The why and how of adaptive immune responses in ischaemic cardiovascular disease

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Abstract

Atherosclerotic cardiovascular disease is a major cause of disability and death worldwide. Most therapeutic approaches target traditional risk factors but ignore the fundamental role of the immune system. This is a huge unmet need. Recent evidence indicates that reducing inflammation may limit cardiovascular events. However, the concomitant increase in the risk of life-threatening infections is a major drawback. In this regard, targeting adaptive immunity could constitute a highly effective and safer approach. In this Review, we address the why and how of the immuno-cardiovascular unit, in health and in atherosclerotic disease. We review and discuss fundamental mechanisms that ensure immune tolerance to cardiovascular tissue, and examine how their disruption promotes disease progression. We identify promising strategies to manipulate the adaptive immune system for patient benefit, including novel biologics and RNA-based vaccination strategies. Finally, we advocate for establishing a molecular classification of atherosclerosis as an important milestone in our quest to radically change the understanding and treatment of atherosclerotic disease.

Dyslipidaemia and elevated low-density lipoprotein (LDL) levels are causally involved in the chronic inflammatory response of the vascular wall[1]. LDL oxidation in the intima and the sterile vascular inflammation associated with it engage adaptive immune responses which profoundly modulate atherosclerosis, from lesion initiation and progression to the occurrence of clinical events[2] (Box 1). Accumulating evidence also involves adaptive immunity in the response to ischaemic heart injury[3], modulating both post-ischaemic cardiac remodelling and atherosclerosis progression[4].

Recent studies provided a proof-of-concept that targeting inflammation may limit the occurrence of CV events[5,6]. Here, we consider the intimate and complex interactions of the immune system with the heart and the vasculature, in health and atherosclerotic disease, with a particular focus on adaptive immune mechanisms and their selective manipulation for patient benefit.

Evidence for adaptive immunity in ischaemic heart disease

The association of adaptive immune responses with human atherosclerotic cardiovascular disease (ACVD) dates back to the 1980s and the discovery that abundant cells in atherosclerotic lesions expressed HLA-DR in association with activated T-lymphocytes[7] and immunoglobulins. This finding was further supported by the local presence of oxLDL along with their specific antibodies and the T-cells that recognise them[8,9]. Subsequently, an overwhelming body of evidence has implicated the adaptive immune system in the development and progression of atherosclerosis[2,10]. More recent studies harnessing the latest developments in mass cytometry and single-cell omics technologies, provided further support for a role of adaptive immunity in ACVD[11].

There is also ample epidemiological evidence supporting a role for adaptive immunity in ACVD, as patients with autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and other rheumatic diseases are at substantially increased risk of atherosclerosis and CV events. The levels of autoantibodies often associate with a higher CV risk, suggesting a detrimental role. Moreover, induction of autoimmunity in mice enhances atherosclerosis[12], indicating a causal role.

Genome-wide association studies [13] also highlight the causal role of inflammation in promoting ACVD[14], and genome-wide network-driven systems biology approaches identified genes involved in B-cell activation as potential key drivers[13]. Intriguingly, HLA genotypes only show weak association with ACVD[15]. However, this should not be interpreted as evidence for the lack of a causal role of autoimmune responses. It is likely that patients with autoimmune disease are severely under-represented in the ACVD case-control cohorts. Furthermore, as discussed in this review, atherosclerosis does not manifest as a classic autoimmune disease with early breakdown of *tolerance* to self-antigens, but rather involves autoimmune responses to altered or modified self or stress-induced neo-self. Breakdown of tolerance, if it occurs, is a secondary and relatively late event in atherosclerosis. More generally, inflammatory responses are highly modulated by environmental factors, and are likely to be more important in rapid ACVD acceleration rather than linear slow progression, making it difficult to capture their causal role using GWAS[16]. Moreover, most GWAS included ACVD patients with a relatively high lifetime CV risk burden, particularly a relatively high cholesterol load. Pre-clinical data indicate that T- and B-cells are much stronger drivers of atherogenesis in mice with low compared to high cholesterol levels[17]. Thus, it is likely that in cohorts where high cholesterol levels are not a strong driver, more immune-related variants could be identified. Our growing understanding of a fragmentation of large disease entities into smaller ones with different molecular profiles suggests that individual patho-mechanistic differences may also be responsible for the residual CV risk

despite optimal treatment of classic CV risk factors. In this regard, a deeper understanding of the interplay of adaptive immunity with the major CV risk factors on an individual level is needed. Finally, the substantially increased risk of cardiovascular inflammation, atherosclerosis progression and CV events after immune checkpoint inhibitor therapy[18] is an important reminder of the major role of adaptive immunity in cardiovascular homeostasis.

Why and how adaptive immunity is involved in atherosclerosis

The immuno-vascular unit in health

Studies employing two-photon intravital microscopy, mass cytometry and single-cell transcriptomics have revealed the presence of several types of resident immune cells of lymphoid (T-cells, B-cells, natural killer and innate lymphoid cells) and myeloid (macrophages, dendritic cells, mast cells) origin in the intima and adventitia of normal arteries[11] (Fig.1). The adventitia is also a site of neuro-immune-vascular cell interactions[19]. The presence of such a vascular-associated lymphoid tissue (VALT) in healthy arteries[20] suggests that it may play a role in immune surveillance, and recent work indicates that it is also involved in maintaining vascular homeostasis and adaptation to haemodynamic cues.

Monocytes/macrophages/dendritic cells: Circulating $Gr1^{dim}$ (mouse), $CD14^{dim}CD16^{+}$ (human) monocytes that crawl on endothelial cells (EC) and patrol the vessels for the presence of danger- or damage-associated molecular patterns (DAMPs), fulfil an immune surveillance programme[21] (Fig.1). The number of patrolling monocytes increases in hypercholesterolemia, and their absence is generally associated with increased atherosclerosis[22], suggesting a protective role.
Resident arterial macrophages originate from embryonic $CX3CR1^{+}$ precursors and, postnatally, from definitive haematopoiesis, and are maintained through local proliferation. They accumulate preferentially at branch points characterised by intimal thickening, suggesting a role for haemodynamic stresses. Their location shapes their resident phenotype and leads to distinct gene signatures for intimal (Mac^{AIR}) versus adventitial macrophages[23]. The latter maintain arterial tone and regulate arterial remodelling in response to blood flow, in part through MMP9- and FXIIIA-dependent regulation of collagen accumulation[24,25] (Fig.1). Intimal macrophages maintain a non-thrombogenic intravascular state[26] and are the earliest foam cells that accumulate in plaques[23]. In mice, intimal macrophages have previously been confounded with vascular-associated dendritic cells (DCs), but it's unclear whether this holds true for human vascular DCs. Resting immature adventitial DCs have been described in healthy human arteries[27] and may play a role in maintaining immune tolerance (Fig.1). Spatial single-cell sequencing will be useful to characterise the various macrophage/DC subtypes and examine whether different subtypes populate distinct vascular beds and perform specific functions.

T-cells, B-cells and fat-associated lymphoid clusters (FALCs): In human arteries, T-cells mainly occur at sites of intimal thickening, whereas adventitial T-cells are found both in human and murine arteries and are thought to home constitutively to the adventitia, in part through an L-selectin-dependent mechanism[28] (Fig.1). Most of these T-cells are αβ CD4+ cells with a few γδ CD4+ cells, as well as CD8+ T-cells. Some express CD25 suggesting an activation state. However, their characterisation remains incomplete.
B-cells are largely absent from the intima but are present in the adventitia of both healthy and atherosclerotic arteries (Fig.1). The fact that adoptively-transferred splenic B-cells also home to the adventitia of recipient non-atherosclerotic aortas[28] further underscores the artery wall

as a physiological niche for B-cells (Fig.1). Arterial homing of B-cells also depends in part on L-selectin[28]. A substantial proportion of T- and B-cells are located in the perivascular adipose tissue (PVAT), and may be organised within FALCs around stromal cells. Unlike secondary lymphoid organs (SLOs), FALC formation is independent of lymphoid tissue-inducer cells and the lymphotoxin-beta receptor pathway, but requires TNFR signalling and the presence of commensal flora. PVAT and FALCs contain substantial amount of innate B1-cells that secrete natural IgM[29], including IgM with specificity for OSE[30]. Part of this B1-cell activation may be driven by innate lymphoid cells type 2 (ILC2)[31], which are major producers of atheroprotective IL5 and IL13[31-33] (Box 2) (Fig.1). The fundamental triggers that initiate immune cell accumulation in normal arteries remain poorly understood (Box 3).

Medial immunoprivilege revisited

The medial layer of the artery wall is highly protected from invasion by effector immune cells. Several mechanisms of *medial immunoprivilege* have been proposed[34]. Passive mechanisms include the presence of elastic laminae and the avascular nature of the media. However, these mechanisms are not entirely consistent with the sustained medial immunoprivilege of advanced plaques, even in the presence of extensive neoangiogenesis, abnormal lymphangiogenesis, and disrupted elastic laminae. Proposed active mechanisms include the relatively low expression of MHC and costimulatory molecules, the high expression of coinhibitory molecules, and the production of immunosuppressive factors, mainly IDO1 and TGF-β, by medial vascular smooth muscle cells (VSMCs). However, VSMCs express high levels of MHC molecules and inflammatory mediators during atherosclerosis, and medial immunoprivilege is preserved in mice with IDO1 deficiency or disruption of TGF-β signalling. Thus, medial immunoprivilege remains a mystery. Peripheral lymphoid stromal cells, particularly follicular dendritic cells (FDCs), play a critical role in *peripheral tolerance*. Their expression of self-antigen induces both deletional *T-cell tolerance*, and non-deletional self-tolerance that maintains antigen-specific regulatory T-cells (Tregs)[35]. Self-antigen expression by FDCs also controls the elimination of self-reactive B-cells, particularly those generated during secondary diversification in the *germinal centre*[36]. Interestingly, these stromal cells share the same peri-vascular precursor as VSMCs and thus express many similar transcripts, both during differentiation and at the mature stage[37]. Here, we propose that the common ontogenic origin of VSMCs and lymphoid stromal cells is the primary mechanism that ensures medial immunoprivilege. A breakdown in lymphoid stromal cell-dependent tolerance or a substantial alteration or loss of VSMCs would be required to disrupt the immunoprivilege of the arterial media.

The immuno-vascular unit in atherosclerotic disease

Given the immunoprivilege of the media, it is unlikely that pathogenic adaptive immune cells are activated in response to arterial media components. Intimal and adventitial T-cell accumulation most likely indicates an adaptive response towards altered components of, or present in, these arterial layers. The oligoclonal T-cell repertoire as well as the limited sets of hypermutated VH regions and inverted κ/λ light chain ratio of B-cells found in atherosclerotic lesions[38,39] suggest an active expansion of antigen-specific T- and B-cell clones. The autoimmune responses most studied in this context are those directed toward lipoprotein components trapped in the artery wall[2,40], and toward proteins expressed by stressed ECs[20]

Adaptive T-cell immunity in atherosclerosis:

How do activated T-cells accumulate in inflamed arteries? Naïve CD4+ T-cell priming in the artery wall[41] is likely to be a marginal phenomenon in the early stages of atherosclerosis. Adventitial resident DCs are more likely than intimal macrophages to migrate to the draining lymph nodes (LNs) for T-cell activation. Therefore, activated T-cells in early lesions are mostly likely recruited as pre-activated (in draining LNs and SLOs) effector memory T-cells (TEM) or Tregs, transmigrating through activated (and potentially antigen-presenting) ECs. Chemokine/chemokine receptor pairs involved in TEM recruitment to the inflamed vasculature (e.g. CX3CL1/CX3CR1) are likely to be different from those involved in Treg recruitment (e.g., CCL5/CCR5[42], CCL1/CCR8[43]) (Fig.1). Once in the artery wall, some of these T-cells may adopt a resident memory phenotype with low levels of S1PR1 and high expression of CD103 and CD69[41]. The latter binds oxLDL, limiting TH17 differentiation while favouring atheroprotective Tregs[44].

LDL particles can also reach SLOs systemically and, in the absence of systemic inflammation (i.e. early atherosclerosis), ApoB peptides are likely to be presented in a tolerogenic manner, generating antigen-specific Tregs rather than T effector cells (Teffs) (Fig.1). This would explain why most ApoB-specific T-cells display a regulatory phenotype in the absence of overt atherosclerosis[45]. It also provides a plausible explanation for the initial surge in Tregs rather than Teffs in response to hypercholesterolemia in mice[46]. This scenario suggests that the first (ApoB-specific) T-cells that accumulate in lesions are likely to be Tregs, which may subsequently switch to helper and effector memory phenotypes with the increased burden of local and systemic inflammation[47]. It is also plausible that ApoB-specific Tregs are instructed in the liver, where ApoB is produced[48].

Different scenarios may arise with different antigens, either sequentially or concomitantly. Among these scenarios is the example of autoimmunity to heat shock proteins (HSPs), where pre-existing immunity to ubiquitous HSP60 (a highly immunogenic microbial antigen) could lead to cross-reactivity with autologous HSP60 (overexpressed on stressed ECs), triggering intimal inflammation[20]. Wick et al. showed that T-cells from early human atherosclerotic lesions are IFNγ-producing memory effector CD4+ cells, and respond to HSP60 and HSP60-derived peptides in vitro[49]. This recall response was associated with the generation of pathogenic anti-HSP60 antibodies. However, the early lesions assessed in that study were from individuals with "inflammatory storm" conditions. The latter may have induced an activated T-cell phenotype, and therefore, it remains unclear whether HSP-specific Teffs are truly predominant in early atherosclerosis. Furthermore, most of these studies have focussed on intimal T-cells, and the antigens that trigger effector T-cell responses in the adventitia remain to be identified.

A shift from regulatory to pathogenic T-cell immunity:

Depletion of CD4+ T-cells accelerates early atherosclerosis in mice[50], supporting the concept that early CD4+ T-cell responses are mostly regulatory and atheroprotective. So, what shifts T-cell responses from regulatory to effector? There's not a particular type of APC shown to selectively promote a Teff versus a Treg phenotype in atherosclerosis[51,52]. Thus, the activation state of the APC and the microenvironment of antigen presentation are paramount. Sustained local inflammation certainly plays a role, activating macrophages and DCs and changing their metabolism and signalling pathways to upregulate costimulatory molecules (e.g., CD40) and pro-inflammatory cytokines (e.g., IL1, IL6) and chemokines (e.g., CCL17), while downregulating coinhibitory molecules (e.g., PDL1) and anti-inflammatory mediators (e.g., IL10, TGF-β)[53-56]. In addition, changes in T-cell metabolism, driven by hypercholesterolemia, alterations in lipid synthesis pathways, hypoxia and other microenvironmental cues, may substantially alter the Tregs' suppressive phenotype[57,58]. Interestingly, sustained activation of Tregs by self-antigens in an inflammatory environment

affects the demethylated region in the *Foxp3* locus, downregulating *Foxp3* expression and destabilising Tregs[59]. These mechanisms could account for the increased plasticity of Tregs[60] and their progressive decline during disease progression, as well as the enrichment of TH1- and TH17-related phenotypes in ApoB-specific T-cells of patients with atherosclerosis compared with those free of ACVD[47] (Fig.1).

The role of the various CD4+ T-cell subsets has been extensively reviewed[2]. Foxp3+ Tregs and IL10-producing TR1 cells are protective, whereas TH1 cells promote disease development. The roles of TH2 and TH17 cells appear to be contextual and most likely depend on the relative abundance of pro- and anti-atherogenic cytokines produced by these T-cells[2,61]. It is important to note that a clear distinction between the various TH archetypes may not be relevant in vivo and it is highly likely that most plaque T-cells display a continuum of helper phenotypes. Indeed, recent single-cell studies in advanced human plaques described an enrichment of chronically activated effector memory T-cells displaying a resident phenotype and enriched in IFN pathways, but TH-cell-specific transcription factors could not be linked to specific clusters[62,63]. There is genetic and experimental evidence for a significant role of IL1, IL6 and IL18 pathways in ACVD. Intriguingly, IL1 and IL6 signalling pathways were more likely to be activated in CD4+ T-cells of asymptomatic compared to symptomatic human carotid plaques[62]. The therapeutic implications of this finding remain unclear.

Many other T-cell subtypes have been suggested to modulate atherosclerosis, but these subsets, and in particular their antigens, remain poorly characterised. The preferential distribution of γδ CD4+ T-cells, NKT cells[64] and MAIT cells in the gut and liver suggests that these subsets are more likely to play a role in atherosclerosis in the context of metabolic syndrome, through their immune and metabolic activities[65]. The role of CD8+ T-cells will require further attention. These cells are enriched in advanced human lesions and display several phenotypes[62,63], including central memory, effector memory and cytotoxic phenotypes, with exhausted CD8+ T-cells being enriched in symptomatic compared with asymptomatic carotid plaques[62]. CD8+ T-cells are suggested to promote atherosclerosis through target-cell lysis or induction of monopoiesis[66]. Their antigen specificity is still largely unknown, although some CD8+ T-cells recognise ApoB100-derived peptides[67]. A Qa-1-restricted regulatory CD8+ T-cell subset was shown to limit atherosclerosis in mice through the control of the T follicular helper (TFH)-germinal centre (GC) B-cell response (see below)[68]. Its relevance to human atherosclerosis remains unexplored.

Adaptive B-cell immunity in atherosclerosis:

Follicular B2-cells represent the major part of B-cells in secondary lymphoid organs and the circulating blood. B2-cells are primarily involved in T-cell-dependent responses, as they differentiate into GC B-cells undergoing class-switching and affinity maturation aided by TFH-cells. Analyses of atherosclerotic arteries support a relative expansion of B2-like over B1-like cells, and the accumulation of activated plasmablasts[11,39]. In advanced disease, some VSMCs and other local stromal cells express high levels of CXCL13 and CCL21 and adopt features of lymphoid tissue organizer-like cells. B-cells in the adventitia become organized in artery tertiary lymphoid organs (ATLOs)[69] that represent a site of recruitment to the artery wall and contain different subsets of B-cells that participate in GC responses[70] (Fig.1). Despite the documented presence of B-cells in the vascular wall, their direct local contribution to atherogenesis is largely unknown. ATLOs are atheroprotective in mice[69]. However, it's unclear whether this is due to protective local B-cell functions or to the predominance of induced immunosuppressive Tregs in ATLOs[69]. Moreover, effector

functions associated with antibody production do not require local B-cells, as specific antibodies produced in the bone marrow or the spleen can reach plaques via the circulation[71,72].

The activation of B2-cells is responsible for the production of class-switched antibodies against modified lipoproteins and other self-components. The help of the TFH, found primarily in B-cell follicles, is required for the generation of memory B-cells and long-lived plasma cells secreting affinity matured class-switched antibodies. TFH-cell deficiency appears to limit atherogenesis[58]. However, this requires further exploration since TFH-cells located outside the GC may be required for both switched and un-switched extra-follicular responses, with potentially different effects on atherosclerosis. Of note, activation of MZ B-cells impairs TFH-cells via upregulation of PDL-1, thereby limiting pro-atherogenic T-cell-dependent responses[73].

Antibody-mediated atherogenic functions of B2-cells: FO B-cells are pro-atherogenic; their preferential depletion with CD20-targeted antibodies or by genetic targeting of the BAFFR pathway reduces atherosclerosis[74-76]. Because FO B-cells participate in GC reactions, the likely explanation for their pro-atherogenic properties is that they contribute to the generation of pathogenic high-affinity IgG antibodies. Hyperlipidaemia in mice promotes a GC response with increased serum levels of IgG2b and IgG2c, as well as OSE-specific IgGs and several classical autoantibodies[77]. Of note, Apoe-/- mice that are unable to make IgGs due to a deficiency of the activation-induced deaminase, which is critical for class-switch recombination, displayed increased total and MDA-specific IgM levels and developed less atherosclerosis[78]. Genetic ablation of GC-derived antibodies greatly reduced lesion size in Apoe-/- mice[71,79]. Moreover, administration of IgG preparation from plasma of atherosclerotic Apoe-/- mice aggravated disease[71]. These data indicate that GC-derived antibodies have the capacity to promote disease. However, only few pathogenic IgG antibody responses have been described, such as anti-HSP60/65 IgGs that bind HSP60 in stressed ECs and promote atherogenesis by antibody-dependent cell-mediated cytotoxicity[20], and IgG autoantibodies to the 78 kDa glucose-regulated protein, which has been described as another endothelial-derived autoantigen[80], that trigger EC activation. Thus, there is still a great need to identify the antigens of GC-derived pathogenic antibodies (Box 4).

Antibody-mediated atheroprotective functions of B2-cells: Besides the atheroprotective properties of B1- and MZ B-derived OSE-specific IgM antibodies[10 81] (see Box 1), GC-derived IgGs may also have protective functions[50], such as promoting SMC proliferation and plaque stability[79]. Passive immunization of Ldlr-/- mice with a recently characterised autoantigen, ALDH4A1, resulted in reduced lipid levels and decreased atherosclerosis[82]. T-cell-dependent IgGs with reactivity for ApoB were also shown to protect form atherosclerosis by promoting LDL clearance and reducing serum cholesterol levels in TCR-transgenic mice[83]. Moreover, despite in vitro studies demonstrating proinflammatory effects of anti-oxLDL IgG in macrophages[84] and several epidemiological studies demonstrating an association of anti-oxLDL IgG titers with ACVD[85], direct experimental evidence of a proatherogenic role in vivo is missing. In contrast, numerous immunization studies in mice and rabbits, which initially aimed at establishing a pro-atherogenic role for anti-oxLDL immune responses, demonstrated a robust induction of T-cell-dependent IgGs and reduced atherosclerosis[10]. For example, immunization with models of OSE generally induced a TH2-biased response, for which IgG1 are surrogate markers[32,86]. While other mechanisms may be at play, there is also direct evidence for an atheroprotective effect of OSE-specific IgGs. Indeed, infusion of monoclonal human IgG1 against the OSEs MDA-LDL and PC protect from atherosclerosis in mice[87,88]. Due to the human origin of the infused IgGs, the

contribution of the IgG subclass cannot be deducted, but it is likely important for the protective capacity of these IgGs. Blockade of the many pro-inflammatory properties of oxLDL may be part of the protective effect, as mice expressing a single-chain version of the anti-PC IgM, E06, developed reduced inflammation and decreased atherosclerosis[89].

Fc receptor-mediated effects of antibodies: To fully understand the contribution of class-switched antibody responses in atherosclerosis, not only antigen specificity, but also the different effector functions of antibody subclasses need to be considered[81]. Key effector functions that also need to be explored in context of the bound antigens are neutralization and clearance of antigens (e.g. of oxLDL and self-antigens), antibody-dependent cell-mediated cytotoxicity (e.g. of HSP60 expressing ECs), complement activation and inflammation, as well as cellular activation. For the latter, the role of Fcγ-receptors has been studied in a series of settings and in general indicated an overall pathogenic role for activating Fcγ receptors and protective effects of the inhibitory FcγRIIB[10,90]. However, these effects are complicated by sexual dimorphism, disease-stage specific effects, cell-type specific differences, and potential non-canonical roles of these receptors[10,90]. Future studies need to further dissect the involvement of Fcγ receptors in mediating IgG effector functions independent of immunomodulatory roles and in an antigen-dependent manner. A clear proatherogenic role has been found for IgE antibodies, which trigger mast cell and macrophage activation via FcεR in the artery wall[91,92]. Whether these effects involve the recognition of specific antigens remains to be shown.

Other effector functions of B-cells: B-cells also contribute to atherosclerosis through cytokine secretion. For example, expression of TNF by B-cells may be in part responsible for the proatherogenic effects of B2-cells[93], and IRA B-cells are GM-CSF-secreting B-cells that promote atherogenesis through GM-CSF-mediated DC activation[94]. The role of B-cell-derived IL10 is still unclear and requires further studies[10]. For all these subsets, it remains unclear if their effects are mediated as simple bystander activities or whether they are associated with certain BCR identities.

Finally, recent studies indicate that the peripheral nervous system is involved in an artery-brain cross-talk impacting local neuro-immune-vascular interactions in the adventitia with important consequences on atherosclerosis progression in mice[19]. The implication of this circuit for human atherosclerosis is currently unknown.

Adaptive immunity in response to cardiac ischaemic injury

Resident DCs and T-cells in normal hearts:

The normal heart harbours several populations of resident macrophages and several populations of DCs, including monocyte-derived DCs, cDC1 and cDC2[95]. The precise spatial distribution of DC subsets is poorly characterised. However, these DCs, namely IRF8-dependent cDC1, drive the generation of autoreactive Tregs that are specific for cardiac self-antigens, particularly α-myosin heavy chain (MYHCA)[96]. This is an important homeostatic mechanism ensuring peripheral non-deletional tolerance to a self-antigen that escapes central negative selection[97] (Fig.2a). T-cells in normal hearts are enriched in selective TRBV-J rearrangements and TRBV gene segments compared to peripheral blood, indicating that they may recognize and tolerise against tissue-specific antigens[98]. Single-cell TCR sequencing will facilitate the characterisation of these T-cells and their antigen specificities. It will also be important to dissect the different steps involved in maintaining peripheral tolerance to cardiac-specific antigens. A well-developed and functional cardiac lymphatic network could

facilitate this immune surveillance task. Biomechanically-induced IL33 may promote/maintain immunosuppressive and reparative ST2+ cardiac Tregs[99]. Furthermore, the heart produces hepatocyte growth factor, which is known to induce immune-regulatory DCs and instructs T-cell cardiotropism by binding to its receptor c-Met in draining LNs, inducing the release of β chemokines and promoting T-cell recruitment through CCR5[100]. This mechanism may be required for cardiac Treg-cell accumulation at steady state, and is consistent with the role of CCR5 in promoting Treg-cell recruitment to the heart after MI, suppressing inflammation and reducing adverse remodelling[101] (Fig.2a).

Adaptive T-cell immunity in response to MI:

Ischaemic injury releases cardiac self-antigens in an inflammatory milieu that promotes maturation and activation of APCs[96] (Fig.2b). The infiltrating monocytes facilitate cardiac self-antigen trafficking to draining LNs, promoting the development of pathogenic autoreactive T-cells[102]. At the exception of pDCs, all other DC subtypes were shown to licence pathogenic autoreactive Th/Teff cells[96,98,103] (Fig.2b). The mechanisms responsible for the recruitment, retention and further activation of pathogenic CD4+ T-cells post-MI remain poorly understood. CX3CR1 expression on human CD4+ T-cells post-MI was associated with their decline in the circulation, potentially attracted to the injured myocardium by increased production of CX3CL1[104]. This is consistent with the mechanisms of tissue recruitment of TEM, but additional mechanisms are likely at play (Fig.2b). CD4+ T-cells of the ischaemic heart show a preferential expansion of TRBV gene segments and TRBV-J rearrangements compared to circulating T-cells[98]. It's still unclear, however, whether these clones are different from those found in non-ischaemic hearts, or whether the same clones that are present before ischaemia switch from naïve and regulatory to effector memory and Th1/Th17 phenotypes. This early CD4+ T-cell activation post-MI is detrimental overall and contributes to increased infarct size and reduced heart function[105]. CD8+ T-cells are also activated and accumulate in the ischaemic heart[98], producing IFNγ and displaying cytotoxicity toward cardiomyocytes[106] (Fig.2b). Although their antigen specificity is still unknown, their granzyme B-dependent[106] detrimental effect[107] requires the recognition of self-antigens in an MHC-I-restricted manner[106]. This is consistent with the detrimental role of CLEC9A-expressing cDC1, which promote CD8+ T-cell cross-priming[107]. Similar to CD4+ T-cells, a role for CX3CR1-CX3CL1 pathway has been proposed in myocardial homing of CD8+ T-cells and their detrimental role[104]. The same group described accelerated senescence of circulating effector memory TEMRA CD8+ T-cells[108]. However, the implication of this finding to post-MI cardiac remodelling is unclear. The role of other T-cell subsets requires further investigation. The TCR repertoire of γδ T-cells becomes significantly restricted in patients with acute MI in association with increased expression of IL17A[109]. CD4-CD8- T-cells also accumulate in large numbers in ischaemic hearts[107] but their significance and role remain unexplored.

The appearance of pathogenic autoreactive T-cells post-MI may constitute a threat to immune homeostasis. However, in most circumstances and in the absence of an autoimmune-prone background, overt cardiac autoimmunity doesn't develop. This may be explained, in part, by the concomitant increase in cardiac self-antigen-specific Tregs[3,99,110]. The ischaemic heart sustains the recruitment of circulating Tregs and their local proliferation and expansion[99], and induces the conversion of recruited T conventional cells into Tregs[3]. However, the mechanisms responsible for these effects are poorly understood. Enhanced release of IL33 from stressed necrotic cells may play a role. Other data point to the presence of a local paracrine/autocrine adenosinergic loop enhancing Treg immunosuppressive effects[105 111]. Several additional hypotheses merit exploration. The hypoxic environment could maintain a

tolerogenic DC phenotype. Furthermore, the site of T-cell priming outside the heart may play a role. Autoreactive T-cell priming by DCs in the spleen after MI has been shown to promote Treg generation[112], whereas DC-dependent priming of T-cells in draining LNs, supported by high levels of HGF and CXCL10, may drive a sustained recruitment of pathogenic T-cells[100] (Fig.2b).

Although CD4+ T-cell depletion is protective at the acute phase of MI[105], total CD4 T-cell deficiency in mice impairs long-term cardiac remodelling and the recovery of heart function, suggesting an overall protective role of CD4+ T-cells[113]. Indeed, a series of experimental studies point to a protective role of Tregs in experimental MI[114,115] and low levels of circulating $CD4^{+}Foxp3^{+}$ Tregs in humans correlate with increased risk of acute coronary events at follow-up[116]. Cardioprotective effects of Tregs are diverse and include the regulation of adaptive and innate immune responses, their impact on stromal cells, modulating fibroblast activation and matrix deposition[115,99], and their regulation of cardiomyocyte apoptosis and proliferation[117]. Despite the relative preservation of Tregs at the acute phase of MI, a subset of Tregs may become dysfunctional and potentially pathogenic over time[118]. Those Tregs appear to have lost their suppressive properties, upregulated the expression of pro-inflammatory mediators (TNF, TNFR1, IFNγ) and acquired anti-angiogenic properties[118]. The molecular pathways responsible for these alterations require further investigation.

B-cell responses to cardiac ischaemic injury:

B-cells, including B1-like and B2-like cells, represent a large portion of resident leukocytes in the myocardium[119,120]. They represent circulating cells that slowly transit through the myocardium to support cardiac homeostasis. Additional resident B1-like cells are found in the pericardial adipose tissue as part of FALCs[121,122] (Fig.2a). Intravascular myocardial B-cells appear to support the expression of MHC-II on resident cardiac macrophages[123]. However, the (patho)physiological relevance of this finding is currently unclear. Studies in mice and rats have shown that within 7 days following MI, more B-cells accumulate in the myocardium and pericardial FALCs[124 121]. Pericardial B-cells produce GM-CSF post-MI and promote CCR7-mediated DC migration and T-cell activation in pericardial adipose tissue, with detrimental consequences on post-MI remodelling and recovery of heart function[121] (Fig.2b). Systemic B-cells and more particularly MZ B-cells also become activated early after MI and their depletion improves cardiac remodelling and the recovery of heart function[124,125]. The detrimental effect of mature B-cells is attributed to their pro-inflammatory role, producing CCL7 and promoting monocyte mobilisation and recruitment[124,125] (Fig.2b). Except for certain natural IgM against non-myosin heavy chain II that promote myocardial damage[126], the immediate effects of B-cells are unlikely to be dependent on specific antibodies. However, following the acute phase, myocardial ischemia triggers GC formation in draining mediastinal LNs[4] (Fig.2b). Such newly-induced autoantibodies, e.g. against cardiac myosin, have been associated with post-MI heart failure[127]. Thus, it is possible that MI leads to the emergence of autoantigens released during myocardial damage, which in turn trigger autoimmune responses and memory B-cells that worsen cardiac function and may even accelerate atherosclerosis post-MI[4]. The latter is particularly relevant in the setting of secondary prevention. However, not all B-cells are detrimental. IL-10-producing B-cells of the pericardial adipose tissue may help in inflammation resolution and heart recovery post-MI[122] (Fig.2).

Targeting adaptive immune responses for patient benefit

A few important points should be considered regardless of which therapeutic strategy is pursued to target the immune response in ACVD (Box 5).

Harnessing the adaptive immune system for patient benefit could be pursued through various modalities. The therapeutic objective is to break the vicious circle of pathogenic adaptive immune activation and promote regulatory homeostatic immunity. Here, we will highlight a few immunomodulatory approaches in development (Fig.3).

Deleting/neutralising pro-atherogenic immunity

Targeting pathogenic B-cells: CD20-mediated B-cell depletion reduces atherosclerosis, MI-remodelling and post-MI accelerated atherosclerosis in experimental models[4,74,75,124]. CD20 antibodies preferentially deplete B2-cells and preserve atheroprotective B1-cells. The RITA-MI phase 1/2a trial tested the safety and tolerability of a single intravenous injection of rituximab in patients with acute STEMI[128]. The treatment led to a rapid and dose-dependent reduction in circulating B-cells and echocardiographic data suggested improved LV-ejection fraction after 6 months. This impact on cardiac remodelling is now being tested in the phase 2b RITA-MI2. A phase 2 trial of Rituximab in patients with systolic heart failure is also underway (NCT03332888).

Belimumab is approved for treatment of SLE. It targets BAFF, a cytokine of the BAFF/APRIL system that is essential for B2-cell survival. While targeting of the BAFFR was atheroprotective in mice[76], BAFF neutralization was proatherogenic due to non-canonical atheroprotective effects of BAFF signalling in myeloid cells that inhibits TLR9-IRF7-dependent inflammatory responses[129]. Thus, caution is warranted regarding this specific B-cell depletion approach.

Several other B-cell depleting agents are being developed (e.g. targeting CD22 and CD19), but they may also deplete protective subsets and data in experimental atherosclerosis are lacking.

Monoclonal anti-OSE antibodies: Neutralizing different OSE, such as MDA or PC, with specific humanized mAb has been successful in preclinical ACVD models[87,88]. The efficacy of anti-MDA antibodies in humans has been tested in the GLACIER trial which assessed aortic ^{18}F-FDG-PET uptake in patients receiving orticumab (human anti-MDA-ApoB100 IgG1) over a period of 12 weeks[130]. While this trial did not show an effect, two randomized double-blind placebo-controlled phase 2 trials with different study design are underway. NCT04776629 investigates the effect of orticumab on coronary plaque burden in psoriatic patients with increased CVD risk, and NCT03991143 investigates the effect of a human IgG1 against PC (ATH3G10) on CV outcomes in STEMI patients. Future approaches could employ RNA-based technologies to produce sustained levels of protective antibodies.

Immunogenic vaccination strategies: A range of vaccination strategies that raise anti-OSEs antibodies are atheroprotective in animal models[40,86,131,132]. In particular, the molecular mimicry between PC and the capsular polysaccharide of *S. pneumoniae* has been exploited to trigger high levels of atheroprotective anti-PC IgM antibodies in Ldlr-/- mice by immunizing them with pneumococcal extracts[131]. This prompted the idea to translate these findings and evaluate if vaccination with approved polyvalent pneumococcal vaccines also induces anti-PC antibodies and reduces cardiovascular events. However, PC is not a major constituent of pneumococcal vaccine preparations, and small studies using the 13-valent conjugate pneumococcal vaccine Prevnar-13 failed to show a robust induction of anti-oxLDL or anti-PC antibodies and special vaccination protocols may be required[133,134]. The effect of the 23-valent vaccine Pneumovax 23 on cardiovascular outcomes (ACS, ischaemic stroke) is

currently tested in the randomized placebo-controlled AUSPICE trial[135]. It will be interesting if this study confirms the cardioprotective signals from a meta-analysis of several observational studies[136].

Re-establishing immune homeostasis

IL2-based therapeutics: Low-dose IL2: The concept of low-dose IL2 therapy in CVD[137] builds on the essential role of IL2 in promoting Tregs, and the exquisite sensitivity of these cells to ultra-low doses of IL2. IL2 also limits the switch of autoreactive Tregs to Teffs that could result from TCR overactivation[59]. A first proof-of-concept safety and biological efficacy trial, LILACS, was completed. Low-dose IL2 significantly expanded Tregs in patients with stable and unstable ischaemic heart disease without adverse events of major concern[138]. A dose of 1.5 x10^6 IU was selected and is currently being tested in IVORY (NCT04241601), a randomised, double-blind, placebo-controlled trial testing the superiority of low-dose IL2 in reducing vascular inflammation in ACS patients. Low-dose IL2 therapy may have protective effects beyond Tregs. In LILCAS, low-dose IL2 increased the activation of ILC2[139], an immune cell population with protective roles in atherosclerosis[31] and cardiac remodelling post-MI[139].

Other IL2 biologics: More targeted approaches to enhance IL2 selectivity are being developed, e.g., an antibody-IL2 conjugate that induces a conformational change resulting in lower affinity for IL2Rα and competitive advantage for Tregs[140]. Other approaches include IL2 muteins with enhanced Treg selectivity[141], some of which are tested in clinical trials (NCT03422627;NCT03451422;NCT03410056). Cell therapy approaches are also in development using engineered T-cells that express defined ligand (normal or mutated IL2)-receptor (mutated versions of the IL2R subunits) complexes, and that signal exclusively together[142].

Cell therapy-based approaches: Chimeric antigen receptor (CAR) expression renders a population of T-cells uniformly specific for a defined antigen and has revolutionised T-cell therapy. Engineered CAR-Tregs that express scFv antibodies directed against atherosclerosis-relevant epitopes, e.g., OSEs, are expected to preferentially home to disease sites and suppress local inflammation. Such CAR-Tregs may also be engineered to express their own IL2, further stabilising them and enhancing their efficacy. Technological breakthroughs allowing for the use of allogeneic cells would overcome major hurdles in translating this technology to the clinic.

Tolerogenic vaccination strategies: The discovery that Tregs are atheroprotective[143] opened the possibility for therapeutic strategies that promote antigen-specific Tregs[144]. Several tolerogenic strategies were tested, particularly using MHC-II-restricted ApoB peptides, and were shown to reduce atherogenesis, likely in a Treg-dependent manner[45,145]. Unfortunately, translation of this knowledge to the clinic has been very slow. This is due to several factors, including but not limited to insufficient knowledge and characterisation of the adaptive immune response in human atherosclerosis, the need for correct HLA class-II matching for peptide-based vaccines, as well as uncertainty about the vaccination route and the type of adjuvant needed to induce protective autoantibodies (while preventing the generation of pathogenic T-cells and autoantibodies), or required to elicit a tolerogenic Treg response. Our increasing knowledge of the adaptive immune response of human atherosclerosis, boosted by the advent of single-cell multiomics technologies, along with the revolution in vaccine design and delivery, as shown by the exceptional efficacy of combining RNA and lipid nanoparticle technologies to combat Covid-19, will help overcome these hurdles and will accelerate the development of various immunogenic and tolerogenic vaccines to prevent and treat ACVD.

Perspectives and conclusion

Despite unequivocal evidence for a fundamental role of the immune system in ACVD, newly developed anti-inflammatory treatments were shown to be incremental or ineffective[5]. We believe that two major areas with fundamental gaps in our knowledge deserve a particular attention.

Despite substantial evidence from GWAS for a major role of inflammation in human atherosclerosis, there's a huge gap in characterising the causal mechanistic pathways. We operate at distance from biology, and we desperately lack the comprehensive cell-type and disease-specific phenotyping information that will enable us to produce useful knowledge.

The second gap is due to our reliance on an outdated morphological classification of atherosclerosis. We need to move towards the medicine of the future and establish a molecular classification of atherosclerosis. This will require comprehensive insight at single-cell resolution, studying healthy and diseased arteries, at all major arterial sites and across space and time. Deep characterisation of antigen-specific T- and B-cells along with their antigens and antibodies must be conducted in a systematic way, at different anatomical sites (arteries, SLOs, ATLOs, blood), in large numbers of individuals and at various time points of the disease process. This effort will be facilitated by the continuous development of advanced single-cell technologies and computational analysis, coupled with powerful antigen screening librairies and advanced proteomics, allowing for the prioritisation of atherosclerosis-specific immune receptors and their relevant antigens and antibodies. These can then be harnessed for the development of transformative diagnostic and therapeutic strategies. The exceptional recent advances in RNA-based and cell-based therapies are expected to further facilitate and accelerate the translation of this knowledge into benefit for patients.

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Figures

Figure 1. The "immuno-vascular unit" in health and atherosclerotic disease

A vascular-associated lymphoid tissue is present in normal healthy arteries and comprises a variety of immune cells, which play essential roles in immune surveillance and vascular homeostasis. Crawling monocytes ($Gr1^{dim}$ in mice, $CD14^{dim}CD16^{+}$ in humans) sense nucleic acids as those released after endothelial cell EC damage, scavenge debris and microvesicles (MVs) and ensure EC protection. Intimal macrophages (Mac^{AIR}) accumulate at sites of intimal thickening (upper right, normal artery) and are the first myeloid cells that scavenge oxidized LDL (oxLDL) in the initial stages of atherosclerosis (middle left, atherosclerotic plaque). This further drives vascular inflammation and leads to the recruitment of inflammatory monocytes ($Gr1^{hi}$ in mice, $CD14^{++}CD16^{-}$ in humans) which give rise to a variety of macrophage subsets. Adventitial macrophages, particularly $Lyve1^{+}$ macrophages (upper right, normal artery) regulate arterial stiffness, in part through the production of MMP9 and FXIIIA. Their specific contribution to atherosclerosis is currently unknown. The adventitia and peri-vascular adipose tissue form a permissive niche for the accumulation of atheroprotective innate lymphoid cells type 2 (ILC2) and innate-like B-cells, organised in fat-associated lymphoid clusters (upper middle, normal artery). ILC2-derived type 2 cytokines (e.g., IL-5 and IL-13) promote B1-cell activation and production of natural IgM antibodies, and contribute to an anti-inflammatory macrophage phenotype. Most dendritic cells (DC) and T-cells of normal arteries are found in the adventitia. T-cells of normal arteries are likely to be enriched in a regulatory phenotype [146] instructed by DCs presenting vascular-associated antigens (in draining lymphoid organs) and maintaining peripheral tolerance. The inflammatory milieu of developing lesions promotes DC maturation, which favours the generation of T helper (Th), effector (Teff) and effector memory (Tem) cells (mostly in draining lymphoid organs) and their recruitment into both the intima and adventitia of atherosclerotic arteries (bottom left). Sustained stimulation of autoreactive Tregs may downregulate Foxp3, promoting their conversion into Th1 and Teff. Some of the Treg and Teff cells acquire a resident memory (TRM) phenotype. Th1, Teff and Tem cells predominate in advanced lesions (bottom right), and activated cytotoxic $CD8^{+}$ T-cells may acquire an exhausted phenotype. Germinal centre (GC) activation in secondary lymphoid organs leads to the production of affinity-matured class-switched (IgG, IgE) antibodies, which accumulate in lesions. Medial vascular smooth muscle cells of advanced lesions produce CXCL13 and CCL21 and may adopt features of lymphoid tissue organizer-like cells, leading to artery tertiary lymphoid organ (ATLO) formation (bottom right). ATLOs are conducive to the generation of Tregs, which play a counter-regulatory atheroprotective role. The diseased adventitia also establishes neuro-immune vascular interactions, which affect lesion progression. VSMC = vascular smooth muscle cells, TFH = T follicular helper, PC = plasma cell.

Figure 2. Adaptive immune responses in the healthy and ischaemic heart

The heart and its adjacent pericardial and adipose tissue harbour several types of immune cells. At steady state (Fig.2a), conventional dendritic cells (cDC), particularly cDC1, home to draining lymph nodes and instruct the generation of tissue-specific (e.g., myosin heavy chain, alpha) regulatory T-cells [146], which establish peripheral tolerance. Heart-derived hepatocyte growth factor (HGF) binds to DC and induces an immune-regulatory phenotype. HGF also promotes chemokine production (e.g., CCL5) by c-MET-expressing T-cells, which drives CCR5-dependent recruitment and cardiac Treg-cell accumulation. Treg immunosuppressive function is further promoted through a local (cardiac) paracrine/autocrine adenosinergic loop, involving CD39/CD73 and adenosine A2A receptor (A2AR), or by engaging ST2 signalling

with heart-derived IL-33. Pericardial innate lymphoid cells type 2 (ILC2)-derived IL-5 and IL-13 activate innate-like B-cells to produce natural IgM antibodies and anti-inflammatory IL-10, which contribute to an anti-inflammatory and reparative macrophage phenotype. During ischaemic injury (Fig.2b), DC acquire an inflammatory phenotype instructing the development of T helper (Th), effector (Teff) and effector memory (Tem) cells, as well as CD8 cytotoxic T-cells. Enhanced production of GM-CSF by pericardial innate-like B-cells further promote DC activation and the generation of Th and Teff cells. These T-cells upregulate CX3CR1 and CXCR3 and their recruitment into the ischaemic heart is facilitated by heart-derived CX3CL1 and CXCL10. Germinal centre (GC) reactions develop in the spleen and draining lymph nodes leading to the production of (heart-specific) autoantibodies with potential detrimental consequences. Splenic marginal zone B-cells (MZB) are activated by the release of damage-associated molecular patterns (DAMPs), including in the form of mitochondria-containing microvesicles (MVs), which activate toll-like receptors (TLR) leading to CCL7 production. The latter promotes inflammatory monocyte mobilisation from the bone marrow, enhancing the accumulation of inflammatory macrophages within the ischaemic myocardium. In most cases, the ischaemic heart is able to maintain the presence of a sufficient number of Tregs to prevent the occurrence of overt full-blown cardiac autoimmunity.

Figure 3. Therapeutic strategies to target adaptive immune responses

The figure, which is not exhaustive, highlights some of the most promising therapeutic strategies to manipulate the adaptive immune system (through neutralising pathogenic or promoting regulatory immunity) for the benefit of individuals with, or at risk of, ischaemic cardiovascular diseases. Current strategies tested in the clinic include the use of a monoclonal antibody (Ab) against CD20 (i.e., rituximab) to deplete mature B-cells, and the use of recombinant (rec) low-dose IL-2 to promote regulatory T-cells (Tregs). Promising strategies in development include the development of CD8 depleting monoclonal antibodies, neutralising antibodies to block pro-inflammatory oxidation specific epitopes (OSE), such as anti-phosphocholine (PC) and anti-malondialdehyde (MDA) Abs, chimeric antigen receptor (CAR)-Tregs targeting OSEs, RNA-based immunogenic vaccines targeting OSEs and RNA-based tolerogenic vaccines using ApoB100-derived peptides. FO B = Follicular B cells, BCR = B cell receptor, GCB = Germinal centre B cells, TFH = T follicular helper cell, TH = T helper cell, Teff = T effector cell, IFNg = Interferon-gamma, DC = dendritic cell, MHC = Major histocompatibility complex, TCR = T cell receptor, TGFb = Transforming growth factor beta.

Boxes

Box 1: OxLDL and OSE-specific immune responses

Once LDL is trapped in the artery wall it becomes oxidized by enzymatic and non-enzymatic processes[1]. The oxidation of LDL contributes to atherogenesis in many ways, which include the generation of bioactive lipids that activate ECs as well as the formation of oxLDL particles that are taken up by macrophages, leading to foam cell formation[1]. OxLDL is also recognized by innate and adaptive immune responses. It can trigger NFκB-dependent expression of proinflammatory chemokines and cytokines as well as components of the NLRP3 inflammasome via TLR4/6 and CD36[147]. Following the uptake of oxidized and aggregated LDL, endosomal leakage of cholesterol crystals can activate the NLRP3 inflammasome [148]. OxLDL uptake by antigen-presenting cells also leads to presentation of ApoB100-derived peptides to specific T-cells[2]. Oxidation of LDL leads to the generation of various oxidation-specific epitopes (OSE)[132]. Oxidation of the polyunsaturated fatty acids of LDL generates highly reactive breakdown products, including malondialdehyde (MDA), 4-hydroxnonenal (4-HNE), or the phosphocholine-containing oxidized phospholipids (PC-oxPL). In turn, these can form covalent adducts with ε-amino groups of ApoB or other proteins with phospholipids. OSE represent prototypic examples of stress-induced neo-self antigens and have the capacity to trigger pro-inflammatory responses in macrophages, such as the expression of cytokines and chemokines, but are also bound by specific antibodies in a hapten-specific manner. A series of studies has shown that OSE represent a class of stress-induced danger-asscoiated molecular pattern (DAMPs) that are recognized by cellular pattern recognition receptors, such as CD36 and SRA-1, as well as soluble pattern recognition proteins, such as complement factor H and germline-encoded natural IgM antibodies, but also as antigens by specific class-switched IgG antibodies[132]. Importantly, the same OSE that are present on oxLDL are also present on a subset of extracellular vesicles and on dying cells that accumulate in atherosclerotic plaques, but also in the context of many other acute and chronic inflammatory settings. Thus, immune responses targeting OSE have an important role in identifying stress-induced self-antigens and promoting their removal. In situations of increased oxidative stress when more OSE are generated, the endogenous clearance or housekeeping mechanisms may become overwhelmed leading to a loss of homeostasis, which would trigger sterile inflammation. Therefore, an inadequate response to OSE is considered to be an important factor contributing to pathological immune activation during atherogenesis.

Box 2: Innate-like B-cells and natural IgM antibodies

Innate-like B-cells represent a special subset of B-cells with important atheroprotective functions in atherosclerosis (reviewed in[10,81,149]). These cells include B1 cells as well as Marginal Zone [39] B-cells and play an important role in host defense and immune homeostasis. B1-cells, which in mice can be divided into B1a- and B1b-cells, differ from conventional B-cells in their developmental origin, activation requirements, and antibody repertoire. Based on these properties, equivalent B cell populations have been suggested to exist in humans as well, though their exact identity is still under debate. While B1-cells they are typically located in serosal cavities, such as peritoneum and pleura, the spleen seems to be critically important for their maintenance. Adoptive transfer of B1a- and B1b-cells in splenectomized or B- and T-cell deficient mice, respectively, protected from lesion formation[150,151]. This effect was dependent on the capacity of B1-cells to secrete IgM antibodies. Indeed, B1-cells are the main source of germline-encoded natural antibodies, which have an important role in the first line of defence against infections as well as in

housekeeping functions by promoting the clearance of dying or damaged cells. The spleen and bone marrow are major sites of B1-cell-derived plasma cells and antibody production, though PVAT has been described as another potentially relevant site[30]. B-cell-specific Id3 deficiency in Apoe KO mice results in increased B1b-cells in PVAT but not the spleen, suggesting local recruitment. The latter is enhanced by CCR6 expression in B1-cells[152]. A large part (>30%) of B1-cell-derived natural IgM have specificity for different types of OSE[72], which explains their involvement in atherosclerosis by neutralizing oxidized lipids of oxLDL, promoting apoptotic cell clearance, and inhibiting the pathogenic activities of OSE+ microvesicles[153,154]. Possibly, also local production of OSE-specific IgM in the aorta is triggered by chronic inflammation and aids the removal of oxidized lipids and dying cells. However, excessive oxLDL generation and increased OSE accumulation during atherogenesis may overwhelm the homeostatic functions of OSE-specific IgM. As a consequence, OSE can trigger inflammatory responses, which may result in inappropriate activation of adaptive immune responses against these same antigens. Several cytokines (IL-5, IL-33), chemokines (CXCR4), and (co-)receptors (Siglec-G, Tim1) have been identified that control B1-cell numbers and plasma levels of OSE-IgM, and thereby modulate atherosclerosis[32,155-157]. Another set of innate-like B-cells are MZ B-cells, which reside in the marginal zone of the spleen where they survey antigens that pass through the red pulp. They are also considered atheroprotective in part by secreting OSE-specific IgM antibodies[158]. Whether these IgM differ functionally from B1-cell derived IgM remains to be investigated. A large part of MZ B-cell-derived hybridomas was suggested to have specificity for OSE. Therefore, the spleen may participate in sensing increased circulating levels of OSE generated during atherosclerosis and in turn produce more neutralizing IgM antibodies. Indeed, inflammasome activation associated with hypercholesterolemia has been shown to occur in the marginal zone where it can trigger B-cell activation[158]. Thus, the involvement of both types of innate-like B-cells and the natural IgM they secrete reflects a response to chronic inflammation that is required to maintain tissue homeostasis by neutralizing stress-induced self-antigens and their accumulation. Another facet of natural IgM may be their ability to modulate BCR-dependent differentiation of B-cells expressing the low-affinity IgE clearing receptor CD23, thereby preventing the accumulation of proatherogenic IgE antibodies[91]. Increased production of self-antigens targeted by natural IgM could interfere with this activity.

Box 3: Mechanisms of immune cell accumulation in healthy arteries

Most resident intimal macrophages accumulate at sites of altered haemodynamics (e.g., reduced shear stress). However, how macrophages accumulate in the adventitia is unclear. We speculate that sudden increases in both blood pressure and shear stress shortly after birth could direct monocyte recruitment through the adventitial microvessels (low shear stress) rather than the intimal side (high shear stress). This could be further promoted by a blood pressure-mediated activation of the vessel wall to express chemokines (e.g., CX3CL1 or CXCL10) known to be involved in adventitial macrophage accumulation[25,159]. Could resident macrophages also sense haemodynamic stress (e.g. pressure) or the consequences thereof and contribute to recruiting other monocyte-derived macrophages?

Similar questions apply to DCs, T- and B-cells that accumulate in healthy arteries. Do they sense components of the artery wall, particularly those from the immunoprivileged media which might be released in response to physiological (e.g., haemodynamic) stresses, and be presented to maintain peripheral tolerance? Do they detect and present lipoprotein components, like ApoA, that cross the arterial wall through outward convection and are removed from the tissue by arterial lymphatics? Could this contribute to maintain a Treg phenotype and limit Treg-cell conversion to pathogenic T-cells[58]? Do resident B-cells in the

adventitia and PVAT mediate housekeeping functions by secreting natural antibodies that prevent the accumulation of damaged vascular wall components? We have recently identified a non-canonical role for the B-cell cytokine APRIL which limits lipoprotein retention and act as a key molecule in vascular homeostasis[160]. Could APRIL be also involved in the immune homeostasis of arteries?

VALT formation, remodelling and function may also be influenced by the lymphatic system, which controls the drainage of macromolecules and may affect the emigration of myeloid and lymphoid cells. Finally, beyond its role in controlling vascular tone, arterial innervation by sympathetic adrenergic and parasympathetic cholinergic nerves could also affect VALT formation and function through adrenergic and cholinergic receptors expressed on immune cells.

Box 4: Some considerations related to B-cell autoimmunity during atherosclerosis

Experimental data in mouse models of atherosclerosis have clearly established the initiation of a germinal centre reaction (GCR) in response to hypercholesterolemia[71,77,79], in association with the development of T-cell dependent antibody responses. It is interesting that most of the B-cell antigens that have been characterized to date represent intracellular antigens that are released or expressed in response to stress (e.g. hsp60, GRP78) or carry lipid-peroxidation derived post-translational modifications (e.g. OSE). Thus, atherosclerosis-related B-cell responses may rather target stress-induced antigens or neo-self antigens, and one may speculate that autoantigens recognized in the context of atherosclerosis may in fact be modified, e.g. by OSE. The mitochondrial origin of one recently identified candidate antigen, ALDH4A1, would certainly facilitate its modification[82]. Intriguingly, hypercholesterolemia in mouse models is also associated with the generation of autoantibodies to classical antigens, such as anti-nuclear antibodies against Ro-52, Ro-60, or SmD1, suggesting a potential loss of tolerance[77]. Moreover, using an autoantigen array, Hutchinson et al recently screened serum IgG1 of Apoe ko mice and identified several potential new candidates, incl. RSPA, PVRL3, GPBP1, and Family with sequence similarity 131 member C (FAM131c)[78]. Indeed, hypercholesterolaemia aggravates immune activation in the presence of an autoimmune background[161]. While autoimmunity is not typically linked to excessive hypercholesterolemia in humans, these data suggest the possibility that if tolerance is broken in the context of atherosclerosis and dyslipidaemia, this in turn could facilitate the activation of autoreactive GCs and B-cells against other self-antigens, consistent with the epitope spreading seen in autoimmune diseases. If and how OSE DAMPs also promote autoreactive B-cells remains to be shown. Interestingly, oxidized phospholipids have been suggested to modulate adaptive immunity by promoting a hyperactive DC state[162]. All these aspects need to be considered when attempts are made to identify relevant B-cell antigens in atherosclerosis.

Box 5: General therapeutic considerations in patients with atherosclerotic disease

Treatment of modifiable classical risk factors will remain an essential component of therapeutic management. This is particularly the case of lipid-lowering therapies, which will continue to evolve with the aim to ensure early, substantial and sustainable reduction of circulating lipid levels. However, not all lipid-lowering therapies are equal in terms of their potential impact on the immuno-inflammatory response, and consideration must be given to whether specific lipid-lowering therapies could be more appropriate than others in particular settings. For example, SLE is characterised by an increased type I interferon response, which reduces the intracellular cholesterol synthesis pathway[163]. This is a vicious circle given that reduced cholesterol synthesis drives more type I interferon production[163]. Will statin therapy

be the most effective strategy to reduce plasma LDL-C levels in SLE, or may patients with SLE derive greater benefit from another LDL-C-lowering (PCSK9-targeted) strategy? We believe this question merits appropriate investigation.

Others have suggested that sustained reduction of circulating lipid levels could make anti-inflammatory therapy redundant. We believe this is unlikely to be the case. A large proportion of individuals recruited in contemporary trials still show a residual inflammatory risk (on-treatment hsCRP above 2 mg/L)[164], and are at substantial lifetime risk of cardiovascular events. Experimental models have established inflammation as an obligatory pathway for atherosclerosis development. Therefore, appropriate targeting of this biological process is expected to yield exceptional benefits.

We would also like to stress the fact that hsCRP, although currently useful, is certainly not the optimal marker to stratify patients for anti-inflammatory therapy. Circulating levels of hsCRP are exclusively dependent on the IL6 pathway. Atherosclerosis is unlikely to be exclusively driven by IL6, and individuals with low hsCRP (<2mg/L) may still mount IL6-independent inflammation and be susceptible to atherosclerosis and cardiovascular complications. Moreover, increased hsCRP may preferentially reflect a state of metabolic inflammation in the liver rather than vascular inflammation. Thus, the optimal circulating marker of plaque inflammation is still to be found. This is particularly true for markers of adaptive immune responses, which will require the development and measurement of antigen-specific responses (e.g., antigen-specific tetramers, autoantibodies).

A final general consideration relates to the increased risk of serious infections that could result from the long-term use of anti-inflammatory therapies. This was indeed responsible for halting the development of canakinumab as a potential therapy for patients with ACVD. We believe the infectious risk to be minimal with the use of therapies that target antigen-specific adaptive immunity compared to therapies that target broad systemic inflammatory pathways. Furthermore, Treg suppression of autoreactive T-cells does not compromise, and may even boost, T-cell immunity directed towards infectious non-self antigens.

Glossary:

T-cell central tolerance: clonal deletion of autoreactive T-cell receptors in the thymus or diversion of these self-reactive cells into an autoreactive Treg-cell lineage.

B-cell central tolerance: clonal deletion involving apoptosis of immature B-cells recognising self-antigens in the bone marrow, and receptor editing (secondary recombination of antibody genes) which reprogrammes the specificity of autoreactive B-cells.

T-cell peripheral tolerance: inactivation of autoreactive effector T-cells (that have escaped central tolerance) through clonal deletion (deletional tolerance) or through conversion to Tregs (non-deletional self-tolerance).

B-cell peripheral tolerance: induction of anergy, and antigen receptor desensitisation by inhibitory receptors, which regulates the survival and activation of B-cells.

Medial immunoprivilege: refers to the sparing of the arterial media of leukocytic infiltrates, particularly adaptive T and B-cells, in most arterial diseases, including atherosclerosis. Medial immunoprivilege is not absolute and may fail under some circumstances.

Germinal centre: A histological structure that forms in the B-cell follicle during an immune response, where B-cells compete for antigen and T-cell help to survive, proliferate and

differentiation into affinity-matured antibody producing plasma cells or memory B-cells. The latter can re-enter the germinal center upon a recall response.

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