The Role of B cells in Atherosclerosis

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Abstract

The cardiovascular system is subject to hyperlipidemic, inflammatory and pro-oxidant stressors. Over time, these factors drive prevalent chronic diseases of which atherosclerosis is most prominent and accounts for the majority of deaths globally. Antibody-producing B cells perform a unique and essential purpose in responses to stress, injury and infection. The power, inducibility and adaptability of the antibody repertoire require an equally complex range of control measures. Defects and chronic perturbations in these checkpoints lead to inappropriate antibody responses, which could play key roles in shaping the development and outcome of atherosclerotic disease. A unique aspect related to atherosclerosis is the prominent role of natural antibodies, specifically those binding oxidized epitopes abundant on modified lipoproteins and cellular debris. In addition, B cells control cellular immune responses through cell-cell contact, antigen presentation and cytokine production, and thereby participate in systemic and local immune responses in atherosclerotic arteries. To date, both pro-and anti-atherogenic properties have been assigned to B cells depending on subsets and how they are functionally targeted. For these reasons, a deeper understanding of the functional influences of B cells on atherosclerotic plaque development is being pursued using new *in vivo* models coupled with novel technologies. Combined with data from human immunotherapeutic and genetic studies, the hope is to provide novel B cell-targeted interventions to prevent inflammation-driven cardiovascular events.

Introduction

Ischemic heart disease and stroke are the major causes of mortality and morbidity globally[1]. The underlying cause responsible for these manifestations is atherosclerosis, a lipid-driven chronic inflammatory disease that causes the formation of a plaque (atheroma) in large and medium size arteries[2]. The central causal risk factor for atherosclerosis is increased plasma low-density lipoprotein (LDL). LDL retention in the subendothelial space is the first step towards the formation of an atherosclerotic plaque. There, LDL undergoes oxidation (OxLDL) and acquires immunogenic properties[3]. Oxidative modification of LDL results in the formation of different lipid peroxidation-derived structures that are recognized as antigens by the immune system and have been termed oxidation-specific epitopes (OSE)[4]. They include phosphocholine (PC)-containing oxidized phospholipids and its degradation product malondialdehyde (MDA). Model antigens carrying OSEs, including MDA-modified LDL (MDA-LDL) and copper-oxidized LDL (CuOx-LDL), are used to study the role of these structures in immune responses[5]. Immunogenic OxLDL triggers a series of inflammatory reactions that involve endothelial cell activation and subsequent non-resolving vascular invasion of various immune cell types, mainly monocytes and T cells[6,7]. This central atherosclerotic process is the hub of an inflammatory network also including systemic inflammation of organs such as liver and adipose tissue, and local adaptive immune responses in vascular adventitia[8,9]. The causal hierarchy of these processes is unlikely to be linear and still under debate.

Several pieces of experimental evidence suggest that the immune system is a major modulator of the initiation and progression of atherosclerosis. Some of the first evidence used mice lacking the monocyte/macrophage survival factor M-CSF, which are resistant to atherosclerosis[10], and *Rag1/2*$^{-/-}$ mice lacking B and T lymphocytes, which display strongly reduced atherosclerosis in the presence of moderate hypercholesterolemia[11,12]. Although the immune phenotype of M-CSF and Rag1/2-deficient mice is severe and impacts multiple pathways, these studies have triggered a multitude of more targeted approaches that confirm the central conclusions of these studies, while revealing the expected complexity of a disease affected by so many body systems. In humans, autoimmune diseases drive inflammatory immune responses, and systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) patients display premature atherosclerosis cannot be explained by classical atherosclerotic risk factors (age, cholesterol, smoking, hypertension)[13]. Recently, the CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study) clinical trial provided definitive proof that immunity is a major arc of this pathology and paves the way for more intensive efforts to translate experimental findings to humans[14]. B cell immunity - both cellular and humoral - has been shown to exhibit a particularly important role in atherosclerotic plaque formation[15,16], with IgM, IgG and IgE as well as regulation of T cell responses affecting the progression of

this disease in either constructive or detrimental modes. We review here the palette of B cell abilities involved in dyslipidemia and atherosclerosis.

B cell receptors, development and subsets

The B cell receptor

B cells exhibit distinct machinery among immune cells. They express B cell receptors (BCRs), which are membrane-bound immunoglobulins (Ig) possessing unique epitope binding sites able to bind antigens (both self and foreign). B cells develop in the bone marrow from hematopoietic precursors through a well-defined set of stages (at least in mice) [17], ultimately maturing in the spleen. Each B cell clone develops a unique BCR via recombinase activation gene (RAG)-dependent sequential Ig gene recombination of available variable (V), diversity (D) and joining (J) genes[18,19]. Recombined heavy and light chain polypeptides together form (in duplicate) the mature BCR and subsequently, secreted antibodies. BCRs form a signalling complex together with key B cell-specific membrane proteins such as CD19, B220, CD22 and Igα (CD79a) and Igβ (CD79b), allowing induction of cell-instructive nuclear factor-κB, phosphatidyl-inositol 3-kinase and mitogen activated protein kinase signalling. BCR signalling plays a key role in all stages of B cell development, differentiation and activation and therefore perturbations are linked to disease modulation. How BCR signalling is affected in different B cell subsets in dyslipidemia remains to be investigated. The final stage of B cell maturation, occurring upon activation, is rapid proliferation (as plasmablasts) and differentiation into plasma cells (Box). There are five main classes of antibodies based on Fc region gene usage, which are IgM, IgG, IgE, IgA and IgD. IgG antibodies are further divided in four different subclasses which are IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2a/c, IgG2b and IgG3 in mice[20]. Immunoglobulin classes also display differences with respect to their structure, secretion capacity and post-translational modifications. For example, while membrane-bound IgM is a monomer, secreted IgM has a pentameric structure. Furthermore, in contrast to the other Ig classes, IgD is typically not secreted in physiological settings[5]. Glycosylation and sialylation of mature antibodies is critical for effector functions and has recently been linked to cardiovascular disease[21,22].

Functional diversity in the B cell system

Different lineages of progenitor cells give rise to distinct B1 and B2 cell lineages. In addition to developmental cues, antigen-specific and other environmental cues create heterogeneity within the mature B cell family, for example by differentiation of B2 cells into marginal zone (MZ) and follicular (FO) cells[17,23]. Broadly, the major lineages of B cells exist to provide specialised capabilities against distinct microbial insults. B1 cells, which in mice can be further subdivided into B1a and B1b subsets, are the major source of naturally occurring antibodies that arise without infection or immunization[24,25]. They patrol mucosal surfaces and provide instant defence and antigen capture, plus opsonisation of invading bacteria but also traffic systemically. B1 cells secreting high levels of antibodies are found in spleen and bone marrow, similar to plasma cells[26]. B1a cells appear more important in spontaneous IgM production whereas B1b cells require some type of induction, however these functional differences are still being investigated[25]. Human B1 cells in peripheral blood were identified by Rothstein and colleagues[27], however the lack of ability to further analyse the human system means that controversy still exists over the equivalence between these cells and murine B1 cells. MZ B cells monitor the blood as it passes through the red pulp and in addition are able to shuttle antigens into the follicles or migrate to T cell zones. In mice, MZ B cells do not leave the spleen like FO B cells[28], whereas in humans MZ-like cells related to IgM memory cells are found in the circulation[29]. A potentially equivalent B cell subtype is also present in the subcapsular sinus of mouse lymph nodes[30]. In contrast, the major role of FO B cells is to combine with antigen-specific follicular helper T cells (Tfh) to become germinal center (GC) B cells to evolve specific, extremely high affinity antibodies that are also class-switched to different Ig isotypes. The GC reaction involves cycles of rapid proliferation of GC ‘centroblasts’ in the dark zone, that then are selected based on competition for antigen, which confers those B cells, referred to as ‘centrocytes’, with the ability to obtain costimulatory signals by presenting antigen peptides to Tfh in the light zone. GC B cells express activation-induced cytidine deaminase (AID), a DNA-mutating enzyme, which creates somatic hypermutation in the BCR region (although off target

mutation also occurs). Thus, multiple subclones emerge during a GC reaction and studies have shown competition leads to clonal evolution towards high affinity clones[31]. The choice between forming memory B cells, forming plasma cells or returning for further rounds of proliferation is beyond the scope of the current review and the reader is directed to other reviews[32]. FO B cells can also respond more directly, in extrafollicular responses, which can involve class-switching of the B cell clones but not affinity maturation. B2 cell-derived plasma cells fight off any trace of invading pathogens systemically through high titres of antibodies secreted strategically from the bone marrow (Box). Whereas B1, MZ or extrafollicular-derived plasma cells tend to be short-lived, FO B cells and GC reactions produce long-lived plasma cells that can maintain antibody titres for decades[32]. In addition, FO B cells differentiate into memory cells[33,34], preserving effective specificities in preparation to more rapidly eliminate future insults from the same pathogen. FO B cells are capable of producing each isotype class of antibody post class-switching, whereas MZ and B1 cells have limited capacity, mostly maintaining IgM class, but also capable of IgG3 production in mice[35]. Each of these subsets appears to contribute to B cell regulation of atherosclerosis.

The differentiation signals driving B cell diversity, and the appropriate markers to identify this heterogeneity, are exquisitely characterized in mice. To a large extent, these subsets have functional equivalents in humans although some markers are distinct and controversy remains over certain subsets, such as B1 cells. B1 cell differentiation signals are thought to depend largely on the temporal appearance earlier during development, including deriving from fetal liver rather than bone marrow precursors, as well as selection of clones with BCR specificity for a constant but restricted repertoire of common (self and foreign) epitopes[25]. Recent studies have shown that distinct waves of progenitors seed the B1 and B2 cell pools during mouse development [36], although more work is still required to understand the functional distinctions of different lineages. B1a cells in particular are derived almost completely from fetal and neonatal progenitors, whereas adult precursors are heavily biased towards B1b cell production[25]. This is of relevance when comparing atherosclerosis studies using germline mice or bone marrow transplant, since the embryonic B1a cell compartment may not be recapitulated with adult bone marrow. B1 cells are distinguished by expression of CD43, although this is also re-expressed on mature plasma cells, which uniquely express syndecan-1. B1a but not B1b cells express CD5, which is expressed by T cells[24]. Peritoneal cavity B1 cells, a common site for B1 cell investigation, also express the myeloid integrin CD11b. The fate of newly formed B2 cells entering the spleen depends primarily on antigen specificity through BCR signalling, combined with other pathways such as the B cell activating factor receptor (BAFFR) pathway[23]. Notch2 dependent signals are essential for MZ differentiation[37]; the MZ B cell phenotype is distinguished as low surface IgE receptor (CD23) and IgD, and high complement receptor 2 (CD21) expression.

The origin(s) of autoimmunity and autoimmune crosstalk in atherosclerosis

Autoimmunity arises in response to a combination of genetic and environmental factors. Genetic defects in the molecular pathways that control immune tolerance checkpoints is one major category[38]. This includes the signalling machinery (e.g. *Ptpn22*) that determines response levels of the cells, death machinery (e.g. *Fas/Fasl*) that ensures swift and appropriate apoptosis, and clearance machinery (e.g. *Mer*, *Mfge8*) that facilitates non-inflammatory removal and non-exposure of autoantigens not screened for centrally. Specific human leukocyte antigen (HLA) alleles also confer higher risks of autoimmunity. Highly autoreactive B cells are primarily removed during checkpoints in the bone marrow (which occurs in the thymus for T cells)[39]. Further checkpoints in the spleen prevent weakly autoreactive cells from entering the germinal center and generating high affinity antibodies. However, highly autoreactive B cells can also arise *de novo* during germinal center reactions to foreign antigens, particularly when the autoantigen is rare, tissue-specific and not present in the germinal center[40]. Alternatively, extrafollicular B cell responses have been proposed as potential sources of autoantibodies in SLE[41]. In the case of atherosclerosis, specificities typically associated with the innate B cell lineages such as anti-MDA and anti-PC IgM may instead emerge in adaptive B2 cell responses as IgG antibody-producing plasma cells. This could impact disease mechanisms by conferring distinct effector functions to these antibodies. In addition, the induction of anti-bacterial heat shock protein (Hsp) antibodies post-infection may result in these antibodies cross-reacting with self-Hsp on stressed endothelial surfaces[42,43]. Classical models of autoimmunity in mice result in enhanced atherosclerosis. Deficiency in Fas – FasL molecules of apoptosis induction results in autoantibody development and leads

to increased atherosclerosis[44-46]. Other models using known lupus-susceptible strains also show enhanced atherosclerosis in combined models[47,48]. This is not the case for all models as, for example, Bim deficiency induced autoimmunity but did not affect atherosclerosis[49]. This may have been due to reduced circulating lipids in the *Bim*$^{-/-}$ atherosclerotic mice, potentially a result of intestinal inflammation. This highlights a common issue in studying a disease driven both by metabolic and immune changes; consideration must always be given to the metabolic impacts of immune modulation as well as those directly associated with atherosclerotic immune responses. It is clear that B cell autoimmunity, as a key pathogenic player in autoimmune diseases, could mediate (indirectly) increased atherosclerosis. More direct roles for B cell autoimmunity arising as part of the immune response associated with atherosclerosis have been hard to separate from the effects of natural antibodies, but new data discussed below highlights the rapid progress being made.

B cell associations with atherosclerosis – *past and present*

Some of the earliest theories of atherosclerotic plaque formation hypothesised the roles of cholesterol, that of Anichkov[50], and of immune cell mediated damage, that of Virchow[51]. In the intervening time, paradigms of atherosclerosis etiology have undergone several iterations. Accumulating evidence over the past three decades has revived Virchow's view, such that the immune system is now seen by many as the "maestro" in the "atherosclerotic plaque orchestra". In fact, nearly 4 decades ago it was found that atherosclerotic plaques contain immunoglobulins[52-54]. In the 90s, seminal work by Joseph Witztum and colleagues demonstrated that plaques and plasma in both mice and humans contain Igs specific for different epitopes on OxLDL, later termed OSE[55]. OSE are also commonly found on apoptotic cell surfaces, microvesicles and by means of molecular mimicry on bacteria[4,56]. It was then shown that OSE antibodies had the capacity to block OxLDL uptake[57-59], which strengthened the hypothesis of a functional role of B cell immunity in atherosclerosis. In addition to Igs in plaques, Hamze *et al* demonstrated that B cells (enriched in plasma cells) secreting IgG and IgA are present in the vascular wall of human atherosclerotic plaques[60]. In line with this, a recent study revealed that aortic preparations from atherosclerotic mice contain three distinct B cell populations (based on the expression levels of B220 and CD43 surface molecules)[61]. The importance of B cell responses in atherogenesis is highlighted by genome-wide association and transcriptomic data pinpointing proliferation and activation status of B cells as important factors in cardiovascular disease (CVD) risk[62]. Clinical studies have also reported a positive association with increased risk for stroke of activated $CD19^{+}CD86^{+}$ B cells, and a negative association with unswitched (IgM-producing) memory B cells, that are also characterized as MZ-like B cells [63,64]. A functional role for B cells in atherosclerosis was first investigated by Caligiuri *et al*. in splenectomised apolipoprotein E deficient (*Apoe*$^{-/-}$) mice that were injected with total splenic B cells[65,66]. The authors showed that transfer of splenic B cells isolated either from wild type or *Apoe*$^{-/-}$ donors decreased lesion size compared to controls. Soon after, a study showed that lethally irradiated LDL receptor deficient (*Ldlr*$^{-/-}$) mice injected with bone marrow from B cell deficient (*μMT*) donors developed increased atherosclerosis compared to controls[67]. Although these studies suggest an overall protective role for B cells in atherosclerosis, it is now recognised that B cells are a very heterogeneous population, comprising several different functional subsets (see above). Distinct knockout models targeting these subsets differentially (Table 1) have revealed a number of pro-atherogenic as well anti-atherogenic potentials (Figure 1 and 2). In addition to studies investigating the importance of the most abundant antibody isotypes, IgM and IgG, there is increasing recognition of the potential importance of IgE. A positive association between IgA antibodies and cardiovascular outcomes in humans was reported[68] but functional roles for IgA in atherosclerosis have yet to be experimentally investigated. B cells may also be key players in adventitial tertiary lymphoid organ-derived responses that regulate advanced atherosclerosis in *Apoe*$^{-/-}$ mice [69,70]. Since ultimately functional molecules/pathways are responsible for these findings, we here focus on the antigens targeted and different B cell effector functions rather than subsets.

Antigens targeted in Atherosclerosis

OSE are central to atherosclerosis and are a target of both natural and adaptive antibodies. ApoB in LDL is a major target that acquires these epitopes in atherosclerosis. OSE in membrane lipids are also exposed on the surface of apoptotic/necrotic cells. Other proteins also acquire OSE adducts, such as extracellular

matrix proteins. For example, basement membrane collagen IV acquires MDA modifications in atherosclerotic plaques; higher levels are associated with myocardial infarction [71] and MDA-collagen IV antibodies are enhanced at baseline in subjects that go on to have MI compared to controls [72]. In diabetic patients, LDL may be subject to alternative modification, such as glycation, and subsequently creation of other neoepitopes. Low levels of methylglyoxal-modified ApoB100 IgM, which derive from human B1 cells, increase the risk for cardiovascular events in both type II-diabetic and non-diabetic patients[73].

Other antigens are probably involved, but much work is still required to demonstrate their importance. The plaque environment may be a strong source for cryptic and neo self-antigens. In addition, plaques are reported to be a source for endogenous toll-like receptor ligands that could switch B cells from tolerance to responsiveness. Oxidized phospholipids may recruit TLRs during CD36-mediated endocytosis of OxLDL[74] and the necrotic core provides nucleic acids that can stimulate TLR7/9. The accumulation of adventitial lymphocytes in both human atherosclerosis and mouse models attests to this process[8,9,75]. Citrullination, a characteristic of arthritic lesions, occurs in atherosclerotic plaques, and anti-CCP antibodies associate with CVD risk even in patients without clinical stage RA[76,77] (Table 2). ApoA1 is an alternative autoantigen, with ApoA1 antibodies potentially binding dysfunctional ApoA1 accumulating in plaque[78] or causing dysfunction in circulating HDL[79]. However, these autoantibodies also cross-react with TLR2[80]. Also, immunization-induced anti-Hsp60 IgG antibodies enhance disease when transferred to non-immunized mice[81]. Autoimmunity is generally associated with a polyspecific autoimmune repertoire. Merched *et al* recently performed a screen for plaque autoantigens using 2D proteomics and found matrix molecules in particular may be targeted specifically in atherosclerosis[82]. This area of research has received comparatively little attention and may be the next big step in progressing the field further. The advent of single cell analysis and next-generation sequence technologies are likely to open the door to these possibilities.

B cell effector mechanisms regulating atherosclerosis

IgM

IgM potently recruits and activates the complement cascade[83] and also binds FcμR, which is found in cell surface and secreted forms[84,85]. IgM may also be important in limiting self-antigen access to BCRs; mice specifically lacking soluble IgM (*sIgM*$^{-/-}$) do not develop the proper B2 cell system and B2 cells display altered BCR signalling[86,87]. Natural IgM antibodies are the best studied antibodies regulating atherosclerosis. Natural antibodies mainly produced by B1 cells contain a large preferential repertoire for OSE in both mice and humans[74,88], probably due to the abundance of OSE and apoptotic/necrotic debris and equivalent epitopes on bacteria. Specificities such as MDA and PC are significantly expanded during murine atherosclerosis[89] and are linked with atheroprotective properties (Table 1). Given the central role proposed for OxLDL and apoptotic/necrotic cell debris in initiating, promoting and sustaining atherosclerotic inflammatory cycles, natural IgM neutralizing these pro-inflammatory epitopes appears to be a key atheroprotective mechanism[5,90-92] (Figure 1), although non-OSE dependent functions for IgM are also now recognised to be important (see below).

In humans, several case-control and prospective clinical studies demonstrate that anti-MDA or anti-PC IgM levels are negatively associated with CVD risk measures, however no association was detected in some cohorts (Table 2). A recent meta-analysis of studies testing relative risk in populations without clinical disease found that both anti-MDA and anti-PC IgM provide a protective influence[93]. Functional insights for the atheroprotective role of anti-OxLDL IgM antibodies were originally obtained by using the E06 IgM that was cloned from the spleens of hypercholesterolemic mice and recognizes oxidized phospholipids (OxPLs). E06 was shown to inhibit OxLDL uptake by macrophages *in vitro* [58]. It was later found that the E06 antibody has an identical CDR3 region as the germline encoded B1 cell-derived T15 clone[94]. *Ldlr*$^{-/-}$ mice immunized with heat-killed pneumococcal extracts display a robust expansion of the PC-binding T15id^{+} IgM clonotype and concomitantly decreased lesion formation[95]. In addition, passive infusion of T15/E06 IgM antibodies reduced vein graft atherosclerosis in *Apoe*$^{-/-}$ mice[96]. However, in another study, infusion of T15 preparations did not impact accelerated atherosclerosis induced by cuffing in *Apoe*$^{-/-}$ mice.[97] A possible explanation may be that these infusions did not lead to a functionally significant change in T15 levels particularly when considering that hypercholesterolemia in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice leads to increased total and anti-

OxLDL plasma IgM[98]. Notably, genetic deficiency of the *V_HS107.1.42* locus, which is essential for the successful production of T15id$^+$ antibodies, did not affect atherosclerosis, probably because the lack of T15/E06 antibodies is compensated by the hypercholesterolemia-driven expansion of alternative anti-OxLDL IgM clones[98]. The fundamental importance of the T15/E06 targets (OxPLs[57]) in triggering proinflammatory responses *in vivo* has been recently demonstrated by Witztum and colleagues, who show that transgenic overexpression of the single chain variable fragment of E06 leads to strong protective effect in both plaque and systemic inflammation in atherosclerotic *Ldlr$^{-/-}$* mice[99]. These studies suggest that increased levels of a T15/E06 single chain variable fragment (lacking classical effector function) can confer an atheroprotective effect. Subsequent alternative strategies that cause expansion of anti-OxLDL IgM Abs have consistently supported the hypothesis that these antibodies are atheroprotective. Ldlr$^{-/-}$ mice deficient in Siglec-G (a highly expressed inhibitory co-receptor in B cells) display greatly enhanced B1a cell numbers and IgM levels (with a preferential expansion of anti-OSE antibodies) and a significant reduction in atherosclerosis with smaller necrotic areas[100]. Apoptotic cell injection led to enhanced IgM and decreased atherosclerosis in intact mice but not in B cell-deficient *Apoe$^{-/-}$* mice[101], further supporting that increased IgM may be, at least in part, responsible for the protective effect seen in these settings[101,102]. Furthermore, infusion of phosphatidylserine-liposomes - potentially mimicking apoptotic cells - induced an expansion of B1a cells and total IgM levels in plasma and atherosclerotic plaques and reduced atherosclerosis[102]. Indeed, transfer of IgM-secreting B1a cells - in contrast to *sIgM$^{-/-}$* B1a cells - into splenectomised *Apoe$^{-/-}$* mice reduced atherosclerotic plaque and necrotic core size[103].

In many of the above studies, total IgM was also expanded in addition to OSE IgM. Total IgM is also a marker of risk; one study recently showed that anti-MDA-LDL IgM was no longer predictive when accounting for total IgM levels[104]. However, it needs to be taken into account that up to 30% of all natural IgM antibodies have been shown to have specificity for OSEs (Chou et al JCI) making this a prominent specificity of total IgM. The protective effect of increasing total IgM levels was also shown upon infusion of polyclonal mouse IgM into atherogenic diet fed *Apoe$^{-/-}$* mice, which reduced atherosclerosis[97]. Thus, besides the OSE-neutralizing role, IgM exhibit additional atheroprotective properties that are instructed by the polyclonal repertoire (derived from both B1 and B2 cells). As discussed in more detail in the following section, we have shown that atherosclerosis-prone *sIgM$^{-/-}$* mice, which develop accelerated atherosclerosis [105,106], exhibit strongly increased IgE levels that are responsible for the aggravated atherosclerosis in this setting[106].

The endogenous triggers for expanded anti-OxLDL IgM responses during atherosclerosis include OxLDL itself as well as cellular debris accumulating in atherosclerotic plaques. OSE IgM may also be expanded downstream of IL-33, an alarmin released upon tissue damage, and/or IL-5. IL-33 is sensed by innate lymphoid cells found in aortic adventitia and fat-associated lymphoid clusters[107,108] which then produce high amounts of IL-5. Thus, it can be speculated that a local axis culminating in IgM production from B1 cells may be a central process protecting against mouse atherosclerosis. Infusion of IL-33 increases IL-5 and anti-OxLDL IgM levels in plasma, and decreases atherosclerosis[109]. However, no effect of ST2 (IL-33 receptor) or IL-33 deficiency on atherosclerosis was observed[110], and the lack of type II innate lymphoid cells was not associated with changes in IgM despite increased atherosclerosis[108]. IL-5 is sufficient to induce antibody-secreting cell (ASC) formation *in vitro* by B1a cells but not by other B cell subsets[89]. Notably, immunization of IL-5 deficient mice with MDA-LDL did not lead to T15/E06 expansion, while it did induce IL-5 secreting T cells and an expansion of T15/E06 in wildtype mice. In humans, plasma IL5 is positively associated with anti-OSE antibodies and negatively with atherosclerosis[111], suggesting that IL-5 may exhibit an atheroprotective role by regulating anti-OxLDL Ab levels. Indeed, *Ldlr$^{-/-}$* mice transplanted with bone marrow from *Il5$^{-/-}$* donors developed increased atherosclerosis and decreased anti-PC IgM[89]. Systemically, Th2-polarized CD4$^+$ T cells may also be important producers of IL-5 and thus also upstream of natural antibody responses. A clinical study found enhanced levels of Th2-polarized T cells and IL-4 production was negatively associated with myocardial infarction[112]. In support of this, anti-CD4 depletion also lead to reduced IgM and enhanced atherosclerosis in *Ldlr$^{-/-}$* mice[113] and *in vitro*, human CD4$^+$ T cells promoted anti-PC IgM production by B cells[114]. However, subcutaneous immunization using human ApoB in alum boosted Th2 responses but was ineffective at modulating atherosclerosis in *Ldlr$^{-/-}$* mice[73,115]. Studies

that assess all of these elements simultaneously may be needed to prove or disprove the importance of this axis.

Overall, the study of IgM antibodies targeting OSE has been seminal in developing current paradigms on fundamental atherosclerotic processes and is now being used as a basis for novel therapeutic strategies.

IgE and receptors

IgE is best known for its capacity to trigger powerful type I hypersensitivity, an allergic reaction occurring upon repeated exposure to an antigen (commonly of environmental origin). This reaction often underlies clinical manifestations such as allergic asthma and anaphylaxis. Thus, IgE is tightly regulated and only present in very small amounts in contrast to other Ig classes. IgE binds to two receptors, FcεRI and FcεRII (or CD23). FcεRI is mainly present on mast cells, basophils and eosinophils, while CD23 is mainly expressed by FO B cells[116]. Several epidemiological studies support a strong proatherogenic role for IgE in CVD (Table 2). We recently demonstrated that antibody-mediated neutralization of free IgE Abs in atherosclerotic mice lacking secreted IgM Abs (*Ldlr*$^{-/-}$*sIgM*$^{-/-}$), which display strongly increased plasma IgE, confers an atheroprotective effect. Furthermore, *Ldlr*$^{-/-}$*sIgM*$^{-/-}$ mice that received anti-IgE treatment contained less activated mast cells in the perivascular area of atherosclerotic plaques[106].

These data suggest that IgE antibodies are proatherogenic via binding to FcεRI receptor on mast cells or macrophages. Mast cells have been shown to promote atherosclerosis[117] by producing IL-6 and IFN-γ[118] and neutrophil recruitment[119]. In addition, mast cell degranulation results in release of histamine, which exhibits a pro-atherogenic effect[120], and may also be responsible for the proatherogenic properties of the IgE-mast cell axis. In agreement with this, *Apoe*$^{-/-}$ mice lacking FcεRI developed decreased atherosclerotic plaque size with decreased necrotic core[121] suggesting that IgE may promote cell death in atherosclerotic lesions. Indeed, stimulation of macrophages with purified IgE led to enhanced apoptosis, which was dependent on the presence of FcεRI receptor[121]. This *in vitro* effect on apoptosis can be reproduced by the increased IgE found in plasma from atherogenic diet-fed *Ldlr* $^{-/-}$*sIgM*$^{-/-}$ mice[106], lending further support to the possibility that this also occurs *in vivo*. Finally, IgE also has the capacity to trigger strong proinflammatory responses in macrophages. For example, short-term stimulation of macrophages with purified IgE or murine plasma with increased IgE levels lead to increased production of IL-6[106,121]. The epitope repertoire of the proatherogenic IgE antibodies is yet to be explored. However, it is likely that the proatherogenic activities of IgE antibodies do not only rely on antigen cross-linking, but can also be exhibited upon engagement of FcεRI receptors by monomeric IgE (i.e. without the need for antigen) that have the capacity to trigger inflammatory cytokine release by mast cells[122,123].

IgG and receptors

IgG is produced as various isotypes: IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2a/c, IgG2b and IgG3 in mice[20]. These differ in affinity for Fcγ receptors and thus have distinct functions (reviewed in[20]). IgG antibodies instruct innate immune cells (macrophages, dendritic cells and granulocytes) to phagocytose antigens, facilitate antigen presentation, polarize function and induce cytokine or antimicrobial proteins and enzymes. Like IgM, IgG also recruits complement molecules to antigenic surfaces (although less potently). Given these multiple potential functions, it is perhaps unsurprising that the influence of IgG on atherosclerosis is still unresolved. Most studies have focused on OxLDL (or OSE) IgG antibodies. Enhanced anti-oxLDL or anti-MDA-LDL IgG antibodies have been linked to the presence of atherosclerosis in some studies, but not consistently in larger studies (Table 2). A recent study found a predictive value for cardiovascular events for anti-OxLDL IgG specifically in participants of black ethnicity in the Dallas Heart Study [124] and a recent meta-analysis also found a positive correlation with future events[93]. Difficulties in overcoming confounding factors may have hampered isolating the specific associations with atherosclerotic disease. OxLDL preparation can be very heterogeneous and so some studies may in fact detect a distinct range of antibodies. Furthermore, anti-MDA and anti-CuOx IgG antibodies have been reported to correlate strongly with pathogen associated IgG levels[125] and more recently, levels also correlate with total IgG levels in patients[104].

These studies are not a reliable basis on which to ascertain the functional importance of these antibodies. Although some *in vitro* studies support a pathogenic role for OxLDL IgG antibodies, the balance of *in vivo* evidence from mouse and other animal immunization models suggests that high affinity anti-OSE IgG responses are atheroprotective[126]. Insoluble immune complexes of IgG antibodies and OxLDL (OxLDL-IC) were more pro-inflammatory to human macrophages than either antibodies or OxLDL alone[127], and another study showed that stimulation with OxLDL-IC immune complexes most closely reproduced the expression profile of human plaque macrophages[128]. Recently, OxLDL-IC were shown to induce inflammasome activation and IL-1β production in mouse bone marrow-derived dendritic cells *in vitro* [129]. However, a case-control study of human circulating apoB-IgG immune complexes in the EPIC-Norfolk cohort - did not find an independent association with CVD after 6 years follow-up[130]. When considering the disease-driving role ascribed to OxLDL, a protective influence of IgG antibodies that could neutralize and enhance clearance of OxLDL is not altogether unexpected. Passive immunization with MDA-ApoB antibodies reduces disease [131], providing direct evidence that this in principal occurs *in vivo*, and suggesting a potential therapeutic strategy (see below). Mechanistically, OxLDL-IC are cleared from the circulation more rapidly than LDL alone, probably through Fc receptor-dependent uptake in the liver/spleen[132]. In several cases, but not all, circulating LDL levels are reduced by high OxLDL IgG levels, suggesting in these cases the effect may be upstream of plaque rather than intrinsic to plaque inflammation[101,133]. An inverse correlation between OxLDL IgG and total cholesterol has also been observed in humans in some studies[125,134]. Active immunization (i.e. repeated injection with adjuvant and antigen) with modified LDL is also protective[135], however, an IgG response may not be the mechanism mediating these effects. These protocols also induce natural IgM antibody response as well as regulatory T cell expansion and can be effective in the absence of a significant IgG response[136]. Alternative tolerization strategies, using non-adjuvanted administration of ApoB peptides, which boost Treg more specifically are also effective in reducing atherosclerosis[137,138].

When considering the overall role of antibodies in atherosclerosis, clearly more antigens than OxLDL or other antigens carrying OSE may be involved (see above). To investigate the role of endogenous antibodies without the need to assume relevant specificities, two recent studies have targeted plasma cells specifically. Mice in which antibody secretion capacity of plasma cells is severely attenuated in all B cell lineages, using *Cd79*-driven Cre deletion of the transcription factor x-box binding protein-1 (XBP1), display reductions in all major antibody isotypes, including IgM levels, and increased atherosclerosis[113]. In contrast, using *Cd23*-cre and deletion of the alternative transcription factor BLIMP1, which led to a robust decrease in plasma cells as well as IgG and IgM, resulted in less atherosclerosis[139]. Differences between these studies include the absence rather than dysfunction of plasma cells and a more severe effect on IgG levels in the BLIMP1 model. One tentative explanation is that the IgG:IgM ratio over a broad range of concentrations could be important. Indeed, small changes in tolerance that increase autoimmune germinal center reactions lead to increased IgG/IgM ratio systemically and in plaques and more atherosclerosis[140]. Some other lines of evidence from mouse studies further support the existence of pathogenic antibodies (Table 1). Transfer of purified total IgG from atherosclerotic but not WT mice was sufficient to accelerate atherosclerosis, providing evidence supporting the hypothesis that IgG antibodies formed specifically in association with atherosclerosis can be pathogenic [139]. This pathogenic role of IgG is consistent with two other studies showing that removing restraints on the GC B cell – Tfh cell response (by lack of MZ B cells)[141] or CD8+ regulatory T cell inhibitory function[140]) leads to significantly enhanced atherosclerosis. However, enhanced IgG responses were only detected in one study; the other suggested alternative pathogenic roles for Tfh (see below)[140,141]. Gaddis *et al* recently targeted GC responses via genetic depletion of Tfh and anti-ICOSL treatment and also showed reduced atherosclerosis, however no role for IgG was investigated[142]. This protective influence of anti-ICOSL was previously observed in two studies, but only in mutant mice where Tfh were enhanced, not in control mice[140,141]. Recently, deletion of the key B cell transcription factor Pax5 in AID-expressing GC B cells, leading to their deletion, reduced atherosclerosis[143]. Thus, either attenuating or enhancing GC B cell responses suggests these responses are pathogenic in mouse atherosclerosis. The antigenic targets and molecular triggers for these GC reactions remain to be determined.

A number of studies have investigated the impact of deleting IgG receptor genes on atherosclerosis, however it is currently unresolved how much of the resulting phenotype depends directly on altered IgG-dependent effects versus altered innate functions of either the receptors or the cells expressing them. Mice deficient in FcγRIII (CD16) develop less atherosclerosis[144,145]. Interestingly, FcγRIII has been shown to bind MDA-LDL directly and so may act like scavenger receptors on macrophages and promote foam cell formation[145]. Several studies have used γ-chain$^{-/-}$ mice, the intracellular signalling domain used by activating Fc receptors, and shown a reduction in atherosclerosis[146,147]. However, γ-chain is also used by a number of other Ig-family receptors including the IL-2 receptor, thus regulating T cell responses, and the C-type lectin Clec4e, which has also been implicated with a pathogenic role[148]. The sole inhibitory IgG receptor, FcγRIIb, is a known autoimmune susceptibility gene, and SNPs in FcγRIIb also influence total IgG levels in humans[149,150]. Conflicting results showing opposite effects on atherosclerosis have been reported using *Fcgr2b*$^{-/-}$ mice[82,151], some but not all of which may have been affected by inadequate backcrossing to remove residual SNPs from the SLE-prone 129 strain[151]. Alternative approaches to using global knockout mice, which will have defects in multiple systems, are required to resolve this impasse.

B cell regulation of cellular immune responses

As a major population in lymphoid and hematopoietic organs, B cells play important roles in development, activation and regulation of cellular immune responses. Disruption of these pathways may be a major mechanism by which B cells regulate atherosclerosis (Figure 2). Most studies to date rely on deletion or depletion of one or many B cell subsets (Table 1). In these studies, antibody-mediated B cell depletion and deletion of BAFF receptor[152-155], that result in similar B2-cell biased deficiency, both result in significant defects in T cell activation and reduced atherosclerosis. B cell depletion with anti-CD20 antibodies resulted in enhanced IL-17-producing T cells[152]. A reduction of IFN-γ was observed and reversal of decreased atherosclerosis was associated with restoration of Th1 immune responses and plaque T cell infiltration [152,153,156], suggesting regulation of this known pro-atherogenic response as the causal pathway. An alternative strategy of deleting the key B cell transcription factor Pax5 in CD23-Cre sensitive cells, primarily mature B2 cells but also some B1 cells, also resulted in reduced atherosclerosis [143]. The effect of FO B cells on T cells could be direct or indirect. Antigen presentation from B cells taking up self-antigens to T cells may be an important interaction that sustains effector memory T cell responses[157,158], i.e. overcoming regulatory T cells. Mice with B cell-restricted MHCII deletion develop less atherosclerosis[139], however this attenuates both T effector responses and T cell-dependent antibody responses. Alternatively, B cells may influence the primary antigen presenting cell, dendritic cells. Innate response activator (IRA) B cells producing GM-CSF were shown to influence splenic cDC maturation and subsequent promotion of Th1 CD4 T cells, leading to enhanced atherosclerosis[159] (Figure 2). IRA B cells expand in response to high fat diet feeding in mice[159] and absolute numbers of spleen cDCs also expand[160]. IRA B cells have also been reported in humans but are yet to be linked to cardiovascular disease[161]. Although not identified as a particular subset, B cell production of TNF, increased in high fat diet-fed mice, was also suggested to enhance atherosclerosis[162]. The authors suggest a local effect of TNF-producing B cells on plaque macrophages, however this is hard to reconcile with the scarcity of B cells and abundance of monocytes/macrophages. Interestingly, in that study μMT/*Apoe*$^{-/-}$ mice had severely reduced atherosclerosis compared to *Apoe*$^{-/-}$ counterparts in a similar way to *Rag2*$^{-/-}$*Apoe*$^{-/-}$ mice. This opposite result to original studies reported with μMT mice suggests different effects between different labs or mouse colonies (Table 1).

In contrast to total B2 cell depletion, genetic deficiency of MZ B cells accelerated atherosclerosis[141]. Chimeric mouse studies revealed that high fat diet feeding triggers an ATF3-dependent upregulation of PD-L1 in MZ B cells, which is key in suppressing Tfh cells. The resulting Tfh displayed key functional differences, such as reduced IL-21 production (Figure 2). Interestingly, antibody levels were not dramatically affected, raising the possibility that Tfh may have other GC-independent pathogenic functions, supported by the fact that Tfh can be found in atherosclerotic aortas[140,142]. Circulating human MZ-like B cells express higher PD-L1 than naïve B cells and upregulated PD-L1 in response to BCR stimulation[141]. These same MZ-like B cells, also termed ‘unswitched memory’ B cells, were reported to be negatively associated with secondary CV events [64]. Patients in the highest tertile of unswitched memory B cells had a

hazard ratio of 0.3 in multivariable models that included adjustment for age and smoking (with which unswitched memory B cells also inversely correlated). In addition to pro-inflammatory cytokines, B cells can also secrete IL-10, which has broad anti-inflammatory properties. Several subsets appear capable of IL-10 secretion, including specialised regulatory B cells[163]. Gjurich *et al* found significantly reduced IL-10 production locally in the aorta of *L-selectin*$^{-/-}$ *Apoe*$^{-/-}$ mice, which have significantly increased atherosclerosis. This was associated to reduced B1a and Breg cell numbers, both high IL-10 producers, whereas Treg numbers were similar. *L-selectin*$^{-/-}$ mice, however, display severe changes in systemic leukocyte trafficking and this study does not address the effect of specifically removing these cells. Indeed, in a model of B cell IL-10 deficiency that showed important roles in SLE and RA, no impact on atherosclerosis was observed[164] (Table 1), despite a very large effect on serum IL-10. Transferring B cells purified from renal lymph nodes, in which IL-10 expression is expanded in atherosclerotic mice, prior to vascular injury led to significantly reduced neointima formation [165]. This effect was maintained when CD21^{hi} CD23^{hi} CD24^{hi} regulatory B cells were used and was partially reversed in the presence of anti-IL-10 antibody treatment. Perhaps B1a cell-specific IL-10 production is important. B1a cells are poorly reconstituted post bone marrow transplant so would have contributed less in both groups of our study[164]. Recently, angiotensin-II (Ang-II) was shown to boost IL-10 production in B cells via direct action on Ang-II type-1 receptor. This action of Ang-II was sufficient to reverse the pro-atherogenic effect of B2 cells in a B cell transfer model[156]. However, in the absence of Ang-II both WT and IL-10$^{-/-}$ B cells had a similar impact on atherosclerosis. Thus, it seems that regulatory B cells may indeed have an important protective potential, however their role (whether this relies on IL-10 or not) is likely contextual.

B cells also consume various cytokines, which are essential for their survival and function but also for other cellular systems that are relevant in atherosclerosis. BAFF, crucial for B cell survival[166], is also recognised by monocytes[167] and macrophages[168] and affects their survival or polarization, respectively, suggesting these B cell-independent functions of BAFF could modulate atherosclerosis. BAFF blockade with an antibody aggravated experimental atherosclerosis despite B2 cell depletion and IgG reduction[169]. The protective effect of BAFF is likely dependent on the alternative receptor for BAFF, TACI, as myeloid-specific TACI deletion enhanced atherosclerosis[169]. TACI, unlike BAFF-R, is not essential for B2 cell development but plays important roles in controlling B2 cell responses[170] and is also expressed in the myeloid compartment[167,168]. TACI is found at the cell surface as well as in association with intracellular TLRs, and is more efficiently activated by multimeric forms of BAFF[171]. Mechanistically, BAFF stimulation dampened TLR9-IRF responses in macrophages. Taken together, these data suggest soluble BAFF exhibits an atheroprotective effect and provide additional mechanistic explanations to the atheroprotective properties of B cell depletion, which elevates BAFF levels[156]. Transgenic overexpression of soluble BAFF from macrophages reduced atherosclerosis, although this may primarily be a result of reduced LDL[133]. An additional prominent example is a soluble cytokine, A Proliferation Inducing Ligand (APRIL), which binds TACI and a third receptor of the so called BAFF-APRIL system, named B cell maturation antigen (BCMA), which is expressed by plasma cells[172]. APRIL is involved in IgA class switching and plasma cell survival[173,174]. Ectopic overexpression of APRIL in atherosclerosis-prone mice lead to a strong increase in peritoneal B1a and B1b cells and in anti-OxLDL IgM levels[175]. Nevertheless, it did not affect atherosclerotic plaque size or necrotic areas but did lead to increased smooth muscle cell content[175] While these data suggest that increased levels of APRIL do not impact atherogenesis, it is important to note that BAFF is still present in this setting and may mask any role of APRIL by competing for binding to their common receptors TACI and BCMA.

Translational spectrum of B cell targeting in atherosclerosis

As mentioned above, B cell responses play a cardinal role in many chronic pathologies that include autoimmune and rheumatic diseases[176] and many of these pathologies are associated with particularly increased CVD risk. The development of therapeutics that modulate B cell immunity has become a strategic priority in the past years. Such therapies may be selectively directed at B cells, as is the case for B cell depleting antibodies, or may affect B cell responses in conjunction with other mechanisms, as is the case for therapies targeting cytokines (IL-6, TNF and IL-1β) or costimulatory pathways (CD40,

ICOS/ICOSL, PD-1/PD-L1). They are all considered as potentially interesting therapies in cardiovascular diseases (reviewed in[177,178]).

B cell depletion. Several B cell depleting agents that target different B cell subsets are already used in the clinic or being tested in clinical trials. Among those, rituximab, an anti-CD20 antibody, was the first and until today the most widely used B cell depletion therapy. Rituximab was first approved by the FDA in 1997 for the treatment of B cell non-Hodgkin lymphoma[179]. It is also now used in autoimmune diseases, predominately in RA[180]. Mechanistically, it cross-links the CD20 receptor present on most B cells, leading to FcγR-mediated cell depletion[181]. The experimental data on the atheroprotective effect of anti-CD20 Ab treatment described above suggest that rituximab would confer a beneficial effect in human atherosclerosis. In addition, anti-CD20 treatment appears to confer beneficial effects on post-ischemic cardiac remodelling in mice[182]. Indeed, the acute setting is the more likely successful therapeutic indication for rituximab or similar therapies. Nevertheless, given the continued use in other diseases with a high cardiovascular risk, human data is gradually emerging on the cardiovascular impacts. A number of safety studies in RA patients included cardiovascular outcomes with up to 12 months follow-up. A recent meta-analysis of these studies found no change in risk of cardiovascular events in rituximab-treated patients but concluded that the limited follow-up time of the studies precludes strong conclusions[183]. Some small studies also investigated carotid intima media thickness (cIMT), with one showing a significant decrease at 6 months in RA patients responding to therapy[184]. Another, with a much lower average baseline cIMT, showed no significant change after 24 months, and a small study of 5 patients showed a transient decrease at 2 weeks but less so at 12 weeks post-rituximab[185] Other studies did not measure atherosclerosis directly but other cardiovascular parameters such as flow-mediated dilatation and arterial stiffness were either reduced or unchanged[183]. Given the presence of RA-related inflammation and its reduction by rituximab, it is hard to translate these findings to the potential effects of rituximab in non-RA patients. However, taking into account the latest mechanistic perspectives from mouse studies, it is important to note that rituximab would also deplete MZ B cells, which exhibit atheroprotective properties[141]. This is important as MZ B cells in humans display distinct functions compared to mice, such as circulating capacity and likely the ability to home into inflammatory areas away from the spleen (e.g. atheromas) where they may play an important protective role[64]. An additional potential side-effect of rituximab is reduction of plasma IgM antibodies that are presumed to display an atheroprotective effect in humans. Collectively, despite promising hints, it remains to be seen if a rituximab-induced B cell depletion would have a beneficial effect in human atherosclerotic CVD.

Belimumab, a neutralizing anti-BAFF antibody, was approved by the FDA in 2011 for the clinical management of SLE patients. Belimumab, the only drug approved for SLE in 50 years[186], blocks BAFF from binding to BAFFR and thereby induces mature B cell depletion and reduces total immunoglobulin production[187]. Belimumab exerts a number of different effects that would be relevant in human atherosclerosis in both positive and detrimental manners. For example, mature B cell depletion and reduction of total IgG levels[188] is likely to confer a beneficial effect while the reduction in total IgM levels[188] may act in the opposite direction. In fact, our studies suggest BAFF exhibits an important atheroprotective effect - outside the B cell compartment - by dampening macrophage proinflammatory responses[169]. Therefore, it is important to monitor the effect of these treatments on CVD in both RA and SLE patients. Such studies would also provide very important insights about the potential impact of B cell depletion in human atherosclerosis.

Anti-IgE. A more precise strategy to modulate B cell immunity involves direct targeting of immunoglobulins. Neutralization of free IgE Abs in atherosclerotic sIgM$^{-/-}$ mice confers an atheroprotective effect[106]. These data suggest that increased levels of IgE Abs may trigger proatherogenic mechanisms and that anti-IgE treatment may be an alternative option against atherosclerotic CVD. This is interesting as Omalizumab, a human anti-IgE antibody that neutralizes free IgE, is used in the clinic for patients with severe asthma who notably also display increased CVD risk[189-191]. The therapeutic value of anti-IgE treatment in CVD may be relevant in other settings that are associated with increased IgE and premature atherosclerosis. For example, autoreactive IgE antibodies have recently been documented to exhibit a pathogenic role in experimental SLE[192] and to be positively associated with disease activity in SLE patients[193].

Vaccination. Boosting (or creating) protective B cell antibody responses is an alternative strategy to treat atherosclerosis, using either passive Ig transfer or active vaccination. Administration of human antibodies raised against immunogenic epitopes of human ApoB100 was effective in reducing atherosclerosis and other cardiovascular disease parameters in pre-clinical models[194,195] and has already been tested in the GLACIER phase-II clinical trial[196]. The effect of anti-MDA-ApoB100 IgG1 injections on ^{18}F-fluorodeoxyglucose (FDG) uptake into plaques on PET/CT imaging, a surrogate for plaque macrophage presence/inflammation after 12 weeks was assessed in stable patients with FDG-positive plaques at baseline. The treatment resulted in no effect on this primary endpoint. In addition to the possibility that this therapeutic approach is ineffective, there are several confounding factors that may have led to the failure of this trial. It is possible that 1) the dose level inside plaques was not sufficient, 2) binding of the antibody to macrophage Fc receptors may have affected metabolism and FDG uptake, and 3) residual systemic inflammation was low since unlike the JUPITER and CANTOS trials subjects were not selected on the basis of high C-reactive protein. Active vaccination trials using MDA-ApoB or ApoB peptides also show promising results in pre-clinical phases (reviewed in [197]), but as discussed above, these strategies may be more effective at boosting regulatory T cell-mediated protection rather than B cell-mediated mechanisms. Since a pneumococcal vaccine was effective in mice at boosting anti-PC IgM and reducing atherosclerosis[95,198], it is interesting to consider this as a therapeutic strategy. A recent meta-analysis of pneumococcal protein vaccine case-control observational studies found an overall odds ratio of 0.88 for ACS in patients over 65[199].

A more detailed understanding of the B cell responses in dyslipidemia along with the rapid development of novel B cell biologicals by the pharmaceutical industry holds great promise for new therapeutic options against atherosclerotic CVD.

Conclusions and future perspectives

The role of the immune system is cardinal in the development and progression of atherosclerosis. However, identifying “sweet-spots” that can be drug-targeted is on one hand a major challenge but also a very attractive aim for the development of new therapeutic strategies against atherosclerosis. Within the complex network of immunity in atherosclerosis, B cells harbour powerful properties with both protective and detrimental impact in atheroma formation and thus represent a promising therapeutic target. Further resolution of the apparent paradoxes arising from the contrasting results reported between seemingly equivalent models will be key to isolating the truly important mechanisms. The research community may need to combine previous models or re-evaluate previous findings in the light of new insights, and reproduce data in different labs to ascertain fully the robustness of the findings. Some specific open questions that still require resolution include 1) which B cell subset is IL-10 production in critical (if at all), 2) does the antigen specificity of FO or MZ B cells matter for their impact on atherosclerosis? And 3) what is the relative contributions of the different mechanisms proposed for the protective effects of B cell depletion, and which of these translates to the human disease? The development of antigen-specific and inducible models will also enable future mechanistic insights. Several biologicals have been developed that allow easy and successful targeting of B cells in the clinic. This was mainly driven by the key role of B cell immunity in several autoimmune diseases, which notably (such as SLE and RA) are also associated with high CVD risk. However, as with autoimmunity, the complexities of mechanistic pathway insight are still holding back molecular insights that would allow the generation of more precise therapies. More intense studies coupled with state of the art technologies and experimental models are needed in order not only to map the network of B cell immunity in atherosclerosis, but to understand how to control the system flux to mitigate disease.

Key Points (3-5 bullet points summary)

- Atherosclerosis is associated with both innate and adaptive immune responses
- Inflammation in atherosclerosis is mainly driven by neo (self-)epitopes present on LDL and dying cells, both recognised by natural antibodies
- B cell responses targeting oxidation-specific epitopes may limit disease whereas other antibodies may have pathogenic consequences
- Antibody-independent roles for B cells such as cytokine production and T cell regulation also contribute to B cell control of atherosclerosis
- B cell depletion therapies and vaccination strategies show promise, however more precise targeting of different B cell functions is an important future goal

Box – B cell transformation into antibody-secreting plasma cells

A B cell is activated by a combination of 1) cognate antigen ligating the B cell receptor, 2) innate signals such as toll-like receptor (TLR4, 7 or 9) ligands and 3) co-stimulatory signals via CD40 and ICOSL and cytokines such as IL-4, IL-5 or IL-21. The absence of inhibitory factors such as pre-existing IgG that recruits FcγRIIb to the B cell receptor complex[149] may also be required. The initial response is rapid proliferation; these cells are plasmablasts and secrete low levels of antibodies[32,200]. B1 cells and marginal zone B cells appear to be held in a poised state at this point, allowing rapid plasma cell formation upon receiving the final trigger (e.g. IL-5 or LPS) without requiring rounds of proliferation[17,89]. B cells subsequently undergo a profound transcriptional reprogramming in the transition to plasma cells[200]. Plasma cells are extreme protein synthesising and secreting factories, with around a 100-fold enhanced secretion rate[201,202]. Transcription at the B cell receptor locus must switch to secretory forms. The coordinated action of transcription factors Blimp-1, interferon response factor-4 and Xbp1 is key in adaptation to this capability[203]. The Xbp1-controlled pathway is part of the unfolded protein response but in plasma cells is co-opted to expand the endoplasmic reticulum, golgi and ribosomal organelles ready for antibody synthesis and secretion. Plasma cells down-regulate CXCR5 and upregulate CXCR4, promoting lymphoid emigration and homing to the bone marrow[200]. B1 antibody secreting cells, only some of which are Blimp-1 dependent, rapidly appear in the splenic red pulp, but also reside in the bone marrow like B2 plasma cells[26]. Plasma cells reside in specific niches adjacent to sinusoids, such that antibodies are secreted directly into the circulation. There, the longevity of plasma cells is defined by competition for survival signals from residual antigen and APRIL, produced by innate immune cells such as eosinophils[204,205].

Table 1 – Mouse models demonstrating atherosclerosis regulation by B cells

Model	Ref(s)	B cell Phenotype	Impact on Atherosclerosis	Proposed mechanism(s)
B cell specific				
µMT	66 162	No B cells	Increased Decreased	Increased OxLDL / OSE
+B2 cell transfer	162		*Increased*	Macrophage TNF production
+TNF$^{-/-}$ B2 cells	162		*No effect*	
+B2 cell transfer	206		*Decreased*	Aortic homing via CCR6
+Breg transfer	165		*Decreased*	B cell IL-10 production
sIgM$^{-/-}$	105,106	No soluble IgM but increased IgE	Increased	IgE activation of mast cells and macrophages
Anti-CD20 Anti-BAFFR	152,153 207	Depletion of mature B2 cells	Decreased	Decreased effector T cell responses
BAFFR$^{-/-}$ *	154, 155,156	Lack of mature B2 cells	Decreased	Decreased effector T cell responses
GM-CSF$^{-/-}$ *	159	No GM-CSF expression	Decreased	Reduced pro-atherogenic cDCs and Th1 cells
IL-10$^{-/-}$ *	164	No IL-10 expression	No difference	n.a.
CD19^{Cre} x Id3$^{fl/fl}$	208	Increased B1b cells	Decreased	B1b cell IgM production
TNF*	*162*	No TNF expression	Decreased	TNF stimulation of plaque macrophages
B cell GITRL^{tg}	209	Enhanced T cell co-stimulation	Decreased	Enhanced regulatory T cells
SiglecG$^{-/-}$ *	100	Increased B1 cell levels and OSE-specific IgM	Decreased	Decreased OxLDL / OSE /dying cell accumulation
CD79^{Cre} x Rbpj $^{fl/fl}$	141	Lack of marginal zone B cells	Increased	Enhanced but defective Tfh formation
CD79^{Cre} x Xbp1 $^{fl/fl}$	113	Attenuated antibody production	Increased	Increased OxLDL / OSE accumulation
CD23^{cre} x Blimp1 $^{fl/fl}$	139	Defective activation, lack of plasma cells	Decreased	Pathogenic effect of IgG from follicular B cells
MHCII$^{-/-}$ *	139	Lack of antigen specific interaction with T cells	Decreased	Pathogenic effect of IgG from follicular B cells and effector T cells
CD40$^{-/-}$ *	139	Lack of co-stimulation e.g. from T cells	Decreased	Pathogenic effect of IgG from follicular B cells and effector T cells
Major B cell phenotype				
Splenectomy	65,103	Reduced B1a cells and IgM	Increased	Increased OxLDL / OSE
IL-5$^{-/-}$	89	Decreased IgM	Increased	Increased OxLDL / OSE
IL-33$^{-/-}$	109	Decreased IgM	Increased	Increased OxLDL / OSE
L-selectin$^{-/-}$	210	Decreased aortic B cell infiltration	Increased	Increased OxLDL / OSE
Qa1$^{-/-}$	140	Increased Tfh – GC formation; increased IgG	Increased	Pathogenic effect of IgG from GC B cells
Rag1$^{-/-}$Apoe$^{-/-}$+ B1b cells	208	Increased B1b cells and IgM	Decreased	B1b cell IgM production
BAFF^{tg}	133	Increased B cell numbers and activation, autoimmunity	Decreased	Anti-OxLDL IgM mediated reduction in systemic LDL levels
APRIL^{tg}	175	Increased B1 cells	No difference	
CD4^{cre}-Bcl6$^{fl/fl}$	142	No Tfh, therefore no GC B cell responses	Decreased	Pathogenic effect of Tfh and/or IgG from GC B cells
Anti-BAFF Ab	169	Depletion of mature B2 cells	Increased	BAFF-TACI signalling represses proatherogenic chemokine production by macrophages

**B cell selectivity achieved with µMT mixed bone marrow chimera approach.*

Table 2 – Summarised association of antibodies with cardiovascular disease in humans

Antibody category	Potential functions		Summary of associations with cardiovascular risk	Example References
	Physiology	Pathology (Atherosclerosis)		
MDA-LDL IgG Ox-LDL IgG	• *Clearance/neutralization of OSE* • *Protection against bacterial insults* • *Endothelial permeability?*	• *Macrophage activation* • *Regulation of foam cell formation*	*Many studies find a positive correlation, however this is rarely independent of other risk factors.* *A recent study found a strong predictive value specifically associated with black ethnicity*	124,130,211,212 124
MDA-ApoB IgG	• *Clearance/neutralization of OSE* • *Protection against bacterial insults* • *Endothelial permeability?*	• *Macrophage activation* • *Regulation of foam cell formation*	*High levels associate with decreased risk of coronary events*	213
MDA-LDL IgM OxLDL-IgM MDA-ApoB IgM	• *Clearance/neutralization of OSE* • *Protection against bacterial insults*	• *Regulation of foam cell formation* • *Enhanced removal of apoptotic/necrotic debris*	*Most studies show a negative correlation with CVD risk, this is mainly independent of other risk factors.* *However, some large studies found no association*	125,130,211,212,214 124,215
PC- IgA & CWPS*-IgA	• *Protection against bacterial insults* • *Regulation of homeostasis of gut flora*	• *Disturbed gut permeability and cholesterol metabolism?*	*Positive association with long term CVD risk*	216
PC IgM	• *Clearance/neutralization of OSE* • *Protection against bacterial insults*	• *Regulation of foam cell formation* • *Enhanced removal of apoptotic/necrotic debris* • *Regulatory T cell function*	*Low levels confer increased risk for future events, particularly in at-risk patients (e.g. stable CAD patients, patients with SLE)*	114,217-220
Classical autoantibodies (RF, ANA, anti-CCP)	*N/A*	• *Increased inflammation, myeloid cell activation* • *Endothelial dysfunction*	*As a diagnostic for RA, CCP positivity is associated independently with increased CVD risk in RA patients.* *RF, ANA and anti-CCP in non-RA patients are also associated with enhanced risk*	76,221,222 76,77
Anti-heat shock protein 65	• *Protection against bacterial insults*	• *Endothelial dysfunction* • *Myeloid cell recruitment and activation*	*In the Bruneck study, anti-hsp60 antibodies correlate with the presence of plaque, even in 15-30 year olds*	42

Table 2 cont.

Antibody	Potential functions	Summary of associations with	Example

category	Physiology	Pathology (Atherosclerosis)	cardiovascular risk	References
ApoA1 IgG	*n/a*	• *Inhibition of HDL function* • *Accumulation in plaques and myeloid cell activation* • *TLR2 ligation*	*In prospective cohort of 5220, increased risk of CAD.*	223
Total serum IgE	• *Mucosal surface immunity / barrier function*	• *Mast cell and eosinophil activation* • *Macrophage activation/cell death*	*Higher IgE increases risk for CAD, unstable plaque and plaque size*	224,225
Total serum IgA	• *Gut barrier function*	• *Disturbed gut permeability and cholesterol metabolism?*	*Positive association with long term CVD risk*	68
Total serum IgM	• *Clearance/neutralization of self-antigens and metabolic debris* • *Protection against bacterial insults* • *Endothelial permeability?*	• *Macrophage activation* • *Regulation of foam cell formation*	*Negative association with long term CVD risk*	104
**Cell wall polysaccharide*				

List of specialist terms

Oxidation-specific epitopes

Lipid moieties, such as malondialdehyde and phosphorylcholine, often found as adducts on biological molecules as a result of oxidative modification.

Class switching

An individual activated B cell clone undergoes recombination at the B cell receptor genomic locus resulting in different constant (Fc) region gene usage and production of a new class (isotype) of antibody.

Affinity maturation

GC B cell clones reactive to a certain antigen hypermutate the BCR variable region and via competitive evolution and natural selection, clones with progressively higher affinity for the antigen emerge.

Germinal center

A localised region of a follicle of a lymphoid organ that arises when antigen-specific B2 cells and T cells combine; B2 cells proliferate rapidly as GC B cells.

Marginal zone

A specialised region of the spleen on the border of the white and red pulp that functions to monitor and filter the blood. The marginal zone is populated by specialised B cell and macrophage subsets.

B cell receptor

The surface antibody protein expressed by each B cell clone. Each clone expresses a unique BCR deriving from random recombination of germline variable region genes.

Natural antibodies

Pre-existing antibodies, mostly of IgM, IgA and/or IgG3 isotypes, that arise early in life independently of the presence of microbes.

BAFF

B cell activating factor of the TNF family; essential for B2 cell development and key in many aspects of B cell physiology, but also regulates other cell types such as macrophages.

B cell depletion therapy

Therapy that comprises a class of monoclonal antibodies that target B cell-specific surface molecules, and so result in antibody dependent cell cytotoxicity.

***Apoe*$^{-/-}$ mice**

Apolipoprotein E is the ligand for the LDL receptor and *Apoe*-deficient mice have high levels of circulating cholesterol (VLDL and triglycerides), in addition to defects in phagocytosis; A widely used model of atherosclerosis.

***Ldlr*$^{-/-}$ mice**

Low density lipoprotein receptor deficient mice are a model of atherosclerosis when fed high cholesterol and fat diets via the inability of the liver to uptake low density lipoprotein.

Efferocytosis

The process of apoptotic cell recognition, uptake via phagocytosis and degradation

Plasma cells

Specialised B cell-derived post-mitotic cells with high protein synthesis and secretion capacity that secrete high amounts of soluble antibodies into the bloodstream.

Antibody secreting cells

A term that encompasses any B cell secreting soluble antibody, including plasmablasts, B1 cells and plasma cells.

Immune complexes

Molecular aggregates of antibodies and their cognate antigen. Immune complex formation is often (but not always) necessary for activation of downstream antibody effector functions.

Figure Legends

Figure 1. B cell effector mechanisms in atherosclerotic plaques. B1 cells are mainly of fetal liver origin whereas B2 cells originate from precursors in the bone marrow, which give rise to immature B cells, following maturation in the spleen leading to formation of FO and MZ B cells. Following exposure to a complex set of stimuli (see box and Figure 2) naïve B cells differentiate to antibody secreting cells (plasmablasts and plasma cells). These cells, which mainly home into the bone marrow, secrete immunoglobulins (Igs) into the blood stream. Activated endothelium overlying atherosclerotic plaques upon dyslipidemia allows the entry of different Igs into the plaque area where they exhibit various functions. In addition, at least in advanced stages of plaque formation, artery tertiary lymphoid organs are also formed (e.g. in the adventitia), which include plasma cell formation in situ leading to production of Igs in the adventitia. A large part of IgM has the capacity to recognize oxidation specific epitopes, which are present on OxLDL and apoptotic debris, and limit OxLDL-induced endothelial activation and OxLDL-induced foam cell formation via scavenger receptors. via complement recruitment On the other hand, IgG antibodies form immune complexes with OxLDL and promote macrophage inflammatory responses. IgE exhibits strong proatherogenic properties by stimulating macrophages and mast cells both in the plaque and in the perivascular area. The role of IgA in atherosclerosis remains elusive. B cells also produce various cytokines such as pro-atherogenic TNF-α or anti-atherogenic IL-10. FO, follicular B cells; MZ, marginal zone B cells; HSP60, heat sock protein 60; OxLDL, oxidized LDL; SRs, scavenger receptors; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-1β, interleukin-1 beta; FcεRI, high affinity IgE receptor, FcγR, Fc-gamma receptors; PC, plasma cell; Tfh, T follicular helper cells; cDCs, conventional dendritic cells.

Figure 2. **B cell responses in lymphoid organs regulating atherosclerosis**. B2 cell responses in atherosclerosis are likely to initiate within lymphoid organs (or potentially vascular adventitia). B2 cells can respond in two main modes, both initially involving a proliferation phase as plasmablasts. Plasmablasts then either directly form plasma cells or first enter the germinal center response allowing affinity maturation of the BCR. Class switching to different antibody isotypes may occur in either case, leading to different antibody effector functions on atherosclerosis (see figure 1). B2 cells may promote or sustain effector $CD4^+$ T cell responses, such as T helper type-1 (Th1) and Th17 responses. High fat diet also expands conventional dendritic cells (cDCs), the main activator of the naive T cell pool, part of which is mediated downstream of innate-response activator (IRA) B cells producing granulocyte-monocyte- colony stimulating factor (GM-CSF). B2 antibody responses formed via germinal center (GC) B cell reactions are now thought to be pathogenic. Recent work has highlighted several important regulators of the pro-atherogenic GC response, as referenced in the text. CD40 and MHCII on follicular (FO) B cells and Bcl6 in $CD4^+$ T cells are important for induction of the GC-T follicular helper (Tfh) axis. Marginal zone (MZ) B cells responding to high fat diet upregulate PD-L1 via ATF3, and PD-L1 dampens ICOSL-driven Tfh differentiation; defective Tfh formed in the absence of MZ B cells may have direct pro-atherogenic effects but these are yet to be defined. T cell expression of Qa1 recruits inhibition from $CD8^+$ regulatory T cells, another layer of Tfh inhibition important in atherosclerosis.

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