $\gamma$ Rs can also bind pentraxins, such as C-reactive protein (CRP). Other Fc $\gamma$  receptors include the neonatal receptor Fc $\gamma$ Rn involved in regulating IgG half-life and the intracellular Fc $\gamma$ -binding E3 ligase tripartite motif-containing 21 (TRIM21).<sup>26</sup> Recent preclinical studies shed light on the complex role of different Fc $\gamma$ R in atherosclerosis.<sup>27</sup>

### 2.2.3 IgA

IgA is the most abundant antibody on mucosal surfaces and the second most abundant antibody in the circulation. IgA exists in two subclasses as a monomeric (usually IgA1) and a dimeric form (usually IgA2), the latter being more common in mucosa. IgA can be actively transported via pIgR, which is required for the transfer of IgA from class-switched IgA+ plasma cells residing in the lamina propria to the luminal mucosal surface of the gut.<sup>18</sup>

IgA acts primarily as a neutralizing antibody (e.g. for bacterial toxins at mucosal barriers), as it only displays very weak opsonizing and complement-engaging abilities. In addition to Fc $\alpha$  $\mu$ R, IgA can act via the Fc $\alpha$ RI (CD89) expressed on many cells including macrophages, dendritic cells (DCs) and Kupffer cells, although its absence in mice has hampered its extensive study. The Fc $\alpha$ RI receptor can mediate phagocytosis and potentially the induction of cell death.<sup>20</sup>

### 2.2.4 IgE

Although IgEs are the least abundant immunoglobulins, they are biologically potent antibodies implicated in allergic responses and parasitic infections. IgEs are present in the circulation, mucosa, and extracellular spaces, where they exert strongly pro-inflammatory functions primarily by binding Fc $\epsilon$ RI receptors on mast cells. This results in their activation, degranulation, and release of pro-inflammatory mediators, such as histamines, IL-6, TNF $\alpha$ , and lipid mediators including prostaglandin D2.<sup>28</sup>

A unique feature of IgE is its ability to bind Fc $\epsilon$ RI with extremely high affinity even in the absence of antigen. Consequently, most IgE exists bound to the surface of mast cells or basophils, where it can rapidly induce Fc $\epsilon$ RI activation and cellular degranulation upon antigen encounter. The second receptor for IgE, Fc $\epsilon$ R2 (CD23), is a structurally unrelated C-type lectin primarily expressed by B cells (follicular and transitional) and eosinophils. It may play a role in the regulation of IgE synthesis and half-life.<sup>28</sup>

## 2.3 Crosstalk with other immune cells

B cells can act as antigen-presenting cells (APCs) to T cells. However, compared to traditional APCs, such as DCs, antigen-presenting abilities of B cells and thus potentially their importance in driving T cell effector functions are limited. Nonetheless, antigen presentation by B cells is essential in driving the interaction between B cells and their cognate T follicular helper (T<sub>FH</sub>) cells in the context of germinal center (GC) reactions.<sup>29</sup>

Upon B-cell activation, B cells take up and process antigen presented on follicular dendritic cells (FDC) via BCR-mediated endocytosis. Presentation of the peptide fragments to T cells via major histocompatibility complex II (MHCII), along with co-stimulatory receptors such as CD40L or inducible T cell costimulator (ICOS), activates cognate T cells and drives their interaction and GC reaction. It is not clear whether antigen presentation by B cells is physiologically important in T cell activation outside the GC.

B cells can secrete various cytokines, some of which may regulate other immune cells and modulate atherosclerosis. For example, some B cells secrete the immunosuppressive cytokines IL-10, IL-35, and TGF $\beta$ . Conversely, they can produce pro-inflammatory cytokines, such as TNF $\alpha$  and IL-6.<sup>30</sup> A specialized subset of innate B cells can secrete cytokines and growth factors that modulate innate immune cell activity.<sup>31</sup> Moreover, B cells can secrete chemokines, such as CCL7 that are involved in the mobilization of immune cells including monocytes in certain conditions.<sup>32</sup> Importantly, B cells may be instrumental in the induction of lymphoid organ structures including tertiary lymphoid organs (TLOs) via the expression of lymphotoxin  $\alpha$ 1 $\beta$ 2.<sup>30</sup>

## 3. B-cell subsets

In analogy to other leukocytes, also different B-cell subsets have been identified based on their origin, anatomical localization, surface marker expression and functions. While the majority of these subsets were characterized in mice due to the difficulty studying the human system, similar cell types were found in humans.

### 3.1 B1 cells

B1 B cells are innate-like B cells that are subdivided into B1a and B1b cells, which are distinguished by their expression of CD5 (CD5<sup>+</sup> B1a, CD5<sup>-</sup> B1b). Moreover, they may differ in their developmental origins, antibody-secreting abilities, and repertoire, although further studies are needed to address the functional heterogeneity and the mechanisms by which antibody secretion is regulated in these cells.<sup>33–35</sup> The primary function of B1 cells is the production of germline-encoded NABs, which are mostly IgM typically of low affinity and broad reactivity.<sup>10,36</sup> Notably, there is increasing evidence that the diversity of the NAb repertoire is altered in the context of ageing<sup>37,38</sup> and in a niche-specific manner,<sup>34,35,38,39</sup> raising the possibility that this may be further affected by hyperlipidaemia or disease. The functional relevance of these repertoire shifts remain to be addressed.

Although B1 cells are well described in mice, their existence in humans is more controversial. Their possible human equivalent, a CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD70<sup>-</sup> B-cell subset, was found in umbilical cord and peripheral blood and is characterized by the spontaneous secretion of germline-like IgM antibodies in a T-cell independent (TI) manner, a skewed BCR repertoire, and tonic intracellular signalling.<sup>40</sup> More recent data suggests considerable phenotypic diversity in the putative human B1 cell pool, which is associated with the ability to secrete antibodies, their

antigen specificity, cytokines production, and crosstalk with other immune cells.<sup>35,41,42</sup>

### 3.2 Follicular B cell

FO B2 cells make up the majority of circulating B cells. They are largely responsible for the generation of T-cell dependent antigen-specific immune responses, where B cells differentiate into GC B cells and undergo affinity maturation and class switching with the help of T<sub>FH</sub> cells. GC B cells can further differentiate into plasma or memory B cells, which specialize in the secretion of vast amounts of antibody or the generation of a rapid immune response upon secondary encounter of an antigen, respectively. In humans, FO B are generally described as B220<sup>+</sup>CD27<sup>+</sup> cells<sup>16,43</sup> (Table 1).

### 3.3 Marginal zone B cell

Like FO B cells, MZ B cells arise from bone marrow (BM)-derived transitional B2 cells. In mice, they are retained in the spleen, where they reside in the outer white pulp adjacent to the marginal sinus. This strategic localization enables MZ B cells to rapidly respond to blood-borne pathogens passing through the red pulp and to shuttle antigens to follicles. MZ B cells can mount T cell-dependent and -independent responses. Thus, they can also give rise to plasmablasts that generate antibodies of different isotypes. Human MZ B cells resemble memory B cells and are not anatomically restricted to the spleen.<sup>16,43</sup>

### 3.4 Other minor B-cell subsets

B cells, such as B-regulatory cells (B<sub>reg</sub>) are functionally defined rather than based on their cellular origin. They secrete anti-inflammatory cytokines including IL-10, IL-35, and TGFβ that are able to induce immunosuppressive T<sub>regs</sub>.<sup>30</sup> In humans, although there is considerable heterogeneity, many types of B<sub>regs</sub> are commonly defined as expressing CD19<sup>+</sup>CD24<sup>hi</sup> while secreting high amounts of IL-10.<sup>43</sup> Innate response activator (IRA) B cells form another small subset of innate-like B1-derived B cells. They are characterized by the secretion of granulocyte-monocyte colony stimulating factor (GM-CSF) and IL-3, which allows them to modulate innate and adaptive immune cell functions.<sup>31</sup>

## 4. B-cell development

### 4.1 Early B-cell development and BCR assembly

B cells develop from common lymphoid progenitors in the BM, where through the activity of Recombination Activating genes 1 and 2 (RAG1 and RAG2), somatic V(D)J recombination of the variable (V), diversity (D), and joining (J) gene segments of the heavy and light chain takes place. Terminal deoxynucleotidyl transferase (TdT) further increases BCR diversity by random alteration of nucleotides at the gene segment junctions.<sup>44</sup>

Given the vast diversity created, some receptors inevitably show binding specificity for self-antigens. Such self-reactive developing B cells are negatively selected and may undergo further receptor editing, clonal deletion in the form of apoptosis, or enter a state of unresponsiveness known as anergy (central tolerance). To ensure no autoreactive B cells survive or are generated during subsequent maturation steps, B cells undergo another round of negative selection in secondary lymphoid organs during the induction of peripheral tolerance. Importantly, if the antigen is not encountered or is present at too low concentrations in primary or secondary lymphoid organs to activate the BCR, B cells may adopt a state of 'immunological ignorance'. This can result in activation of B-cell clones upon encounter of these antigens, which might occur upon disruption of homeostasis or following the generation of neo-epitopes.<sup>45</sup>

### 4.2 B-cell differentiation

Immature B cells exit the BM and migrate to the spleen as transitional B cells, where they differentiate into MZ or FO B2 cells. This fate decision is primarily driven by BCR signal strength, but is likely also influenced by other pathways, such as Notch2, BAFFR, NF-κB, and TLR signalling.<sup>16,19,23,46</sup> Furthermore, a role for BCR specificity in driving B-cell differentiation has been suggested.<sup>47</sup> For example, self-reactivity drives positive selection in B1 cells, which may account for the high proportion of self-reactive clones found within the natural IgM repertoire.<sup>36</sup> It is currently not clear if and how the crosstalk and potential synergistic signalling between pathways may be altered in the context of atherosclerosis or dyslipidaemia.

Unlike B2 cells, which arise from the BM throughout life, B1 cells are generally thought to derive from foetal progenitors and persist by self-renewal. Although it has been suggested that B1 cells can arise from BM-derived cells in some settings, it appears that this process is slow, and BM-derived B1 cells may not fully recapitulate the function of *bona fide* B1 cells.<sup>36</sup> This is important to take into consideration in the

**Table 1** Surface markers of murine B-cell subsets and their potential human equivalents

Cellular subset	Surface markers (mouse)	Surface markers (possible human equivalent)
Immature B cells	CD19 <sup>+</sup> IgM <sup>hi</sup> IgD <sup>+</sup> CD93 <sup>+</sup>	CD19 <sup>+</sup> CD20 <sup>+</sup> CD27 <sup>+</sup> CD24 <sup>hi</sup>
B1a cell	CD19 <sup>+</sup> B220 <sup>int</sup> IgM <sup>hi</sup> IgD <sup>low</sup> CD23 <sup>low</sup> CD11b <sup>+</sup> CD43 <sup>b</sup> CD5 <sup>+</sup>	CD20 <sup>+</sup> CD27 <sup>+</sup> CD43 <sup>+</sup> CD70 <sup>-</sup>
B1b cell	CD19 <sup>+</sup> B220 <sup>int</sup> IgM <sup>hi</sup> IgD <sup>low</sup> CD23 <sup>low</sup> CD11b <sup>+</sup> CD43 <sup>b</sup> CD5 <sup>-</sup>	
Follicular B cell	CD19 <sup>+</sup> B220 <sup>+</sup> CD43 <sup>-</sup> IgM <sup>low</sup> IgD <sup>hi</sup> CD23 <sup>+</sup> CD21 <sup>int</sup> CD1d <sup>-</sup>	CD19 <sup>+</sup> CD20 <sup>+</sup> IgD <sup>+</sup> CD27 <sup>+/-</sup>
Marginal zone B cell <sup>c</sup>	CD19 <sup>+</sup> B220 <sup>+</sup> CD43 <sup>-</sup> IgM <sup>hi</sup> IgD <sup>low</sup> CD23 <sup>-</sup> CD21 <sup>hi</sup> CD1d <sup>hi</sup>	IgM <sup>+</sup> IgD <sup>+</sup> CD27 <sup>+</sup> CD1c <sup>+</sup> CD21 <sup>high</sup> CD23 <sup>-</sup>
Germinal centre B cell	CD19 <sup>+</sup> B220 <sup>+</sup> GL7 <sup>+</sup> CD95 <sup>+</sup>	
Plasma cell	CD19 <sup>+</sup> B220 <sup>-</sup> CD138 <sup>+</sup>	IgD <sup>-</sup> CD27 <sup>hi</sup> CD38 <sup>hi</sup> CD138 <sup>+</sup>
Plasma blast	CD19 <sup>+</sup> B220 <sup>+</sup> CD138 <sup>+</sup>	IgD <sup>-</sup> CD27 <sup>+</sup> CD38 <sup>hi</sup> CD138 <sup>-</sup>
Memory B cell <sup>c</sup>	CD19 <sup>+</sup> B220 <sup>+</sup> CD38 <sup>+</sup> CD73 <sup>+</sup> CD80 <sup>+</sup>	IgD <sup>-</sup> CD20 <sup>+</sup> CD27 <sup>+</sup>

<sup>a</sup>Expression in peritoneal cavity.

<sup>b</sup>Expression in spleen.

<sup>c</sup>Marginal zone B cells in humans potentially similar to memory B cells<sup>16</sup>.

experimental design of murine studies, where the use of BM transplantation is particularly common in the study of immune-related pathways.

### 4.3 The germinal center reaction and the generation of antibody-secreting cells

Upon activation by their cognate antigens, B cells can undergo proliferation and differentiate into antibody-secreting cells. Early responses often include extrafollicular responses and the formation of plasmablasts that allow a rapid, low-affinity antibody response. However, some activated B cells in follicles can enter GC responses,<sup>46</sup> which have recently been shown to be critical in the modulation of atherosclerosis.<sup>48,49</sup>

This process takes place in secondary lymphoid tissues. GCs give rise to highly specific antibodies in a process termed affinity maturation, which is mediated by activation-induced cytidine deaminase (AID) that induces somatic hypermutation in the CDRs of antigen-binding sites of immunoglobulin V regions. GCs are interspersed with T<sub>FH</sub> and FDC. FDC efficiently present antigens that can be bound by the BCR and internalized for antigen processing and presentation on their MHCII to T<sub>FH</sub> cells recognizing the linked antigens. T<sub>FH</sub> cells also present co-stimulatory molecules, such as CD40L, to B cells, which further induces their activation. As the B cells compete both for antigen on FDCs and for T<sub>FH</sub> help, only those B cells with high-affinity immunoglobulin receptors will survive and potentially undergo further rounds of proliferation and affinity maturation during cyclic re-entry. This process is termed clonal selection.<sup>46</sup>

In the GC, B cells can undergo isotype switching following activation. This class switch recombination (CSR) is another somatic recombination process driven by AID. It is thought to be induced by a combination of signals including BCR signalling, co-stimulatory pathways, such as CD40, TLRs as well as cytokines, which appear to be instrumental in the induction of different isotypes.<sup>19,46</sup>

Following affinity maturation, cells eventually exit GCs as memory B or plasma cells. This fate decision depends on BCR signalling and T-cell help.<sup>19,46,50,51</sup> Memory B cells represent a quiescent long-lived population that upon antigen encounter rapidly differentiates into antibody-secreting plasma cells.<sup>51</sup>

Plasma cell fate is dependent on a down-regulation of the transcription factors Paired Box 5 (PAX5) and B-cell lymphoma protein 6 (BCL6) and an induction of the B-lymphocyte-induced maturation protein-1 (BLIMP1; encoded by *Prdm1*), Interferon regulatory factor 4 (IRF4), and X-box binding protein 1 (XBP1) transcription factors. These allow plasma cells to adapt their cellular machinery to synthesize and secrete vast amounts of antibody. At the same time, plasma cells up-regulate the expression of CXCR4, which facilitates migration to the BM where they can become long-lived plasma cells that continuously secrete antibodies.<sup>19,35,51</sup>

Notably, some antigens activate B cells in a TI manner, which generally does not involve GC reactions. TI-1 responses are driven by TLR ligands, while TI-2 antigens typically comprise highly repetitive structures, such as OSE. B1 cells and MZ B cells play prominent roles in these TI responses, which involve mostly IgM and sometimes IgG3.<sup>19</sup>

## 5. B cells in ischaemic cardiovascular disease

### 5.1 The role of B cells in atherosclerosis and MI

Early immunohistological characterizations of atherosclerotic aortas reported the presence of B cells in the adventitia, but not in

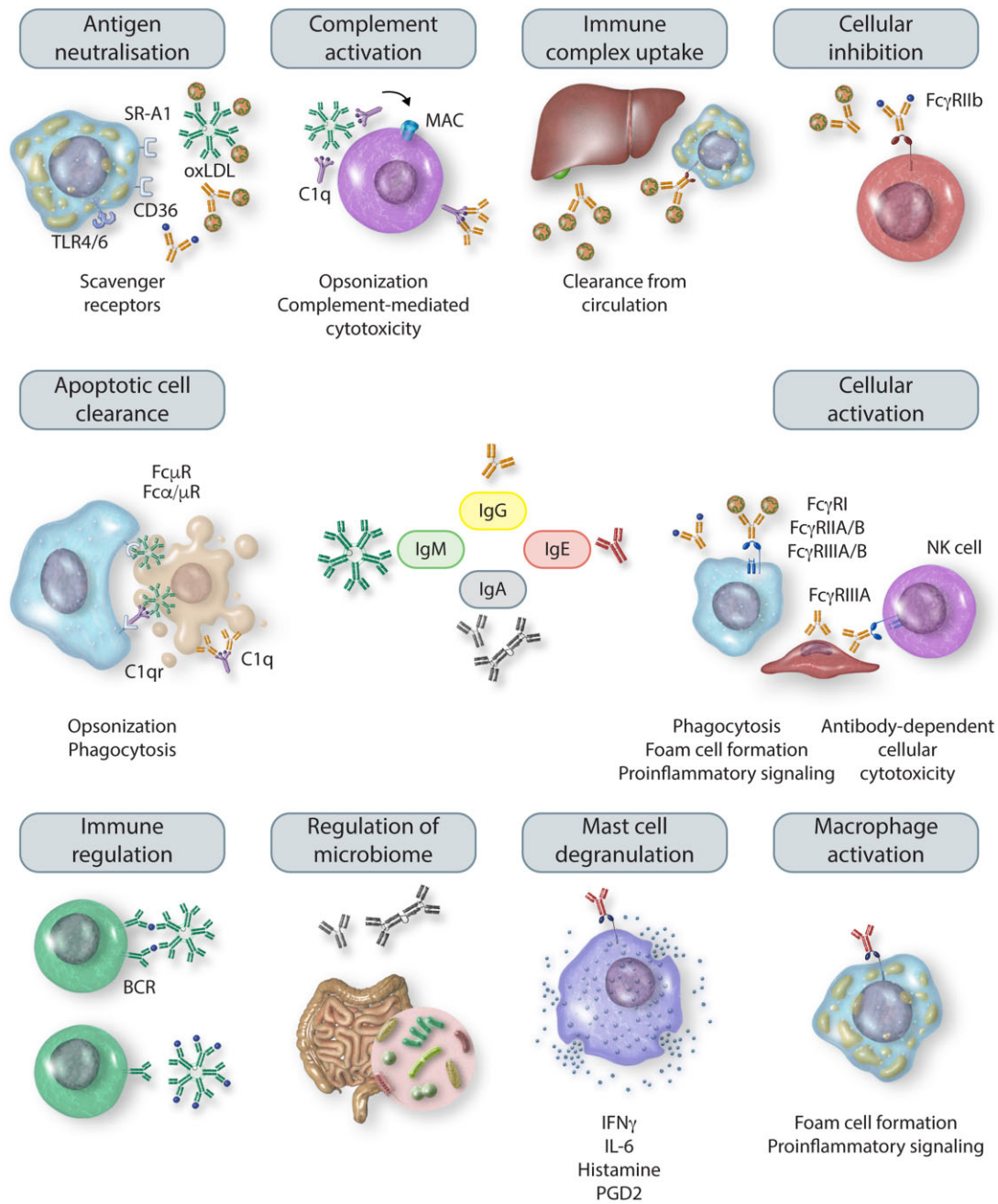
atheromatous plaques.<sup>52</sup> Consistent with this, it was found that only 1% of intimal cells of coronary atherectomy specimens are B cells in contrast to T cells (11%) or monocytes (12%).<sup>53</sup> B cells are typically found in lymphoid follicles containing T cells, DCs, and macrophages in the adventitia surrounding atherosclerotic aortas, suggesting local immune responses.<sup>54</sup> A recent examination of 246 human coronary plaques revealed increased numbers of B cells in perivascular adipose tissue that correlated with the extent of obstruction.<sup>55</sup> Interestingly, B cells in human perivascular adipose tissues also include CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B cells, the previously proposed human equivalent of B1 cells.<sup>56</sup> However, analyses of infiltrating B cells of human carotid plaques demonstrated predominance of B2-like CD20<sup>+</sup> activated plasmablasts.<sup>57</sup> Notably, resident B cells exhibited frequently IgA or IgG isotypes, inverted  $\lambda/\kappa$  light chain ratios and limited sets of hypermutated V<sub>H</sub> regions, indicative of oligoclonal antigen-driven B-cell responses.

Several studies assessed the involvement of B-cell immunity in ACVD by studying the association of different circulating B-cell subsets with the extent of disease and/or cardiovascular events. For example, higher frequencies of CD40<sup>+</sup>CD19<sup>+</sup> B cells were associated with a higher stroke event-free survival, while numbers of CD86<sup>+</sup>CD19<sup>+</sup> B cells were associated with higher risk for acute cardiovascular events.<sup>58</sup> Higher levels of unswitched (IgD<sup>+</sup>) and switched (IgD<sup>-</sup>) CD27<sup>+</sup> memory B cells were associated with lower risk for cardiovascular events in patients undergoing carotid atherectomy.<sup>59</sup> Unswitched memory B cells of these patients also had higher surface IgM expression, suggesting increased IgM production. In another study, CXCR4 expression on CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B cells correlated with potentially protective IgM against malondialdehyde (MDA)-LDL levels in serum and was inversely associated with plaque burden.<sup>35</sup> While these analyses are restricted to B cells in the circulation and may not reflect antigen-specific cells responding to plaque antigens, they shed light on the association of certain B-cell functions with ACVD.

We and others have recently reviewed the functional role of B cells in the pathogenesis of atherosclerosis in detail<sup>8–10</sup> (Figure 1). First experimental evidence came from Caligiuri *et al.*<sup>60</sup> and Major *et al.*<sup>61</sup> who found that splenectomy-induced<sup>60</sup> and genetic B-cell deficiency<sup>61</sup> associated with increased atherosclerosis in mice, which was reversed by adoptive splenic B-cell transfer.<sup>60</sup> Subsequent studies have dissected the effects of individual B-cell subsets and effector functions.

B1 cells are considered the primary mediators of the atheroprotective effects of B cells. Increases in B1 cells and IgM ameliorate atherosclerosis in murine studies while their reduction exacerbates atherosclerosis.<sup>8–10</sup> Both B1a cells and B1b cells are now recognized to have beneficial effects in atherogenesis, and their protective effects depend on the presence of sIgM.<sup>62,63</sup> B2 cells were initially viewed to be proatherogenic after preferential B2-cell depletion using CD20-targeted antibodies<sup>64,65</sup> or by targeting the BAFF<sup>66,67</sup> pathway reduced atherosclerosis. However, recent reports suggest MZ B cells can exert protective effects by regulating T<sub>FH</sub> cells<sup>68</sup> and potentially by secreting IgM.<sup>69</sup> In contrast, FO B cells are considered proatherogenic potentially due to their ability to undergo GC reactions and form class-switched plasma cells.<sup>49,70</sup> Interestingly, such T-cell dependent B-cell responses can also arise in adventitial TLOs (ATLOs), which form around advanced atherosclerotic lesions and have been proposed to have atheroprotective functions.<sup>71</sup>

In contrast to the arterial intima, a large proportion of cardiac leukocytes are B cells,<sup>72</sup> suggesting a role in cardiac homeostasis<sup>73</sup> and their involvement in the response to injury following MI. B cells in the heart and pericardial adipose tissue appear heterogeneous, encompassing B2-like, B1a-like, B1b-like, and GM-CSF-secreting IRA B cells.<sup>72,74</sup> Upon myocardial damage, there is an influx of B cells into cardiac tissue within 1–7 days



**Figure 2** Antibody effector functions and their potential functions in ACVD. Different isotypes (IgM, IgG, IgE, and IgA) have different functions. Most antibodies can cause antigen neutralization. For example, IgM (and IgG) binding of oxLDL can neutralize its TLR-dependent pro-inflammatory properties and blocks scavenger receptor-mediated foam cell formation. Opsonization with antibodies can lead to receptor-mediated phagocytic engulfment by Fc- and complement-mechanisms resulting in different cellular responses. Complement activation can also induce CDC. Immune complex formation, e.g. with oxLDL, may facilitate their clearance from the circulation. Antibodies can also activate or in rare cases inhibit cellular activation via Fc receptors. NK cell activation can cause ADCC. IgE can activate macrophages or mast cell degranulation and pro-inflammatory mediator release. IgA may play a role in modulating the microbiota. Antibodies, particularly IgM, may regulate BCR signal strength by antigen sequestration or crosslinking.

of MI, although the temporal dynamics and localization of the cells differed slightly between models.<sup>9,32,74,75</sup> Zougari *et al.*<sup>32</sup> were the first to show a functional, potentially detrimental role for B cells in acute MI using *Baffr*<sup>-/-</sup> and anti-CD20- or anti-BAFF-mediated global B2 cell depletion models. B cells were shown to induce CCL7-mediated inflammatory monocyte recruitment upon MI, with B2 cell depletion resulting in reduced myocardial injury and improved cardiac function.<sup>32</sup> In agreement, Horckmans *et al.*<sup>74</sup> found that using *Cb2*<sup>-/-</sup> mice with increased B-cell numbers exacerbated cardiac fibrosis, which may be driven by the accumulation of GM-CSF producing IRA-like B cells inducing DC and subsequent T cell expansion in pericardiac adipose tissue. Antibody-mediated depletion of global B cells or GM-CSF neutralization abrogated this effect.<sup>74</sup> Furthermore, it was recently shown that the cardioprotective effects of the anti-inflammatory drug pirfenidone may be partially mediated by reductions in cardiac B cells in a murine model of ischaemia-reperfusion injury.<sup>72</sup> In contrast to these studies, it was shown that B cells preserved cardiac function and reduced cardiomyocyte apoptosis in rats receiving intramyocardial injections of different BM mononuclear cell fractions isolated from isogenic rats after coronary artery ligation-induced MI.<sup>76</sup>

While these studies establish a role for B cells in modulating the response to MI in general, the role of B cells that localize to the heart during myocardial injury is not fully understood. Interestingly, a recent study investigating the effect of ablation of cardiotropic CXCR5<sup>+</sup> B cells via CXCL13 neutralization or CXCR5 deficiency showed no effect on cardiac function following MI,<sup>77</sup> suggesting that the previously observed effects of B-cell depletion on cardiac function may be mediated primarily by systemic effects, such as the production of soluble factors or antibodies. In line with this, MI is accompanied by an expansion in heart-draining mediastinal lymph nodes in mouse and rat MI models, which is associated with increased GC formation, class switching, and oligoclonal B-cell expansion,<sup>77,78</sup> indicating antigen-driven responses to myocardial injury. Indeed, the increased prevalence of autoantibodies against cardiac proteins, such as cardiac myosin and troponin I during MI-associated heart failure is well established.<sup>78–80</sup> Moreover, an unbiased screen using 56-day post-MI plasma of mice also identified IgG antibodies with reactivity for myocardial scar tissue.<sup>81</sup> The identification of further antigens driving such responses and their impact on cardiac remodelling following MI are an active area of research. While global antibody deficiency was found to improve adverse cardiac remodelling and preserve cardiac function in preclinical studies using mice deficient in sIgM and AID,<sup>81</sup> the role of individual antibody subsets or of antibodies recognizing specific epitopes is less clear. *Rag1*<sup>-/-</sup> mice with global B- and T-cell deficiency or *Cd21*<sup>-/-</sup> that have a proposed altered IgM repertoire develop reduced infarct sizes following myocardial I/R injury, which is abrogated by the passive administration of wild type mouse-derived pooled polyclonal IgM,<sup>82</sup> suggesting that IgM can mediate myocardial damage. B-cell-derived soluble factors other than antibodies may also contribute to the role of B cells in the response to MI and reperfusion injury. For example, B-cell-specific deletion of IL-10 was found to exacerbate myocardial injury in a murine MI model, which may be mediated by the modulation of the resolution of inflammation by IL-10-producing pericardiac adipose tissue-resident CD5<sup>+</sup> B cells.<sup>83</sup>

Intriguingly, B cells may also exacerbate MI-accelerated atherosclerosis, a process that has been shown to be dependent on monocyte accumulation. Kyaw *et al.*<sup>84</sup> suggest that increased atherosclerosis following MI is primarily driven by B cells due to the induction of autoreactive B-cell memory resulting in vascular damage and atherosclerosis development. Anti-CD20-mediated B2 cell depletion abrogated the increased

lesion formation, while adoptive transfer of B cells from mice with previous MI greatly accelerated lesion formation in Western-diet fed *ApoE*<sup>-/-</sup> mice compared to B cells from sham-treated mice.

Below, we discuss the evidence for the roles of different B-cell effector functions, focusing primarily on B-cell-derived humoral immunity.

## 5.2 The role of humoral immunity

### 5.2.1 Ischaemic cardiovascular disease-associated antigens

In contrast to B cells, immunoglobulins are abundantly present in the intima of atherosclerotic lesions. Studies in the late 1970s documented the presence of IgM, IgG, and IgA in human intimal atherosclerotic plaque extracts using immune-electrophoresis.<sup>85</sup> Similarly, Parums and Mitchinson<sup>86</sup> described the presence of IgG and IgM in inflamed atherosclerotic plaques, proposing that they are locally produced by plasma cells present in areas of advanced lesions where the media is disrupted. Moreover, Vlaicu *et al.*<sup>87</sup> documented preferential retention of immunoglobulins in the intima suggesting direct recognition of antigens.

Autoantibodies against different types of antigens have been proposed for ACVD, including lipoprotein- and vascular wall-associated (neo-)antigens as well as classical autoantigens of autoimmunity. The possibility that patients with atherosclerosis have higher levels of circulating autoantibodies reacting with antigens present in cardiovascular tissues has been studied for decades. For example, in 1973, Golod<sup>88</sup> found that sera of atherosclerotic patients exhibited higher reactivity with affected aortic and myocardial tissue extracts. Since then, several antigens have been identified and proposed to trigger immune responses in ACVD and to represent targets of disease-modulating antibodies (Table 2).

**Table 2** Known antigens targeted by specific antibodies in ACVD

Group	Source	Antigen/epitope	Ref.
Lipoproteins	oxLDL/OSE	Copper-oxidized LDL	2
		MDA-LDL	
		PC-BSA	
		MDA-BSA	
	ApoB	Native p45 and MDA-p45	93
		Native p210 and MDA-p210	94
	ApoA1	MGO-p220	92
	ApoA1	ApoA1	150
Vascular wall	Stressed ECs	Hsp60	24
		GRP78	25
	Matrix proteins	MDA-modified collagen IV	90
		MDA-modified fibronectin	91
Others	Mitochondria	ALDH4A1	101
Classical autoantigens		CCP	105
		$\beta$ 2GP1	95,105
		CL	

OxLDL, oxidized LDL; OSE, oxidation-specific epitopes; MDA-LDL, malondialdehyde-modified LDL; PC-BSA, phosphocholine-modified BSA; ApoB, apolipoprotein B; MGO, methylglyoxal; ApoA1, apolipoprotein A-1; Hsp60, heat shock protein 60; GRP78, 78 kDa glucose-regulated protein; ALDH4A1, aldehyde dehydrogenase 4 family member A1; CCP, cyclic citrullinated protein;  $\beta$ 2GP1,  $\beta$ 2 glycoprotein 1; CL, cardiolipin.



A major amount of work has focused on antigens derived from both lipid and protein moieties of LDL and most work have focused on oxLDL. Characterization of the antigen specificity of IgG isolates from plaques showed that a significant portion binds oxLDL.<sup>89</sup> Moreover, immune complexes isolated from plaques contained apolipoprotein B (ApoB), consistent with local reactivity with modified LDL. OSE represent the immunogenic determinants of oxLDL. They are a heterogeneous group of lipid peroxidation-derived products that modify endogenous structures, resulting in the generation of neo-epitopes that can act as DAMPs, but can also be recognized by specific antibodies.<sup>2</sup> For example, oxidation of the polyunsaturated fatty acids of LDL can give rise to highly reactive degradation products that form covalent adducts with ApoB or other LDL phospholipids. The best studied OSE include malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), as well as the remaining oxidized phospholipid (oxPL)-containing phosphocholine (PC). Importantly, OSEs are also found on the surface of dying cells, cellular debris, and microvesicles, and can form adducts on other vascular proteins, such as MDA-modified collagen type IV<sup>90</sup> and fibronectin.<sup>91</sup> Antibody responses to OSE are typically studied using model antigens, such as MDA-modified LDL and copper-oxidized LDL (CuOx-LDL), but proteins, such as BSA conjugated with MDA or PC, are also used. Additionally, protein modifications with advanced glycation end products, such as methylglyoxal (MGO), have been proposed.<sup>92</sup> On the other hand, immunogenic peptides derived from ApoB, termed p45 and p210, have also been identified as antigens.<sup>93,94</sup>

Another group of antigens include classical autoantigens that are well known from autoimmune diseases, such SLE and RA. These include double stranded DNA (dsDNA), cyclic citrullinated proteins (CCP), targets of anti-phospholipid antibodies, such as  $\beta$ 2-glycoprotein 1 ( $\beta$ 2GP1) and cardiolipin (CL), and apolipoprotein A-I (Apo-AI). Some of the anti-phospholipid antibodies may however target epitopes generated by oxidative modifications.<sup>95</sup> Similarly, Apo-AI, the major protein of high-density lipoprotein (HDL), may be modified or oxidized, rendering it immunogenic and dysfunctional.<sup>96,97</sup>

Immune responses elicited by bacterial and viral antigens also modulate lesion progression. PC is present in the capsular polysaccharide of *Streptococcus pneumoniae* and several other pathogens,<sup>98,99</sup> but is also an OSE present on oxLDL.<sup>100</sup> Due to this molecular mimicry the same PC-specific antibodies can bind oxLDL, dying cells, and pneumococci. Another antimicrobial immune response cross-reactive with endogenous antigens is the response against mycobacterial heat shock protein 65 (HSP65) targeting endogenous HSP60, which is expressed by endothelial cells that were exposed to classical risk factors of atherosclerosis.<sup>24</sup> These cross-reactive antibodies have been shown to damage the endothelium.<sup>24</sup>

More recently, the 78 kDa glucose-regulated protein (GRP78) has been described as another endothelial-derived auto-antigen.<sup>25</sup> It is an ER molecular chaperone that is translocated to the cell surface in response to ER stress, e.g. triggered by hypercholesterolaemia. Antibodies to the GRP78 on the cell surface promote the expression of adhesion molecules ICAM-1 and VCAM-1 in an NF- $\kappa$ B dependent manner. *ApoE*<sup>-/-</sup> mice develop antibodies against GRP78, which is increased when mice are fed an atherogenic diet, and immunization with GRP78 induced autoantibodies that correlate with lesion size.<sup>25</sup>

Recently, Lorenzo et al.<sup>101</sup> characterized the antibody repertoire of GC B and plasma cells isolated from atherosclerotic *Ldlr*<sup>-/-</sup> mice. Of 56 unique antibodies identified, 18 showed reactivity against the plaque, and one clone, termed A12, was characterized in detail. Immunoprecipitation of plaque extracts with A12 and proteomic analysis

revealed binding to the mitochondrial matrix protein aldehyde dehydrogenase 4 family member A1 (ALDH4A1), which was found to be elevated in the plasma during atherosclerosis. Intriguingly, passive immunization with the A12 antibody resulted in reduced lesion formation and an improved lipid profile in *Ldlr*<sup>-/-</sup> mice, demonstrating potential disease-modulating activity of this antibody,<sup>101</sup> although the precise mechanisms remain to be addressed. Although not evaluated in this study, it is interesting to consider the origin of ALDH4A1 autoreactivity. Being a mitochondrial protein usually localized to the mitochondrial niche, it is possible that the increased cell death occurring in the context of atherosclerosis may expose the antigen, resulting in an autoantibody response. It is conceivable that comparable mechanisms could occur for other physiologically intracellular putative antigens.

It will be interesting to identify additional atherosclerosis-associated antigens and their role in disease development. Emerging high-throughput methods to analyse antibody repertoires on a single cell level may lead to the discovery of novel antigen specificities, which may provide us with novel tools to therapeutically target CVD in an antigen-specific manner.

Different types of antibody responses against these antigens have been found and depending on the antigen and the class of antibodies, protective and pathogenic effects occur.

### 5.2.2 IgM

Only two studies assessed the association of total IgM levels with ACVD in humans. High baseline IgM levels were found to be associated with freedom of cardiovascular events in one study,<sup>102</sup> while a nested case-control study showed no association.<sup>103</sup> In contrast, many studies have explored the association of IgM antibodies to oxLDL or OSE. Most of these studies found an inverse association of IgM antibodies to CuOx-LDL and MDA-LDL with the extent of carotid and coronary atherosclerosis and events, although not always independent of classical risk factors.<sup>104</sup> Recently, van den Berg et al.<sup>104</sup> summarized all available data on cohort, case-control, and cross-sectional studies and found a clear relationship of low anti-oxLDL IgM levels with severe CAD and—to a lesser extent—events.

A limitation of using oxLDL in serological studies is the heterogeneity of these preparations and the potential lack of reproducibility. Thus, preparations of MDA- or PC-modified proteins or peptides have been used, which enable the assessment of more defined antibody responses. For example, antibodies against MDA-modified ApoB peptides have been identified. IgM levels against MDA-modified p45 and p210 were found to be inversely associated with atherosclerosis and future events,<sup>93,105</sup> though one study found increased carotid intima media thickness (cIMT) progression in patients with high anti-MDA-p210 IgM.<sup>106</sup> Notably, some of the associations were also found when native ApoB peptides were used as antigens.<sup>94,107</sup> Moreover, low levels of IgM against the methylglyoxal-modified ApoB peptide 220 were also found to predict CV events.<sup>92</sup> Finally, low levels of anti-PC IgM have also been shown to increase the risk for CAD and events particularly in SLE patients.<sup>105,108</sup>

Based on preclinical models, there is ample evidence for atheroprotective functions of IgM. A particularly important role has been proposed for germline-encoded, primarily B1 cell-derived NABs, a large portion of which show specificity for OSE in mice and humans.<sup>21</sup> Furthermore, IgM antibody levels, particularly those directed against OSE, are further increased during atherosclerosis, possibly reflecting the increased abundance of such epitopes.<sup>109</sup>

Although it is likely that the beneficial effect of IgM is not solely due to IgMs directed against OSE, several preclinical studies have shown protective effects for IgM in general. *Ldlr*<sup>-/-</sup> mice deficient in sIgM show increased lesion formation.<sup>110,111</sup> In contrast, adoptive transfer of B1a cells to splenectomized Western-diet fed *Apoe*<sup>-/-</sup> abrogated the splenectomy-induced increase in atherosclerosis in a sIgM-dependent manner.<sup>62</sup> Infusion of polyclonal IgM reduces atherosclerosis in *Apoe*<sup>-/-</sup> mice.<sup>112</sup> Deficiency of the inhibitory receptor Siglec-G results in a selective expansion of B1 cells and an associated increase in IgM, thereby reducing atherosclerotic lesion formation and hepatic inflammation.<sup>113</sup> Furthermore, injection of apoptotic cells or phosphatidylserine-carrying liposomes mimicking apoptotic cells induces a sterile inflammatory response associated with increased IgM production and reduced atherosclerosis.<sup>69,114</sup> Similarly, injecting an antibody against the co-stimulatory molecule T-cell immunoglobulin and mucin domain-1 (TIM-1), triggers an induction of B1 cells and increased IgM levels resulting in reduced lesion formation.<sup>115</sup> Interestingly, a recent study investigating the role of antibody class switching using *Apoe*<sup>-/-</sup> *Aid*<sup>-/-</sup> mice fed an atherogenic diet for 16 weeks found these mice to have significantly elevated total and particularly MDA-LDL-specific IgM levels and reduced atherosclerotic lesion formation. High MDA-LDL-specific IgMs were also found to be associated with decreased levels of autoantibodies against self-antigens,<sup>116</sup> suggesting that OSE-specific IgM can protect from the generation of potentially proatherogenic autoantibodies.

Much of our knowledge on IgM against OSE stems from the work of Witztum and colleagues,<sup>117</sup> who isolated the antibody E06 against oxPLs of oxLDL from the spleens of atherosclerotic mice. It was later found to share the idiotype of T15 antibodies<sup>100</sup> that bind PC of the capsular polysaccharide of *S. pneumoniae* and protect from pneumococcal infections.<sup>118</sup> OSE-directed IgMs, such as E06/T15 may exert their function via neutralization of oxLDL and prevention of its uptake by macrophages via scavenger receptor-mediated mechanisms, which has been found to reduce foam cell formation.<sup>2,119</sup> Intriguingly, Que *et al.*<sup>120</sup> could recently show that the expression of the single-chain variable fragment of E06 (scFv-E06) was sufficient to strongly suppress atherosclerosis development at multiple disease stages in *Ldlr*<sup>-/-</sup> mice. Mechanistically, the scFv-E06 fragment prevented oxPL-induced inflammatory signalling and uptake by macrophages, consistent with the effect of full size E06 antibodies.<sup>119,121</sup> E06/T15 IgM were also shown to inhibit adhesion molecule expression and pro-inflammatory cytokine secretion by endothelial cells and macrophages induced by other OSE-carriers, such as apoptotic cells and microvesicles.<sup>122–124</sup> In addition, E06/T15 IgM have also been shown to promote the clearance of apoptotic cells aided by C1q,<sup>125</sup> which is a critical mechanism to prevent apoptotic cell accumulation in plaques. Similar activities have also been found for MDA-specific natural IgM.<sup>21,126,127</sup>

Experimental models resulting in increased abundance of such antibodies against OSE typically result in reduced lesion formation. The passive administration of IgM against PC reduced atherosclerosis in *Apoe*<sup>-/-</sup> in a vein-graft induced model.<sup>128</sup> Furthermore, active immunization strategies using oxLDL increased the levels of IgM directed against these epitopes, which was associated with atheroprotective effects in preclinical models, although some of these studies were likely also associated with antibody-independent T-cell-mediated protective effects.<sup>2,129</sup> Intriguingly, a similar effect was achieved by immunization of *Ldlr*<sup>-/-</sup> mice with heat-killed pneumococci, which due to molecular mimicry induced robust IgM reacting with PC and oxLDL and decreased lesion formation.<sup>98</sup> A similar finding could be reproduced using immunization with PC-conjugated KLH.<sup>130</sup>

Despite this evidence for protective functions of T15-idiotypic IgM, another study showed no benefit of administration of a T15-IgM to *Apoe*<sup>-/-</sup> mice.<sup>112</sup> Similarly, deletion of the V1 gene that is essential for the formation of T15-idiotypic antibodies in *Ldlr*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> did not result in changes in lesion formation and surprisingly showed no differences in the levels of antibodies against atherosclerosis-associated antigens including oxLDL.<sup>131</sup> This raises the possibility that the induction of IgM responses against OSE in the setting of hypercholesterolaemia<sup>109</sup> can mask the presence of exogenously administered T15-idiotypic antibodies or compensate for the lack of V1 chain-dependent T15-idiotypic antibodies. In support of this, a recent study looking at the repertoires of murine IgM-producing B1 cells found that the T15 idiotype occurred only relatively infrequently, while other PC- and oxPL-binding heavy chain CDR3 regions were much more common.<sup>34</sup> Although this study was carried out in non-atherosclerotic C57BL/6J mice, it suggests that other OSE-specific antibodies may take over and even predominate.

While OSE-IgM represent some of the most prevalent antibodies from birth, their levels are further increased by several triggers, as described above. Increases in OSE-IgM and atheroprotection associated with adoptive transfer of B1a cells into splenectomised *Apoe*<sup>-/-</sup> were dependent on the presence of TLR4 and Myeloid differentiation primary response 88, suggesting that the induction of the IgM response may be dependent on the sensing of environmental triggers by pattern recognition receptors.<sup>132</sup> Other immunomodulatory receptors, such as the B-cell inhibitory co-receptor Siglec-G, can also modulate IgM production, as deficiency in Siglec-G results in increased OSE-IgM levels and consequently reduced lesion formation and decreased necrotic core formation, consistent with a pro-efferocytic capacity of OSE-IgM.<sup>113</sup> Moreover, cytokine signalling axes can regulate the induction of atheroprotective IgM. For example, immunization with MDA-LDL has been shown to induce the secretion of the Th2-derived cytokine IL-5. Induction of IgM in this setting was abrogated in the context of IL-5 deficiency, suggesting that IL-5 represents a key factor in the induction of OSE-specific IgM.<sup>133</sup> The secretion of IL-5 can also be induced in a subset of innate lymphoid cells (ILCs) upon sensing the tissue damage-associated alarmin IL-33,<sup>134</sup> potentially providing a further link between innate immune response to cellular damage and the induction of an antibody response to OSE. Signals driving the localization and trafficking of B cells may also play important roles in the regulation of IgM levels. The chemokine receptor CXCR4 has been shown to be a critical regulator of B1a cell numbers and plasma IgM levels via the regulation of homing of B1a-derived cells to the BM.<sup>35</sup> In line with this, B-cell-specific deletion of CXCR4 resulted in strongly decreased BM B1 cell numbers and plasma IgM levels, which was associated with exacerbated atherosclerosis development.<sup>135</sup> Recently, a role for the MHC-related protein CD1d, which is primarily involved in the presentation of lipid-derived antigens to NKT cells, in the regulation of E06 has been suggested. Interestingly, deficiency of CD1d resulted in a selective increase in splenic E06-idiotype antibody-secreting cells, while global B1 cell numbers or total IgM levels were unchanged.<sup>136</sup>

In contrast to the clear protective role of IgM in atherosclerosis, certain IgM antibodies may play a detrimental role in post-MI remodelling. Circulating natural IgM has been suggested to exacerbate post-MI I/R injury<sup>82</sup> and non-muscle myosin heavy chain II has been identified as target self-antigen.<sup>137</sup> Blockade of this self-antigen with a synthetic peptide prevented IgM binding and reduced post-MI injury.<sup>138</sup> *Aid*<sup>-/-</sup> *sIgM*<sup>-/-</sup> mice that are unable to make functional antibodies showed reduced infarct size, attenuated inflammation and remodelling, and improved left ventricular (LV) function in a coronary ligation model.<sup>81</sup> Thus, in the context of cardiac I/R injury, IgM against specific self-antigens can trigger local

complement activation and worsen the outcome. Interestingly, E06-scFv expressing transgenic mice<sup>120</sup> developed decreased infarct sizes during experimentally induced MI-associated ischaemia-reperfusion injury,<sup>139</sup> suggesting that antibodies targeting OSE may also play a role in limiting the post-ischaemic inflammatory response induced following cardiomyocyte death during MI and particularly reperfusion injury, which involves myocardial damage driven by the formation of reactive oxygen species that may lead to the generation of OSE.<sup>139</sup>

### 5.2.3 IgG

Given the multiple subtypes of IgGs and Fc receptors, their differing affinities for Fc $\gamma$  receptors with sometimes opposing functions expressed in varying amounts on the surface of different effector cells, as well as their additional receptor-independent functions, it has been particularly challenging to dissect the functional role of IgGs in ACVD. It is likely that the effects depend on IgG subclass and antigen specificity.

Two studies assessing total IgG levels in human CVD cohorts gave opposite results. While Khamis et al.<sup>102</sup> found high baseline IgG levels predict fewer cardiovascular events, another study showed that high IgG levels are associated with MI and cardiac death in hyperlipidaemic men.<sup>103</sup> Data on total IgG subclasses are not available. The association with oxLDL-specific IgG is also complex. While some studies found higher baseline IgG levels to MDA-LDL to predict coronary events,<sup>140</sup> others failed to show an independent association.<sup>141–143</sup> Nevertheless, a meta-analysis of seven studies concluded an increased odds ratio (OR = 1.25; 95% CI: 1.11–1.41) for cardiovascular outcomes in patients with elevated anti-oxLDL IgG. Consistent with the latter, circulating IgG immune complexes containing MDA-LDL were found to predict cardiovascular events in type 1 and type 2 diabetics.<sup>144,145</sup> In contrast, IgG levels against native and MDA-modified p45 and p210 were found to be inversely associated with atherosclerosis and future events.<sup>93,94,106,107,146</sup>

Definite proatherogenic roles have been assigned to IgG anti-HSP60/65 antibodies, which represent cross-reactive antibodies that are mounted against microbial HSP65 antigens that can cross-react with stress-induced endogenous HSP60 expressed in endothelial cells triggering endothelial damage.<sup>147</sup> IgG titres to mycobacterial HSP65 have been shown to be increased in patients with carotid atherosclerosis.<sup>148,149</sup> Classical autoantibody responses have also been found to associate with ACVD risk. These include rheumatoid factor, anti-nuclear antibodies, the anti-CCP, and anti-phospholipid antibodies, such as anti-cardiolipin and anti- $\beta$ 2GPI.<sup>105</sup> However, it is unclear if these only reflect the underlying disease condition and the associated cardiovascular risk. Finally, autoantibodies against ApoA1 that impair the anti-atherogenic properties of HDL on and trigger innate immune responses via TLR2/TLR4/CD14 complex are independently associated with CAD.<sup>150</sup>

Most IgG in the setting of atherosclerosis arise from GCs within secondary lymphoid organs or ATLOs, which form in atherosclerotic mice over time<sup>48,49,69,71,101,151</sup> and may be driven by hyperlipidaemia.<sup>152</sup> Initial evidence for a potential proatherogenic role of GC reactions came from studies targeting T<sub>FH</sub> cells. Depletion of T<sub>FH</sub> cells reduces atherosclerosis in mice,<sup>48,151</sup> while enhancing T<sub>FH</sub> activity by ablation of T<sub>FH</sub>-suppressing CD8+ Tregs<sup>48</sup> or MZ B cells<sup>68</sup> increases lesion formation. However, these studies did not target GC B cells specifically. Centa et al.<sup>49</sup> recently dissected the effect of GC-derived IgG responses on atherosclerosis. Using *Aicda*<sup>Cre/+</sup> *Pax5*<sup>fl/fl</sup> mice, they employed an elegant model to specifically ablate GC-derived B cells including plasma cells, while preserving B1 and B2 cells that have not entered the GC reaction as well as total IgMs and those directed against oxLDL. *ApoE*<sup>-/-</sup> mice deficient in GC-derived

cells and total as well as OSE-specific IgG showed greatly reduced lesion formation, suggesting an overall proatherogenic role for GC-derived antibodies.<sup>49,152</sup> However, GC-derived IgGs were also found to promote plaque stability, potentially by promoting smooth muscle cell proliferation in a Fc $\gamma$ -dependent manner,<sup>49</sup> highlighting the potential heterogeneous effects of IgGs on atherogenesis.

Other studies focused on the role of plasma cells and plasma cell-derived antibodies. Administration of polyclonal immunoglobulin preparations reduced lesion formation in *ApoE*<sup>-/-</sup> *Ldlr*<sup>-/-</sup><sup>153</sup> and in *ApoE*<sup>-/-</sup> mice.<sup>154</sup> In line with the proposed overall proatherogenic function of GC B cells, conditional deletion of the plasma cell differentiation-driving transcriptional repressor BLIMP1 (*Prdm1*) in CD19-expressing B cells<sup>49</sup> and in CD23-expressing cells,<sup>70</sup> which efficiently reduced plasma cell numbers, IgG as well as IgM levels, significantly decreased lesion formation. In contrast, Sage et al.<sup>155</sup> showed that B-cell-specific deletion of XBP1, an ER-stress associated transcription factor that is required for the adoption of a highly secretory phenotype and differentiation to antibody-secreting plasma cells, resulted in increased plaque size in *Ldlr*<sup>-/-</sup> in a BM transplantation setting. Potential contributing factors to the discrepancies between the studies may be the experimental setting, the use of different Cre-recombinase expressing strains and the fact that unlike BLIMP1 deficiency, XBP1 deficiency results in a functional defect in plasma cells rather than a reduction in numbers.

While these above studies address the effects of global antibody deficiency, it is important to note that the effects of IgG likely depend on antigen specificity. Atherosclerosis and dyslipidaemia affects the composition of the IgG pool<sup>152</sup> which may modulate atherogenesis. In line with this, IgG purified from the plasma of *ApoE*<sup>-/-</sup> mice but not from non-atherosclerotic wild-type mice exacerbated disease in *Ldlr*<sup>-/-</sup> *Cd2*<sup>cre</sup> *Prdm1*<sup>fl/fl</sup> mice.<sup>70</sup> Additionally, transferred IgG accumulated in the plaques of recipient mice, suggesting that some IgGs generated in atherosclerotic mice react with antigens in atherosclerotic lesions.

In contrast, anti-OSE IgG may have atheroprotective effects in mice. For example, infusion of human IgG1 against MDA-modified ApoB100 peptides reduces atherosclerosis in a dose-dependent manner in *ApoE*<sup>-/-</sup> mice<sup>156</sup> and induces plaque regression in *Apobec1*<sup>-/-</sup> *Ldlr*<sup>-/-</sup> mice.<sup>93,157</sup> Similarly, immunization with an MDA-modified ApoB100 peptide and fibronectin induce a robust IgG response and reduces lesion formation.<sup>158,159</sup> Furthermore, a monoclonal IgG1 antibody against PC (T15) reduced lesion development in a murine femoral cuff atherosclerosis model<sup>160</sup> and reduced lesion inflammation in atherosclerotic *Ldlr*<sup>-/-</sup> ApoB<sup>100</sup> mice.<sup>161</sup> Similarly, passive administration of this antibody to hypercholesterolaemic APOE\*3 Leiden mice with experimental MI-associated I/R injury attenuated the immediate post-ischaemic recruitment of pro-inflammatory Ly6C<sup>high</sup> monocytes, thus reducing lesion size at a late timepoint and improving cardiac remodelling and function.<sup>162</sup> The mechanisms may involve neutralization of OSE arising in the context of vascular or cardiomyocyte damage to limit their ability to induce pro-inflammatory responses, as human PBMCs treated with oxLDL complexed with IgG against a MDA-modified ApoB100-peptide showed significantly reduced TNF $\alpha$  secretion and increased IL-10 secretion compared to oxLDL-treated cells.<sup>163</sup> Interestingly, in another experiment atherosclerosis-prone mice expressing a human ApoB-specific T cell receptor developed IgG against ApoB, resulting in reduced LDL cholesterol and decreased lesions, suggesting antibody-mediated clearance of LDL.<sup>164</sup>

Our understanding of the role of individual Fc $\gamma$  receptors is complicated by the broad and heterogeneous expression across cell types and the significant crosstalk between these receptors and others, which

affects the net effect of their engagement. Furthermore, there are considerable differences between the human and murine system, which makes the translatability of findings difficult.<sup>165</sup> Nonetheless, several murine studies have addressed the role of individual Fc $\gamma$  receptors. Deletion of the Fc receptor  $\gamma$  (FcR $\gamma$ ) chain, which is essential for signal transduction in most activating Fc $\gamma$  receptors, has been shown to reduce atherosclerosis development in several models,<sup>166–169</sup> suggesting an overall proatherogenic function of activating Fc $\gamma$  receptors. However, as the deletion of the FcR $\gamma$  chain resulted in relative increases in the inhibitory Fc $\gamma$ R1b,<sup>166</sup> it is also possible that Fc $\gamma$ R deficiency caused a shift towards a more immunosuppressive, potentially atheroprotective phenotype in effector cells.

Other studies have assessed the role of individual receptors. Deficiency in the Fc $\gamma$ R1b activating receptor resulted in reduced atherosclerosis independent of sex in *Ldlr*<sup>-/-</sup> mice fed an atherogenic diet for 24 and 14, but not 6 weeks<sup>170</sup> and in *Apoe*<sup>-/-</sup> mice fed a Western diet for 10 weeks.<sup>171</sup> However, another study demonstrated such atheroprotective effects in female *Apoe*<sup>-/-</sup> *Fcgr3*<sup>-/-</sup> mice only at an early disease stage (4 weeks diet) but increases in atherosclerosis at later disease stages (14, 24 weeks diet),<sup>172</sup> suggesting potential disease-stage-specific effects of Fc $\gamma$ R1b. Interestingly, Fc $\gamma$ R1b has been suggested as a scavenger receptor for MDA-LDL mediating its uptake and foam cell formation<sup>173</sup> thus adding another layer of complexity to the role of Fc $\gamma$ Rs in atherosclerosis.

This complexity also becomes evident studying the role of the inhibitory Fc $\gamma$ R1b. Initial studies suggested that Fc $\gamma$ R1b deficiency results in increased lesion formation in chow-fed male *Apoe*<sup>-/-</sup> *Fcgr2b*<sup>-/-</sup> mice<sup>174</sup> and in female *Ldlr*<sup>-/-</sup> mice transplanted with *Fcgr2b*<sup>-/-</sup> or wild-type BM fed a Western diet for 8<sup>175</sup> or 14 weeks.<sup>176</sup> In contrast, two separate studies showed reduced atherosclerosis in male and female *Apoe*<sup>-/-</sup> mice with global and haematopoietic *Fcgr2b*<sup>-/-</sup> deficiency.<sup>177</sup> Bagchi-Chakraborty *et al.*<sup>178</sup> employed B-cell-specific models of *Fcgr2b* overexpression or GC-specific reduced expression. Intriguingly, they found that Fc $\gamma$ R1b had differential effects on B-cell responses and atherosclerosis in males and females. B-cell-specific Fc $\gamma$ R1b overexpression reduced GC formation, plasma cell differentiation, and selectively reduced IgG levels, in line with the important role of Fc $\gamma$ R1b in B-cell development, in male mice. In contrast, females with B-cell-specific Fc $\gamma$ R1b overexpression showed reduced GC formation, but also strongly decreased B1 cell-derived IgM levels. Consequently, atherosclerotic lesion size was decreased in both *Ldlr*<sup>-/-</sup> and *Apoe*<sup>-/-</sup>-based models in males, but there was increased plaque formation in females. This finding was mirrored in experiments using a Fc $\gamma$ R1b mutant (Fc $\gamma$ R1b<sup>ΔAP1</sup>) that lacks the ability to up-regulate its expression in GCs, thus leading to increased GC formation. While male Fc $\gamma$ R1b<sup>ΔAP1</sup> mice showed increased GC formation and IgG2c levels exacerbating atherosclerotic lesion formation, the Fc $\gamma$ R1b<sup>ΔAP1</sup> mutant had no effect in females. Remarkably, the authors found intrinsic differences in the response of B1 cells between males and females, with B1 cells from female *Apoe*<sup>-/-</sup> mice showing an increased cellular turnover and decreased sensitivity to IL-5 and BAFF, as evidenced by decreased IgM secretion upon stimulation. Therefore, the effect of Fc $\gamma$ R1b may additionally depend on sex, underscoring the importance of taking sex into consideration in the experimental design in the study of immunology and metabolic diseases.

### 5.2.4 IgE

Several studies have shown a link between IgE and human CAD.<sup>8</sup> Furthermore, human patients with MI or unstable angina-pectoris show increased plasma IgE levels and an enrichment of IgE and *FCER1* expression in atherosclerotic lesions,<sup>179</sup> suggesting potential proatherogenic roles for IgE and its effector functions. Indeed, FcεR1  $\alpha$ -chain deficiency reduced lesion and necrotic core formation in *Apoe*<sup>-/-</sup> mice, which was associated with reduced macrophage activation and increased macrophage apoptosis *in vitro*.<sup>179</sup> In line with this, genetic IgE deficiency was recently shown to reduce atherosclerosis in *Apoe*<sup>-/-</sup> mice.<sup>180</sup> This was also associated with reduced plaque inflammation and a shift towards more reparative M2 macrophage phenotypes, although this may also be associated with the less advanced plaque phenotype.<sup>180</sup>

Given the prominent role of IgE in the activation of mast cells, it is possible that IgE can mediate proatherogenic effects via induction of mast cell degranulation, which is associated with increased atherosclerosis<sup>181</sup> potentially via the release of histamine,<sup>182</sup> IL-6, and IFN $\gamma$ .<sup>183</sup> Additionally, systemic mast cell activation via repeated administration of anti-DNP IgE followed by DNP-HSA antigen to B-cell deficient  *$\mu$ MT*<sup>-/-</sup> *Apoe*<sup>-/-</sup> mice showed increased lesion formation that was mediated by increased systemic mast cell activation and consequent neutrophil recruitment.<sup>184</sup> In agreement with this data, we have previously shown that the increase in atherosclerosis observed in sIgM-deficient *Ldlr*<sup>-/-</sup> mice may be mediated by increases in plasma IgE levels, as neutralization of IgE levels in these mice abrogated enhanced lesion formation. IgE neutralization was also associated with reduced numbers of activated mast cells in the plaques of these mice.<sup>111</sup> Surprisingly, the study by Zhang *et al.*<sup>180</sup> found no difference in mast cell activation in *Ige*<sup>-/-</sup> *Apoe*<sup>-/-</sup> mice, suggesting that the putatively proatherogenic effects of IgE may be mediated by other mechanisms such as their effect on macrophages.

It remains to be determined whether IgE modulates atherosclerosis development primarily via mast cells or macrophages. Additionally, it is not clear whether the effects are dependent on antigen specificity, given that IgE can bind FcεR1 receptors with high affinity in monomeric form.<sup>185</sup> Interestingly, sensitization to Galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal) by tick bites in endemic areas has been proposed to trigger specific IgE, which could enhance an inflammatory response to dietary glycolipids.<sup>186</sup> The presence of anti- $\alpha$ -Gal IgE has been found to be associated with increased atheroma burden and unstable plaques in patients <65 years of age.<sup>186</sup>

### 5.2.5 IgA

Although some epidemiological evidence for associations between IgA and cardiovascular disease in human exists,<sup>103,187</sup> so far, the functional role of IgA has not been explored in preclinical studies. Given the importance of IgA in mucosal immunity, it is likely that IgA plays a role in the crosstalk with the intestinal microbiome. There is emerging evidence for an effect of the microbiome on cardiovascular disease.<sup>188</sup> Furthermore, IgA has been implicated in the pathogenesis of fatty liver disease, which represents a common co-morbidity and shares many pathophysiological features with atherosclerosis, including the increased emergence of OSE and lipid-driven inflammatory responses.<sup>127</sup> It will be interesting to see in the future whether and by which mechanisms IgA could play a role in atherogenesis. Of note, high levels of IgA against PC were found to predict long-term CV risk.<sup>189</sup>

## 5.3. The role of crosstalk with other immune cells

### 5.3.1 Antigen presentation

As discussed, MHCII is required for the interaction between T cells and B cells, and thus important for the generation of the GC reaction and affinity-matured antibodies. While global MHCII deficiency exacerbates atherosclerosis,<sup>190</sup> the role of B-cell-specific MHCII is less clear. B-cell-specific MHCII deficiency in a BM chimaeric approach using 80%  $\mu$ MT<sup>-/-</sup> BM and 20% MHCII<sup>-/-</sup> or wild-type BM reduced atherosclerosis development,<sup>70</sup> which was mirrored in the failure of adoptively transferred MHCII<sup>-/-</sup> B2 cells to exacerbate atherosclerosis in  $\mu$ MT<sup>-/-</sup> ApoE<sup>-/-</sup> mice.<sup>191</sup> In contrast, no difference in lesion size was observed in a conditional *Cd19<sup>cre/+</sup> MHCII<sup>fl/fl</sup>* model.<sup>192</sup> However, importantly, insertion of Cre into the CD19 locus abrogates the expression of functional CD19, resulting in haploinsufficiency. Given the central role of CD19 as a B-cell co-stimulatory receptor involved in BCR signalling, it is possible that any effects of the conditional MHCII deletion in this study may have been masked by differences in B-cell activity. Therefore, the experimental findings to date suggest a potential proatherogenic role for B-cell-specific MHCII.

### 5.3.2 Co-stimulatory pathways

Notably, CD40-CD40L has been shown to play roles in a variety of cell types that have different roles in atherosclerosis.<sup>193</sup> B-cell-specific deficiency of CD40 resulted in decreased atherosclerosis.<sup>70</sup> Interestingly, unlike wild-type B2 cells, adoptive transfer of CD40-deficient B2 cells to  $\mu$ MT<sup>-/-</sup> ApoE<sup>-/-</sup> mice failed to increase atherosclerosis development,<sup>191</sup> suggesting that the interaction between CD40 on B2 cells and CD4+ T cells is important in driving B2 cell-mediated proatherogenic effects. On the other hand, CD40+ B cells may have a protective effect in humans<sup>58</sup> and CD40 may be required for immunosuppressive B-cell functions and B<sub>reg</sub> differentiation.<sup>30</sup>

### 5.3.3 B-cell-derived cytokines

IL-10 is an important immunosuppressive cytokine. Global IL-10 deficiency has been shown to increase atherosclerosis.<sup>194</sup> However, in B cells, the function is less clear. Using a B-cell-chimaeric BM transplantation approach to induce B-cell-specific IL-10 deficiency, Sage et al.<sup>195</sup> showed no difference in lesion formation upon IL-10 deficiency. In contrast, one study showed beneficial effects of transferring IL-10-expressing B cells from atherosclerotic mice in a cuff-induced arterial injury model.<sup>196</sup> Moreover, it was found that angiotensin II can synergise with BAFF to induce IL-10 expression in B cells, which could counteract the proatherogenic effects of adoptive B2 cell transfer.<sup>197</sup>

GM-CSF can be secreted by IRA B cells. Hilgendorf et al.<sup>31</sup> showed that IRA B cells are induced during atherosclerosis, where they promote extramedullary haematopoiesis and the expansion of DCs, thus indirectly affecting Th1 differentiation. This proatherogenic function was shown to be dependent on GM-CSF. Interestingly, IgM+ IRA B cells were also expanded in humans with severe coronary and peripheral artery disease.

In addition to the proatherogenic roles of TNF $\alpha$ ,<sup>1</sup> Kyaw and colleagues have found that B-cell-specific TNF $\alpha$  deficiency in a B-cell-chimaeric BM transplantation model (80%  $\mu$ MT<sup>-/-</sup>/20%TNF $\alpha$ <sup>-/-</sup>) reduced lesion size, inflammation and necrotic core formation.<sup>198</sup> Interestingly, adoptively transferred TNF $\alpha$ -deficient B2 cells failed to exacerbate atherosclerosis in ApoE<sup>-/-</sup>  $\mu$ MT<sup>-/-</sup> or ApoE<sup>-/-</sup> Rag2<sup>-/-</sup>  $\gamma$ C<sup>-/-</sup> mice<sup>198</sup> suggesting that the proatherogenic effects of B2 cells may be at least partially mediated by TNF $\alpha$ .

## 6. B-cell and humoral immunity-directed therapy in ischaemic cardiovascular disease

Although much less is known about the role of B cells in human ACVD and available data are primarily of correlative nature, preclinical data have paved the way for clinical trials testing the role of B-cell-based immune interventions. Clearly, differences between mice and humans with respect to subset definition and functional states are a limiting factor for translational studies, but emerging technologies (including single cell-based techniques) will be helpful in addressing these challenges and will allow a better distinction of human B-cell subsets in this regard.<sup>199</sup> Moreover, a major step forward to the understanding of the functional role of B cells also in human disease will be the unbiased characterization of specific antigens recognized by different B-cell subsets in the context of ACVD, which can now be achieved by high throughput BCR sequencing methods.<sup>200</sup> Still, a number of clinical studies targeting B cells or known antigens have been initiated.

### 6.1 B-cell depletion

Given the suggested proatherogenic function of follicular B cells, B-cell targeted therapy may provide an attractive future avenue for the treatment of high-risk patients.<sup>201</sup> There is evidence from preclinical studies that suggest a protective effect of CD20-mediated B-cell depletion both in atherosclerosis<sup>64,65,84</sup> and MI.<sup>32</sup>

Rituximab is a CD20-targeting monoclonal antibody approved for the treatment of several B-cell-derived malignancies and autoimmune diseases. In line with preclinical data, several small-scale human clinical studies in patients receiving rituximab could show improvements in cardiovascular parameters including decreased arterial and carotid IMT and improved flow-mediated dilation.<sup>201</sup> Additionally, administration of rituximab to patients following kidney transplantation was shown to decrease the risk of transplantation-associated atherosclerotic disease.<sup>202</sup> However, a meta-analysis of randomized controlled trials involving rituximab did not show benefits on short-term cardiovascular adverse events,<sup>203</sup> although long-term follow-up was not available for most studies analysed.

Although the study design did not include formal assessment of cardiovascular outcomes, the recently completed rituximab in patients with acute ST-elevation myocardial infarction (RITA-MI) trial, a prospective, open-label, single-arm phase I/IIa trial assessing the tolerability of a single intravenous injection of different doses of rituximab within 48 h of STEMI onset, has shown an excellent safety profile. Treatment was associated with robust, dose-dependent B cell decreases that persisted throughout the 6-month follow-up period. Importantly, treatment did not result in profound changes in plasma immunoglobulin levels. Echocardiographic data that were available for half the study participants showed an encouraging trend towards improved LV ejection fraction at follow-up.<sup>204</sup> Although the phase I/IIa study is limited by the relatively small size of the study and lack of a placebo-treated control group, the treatment appears safe and well-tolerated, and suggests potentially beneficial effects on cardiovascular parameters, thus warranting further investigation of the effects of a single administration of selected doses of rituximab after STEMI on LV dysfunction and cardiac remodelling in the phase IIb RITA-MI2 trial.

## 6.2 Humoral immunity

The large number of preclinical studies demonstrating an atheroprotective effect of immunization with LDL antigens has sparked the idea of developing a vaccine against atherosclerosis.<sup>129</sup> In particular immunization approaches with oxLDL and OSE trigger robust and lasting antibody responses that correlate with atheroprotection. The latter may be needed to have an impact on disease progression, as the phase II GLACIER trial showed no clinical benefit in patients receiving a recombinant IgG1 antibody against MDA-modified ApoB100 (MLDL1278a, orticumab) as evaluated by FDG-PET—although the study may have been limited by its short follow-up and lack of coronary artery assessment.<sup>205</sup> Another randomized double-blind placebo-controlled phase II trial (NCT04776629) of the same antibody is currently ongoing in a cohort of psoriatic patients with elevated cardiometabolic risk, during which coronary artery inflammation and plaque burden will be assessed by coronary computed tomographic angiography. Furthermore, a human IgG1 antibody against PC (ATH3G10) is being investigated in patients with acute ST-segment elevation in a randomized double-blind placebo-controlled phase II trial (NCT03991143).

Exploiting active vaccination strategies may represent a realistic alternative. For example, induction of antibodies against OSE may also be achieved with the use of PC-containing pneumococcal polysaccharide vaccines, although whether this can affect disease progression is not clear. While a meta-analysis has shown mildly reduced acute coronary syndrome-associated events in elderly populations,<sup>206</sup> a placebo-controlled randomized controlled trial assessing the effect of the pneumococcal vaccine Pneumovax23 on the occurrence of acute coronary syndrome and ischaemic stroke in a cohort of 6000 patients with increased cardiovascular risk is currently under way (The Australian Study for the Prevention through Immunization of Cardiovascular Events; ACTRN12615000536561).<sup>207</sup> Moreover, a recent randomized placebo-controlled trial in healthy volunteers using vaccination with the 13-valent conjugate pneumococcal vaccine Prevnar-13 suggested an induction of anti-oxLDL antibodies only when certain vaccination regimens are followed.<sup>208</sup> However, several aspects need to be considered for vaccinations: (i) the identification of the right B-cell epitopes that induce neutralizing antibodies. oxPLs or other pro-inflammatory OSE are attractive in this regard, as blocking their activities has been shown to be protective in preclinical models and we have previously generated peptide mimotopes of MDA-epitopes that could be used as standardized antigens to induce MDA-specific antibody responses.<sup>209</sup> (ii) The right choice of vaccine platforms and adjuvants that ensure the right type of antibody responses with sustained titres; and (iii) accurate biomarker assays that allow monitoring these. Although the development of a vaccine against ACVD may sound futuristic, the general excellent safety profile of vaccines further argues for it.

## 7. Outlook

Our insights into the contribution of different B-cell subsets and their functions as well as antibody classes in ACVD have rapidly increased in the last years. Despite some advances, major efforts should be put into the identification and better characterization of B-cell responses against known and newly discovered antigens. In this regard, what are the specific characteristics of ACVD-associated antigens? How do they relate to classical risk factors, such as dyslipidaemia and what is the contribution of the local tissue environment, e.g. epitopes that are only exposed and/or post-translationally modified in response to stress? Moreover, does the

nature of such antigens in the context of the dyslipidaemic environment affect their ability to activate B cells or make them more prone to triggering autoantibody responses? We also need to learn much more about the impact these immune responses have in other aspects of ACVD, such as during MI and post-MI remodelling. Finally, how does all our knowledge from preclinical models relate to human disease? Insights from ongoing intervention studies targeting B cells and detailed characterization of the immune responses in humans using emerging technologies could have a major impact in this.

## Funding

F.P. is supported by the Doctoral program Cell Communication in Health and Disease (FWF\_VW1205-B09) funded by the Austrian Science Fund (FWF) and C.J.B. by grants of the Austrian Science Fund (FWF SFB F54) and the Leducq Foundation (Transatlantic Network of Excellence; TNE-20CVD03). Z.M. is supported by a Chair from the British Heart Foundation (BHF, UK).

**Conflict of interest:** none declared.

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