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Immunity and Inflammation in atherosclerosis

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Abstract

There is now overwhelming experimental and clinical evidence that atherosclerosis is a chronic inflammatory disease. Lessons from genome-wide association studies, advanced *in vivo* imaging techniques, transgenic lineage tracing mice, and clinical interventional studies have shown that both innate and adaptive immune mechanisms can accelerate or curb atherosclerosis. Here, we summarize and discuss the pathogenesis of atherosclerosis with a focus on adaptive immunity. We discuss some limitations of animal models and the need for models that are tailored to better translate to human atherosclerosis and ultimately progress in prevention and treatment.

Keywords

Atherosclerosis; immunology; inflammation; T-cells; B-cells; myeloid cells; vaccination; LDL

Subject Terms:

Atherosclerosis; inflammation

Introduction

Atherosclerosis is the most common underlying pathology of coronary artery disease (CAD), peripheral artery disease (PAD), and cerebrovascular disease[1, 2]. The chronic build-up of vessel-occluding plaques in the subendothelial intimal layer of large and medium sized arteries eventually results in significant stenosis that restricts blood flow and causes critical tissue hypoxia[3]. The most common complications, myocardial infarction (MI) and stroke, are caused by spontaneous thrombotic vessel occlusion and represent the most common cause of death worldwide[4, 5]. Current clinical guidelines focus on the treatment of these complications[6]. Clinically used therapies that efficiently prevent or curb the progression of atherosclerosis are limited to drugs that lower low-density lipoprotein (LDL) cholesterol.

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Traditionally, atherosclerosis was regarded as a cholesterol storage disease caused by the retention of lipoproteins including LDL in the intimal space of arteries. Retained LDL is modified and taken up by scavenger receptor-mediated phagocytosis. This process results in the continuous growth of fatty infiltrates rich in inflammatory leukocytes that macroscopically appear as plaques. Levels of plasma cholesterol, LDL cholesterol, and apolipoproteins, including Apolipoprotein B (ApoB), are highly correlated with clinical atherosclerosis[7, 8]. Mice along with other animal models suggest causality: Elevating plasma cholesterol levels, as achieved by genetic knock-outs of LDL-receptor (LDLR) or Apolipoprotein E (ApoE) in mice, causes atherosclerosis in C57BL/6 mice that otherwise do not develop spontaneous disease[9, 10]. Genome-wide association studies (GWAS) have correlated many single nucleotide polymorphisms (SNPs) in or near the genes encoding lipid-associated proteins. Examples include *LDLR*, *APOB* and proprotein convertase subtilisin/kexin type 9 (*PCSK9*), which modulate LDL cholesterol levels, as risk factors in atherosclerosis and MI[11, 12]. In addition, atherosclerosis is accompanied by a chronic, low-grade inflammatory response that attracts cells of the innate and adaptive immune systems into the atherosclerotic plaque[3], some of them recognizing ApoB, the core protein of LDL particles. Thus, atherosclerosis is a chronic inflammatory disease with an autoimmune component[13]. This autoimmune response is clinically best documented by antibodies against LDL and other atherosclerosis antigens, which are found in all patients and animal models. In many studies, low-affinity ‘natural’ antibodies against oxidation epitopes in LDL were found to be negatively correlated with atherosclerosis, while high-affinity antibodies secreted by IgG-producing plasma cells were positively correlated[14]. Here, we will summarize and discuss the adaptive autoimmune mechanisms that accompany and modify atherosclerotic disease.

LDL accumulation initiates vascular inflammation

The atherogenic process starts with the accumulation of several plasma lipoproteins in the subendothelial space at sites of flow perturbation and endothelial dysfunction. This is best documented for LDL, whose accumulation correlates with classical risk factors, such as smoking, hypertension, and metabolic dysregulation in obesity and diabetes[15]. In the intima, LDL undergoes oxidative modifications by reactive-oxygen species (ROS), which promote the uptake of oxLDL into macrophages[16]. In addition, oxidized phospholipids *per se* trigger inflammation of the arterial wall[17] by binding to Toll-like receptors (TLRs), a group of widely expressed pattern-recognition receptors (PRRs) that cause pro-inflammatory signaling[18]. Clinically, oxLDL is a marker of plaque inflammation[19]. Native LDL can also be taken up by macrophages by micropinocytosis, or in its aggregated form as cholesterol complexes or -crystals by phagocytosis. The sustained influx of cholesterol eventually exceeds the phagocytes’ metabolic capacity and intracellular lipid droplets form. Microscopically, cholesterol-laden macrophages are ‘foam cells’. Cholesterol loading is thought to cause a myeloid cell response with pro-inflammatory cytokine secretion, *in situ* macrophage proliferation, and further recruitment of myeloid cells (summarized in[20]). A clinically important consequence of cholesterol loading is the formation of intracellular cholesterol microcrystals that activate the inflammasome, a molecular machinery comprising molecules of the cytosolic-nucleotide binding domain and leucine-rich repeat gene family (NLRP3) that cleaves pro-IL-1β into its biologically active form[21]. IL-1β serves as an

inflammatory master cytokine that enhances the expression of many pro-inflammatory cytokines, as well as of CRP[22]. Notably, attenuating cholesterol storage and enhancing cholesterol efflux pathways may favor the resolution of plaque inflammation end even promote plaque regression (summarized in[23]). The myeloid response is accompanied by the infiltration of cells of the adaptive immune system, B and T cells[24, 25]. Notably, the plaque's growing content of myeloid cells and lymphocytes correlates with clinical complications and may predispose for future thromboembolic events caused by large cellular infiltrates and a thin fibrous cap ('unstable plaque')[26, 27].

Evidence for an autoimmune response in atherosclerosis

The presence of T and B cells in the plaque[28] sparked the hypothesis that atherosclerosis includes an autoimmune response. Adaptive immunity in infection and autoimmunity proceeds by a humoral arm that comprises specific antibodies against the antigen secreted by plasma cells, and a cellular arm with T cells that either activate B cells during co-stimulation or differentiate into effector T cells with pro- or anti-inflammatory cytokine production[29]. $CD8^+$ and $CD4^+$ T cells only initiate immune responses to peptides presented MHC-I on all nucleated cells or MHC-II on antigen-presenting cells (APCs), respectively. Such responses are MHC-restricted, i.e. they only occur in individuals expressing a specific MHC-allele with the ability to bind the relevant peptide epitope. Binding of a specific T cell receptor (TCR) concomitant with co-stimulatory events provided by APCs activates T cells and causes their clonal proliferation[30]. In mouse atherosclerosis, 2-photon microscopy has revealed an increased rate of APC-$CD4^+$ T-helper cell interactions in the plaque specifically in the setting of hypercholesterolemia that resulted in pro-inflammatory cytokine secretion[31]. In addition, T-helper cells show an increasing maturation into antigen-experienced effector/memory (T_{EM}) and central-memory (T_{CM}) T cells in the lymph nodes (Figure 1a) that is also observed in atherosclerotic plaques[28, 31]. Sequencing of the TCR revealed an oligoclonal origin of lesional T cells[32, 33] suggesting that some (antigen-specific) T cell clones actively expand in the plaque. The enhanced activation of T cells is accompanied by an expansion of lymph nodes draining the atherosclerotic aorta in aged atherosclerotic *Apoe*$^{-/-}$ mice (Figure 1b) and a local and systemic pro-inflammatory response that is further enhanced by a hypercholesterolemia-inducing diet[34-36]. These findings support the concept that specific antigens drive an immune-response in the aorta and lymph nodes during atherosclerosis.

LDL – an autoantigen within the plaque

Of all candidates that may serve as B- and T cell activating antigens, plasma levels of LDL and its core protein ApoB show the strongest clinical and causal link with atherosclerosis in humans[37]. ApoB-containing triglyceride-rich remnant particles also show a strong association with CVD, inflammation, and immune pathways[7]. Indeed, LDL as (auto-) antigen was first suggested by Gero et al in 1959: immunization with LDL protected against atherosclerosis in rabbits[38], suggesting that autoimmune response against LDL can be atheroprotective[39]. Many $CD4^+$ T cells in human plaques recognize oxLDL[40] by binding to MHC-presented peptide epitopes from ApoB-100[41, 42]. A tetramer of recombinant MHC-molecules loaded with an ApoB-derived peptide – a tool to detect antigen-specific T-helper cells *in vivo*[43] – found a naturally occurring population of $CD4^+$ T cells in the blood that

recognizes the human peptide $ApoB_{3036-3050}$[42]. Furthermore, atherosclerosis is accompanied by IgG-antibodies against LDL, oxLDL, and ApoB[44]. Collectively, these findings strongly suggest LDL as a relevant self-antigen that drives an autoimmune response against self-proteins in the atherosclerotic plaque. Besides LDL/ApoB, heat shock proteins (HSPs)[45-47] and some foreign peptides from pathogens such as *Cytomegalovirus* (CMV), *Hepatitis C Virus* (HCV), *Human Immunodeficiency Virus* (HIV), *Human Papilloma Virus* (HPV), and others[48-50] have been proposed as atherosclerosis-relevant antigens.

T-helper cell dependent immunity in atherosclerosis

Early evidence from immunohistochemistry studies[28, 51], more recent scRNAseq[24, 52], and CyTOF approaches[24, 53] have estimated that ~ 25-38 % of all leukocytes in mouse aortic and human atherosclerotic plaques are $CD3^+$ T cells, with $CD3^+CD4^+$ T-helper cells accounting for ~ 10 %. T cells predominantly populate atherosclerotic lesions with an enrichment in the fibrous cap[28, 51], but are also found in the adventitia of older lesions[24, 54]. Their recruitment to the plaque occurs via chemokine receptors C-C chemokine receptor type 5 (CCR5), -X-C Motif Chemokine Receptor 6 (CXCR6), and others[55, 56]. $CD4^+$ T cells are critical regulators of the adaptive immune response with the ability to differentiate into distinct T-helper subtypes that can either be immune-dampening or activating to other T cells, exert direct anti- or pro-inflammatory effects on tissue resident cells, provide B cell help to induce the production of high-affinity IgG antibodies, or exhibit cytolytic activity[29] (Figure 2). Thus, the function of T-helper cells in atherosclerosis is multi-faceted and depends on specific transcriptional programs and patterns of cytokine secretion that can either fuel or attenuate atherosclerosis. Early evidence from Rag-1 deficient mice, which cannot produce mature T- and B cells, suggested a pathogenic role for T and B lymphocytes only in early atherosclerosis with moderately enhanced lipid levels, but not in severely hypercholesteremic $Apoe^{-/-}$ mice[57, 58]. Genetic absence of T cells in athymic *nu/nu* mice or a depletion of $CD4^+$ T cells by anti-CD4 antibodies protected from lesion development[59]. After antigen presentation by APCs, lesional T cells differentiate into functionally distinct T-helper subtype (T_H) −1, −2, −17, T-regulatory cells (T_{reg}), T-follicular helper cells (T_{FH}) and Type 1 regulatory (T_R1) cells[60]. Atherosclerosis is a known T_H1-disease. Many $CD4^+$ T cells in the plaque express the pro-inflammatory, T_H1-associated cytokines IFN-γ, IL-2, IL-3, TNF, and lymphotoxin (LT), which can activate macrophages, T cells, and other components of the plaque, and thereby aggravate the inflammatory response[61]. T cells that express the plaque-homing chemokine receptor CCR5 in lymph nodes, and T cells from atherosclerotic lesions secrete IFN-γ and express T-bet, the T_H1-lineage defining transcription factor[55, 62]. Knocking out IFN-γ, its receptor, or T-bet protects mice from atherosclerosis[63-65]. IFN-γ may directly reduce plaque stability by inhibiting smooth muscle cell proliferation[66], affecting macrophage polarization, and modulating cardiovascular risk factors[67]. On the other hand, regulatory $CD4^+$ T cells (T_{regs}) that express the transcription factor FoxP3 and the high affinity IL-2 receptor CD25 protect mice from atherosclerosis[68, 69]. T_{regs} exert their atheroprotective properties by secreting the anti-inflammatory cytokine IL-10[70], plaque-stabilizing TGF-β[71], and by suppressing the proliferation of pro-inflammatory T-effector cells[72]. Atheroprotective effects of *in-vivo* treatment with IL-2 complexes[73] and anti-CD3 treatment[74, 75] have been attributed to a relative increase of T_{regs}. In addition, T_R1 cells that lack FoxP3 expression but express

CD49b and Lag-3 secrete IL-10 and are atheroprotective[76, 77]. In the atherosclerotic plaque, a substantial proportion of T cells moreover express transcripts for the T_H2 cytokines IL-4, IL-5, and IL-13[24]. In contrast to abdominal aortic aneurysm formation, which is a clear T_H2-dependent disease[78] and the negative correlation of IL-4 with clinical atherosclerosis[79], the relevance of T_H-2 immunity in atherosclerosis remains unclear. The T_H2 cytokine IL-4 antagonizes T_H1 responses and diminished lesion formation in one study[80], while depletion of IL-4 has also been reported to be atheroprotective[81]. Likewise, the role of T_H17 cells in atherosclerosis is controversial: Deletion or neutralization of the master cytokine IL-17 protected from atherosclerosis[82-84], while other studies reported that T_H17 immunity protected from atherosclerosis and induced a stable plaque phenotype[85-87]. T-follicular helper cells (T_{FH}), which are required to co-stimulate B cells and to induce an Ig-class switch, have also been proposed to be pro-atherogenic[88] or to protect from atherosclerosis by secreting LDL-lowering/neutralizing anti-LDL/ApoB secreting antibodies[89]. The different findings in these studies may reflect different but unknown antigen specificities of the T cells studied.

It is noteworthy that antigen presentation initiates and modulates the $CD4^+$ T-helper cell response atherosclerosis. T cell activation in an antigen-specific manner is an exclusive consequence of antigen-presenting cells (APCs) that present antigenic peptides displayed on MHC molecules[29]. Blocking MHC-II during co-stimulation or on APCs abrogates the downstream $CD4^+$ T cell response[31, 90]. T cell immune responses are typically initiated by antigen-loaded dendritic cells (DCs) migrating to lymph nodes. Several cells in the atherosclerotic plaque act as APCs for recall responses of antigen-experienced effector and memory T cells, including macrophages in the plaque, B cells in the adventitia, along with conventional DCs and plasmacytoid dendritic cells (pDCs). Depending on co-stimulatory signals and cytokines provided by these APCs, the immune response can be skewed into a tolerogenic (immune-suppressive) or an immunogenic response (summarized in[91, 92]).

The role of other T cell subsets remains less well defined. It has been suggested that MHC-I dependent cytotoxic $CD8^+$ T cells contribute to plaque inflammation and the build-up of the necrotic core[93, 94], but antigen specificity has not been considered[95]. Natural killer (NK) cells regulate antigen-specific T cell immunity besides the killing of infected and tumor cells. They are detected at low frequencies in the plaque and may therefore modulate atherosclerosis[96]. In contrast to earlier studies, a recent report, however, suggested that NK cells do not affect atherogenesis[97-100]. In addition, CD1d-restricted NK-cells can recognize glycolipid antigens. Some NKT cell subsets were reported to aggravate atherosclerosis, but the atherosclerosis-relevant glycolipids detected by these NKT-cells remain unknown[101-103].

The function of ApoB-specific, auto-reactive T-helper cells

It has been challenging to determine the phenotype of ApoB-specific $CD4^+$ T cells, i.e. the fraction of T cells with a TCR recognizing ApoB-peptides presented on MHC-II, within the pool of all lesional T cells. In animal models, ApoB-specific T cells have been expanded *in-vitro* or by vaccination against LDL or ApoB-peptides *in-vivo*. A direct transfer of vaccination-induced T cells in one study aggravated atherosclerotic disease[104]. T cells re-stimulated with oxLDL *ex-vivo* promoted atherosclerotic disease after adoptive transfer in a

model of immunodeficient scid/*Apoe*$^{-/-}$ mice[105]. Neutralization of T cells that responded to oxLDL stimulation by a monoclonal antibody directed against the TCRBV31 chain protected from atherosclerosis[106], suggestive of a pro-atherogenic function of ApoB-reactive T cells. However, more recent technologies to specifically detect ApoB-specific T cells *in-vivo* suggest the opposite: Tracking of ApoB-reactive T cells in mice and humans suggests that a majority of antigen-specific T cells are immunosuppressive T_{regs}[42]. This is consistent with recent work from Gistera et al. who transferred ApoB-reactive T cells from a mouse with a transgenic TCR directed against oxLDL/ApoB, which protected from atherosclerosis[89]. These mixed results obscure the exact function of antigen-specific T cells. Likely, their phenotype and function depend on presented peptides, the microenvironment, and cytokine milieu, which potentially affects T cell polarization. Some pro-atherogenic antigen-specific T cells[104, 106] were isolated and cloned for *in vitro* assays by a procedure known to pre-dispose and select pathogenic T_H1 and to neglect T_{reg} clones. It is possible that the population of antigen-specific T cells may be multi-potent to give rise to several T_H-lineages *in-vivo* – an idea consistent with the recent observation that MHC-II multimer selected ApoB-reactive T cells can express several T_H-defining transcription factors simultaneously[42].

The T_{reg}-switch hypothesis – how protective immunity turns into a pathogenic response

The notion that atherosclerosis has an autoimmune component raised the question whether atherosclerosis is prevented in an antigen-specific manner by ApoB-reactive T_{regs}[39] in healthy individuals. T_{regs} prevent the onset on autoimmune disease[107]. Naturally occurring T_{regs} are generated in the thymus (nT_{regs}) and peripheral are induced T_{regs} from naïve T cells (iT_{regs}). Despite the proven atheroprotective role of bulk T_{regs}[68, 69] it has been unclear whether T_{regs} reactive to ApoB exist and how these may contribute to disease. Interestingly, ample clinical data suggest a strong inverse relationship between T_{regs} and atherosclerosis: Numbers of T_{regs} and IL-10 expression are lower in patients with myocardial infarction[108, 109]. Low T_{reg} numbers predict cardiovascular events[110]. Blood T_{reg} numbers in established murine atherosclerosis decline in later disease, while effector T cells increase[36] (Figure 3a). However, in subclinical human atherosclerosis, T_{reg} numbers correlate positively with LDL[111]. Likewise, in mice hypercholesterolemia initially favors the differentiation of T_{regs}[112], an effect that may be a counter-regulatory response to enhanced inflammation[36], intracellular lipid accumulation[113], or an antigen-specific response. The latter hypothesis was supported by enhanced T cell receptor (TCR) downstream signaling events in hypercholesterolemic mice[114], suggesting that a sub-population of T cells responds to antigens associated with increased LDL levels or to components of LDL particles itself. Thus, these data indirectly suggest the existence of LDL/ApoB-reactive T_{regs} that bear a TCR specifically responding to ApoB auto-peptides. These cells respond when the corresponding peptides are presented by MHC-II molecules by various APCs. Indeed, we directly demonstrated the existence of such ApoB-reactive T cells by MHC-II tetramers loaded with the human and mouse auto-peptide $ApoB_{3036\text{-}3050}$. Using this tool, we showed that among all $ApoB_{3036\text{-}3050}$-reactive $CD4^+$ T-cells in patients free of cardiovascular disease, two thirds exclusively expressed FoxP3, indicative of a large population of ApoB-reactive T_{regs}. In patients with subclinical atherosclerosis, the percentage of exclusively FoxP3-positive T cells declined to ~ 30%, while a substantial proportion of the remaining

FoxP3$^+$ T cells acquired simultaneous expression of ROR-γT or T-bet, the T_H17 and T_H1-defining transcription factors, respectively[42]. These observations, along with the diminished pool of T_{regs} in later mouse and human disease, support the idea that the immunosuppressive phenotype of T_{regs} disappears as atherosclerosis progresses. Consist with this hypothesis, T_{regs} in late atherosclerosis in mice simultaneously express T-bet, lose their ability to regulate and to protect from atherosclerosis, while retaining some phenotypic similarity with T_{regs}, such as some residual expression of FoxP3[36, 55, 62]. Gaddis et al. recently proposed that FoxP3 expression may be lost in favor of the transcription factor Bcl-6, the defining transcription factor for follicular-helper T cells[88]. Adoptively transferred ApoB$^+$ T-helper cells turned into T_{FH} cells after adoptive transfer[89]. In other autoimmune conditions, such as experimental autoimmune encephalitis (EAE) and arthritis, an instability of FoxP3 expression triggers the formation of antigen-specific, but dysfunctional, partially non-protective former T_{regs} (exT_{regs})[115-117]. The instability of FoxP3 may be caused by increased methylation of the FoxP3 locus, which is observed in patients with severe cardiovascular disease[118] and that may be prevented by modifications of lipid metabolism or anti-cytokine interventions[88, 115]. In addition, the function of FoxP3 may be regulated by alternative splicing favoring pathogenic transcriptional programs[119]. These data suggest that the initial protective immune response by T_{regs} switches into a pathogenic response as atherosclerosis progresses[39] (Figure 3b,c).

Pro- and anti-atherogenic B cell responses in atherosclerosis

Classically, two types of B cells can be distinguished: B1 cells that are part of the innate immune system and secrete germ-line encoded IgM antibodies in a T cell independent manner, and B2 cells that need to be activated by T follicular helper cells (T_{FH}) to differentiate into plasma cells that secrete IgG antibodies. In infection and vaccination against pathogens, B cell-derived plasma cells secrete IgG antibodies that neutralize or opsonize bacteria and viruses[29]. In addition, B cells can secrete numerous cytokines that distinctly affect inflammation. Examples include IRA-B cells, which are pro-atherogenic and secrete GM-CSF to drive myeloid cell activation and to induce pro-atherogenic T_H1 immunity[120]. B-regulatory cells (B_{reg}) secrete the anti-inflammatory cytokine IL-10 and induce protective T-regulatory cells or directly act anti-inflammatory[121], although the relevance for atherosclerosis is controversial[122]. The role of other cytokine producing B-effector (Be) cells is unclear. Only a few B cells are found in the atherosclerotic plaque[24]; the majority of B cells reside in the adventitia, in particular in aged atherosclerotic animals, where arterial tertiary lymphoid organs (ATLOs) form[123]. B cells in the spleen respond to a high cholesterol diet[124], suggesting local and systemic B cell responses in atherosclerosis. Global gain and loss of function experiments have suggested an overall protective role of B cells[125, 126]. In general, innate B1 response seem to be atheroprotective and adaptive B2 responses pro-atherogenic (Figure 4):

B1 cells: B 1 cells represent a first-line, innate defense against common pathogens. In mice, they are characterized as CD11b$^+$CD43$^+$CD23$^-$B220^{low}CD19$^+$ cells and may be sub-divided into B1a and B1b cells depending on their location and surface markers[127]. Typically, most B1 cells reside in the peritoneal cavity. In the atherosclerotic plaque, a few CD11b$^+$ $B220_{neg}$CD19$^+$ B1-like cells are found that further decrease in more advanced

disease[24]. B1 cells secrete germ-line encoded IgM. Typically, B1-derived IgM recognize phosphocholine (PC) head groups of polysaccharides in the wall of bacteria, such as *S. pneumoniae*. The same IgMs also bind oxidation-specific neo-epitopes on LDL and epitopes on apoptotic cells[128-130]. Oxidative neo-epitopes also seem to be generated in the spleen during sterile inflammation[131]. In cardiovascular disease, IgM recognizing epitopes on LDL or ApoB are inversely correlated with atherosclerosis, complications, and outcome[132-138]. It has been shown that IgMs directed against oxLDL inhibit its uptake by macrophages and prevent myeloid-cell inflammation[139, 140]. Consistently, several studies with gain- and loss-of-function experiments have established an atheroprotective role for B1 cells[141-145].

B2 cells: IgG antibodies originate from plasma cells that have undergone B cell maturation with the help of T_{FH} cells in germinal centers, which causes a switch from low-affinity IgM to high-affinity IgG[146]. IgG antibody titers to native and oxidized LDL or ApoB are positively correlated with atherosclerotic disease in mice and humans[133, 147-149]. Inhibiting B2 cells is reportedly atheroprotective[150-152], while specifically interfering with plasma cell functioning seems to be proatherogenic[153]. The role of IgG antibodies in atherosclerosis is controversial: It was suggested that IgGs against ApoB aggravate[154] or protect from atherosclerosis[155, 156]. A clinical phase II study (Goal of Oxidized LDL and Activated Macrophage Inhibition by Exposure to a Recombinant Antibody, 'GLACIER') using a monoclonal IgG antibody against a human ApoB-peptide failed to show its expected atheroprotective effect[157]. The design of the study with the use of 8F-fluorodeoxyglucose (FDG) PET-imaging as surrogate for plaque inflammation instead of cardiovascular endpoints, the short observation period of 85 days, and the small study population may have contributed to its lack of efficacy.

Vaccination against atherosclerosis – a translatable strategy?

The discovery of the autoimmune component of atherosclerosis has sparked the idea of immunizing with LDL or peptides from ApoB to prevent atherosclerosis by inducing or maintaining the traits of protective immunity against ApoB. Almost 60 years ago, it was shown that rabbits develop smaller atherosclerotic lesions after subcutaneous injection of LDL[38]. That vaccination with LDL can be atheroprotective was confirmed in a variety of species, LDL preparations, routes, and adjuvants[158-160]. At least seven MHC-II-restricted peptides from ApoB, which contains the immunodominant epitopes of LDL, are protecting from atherosclerosis when used in vaccines: p3, p6, p101, p102, p103, p18, p210[42, 161-163]. An ongoing challenge is to decipher the mechanism of action, which is critically required to define vaccination protocols translatable to humans. It has been proposed that either T_{regs}[42, 164-167], IL-10[42, 161, 167, 168], or vaccination-induced IgG-antibodies may confer atheroprotection, depending on doses, routes, and adjuvants used[44]. Recent studies, however, suggest that atheroprotection does not require IgG-antibodies[169] and primarily proceeds by IL-10^+ ApoB-specific T_{regs}[42].

Whether vaccination strategies can be translated to humans remains unclear. A first step towards a translatable approach was the identification of human ApoB-peptide epitopes accessible to immunomodulation in two recent studies[42, 170]. In mice, ApoB-peptides have been delivered in the non-translatable classical adjuvants Complete Freund's Adjuvant

(CFA), an emulsion of mineral oil supplemented with inactivated mycobacteria, or Incomplete Freund's Adjuvant (IFA), which lacks the mycobacteria component of CFA. Subcutaneous or intraperitoneal injections of both, CFA and IFA, were shown to elicit non-specific inflammation[171, 172]. This limitation was recently overcome by the discovery that a squalene oil, a class of adjuvants already used in clinical practice, can be used as an adjuvant for ApoB-peptides[169]. In addition, it remains unclear whether vaccination is effective in established atherosclerosis as most studies tested the prevention of *de-novo* atherosclerosis in rodents.

Limitations of animal models

The principles of the cellular and humoral adaptive immune response in experimental murine atherosclerosis have been established. The efficacy of anti-inflammatory therapy in human atherosclerosis has been validated in the CANTOS trial recently. However, significant challenges remain for the translation of animal studies to humans. First, mice, which represent the most widely employed atherosclerosis model, neither develop spontaneous atherosclerosis, nor do atherosclerotic knockout mice develop coronary artery disease. In addition, spontaneous atherothrombotic events resembling heart attacks and strokes do not occur in atherosclerotic mice. Also, blood lipoprotein profiles in mice are unlike those in humans, even in the genetic atherosclerosis models. Second, most atherosclerosis studies are conducted in a single mouse strain (C57BL/6) that cannot capture the genetic diversity seen in humans. Genetic diversity is known to modulate the response against antigens and atherosclerosis-relevant stimuli within a spectrum from pro- to anti-inflammatory[173174]. Third, some cytokines and immune receptors are not conserved between mice and humans, because the immune systems of both species are under intense evolutionary pressure. Fourth, mice represent a simplified model system for antigen presentation and recognition. Unlike humans, mice are housed in specific-pathogen free (SPF) facilities, which neglects the likely pathogen-driven activation, antigenic repertoire, and differentiation of immune cells[175]. While C57BL/6 mice bear just one MHC-II allele/molecule (I-A^b), humans express several alleles of a large pool of different MHC-II variants that are termed human leukocyte antigens (HLA) with over 10,000 different HLA allelic forms. This extreme variability renders the direction and amplitude of autoimmunity in humans difficult to predict.

Clinical considerations

Decreasing LDL levels and attenuating the inflammatory response represent the two fundamental therapeutic strategies against atherosclerosis available today. The most successful causal medication as measured by event-free person years are inhibitors of endogenous cholesterol synthesis by the HMG-CoA reductase (statins)[176, 177], which lower LDL-cholesterol and have pleiotropic anti-inflammatory effects beyond what can be expected from the reduction of LDL[178]. Statins can prevent, reduce, and even reverse atherosclerotic plaque burden[179]. Monoclonal antibodies to Proprotein convertase subtilisin/kexin type 9 (PCSK9) lower LDL-cholesterol even more dramatically by blocking LDL degradation[180, 181] without apparent impact on levels of CRP levels[182], a biomarker of systemic inflammation. However, even after LDL-lowering with statins and PCSK9-inhibition, a substantial residual inflammatory risk remains[183]. These observations have

established the distinct, but overlapping, roles of inflammation- and lipid associated risk. Low-dose treatment with the anti-proliferative and anti-inflammatory drug colchicine prevented cardiovascular events in a small prospective clinical trial[184]. In addition, the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) showed that inhibition of inflammation by the Interleukin 1-β (IL1-β) antibody canakinumab reduced cardiovascular end-points in patients with established atherosclerosis by 15%[185]. Strikingly, these observations have proven the inflammatory hypothesis on a conceptual basis, yet it is unclear, which patients may benefit from novel anti-inflammatory therapies: First, inhibition of IL1-β impaired host defense, which was reflected by an increased incidence of lethal infections[185]. Second, the recent Cardiovascular Inflammation Reduction Trial (CIRT) that tested low-dose anti-inflammatory methotrexate in patients with coronary heart disease did not reach its endpoints[186]. This lack of efficacy was partially explained by the inclusion of patients at low inflammatory risk and calls for a future personalized risk stratification (inflammatory versus lipid risk) and treatment once anti-inflammatory therapy is available in clinical practice. Whether the autoimmune component of atherosclerosis may already be addressable by unspecific anti-inflammatory therapy is currently unknown. However, vaccination and immunomodulation may provide a future antigen-specific therapy that is unlikely to impair host defense. The first validation of MHC-II tetramers to quantify the ApoB-reactive T cell responses[42] and the measurement of auto-antibodies[187] in humans may provide feasible risk stratification tools in the challenge to define patients at a high immune risk for atherosclerosis in future.

Conclusion

Atherosclerosis is a chronic inflammatory disease of the vessel wall that is largely driven by an innate immune response through myeloid cells as monocytes and macrophages. Autoimmunity against ApoB and other antigens involve $CD4^+$ T-helper cells that instruct myeloid cells and antigen-specific antibodies that may directly modify the pathogenicity of these antigens. This autoimmune response is detectable in humans and animal models with atherosclerosis. While the classical perception is that autoimmunity is pathogenic *per se*, recent evidence suggests that ApoB-specific $CD4^+$ T-helper cells are already detectable in subjects without clinical atherosclerosis, where many of them show atheroprotective features. As atherosclerosis progresses, the protective auto-immune response converts into a pathogenic one. It is unknown whether this switch in functionality represents a cause or a consequence of atherosclerosis and inflammation. It is clear that the adaptive immune system in atherosclerosis can be pro- or anti-inflammatory and thus pro- or anti-atherogenic. Manipulating the adaptive immune system by immunomodulatory strategies or vaccination is an attractive concept. Limitations in the predictive power of animal models and a lack of a full understanding of the role of auto-antibodies, B- and T cells present formidable hurdles to clinical translation.

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Non-Standard Abbreviations and Acronyms

scRNAseq	Single-cell RNA sequencing
CyTOF	Cytometry by Time of Flight (mass cytometry)
T_H	T-helper cell
T_{reg}	T-regulatory cell
ATLO	Artery tertiary lymphoid organ
FACS	Fluorescence-activated cell sorting
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
ApoE	Apolipoprotein E
PCSK9	Proprotein convertase subtilisin/kexin type 9
GWAS	Genome-wide association studies
CRP	C-reactive protein
MHC	Major Histocompatibility complex
HDL	High-density lipoprotein

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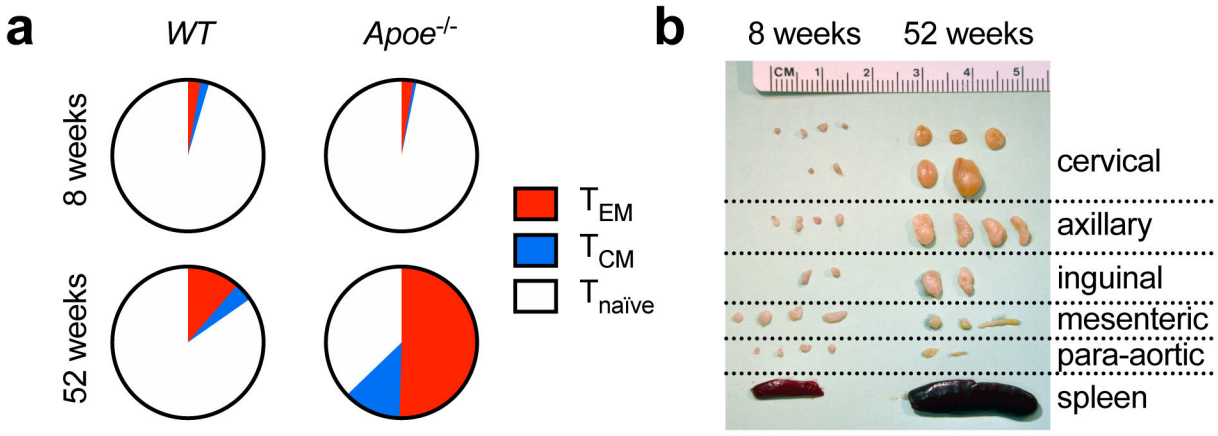

Figure 1: Activation of T cells is a hallmark of atherosclerosis.

(**a**) During feeding with a Western Diet (WD), $CD4^+$ T-helper cells from atherosclerosis-prone *Apoe*$^{-/-}$ build-up a significant immune memory with more than one half of T cells express markers of $CD62L^-$ $CD44^+$ T-effector memory cells (T_{EM}) and $CD62L^+$ $CD44^+$ central-memory cells (T_{CM}) when compared to atherosclerosis-free wildtype (*WT*) mice. (**b**) Along with enhanced T cell activation, lymph nodes draining the aorta and supra-aortic arteries (cervical, axillary lymph nodes) massively increase in size. *Courtesy of D. Wolf and K. Ley*

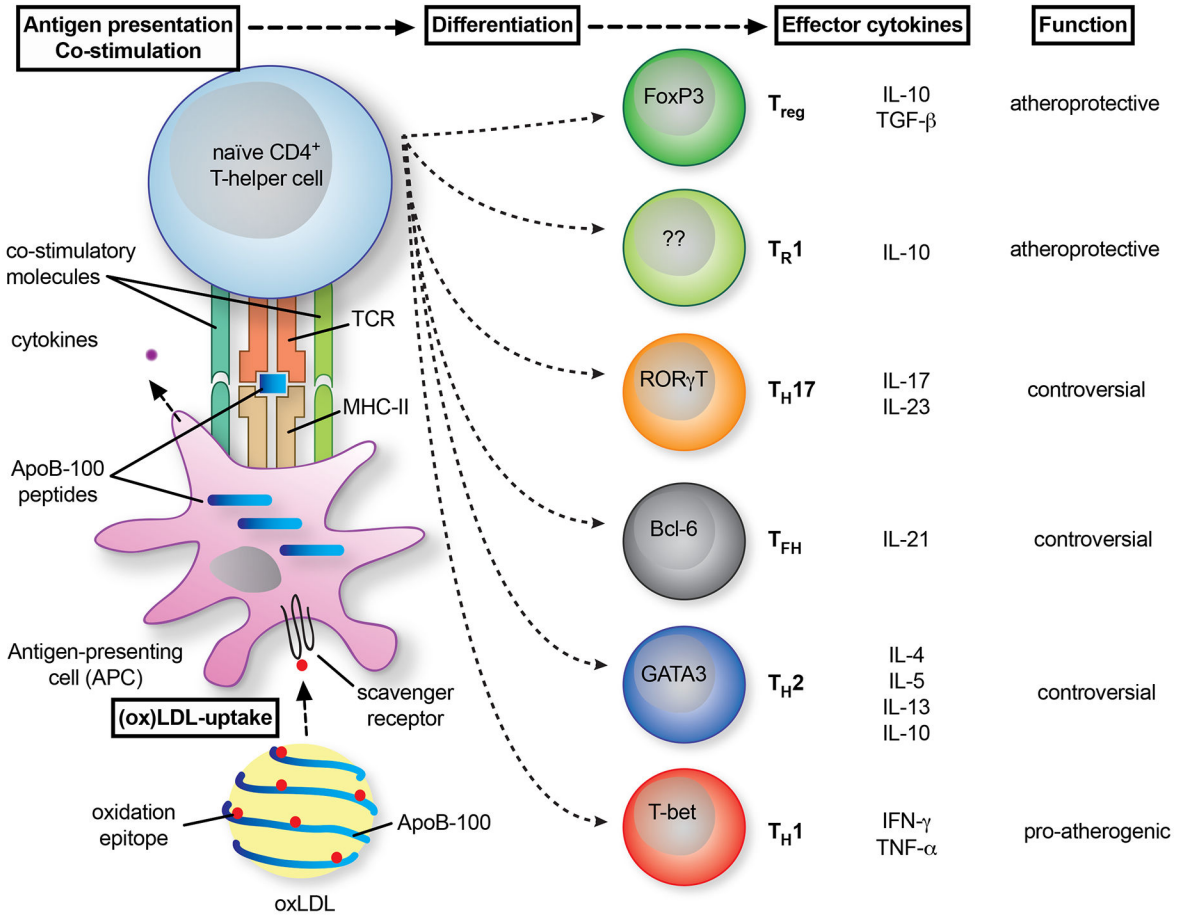

Figure 2: T cell polarization in atherosclerosis.

Naïve T helper cells (T_H) acquire the complete phenotype of an effector T cell in the plaque after presentation of antigenic peptides from ApoB by antigen-presenting cells (APCs). Therefore, an APC takes up (oxidized) LDL-cholesterol particles, processes, and presents peptides from ApoB on MHC-II. The T cell recognized this complex by a specific T cell receptor (TCR). This process is guided by the binding of co-stimulatory ligands to their corresponding receptors on T cells. As a result of co-stimulatory signals and cytokines secreted by the APC, T cells express transcription factors (denoted in the cells) that favor the differentiation into distinct T_H-types. These express specific cytokines that can either act in an atheroprotection or pro-atherogenic manner. The relevance for atherosclerosis remains controversial for some T_H-phenotypes.

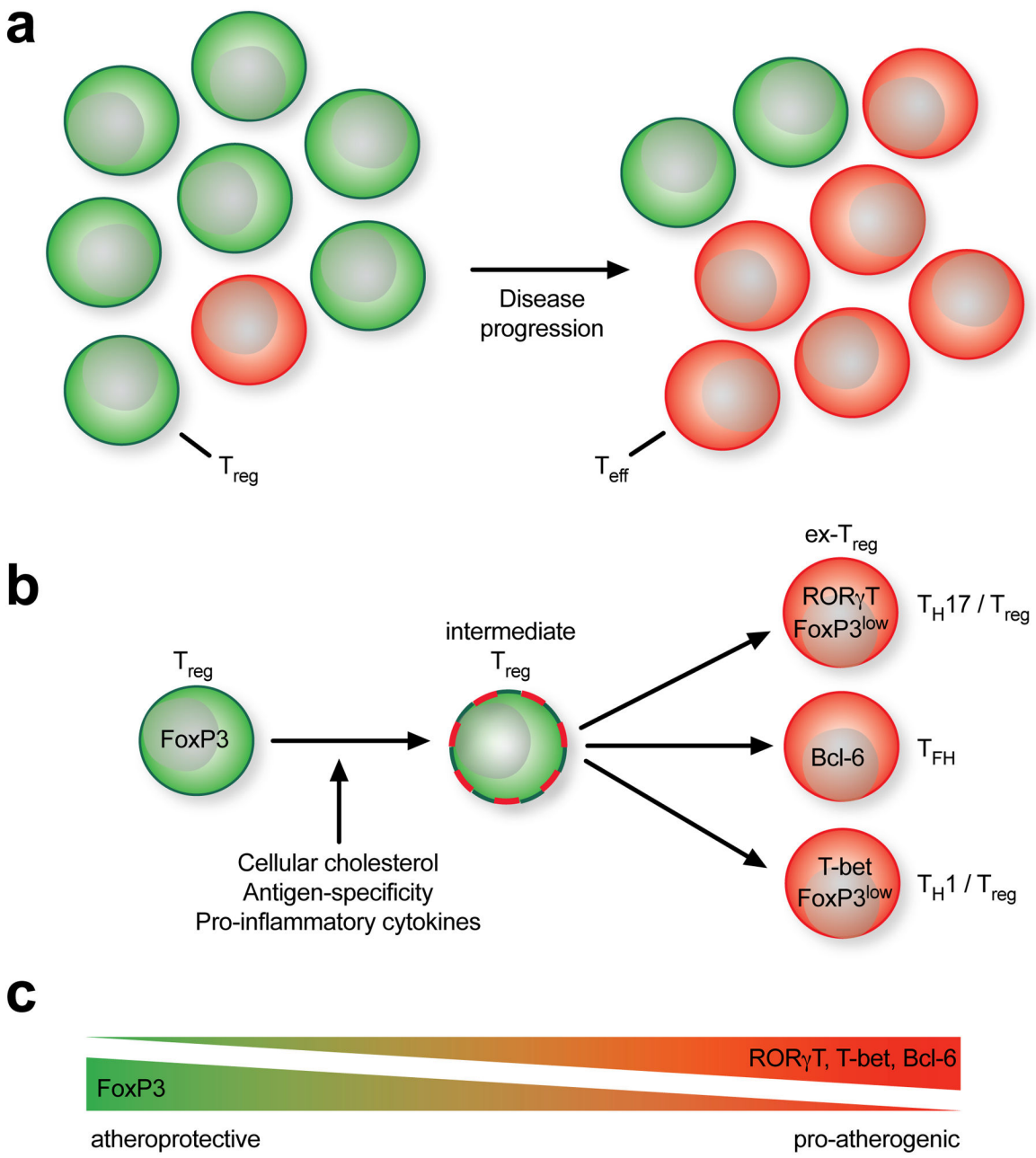

Figure 3: Decline of protective T-regulatory cells (T_{reg}) in the course of atherosclerosis.

(**a**) As disease progresses, the pool of T_{reg}-dominated antigen-specific T cells is overwhelmed by effector T cells (T_{eff}) with a presumably pro-atherogenic function. (**b**) Over time, T_{regs} expressing their defining transcription factor FoxP3 start to express alternative T_H-transcription factors, such as RORγ-T (T_H17), Bcl-6 (T_{FH}), or T-bet (T_H1). FoxP3 either remains co-expressed or disappears. Likely, this switch into FoxP3-low expressed or -negative exT_{regs} may be caused by antigen-specificity of the T cell, the cytokine milieu in the atherosclerotic plaque, or the loading of the T cell with intracellular cholesterol. (c) These observations have built the concept of an increasing replacement of (athero-) protective immunity with a pro-atherogenic response.

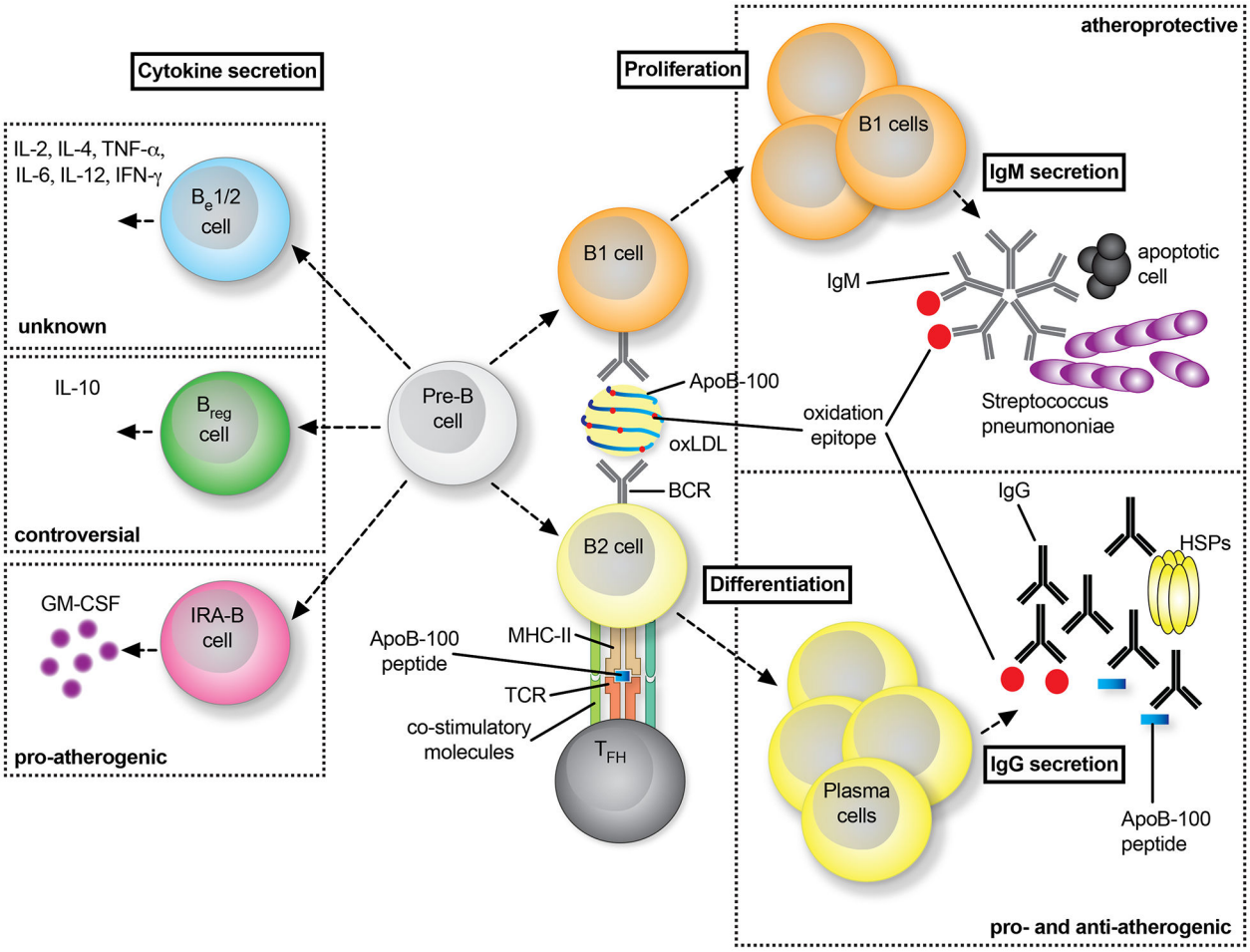

Figure 4: Distinct role of B cells in atherosclerosis.

B cells on a developmental stage (Pre-B) turn into innate-like B1 cells or adaptive, conventional B2 cells (right panel). B1 cells recognize epitopes on LDL and oxLDL particles, which leads to their activation and expression of low-affinity IgM antibodies by proliferation. Often, these IgM show cross-reactivity with epitopes on bacteria such as *Streptococcus pneumoniae* or on apoptotic cells. Interfering with B1 functionality aggravates atherosclerosis. B2 cells require co-stimulation by T_{FH} cells by MHC-II:peptide:TCR interactions and co-stimulatory signaling events to fully differentiate into plasma cells that express high-affinity IgG antibodies against atherogenic antigens, such as ApoB, oxLDL, or heat-shock proteins (HSP). Neutralizing B2 cells is atheroprotective, while the role of IgG-antibodies remains controversial with reported pro- and anti-atherogenic functions. Independent of the classification of B1/2 cells, distinct B cell subsets have been shown to express non-exclusive sets of cytokines, which allows the definition of cytokine-secreting B-effector (Be) −1 and −2 cells, regulatory B cells (B_{regs}), and innate-response activator (IRA) B cells (left panel).