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## B Cells and Humoral Immunity in Atherosclerosis

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### Abstract

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

### Keywords

antibodies; atherosclerosis; B-lymphocytes; complement system proteins; immunity, humoral; immunoglobulin M

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Atherosclerosis is a chronic inflammatory disease of large- and medium-sized arteries. It is characterized by the deposition and trapping of low-density lipoproteins (LDLs) in the artery wall, which then undergo a variety of changes, both enzymatic and nonenzymatic, that lead to a cascade of inflammatory responses followed by the recruitment of mostly macrophages and T cells.<sup>1</sup> The accumulation of LDL in the subendothelial space makes it susceptible to oxidation by various processes, and the resultant formation of oxidized LDL (OxLDL) has been suggested to be the key event in driving the subsequent inflammation that characterizes atherosclerosis.<sup>2</sup> Infiltrating macrophages take up OxLDL via scavenger receptors leading to the formation of lipid-laden foam cells, which are the hallmark cells of atherosclerotic

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lesions. With plaque progression, many foam cells undergo apoptosis resulting in the formation of an acellular necrotic core that is full of lipid gruel and cellular debris. Once the fibrous cap covering the necrotic core erodes or ruptures, allowing contact between the exposed material and the circulating coagulation system, thrombus formation is triggered, resulting in clinical events such as stroke and myocardial infarction,<sup>3,4</sup> which are leading causes of death globally.<sup>5</sup>

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

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

Adaptive immunity also plays an important role in lesion development. Antibodies with specificity for plaque antigens (eg, OxLDL) and activated T cells are present in human lesions.<sup>12,13</sup> Important experimental evidence regarding the involvement of adaptive immunity came from studies with atherosclerosis-prone LDL receptor (*Ldlr*<sup>-/-</sup>) or apolipoprotein E-deficient (*ApoE*<sup>-/-</sup>) mice that lack both functional T and B cells as a result of recombination activating gene deficiency or a severe combined immunodeficiency background.<sup>14-16</sup> These mice were found to exhibit significantly less lesion development indicating an important role for adaptive immunity. However, lesions still develop in these mice, and in the face of marked hypercholesterolemia, the immune-deficient mice develop similar lesions compared with those with intact adaptive immunity. Thus, although not a prerequisite for lesion development, adaptive immunity has an important modulatory role that can both enhance and retard lesion progression. There is now extensive evidence that Th1 responses and interferon- $\gamma$  (IFN- $\gamma$ ) in particular are critical drivers of lesion formation. These effects are primarily mediated by CD4<sup>+</sup> T cells, but CD8<sup>+</sup> T cells seem to play a critical role as well.<sup>9</sup> Compelling evidence also demonstrates that the activity of proatherogenic T-cell subsets is tightly regulated by regulatory T cells, which act via multiple mechanisms and may directly alter plaque characteristics by the secretion of the fibrogenic cytokine transforming growth factor- $\beta$ .<sup>8</sup> The roles of other T-cell subsets such as Th17 and Th2 are less consistent, and their effects may be best understood by the cytokines they secrete and how these function in relationship to IFN- $\gamma$ -secreting Th1 cells. In contrast to the pronounced presence of T cells within lesions, only few B cells can be detected.<sup>17</sup> B cells participate in lymphocyte infiltrates of the adventitia surrounding affected arteries, which can be organized in tertiary lymphoid organs that have been suggested to regulate the inflammatory response of lesions.<sup>18</sup> Emerging data reviewed by Perry et al<sup>19</sup> and Kyaw et al<sup>20</sup> have recently identified an important role for B cells in atherogenesis. The potential mechanisms by which B cells may affect atherosclerosis include the production of immunoglobulins and the subsequent formation of immune complexes with their cognate antigens. Antibodies with specificity for plaque antigens, such as OxLDL, have been detected in atherosclerotic lesions, and plasma antibody titers to OSEs and other antigens such as Hsp60 have been shown to correlate with several clinical manifestations of atherosclerosis.<sup>12</sup> Depending on the class of immunoglobulin, this can lead to the engagement of different Fc receptors resulting in cellular activation or inhibition. Moreover, antibodies may also provide an important function by neutralizing antigens and promoting their clearance. In addition, B cells mediate functions independent of antibody production, such as antigen presentation and the release of cytokines, including interleukin-10 (IL-10). In this review, we will discuss how different B-cell subsets may affect atherogenesis and particularly focus on the role of humoral immunity in mediating some of these effects.

## B Cells Play a Role in Atherosclerosis

A recent network-driven integrative analyses of data from genome-wide association studies and whole blood gene expression profiles from Framingham Heart Study participants identified B-cell immune responses as causative in coronary heart disease (CHD).<sup>21</sup> Based on differential gene expression in whole blood extracts between healthy controls and patients with CHD, the authors identified modules enriched in genes involved in B-cell

function, which were integrated with tissue-specific Bayesian networks and protein–protein interaction networks and led to the identification of B-cell–related genes as key drivers in CHD.

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

The apparent discrepancy between the studies described above may be rooted in part in the differential roles of unique B-cell subsets (Table 1). Extensive studies of the peripheral blood, peritoneal cavity, spleen, and other lymphoid tissues have identified B1 and B2 cells as the 2 main B-cell subsets in mice based on their developmental origin. B2 cells, which represent the vast majority of B cells, are bone marrow derived and include follicular as well as marginal zone B cells. B1 cells, which can be further divided into B1a and B1b based on their surface marker expression, seem to be a developmentally and functionally distinct subset that is localized primarily in the peritoneal and pleural cavities.<sup>26,27</sup> Although B1a cells seem to be atheroprotective, the role of B2 cells in atherosclerosis is still debatable although the majority of studies suggest a proatherogenic role (Table 2 and see below). In addition, a distinct B1-cell subset termed innate response activator (IRA) that responds to lipopolysaccharide stimulation by secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) has recently been identified.<sup>33</sup> It has been shown to play a vital role in protection against sepsis (Table 1). IRA B cells seem to be phenotypically distinct from other B1 cells because they also express the immature B-cell marker CD93. Moreover, in contrast to B1 cells, IRA B-cell survival is dependent on B-cell–activating factor receptor (BAFFR) signaling because these cells are depleted in BAFFR-deficient mice. Hilgendorf et al<sup>32</sup> showed that cholesterol-fed *Ldlr*<sup>-/-</sup> mice deficient in GM-CSF expressing B cells lack IRA B cells and develop reduced atherosclerosis. The authors propose that IRA B cells promote atherosclerosis by stimulating the expansion of mature dendritic cells that results in the generation of IFN- $\gamma$ –secreting Th1 cells, accompanied by a switch from IgG1 to IgG2c antibodies against OxLDL. Moreover, these cells are increased in the spleen of patients with symptomatic compared with nonsymptomatic cardiovascular disease (CVD).<sup>32</sup> Finally, regulatory B cells with a distinct surface marker expression profile have been identified and

are being discussed as modulators of autoimmunity, for example, by the secretion of IL-10 or direct interaction with pathogenic T cells.<sup>34</sup> The role of regulatory B cells in atherogenesis remains to be identified.

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

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

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

In contrast to the studies discussed above, McNamara and colleagues found that adoptive transfer of splenic B2 cells from *ApoE*<sup>-/-</sup> mice into cholesterol-fed  $\mu$ MT/*ApoE*<sup>-/-</sup> mice resulted in robust protection from atherosclerosis. In addition, they found that the effect was absent when B cells from *ApoE*<sup>-/-</sup> mice, which were also deficient in the transcription factor inhibitor of differentiation-3 (Id3), were transferred.<sup>28</sup> Of note, transferred *Id3*<sup>-/-</sup> B cells failed to home to aortic sites, which was accompanied by decreased chemokine receptor (CCR)6 expression on Id3-deficient B cells. A possible explanation for these contradictory data could be the different numbers and different genetic background (wild type versus *ApoE*<sup>-/-</sup>) of transferred B2 cells, which may affect their fate and function in the recipient. Moreover, differences in the purity of transferred cells may also have contributed to these conflicting results. One may even speculate that the CD43<sup>-</sup> B-cell preparations also contained potentially atheroprotective regulatory B cells and that an increased frequency of such cells could have contributed to the beneficial outcome in the study by McNamara and colleagues, for example, via IL-10 secretion.

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

B1a cells on the contrary, which represent the much smaller subset of B cells, clearly protect from atherosclerosis in mice. It is currently not known whether B1b cells have the same atheroprotective potential as B1a cells. An intact spleen has been shown to be required for B1a cell maintenance, because splenectomized or genetically asplenic mice have significantly lower B1 cell numbers in the circulation and peritoneal cavities.<sup>40</sup> Moreover, it has been shown that the splenic microenvironment enhances the antibody production capacity of B1 cells.<sup>41</sup> Thus, splenectomy may result in the loss of this potentially atheroprotective B-cell subset. Indeed, Kyaw et al<sup>29</sup> showed that the peritoneal B1a cells of splenectomized *ApoE*<sup>-/-</sup> mice are reduced by 50%. Importantly, transfer of peritoneal B1a cells but not splenic B2 cells into splenectomized *ApoE*<sup>-/-</sup> mice resulted in an amelioration of the splenectomy-induced accelerated atherosclerosis.<sup>29</sup> Interestingly, in a long-term follow-up study on the cause of death of 740 World War II American servicemen who underwent splenectomy consequent to trauma, the risk of death attributable to ischemic heart disease was found to be 2-fold higher, suggesting a similar net protective effect of splenic cells in humans as well.<sup>42</sup> A human B1 cell equivalent has been recently described by Griffin et al<sup>43</sup> applying the criteria of spontaneous IgM secretion, efficient T-cell stimulation, and tonic intracellular signaling. Based on these criteria, they identified a small population of B1 cells present in umbilical cord and adult peripheral blood that express the unique phenotype of CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD70<sup>-</sup>.<sup>43</sup> A recent study, however, has challenged this fact, as Covens et al<sup>44</sup> analyzed characteristics of this newly described B-cell population and found them to have a gene expression signature more similar to preplasmablasts than to murine B1 cells. Interestingly, this population of B cells was particularly enriched in umbilical cord blood and found to decline with age. It is tempting to speculate that such a decline correlates with a potential loss of protective capacities resulting

in increased cardiovascular risk. Because the secretion of natural IgM antibodies is one of the major functions of B1 cells, it has been hypothesized that the atheroprotective effect of B1 cells depends on the secretion of IgM antibodies. Splenectomy of *ApoE*<sup>-/-</sup> mice is associated with decreased IgM levels, and patients subjected to splenectomy after trauma have been reported to have significantly lower serum IgM titers.<sup>45</sup> Of note, unlike transfer of wild-type B1a cells, B1a cells isolated from *sIgM*<sup>-/-</sup> donor mice, which express but do not secrete IgM, failed to attenuate the accelerated atherosclerosis in splenectomized mice, demonstrating that the IgM conferred the atheroprotection.<sup>29</sup> This is particularly interesting because we and others have previously characterized atheroprotective properties of specific B1 cell-derived natural IgM antibodies.<sup>46–50</sup>

## IgM: The House Keeper

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

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

E06 could bind to OxLDL and prevent its binding to CD36 and SR-B1 of macrophages and thus inhibit OxLDL uptake and prevent foam cell formation in vitro.<sup>69</sup> These and other data demonstrated that the phosphocholine of OxPL is a ligand for CD36 and mediated the uptake of OxLDL by macrophages.<sup>11</sup>

Subsequently, sequence analysis of the CDR3 region of E06 revealed a germline sequence, which was 100% identical in both the V<sub>L</sub> and V<sub>H</sub> chains to that of the prototypic B1 cell–derived NAb T15, which is an IgA. T15 has specificity for phosphocholine, which is also a prominent constituent of the capsular polysaccharide of *Streptococcus pneumoniae* (but is not part of a phospholipid) and has been shown to provide optimal protection to mice from pneumococcal infections.<sup>50,70,71</sup> We first demonstrated an atheroprotective function of T15/E06 IgM in *Ldlr*<sup>-/-</sup> mice that were immunized with heat-killed pneumococcal extracts and fed an atherogenic diet for 16 weeks. This immunization resulted in high anti-OxLDL IgM titers attributable to a near monoclonal expansion of T15id<sup>+</sup> IgM and concomitantly decreased lesion formation.<sup>47</sup> Plasma from these mice were able to block the uptake of OxLDL by macrophages effectively. Subsequently, Faria-Neto et al<sup>49</sup> confirmed the atheroprotective function of T15/E06 by showing that passive infusion of T15/E06 IgM antibodies reduced vein graft atherosclerosis in *ApoE*<sup>-/-</sup> mice. Moreover, several atheroprotective interventions are associated with an increase in T15/E06 IgM levels. For example, we could show that the atheroprotective immunization with homologous MDA-LDL resulted not only in the generation of high titers of antibodies against MDA-LDL, but also in the expansion of T15/E06 IgM. We found that this effect was dependent on the induction of IL-5, because MDA-LDL immunization of *Il5*<sup>-/-</sup> mice did not show an expansion of T15/E06 IgM. Moreover, IL-5–deficient *Ldlr*<sup>-/-</sup> bone marrow chimeras had significantly lower T15/E06 levels and developed accelerated atherosclerosis. The potential atheroprotective function of IL-5 and its role in modulating anti-OxLDL IgM levels was further confirmed in a human population, in which low serum IL-5 levels were associated with increased carotid intima–media thickness and low anti-OxLDL IgM levels.<sup>72,73</sup> Of note, in a genome-wide association study analysis of 15 596 patients with coronary artery disease and 34 992 controls, a single-nucleotide polymorphism (SNP) variant in the 3′ untranslated region of the *IL-5* gene was associated with increased risk of coronary artery disease.<sup>74</sup>

The mechanisms that underlie the protective properties of T15/E06 IgM are not entirely clear. As noted above, experiments performed in vitro have shown that T15/E06 prevents uptake of OxLDL by binding to the phosphocholine of OxPL, thereby inhibiting foam cell formation.<sup>46,47</sup> An additional mechanism by which T15/E06 IgM may limit plaque burden is by limiting the accumulation of apoptotic cells in developing lesions through the recognition of phosphocholine of OxPL formed on apoptotic cell surfaces.<sup>65</sup> Impaired efferocytosis has been linked to enhanced atherogenesis, and T15/E06 has the capacity to promote apoptotic cell clearance by macrophages in a C1q-dependent manner.<sup>64,75,76</sup> Finally, a key protective function is found in the ability of T15/E06 IgM to neutralize proinflammatory gene expression induced by OxPL present in OxLDL and the membranes of apoptotic cells.<sup>11,48</sup> For example, T15/E06 has been shown to inhibit IL-8 and adhesion molecule expression in endothelial cells stimulated with apoptotic cells or blebs and decreased monocyte



adherence.<sup>48,77</sup> Moreover, T15/E06 was able to abrogate the recognition of POVPC (1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine) (an OxPL) by the macrophage scavenger receptor CD36,<sup>69</sup> which is also critically involved in the proinflammatory response of macrophages to OxPL by cooperating with Toll-like receptor 4 and 6.<sup>78</sup> Indeed, T15/E06 IgM has been found to prevent OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine)-induced IL-6 secretion by macrophages<sup>79</sup> and to block the ability of OxPL to decrease macrophage phagocytosis.<sup>80</sup> Many more natural IgMs with specificity for other OSEs exist, which represent a prominent fraction (20%–30%) of all natural IgMs in mice and humans.<sup>66</sup> For example, IgM with specificity for malondialdehyde epitopes, such as the natural IgM NA17, also bind apoptotic cells and enhance the in vivo clearance of injected apoptotic cells by peritoneal macrophages.<sup>66</sup> Malondialdehyde represents another important danger signal that is present in atherosclerotic lesions and promotes inflammatory cytokine expression in vivo.<sup>66</sup> Because of the prominent presence of malondialdehyde adducts in lesions, malondialdehyde-specific IgM may have a particularly important role in atheroprotection. Indeed, we have shown that the atheroprotective immunization of animal models of atherosclerosis with autologous MDA-LDL also leads to the induction of high titered IgM antibodies against MDA-LDL, which may in part be responsible for the protective effect of immunization.<sup>72</sup>

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

In this regard, immunoregulatory properties of IgM may be of particular importance. Natural IgMs possess a biased repertoire toward (neo)self antigens, and this interaction may be a critical factor for immune homeostasis. Mice deficient in sIgM display strong differences in their splenic B-cell populations with increased B1 and marginal zone cells and decreased follicular B cells, likely as a result of impaired B-cell receptor signaling. Moreover, IgG2a

and IgG3 as well as IgA plasma levels are increased in *sIgM*<sup>-/-</sup> mice, which also show impaired IgG antibody responses at least to suboptimal doses of a T-cell-dependent antigen.<sup>82–85</sup> Paradoxically, Lewis et al<sup>81</sup> reported decreased IgG2a/c titers against CuOx-LDL in atherosclerotic *sIgM*<sup>-/-</sup>*Ldlr*<sup>-/-</sup> mice, which may reflect increased immune complex formation with OxLDL in the absence of circulating IgM. Interestingly, infusion of polyclonal IgM into *Apoe*<sup>-/-</sup> mice during the last 4 weeks of a 29-week atherogenic diet has been reported to delay lesion formation, whereas infusion of a monoclonal T15id<sup>+</sup> IgM preparation failed to do so in this setting.<sup>86</sup> Atheroprotection in mice receiving polyclonal IgM was associated with a decreased frequency of CD4<sup>+</sup> cells in the spleen. These data suggest that immunoregulatory functions of IgM, which may require the full repertoire of IgM specificities, provide an additional atheroprotective mechanism. In this regard, it is worthwhile to note that the repertoire (antibody specificities) of IgM antibodies differs between different B-cell subsets. For example, B1a cell-derived IgMs are more restricted to a germline-encoded repertoire, whereas the specificities of B1b cell-derived IgM can be more adaptive to antigen challenge.<sup>27</sup> Therefore, insights into the contribution of the polyclonal repertoire of natural IgM as well as the role of IgM with defined specificities, such as phosphocholine and malondialdehyde, will be critical for the understanding of the atheroprotective mechanism of IgM.

### IgG: Old Friend or Foe?

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

Despite all these caveats, IgG antibodies have often been suggested to be proatherogenic. However, there is little experimental evidence for this or even any functional role of IgG antibodies in atherosclerosis. Apart from the recent mouse studies, in which B-cell depletion was associated with decreased atherosclerosis and a profound reduction of both total and OxLDL-specific IgG antibodies and to a lesser extent IgM antibodies,<sup>24,25,30,31,39</sup> their role has been mostly implicated from immunization studies with either Hsp65 or models of

OxLDL. For example, immunization of normocholesterolemic rabbits with Hsp65 induced arteritis even in the absence of elevated serum cholesterol levels and accelerated atherosclerosis on a cholesterol-enriched diet.<sup>92</sup> These effects were reproduced in studies using *Ldlr*<sup>-/-</sup> mice fed a regular chow diet, which developed high anti-Hsp65 IgG antibodies and increased atherosclerotic lesions.<sup>93</sup> A proatherogenic effect of anti-Hsp65 IgG was also demonstrated by George et al,<sup>94</sup> who showed that intraperitoneal injections of chow-fed *Ldlr*<sup>-/-</sup> mice with IgG preparations from Hsp65-immunized mice promoted fatty streak formation. These data suggest that at least in part Hsp65-specific IgGs have proatherogenic properties, for example, by damaging Hsp60-expressing endothelial cells<sup>95</sup> (Figure 2).

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

However, there are only limited insights into the mechanisms by which these anti-OxLDL IgGs act in vivo (Figure 2). Based on in vitro analysis of plasma from mice with high IgG titers to MDA-LDL, it seems that such IgGs have the capacity to block the binding of OxLDL to macrophages in vitro, but whether this occurs in vivo is unknown. On the contrary, Schioppa et al<sup>101</sup> reported that the recombinant human MDALDL-specific IgG1 that inhibited atherosclerosis actually increased uptake of OxLDL by macrophages in vitro. Of importance, the contributing role of Fc effector functions of these anti-OxLDL IgG antibodies is still elusive. Fcγ receptors, which bind to the Fc portion of antibodies, are critical mediators of functions of IgG, and several studies in mouse models of atherosclerosis have addressed the global role of Fcγ receptors. Two categories of Fcγ receptors exist: the activating receptors that include the FcγRI, FcγRIIA, FcγRIII, and on the

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

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

## IgE: The Underestimated Player

IgE antibodies have been extensively studied in allergy and asthma, where they are considered critical mediators of these pathologies. Little is known about their role in atherosclerosis, although a few epidemiological studies exist that suggest a contribution for this usually tightly controlled immunoglobulin. For example, Kovanen et al<sup>108</sup> found that high IgE levels are a prognostic factor for myocardial infarction and cardiac death in dyslipidemic men in the Helsinki Heart Study. This association was later confirmed by Wang et al,<sup>109</sup> who found that patients with CHD have elevated IgE levels compared with subjects without CHD. Moreover, in this study, IgE levels were positively correlated with the CVD severity, with patients with acute myocardial infarction having the highest serum IgE levels compared with patients with unstable angina pectoris and stable angina pectoris.

The association with IgE was independent of sex, age, body mass index, hypertension, diabetes mellitus, or serum lipid profiles. This study and several others, including the one by Kovanen et al,<sup>108</sup> have also shown that IgE levels were directly associated with smoking. Therefore, IgE may be involved in the increased risk of atherosclerosis associated with smoking. Thus, epidemiological data suggest a proatherogenic role for IgE antibodies.

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

Other FcεRI-mediated effects may also be operative because IgE antibodies are a major stimulus for mast cells, which have been implicated in atherosclerosis and in the destabilization of lesions in particular.<sup>111</sup> Studies in atherosclerotic *Ldlr*<sup>-/-</sup> mice have shown that mast cell deficiency results in reduced lesion formation and that this may be promoted by mast cell-derived IL-6 and IFN-γ.<sup>112</sup> Another study by Bot et al<sup>113</sup> also demonstrated a proatherogenic effect of mast cell activation in *ApoE*<sup>-/-</sup> mice and a role for mast cell degranulation in particular. In line with this, in vitro degranulation of mast cells by IgE treatment has been shown to promote OxLDL uptake by macrophages in coculture experiments.<sup>114</sup> Thus, mast cells may be a critical mediator of the proatherogenic effects of IgE binding to FcεRI (Figure 2). Interestingly, galectin-3 was found to be upregulated in atherosclerotic lesions of rabbits and humans. *ApoE*<sup>-/-</sup> mice deficient in galectin-3 or treated with a galectin-3 inhibitor were shown to develop significantly less atherosclerosis.<sup>115,116</sup> Although galectin-3 has many other potential functions, its proatherogenic effect may in part be mediated by its ability to bind and cross-link IgE antibodies. Critical insights into a direct role of IgE in atherosclerosis are still missing. Moreover, it will be important to demonstrate whether such effects are dependent on certain antibody specificities for relevant antigens, for example, OxLDL, or whether they are antigen independent.<sup>117,118</sup>

## IgA: Waiting to Go on Stage

IgA immunoglobulins have been selected to provide the first line of defense in mucosal areas (mucosal IgA). On the contrary, IgA antibodies are also found in the circulation. In humans, there are 2 classes of IgA, IgA1 and IgA2, whereas in mice, only 1 class exists. In addition, the human secreted IgA comprised monomeric IgA1 and IgA2, whereas in mice, the circulating IgA antibodies form dimers and oligomers.<sup>119</sup> There is little information about the role of IgA antibodies in CVD. Two studies by Muscari et al<sup>120,121</sup> found elevated IgA levels in patients with advanced vascular disease and myocardial infarction,

respectively. Moreover, Kovanen et al<sup>108</sup> reported IgA levels to be correlated with myocardial infarction and cardiac death in dyslipidemic men after adjustment for CVD risk factors. However, there are no mechanistic studies available with respect to IgA antibodies in atherosclerosis. Recent insights on the effects of the gut microbiome on CVD,<sup>122</sup> however, could suggest a potentially important mechanism by which IgA antibodies could be modulated to impact atherogenesis.

## Complement in Atherosclerosis

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

Thus, studies on the role of complement components do not allow any conclusion on immunoglobulin effector functions in atherosclerosis. In fact, homeostatic functions of complement may be of great relevance. This should also include regulators of complement activation, such as C4bp and complement factor H (CFH), which are present in atherosclerotic lesions and may also modulate immunoglobulin function.<sup>129-131</sup> CFH provides cofactor activity for factor I-mediated degradation of C3b into iC3b fragments, and

deposition of iC3b on the surface of apoptotic cells has been shown to promote anti-inflammatory clearance mechanisms.<sup>132</sup> Our recent discovery that CFH binds malondialdehyde epitopes on cellular debris and inhibits their proinflammatory effects shines light on a potential function in atherosclerosis. In fact, we showed that the malondialdehyde-binding sites in CFH are localized to domains that are also hot spots of disease-associated mutations. For example, a common SNP rs1061170, which is highly associated with an increased risk for age-related macular degeneration, significantly impairs the binding of CFH to malondialdehyde. Although meta-analyses of clinical studies involving  $\approx 48\,000$  individuals do not support a role for this specific SNP in CVD,<sup>133</sup> it is tempting to speculate that the combination of several rare SNPs in the malondialdehyde-binding sites results in functional alterations of CFH binding to malondialdehyde. Such SNPs might not be detected in genetic association studies because of their low frequencies. In this regard, it will be interesting to evaluate the association of CFH binding to malondialdehyde with CVD in large clinical cohorts.

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

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

## Concluding Remarks

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

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## Nonstandard Abbreviations and Acronyms

<i>ApoE</i> <sup>-/-</sup>	apolipoprotein E deficient
<b>BAFF</b>	B-cell-activating factor
<b>BAFFR</b>	B-cell-activating factor receptor
<b>CFH</b>	complement factor H
<b>CHD</b>	coronary heart disease
<b>CuOx-LDL</b>	copper-oxidized LDL
<b>CVD</b>	cardiovascular disease
<b>Hsp60</b>	heat shock protein 60
<b>IFN-<math>\gamma</math></b>	interferon- $\gamma$
<b>IL-10</b>	interleukin-10
<b>IRA</b>	innate response activator
<b>LDL</b>	low-density lipoprotein
<i>Ldlr</i> <sup>-/-</sup>	low-density lipoprotein receptor-deficient
<b>MDA-LDL</b>	malondialdehyde-modified LDL
<b>NAbs</b>	natural antibodies
<b>OSE</b>	oxidation-specific epitope
<b>OxLDL</b>	oxidized LDL



<b>OxPL</b>	oxidized phospholipid
<b>SLE</b>	systemic lupus erythematosus
<b>SNP</b>	single-nucleotide polymorphism

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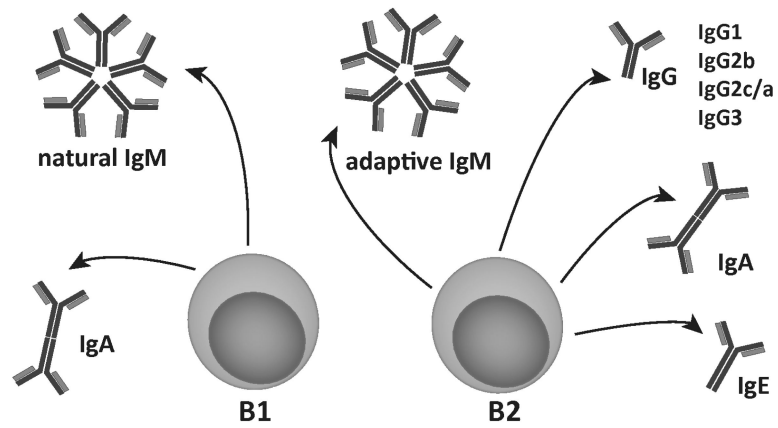
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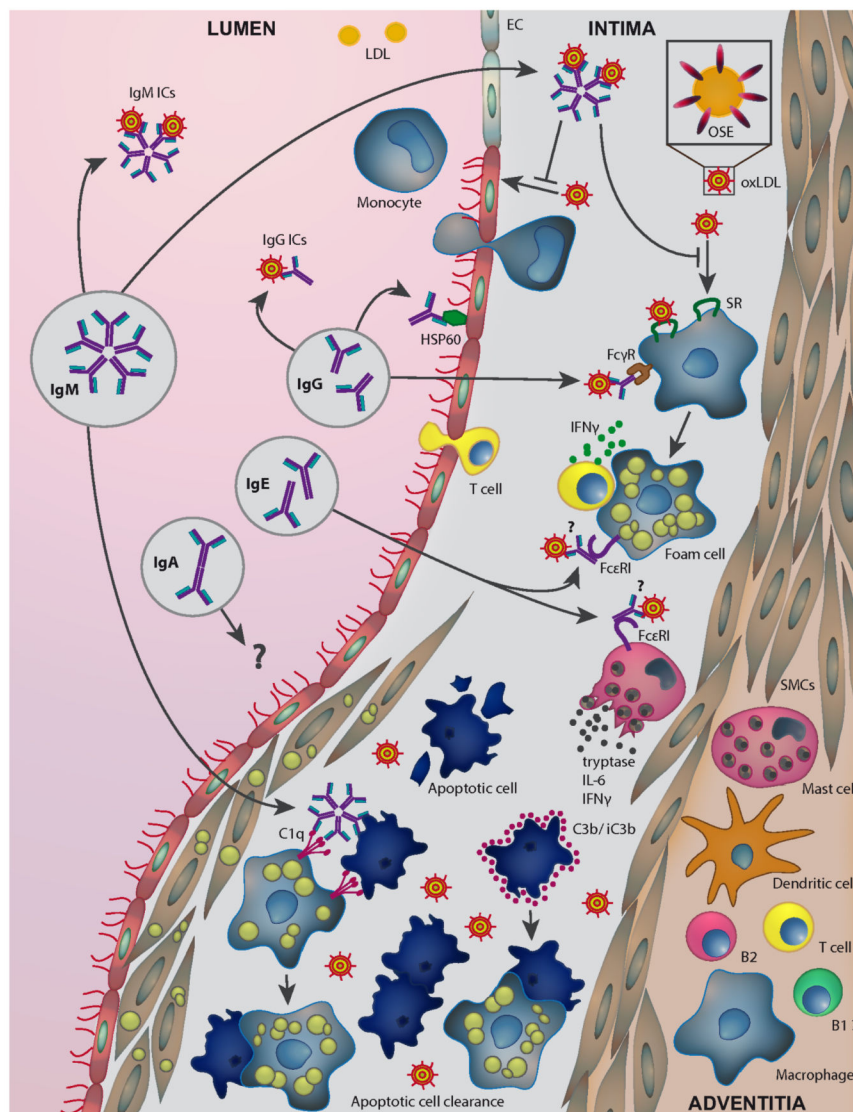


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**Figure 1. B1 and B2 cells have different immunoglobulin production profiles in mice**

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



### Figure 2. Role of immunoglobulins in lesion development

Different immunoglobulins are present in atherosclerotic plaques, which are directed against relevant antigens such as heat shock proteins (HSPs) and oxidized low-density lipoproteins (OxLDLs). IgM antibodies may mediate atheroprotection by neutralizing the proinflammatory properties of OxLDL, inhibiting the uptake of OxLDL by macrophages, and by promoting apoptotic cell clearance. Their protective capacity may be largely dependent on their ability to recognize oxidation-specific epitopes (OSEs) present on both OxLDL and apoptotic cells. OxLDL-specific IgG antibodies could activate macrophages via Fc $\gamma$  receptor engagement, thereby promoting atherogenesis, but may also exhibit protective neutralizing capacities. IgM and IgG immune complexes with LDL carrying OSEs are also found in the circulation and may promote clearance of proatherogenic LDL particles. HSP60/65-specific IgG recognize stressed endothelial cells and induce damage via antibody-dependent cellular cytotoxicity. IgE antibodies activate mast cells and macrophages via the engagement Fc $\epsilon$ RI resulting in plaque destabilization. Specificities of

IgE for atherosclerosis antigens, for example, OxLDL, remain to be shown. The role of IgA antibodies in atherosclerosis is still elusive. Opsonization of apoptotic cells with C3b and iC3b enhances their uptake by macrophages. EC indicates endothelial cells; IC, immune complexes; IFN- $\gamma$  interferon- $\gamma$ ; IL-6, interleukin-6; SMCs, smooth muscle cells; and SR, scavenger receptors.

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

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

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

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

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

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

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

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

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

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