Therapeutic strategies targeting inflammation and the immune system in atherosclerosis: how to proceed?

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Abstract | Atherosclerosis is a chronic inflammatory disease of the arterial wall, where the formation of plaques containing lipid, connective tissue and immune cells takes place in the intima of large-sized and medium-sized arteries. Over the past decade, a substantial decrease in cardiovascular mortality has been achieved, largely due to cholesterol-lowering regimes and therapies targeting other traditional risk factors of cardiovascular disease (CVD), such as hypertension, smoking, diabetes mellitus and obesity. However, the overall benefits of risk factor targeting have stagnated, and a huge burden of CVD remains. More recently, the indispensable role of immunological components in the establishment and chronicity of atherosclerosis have come to the forefront as clinical targets, with emerging proof-of-principle studies demonstrating the benefit of targeting inflammation in CVD, while also uncovering key issues that targeting the immune system presents. In this review, we provide an overview of the latest developments concerning the role of the immune system in atherosclerosis, preclinical research and clinical trials. We also identify important challenges that need to be addressed in order to advance the field, including patient selection, identification of responders and non-responders, implementation of immune-phenotyping and surrogate end points for vascular inflammation. Finally, we provide strategic guidance for the translation of novel targets in immunotherapy into improvements in the clinic.

Introduction

Atherosclerosis, the major cause of cardiovascular disease (CVD), is a chronic inflammatory disease triggered by the accumulation of cholesterol-containing low-density lipoprotein (LDL) particles in the arterial wall[1]. The gold standard of treatment is the prevention of cardiovascular events by targeting modifiable risk factors and the re-establishment of arterial flow by percutaneous or surgical procedures[2,3]. However, the therapeutic benefit of this strategy on cardiovascular outcome has stagnated and a huge burden of CVD remains[4].

Attilio Maseri (1935-2021) was one of the first to foresee the importance of inflammation as a component of the pathogenesis of acute coronary syndromes (NEJM CRP and Circulation IL6). Over the last 35 years evidence for the role of inflammation in atherosclerosis has accumulated (FIG 1). The arterial wall is populated by various immune cells, both in health and disease[5–7]. The innate immune system is the first line of defence against invading pathogens (PAMPS) and endogenous danger signals (DAMPS)[8] and is usually initiated by pattern recognition receptors, including Toll-Like Receptors (TLRs), and the inflammasome[9]. The innate immune response induces activation of macrophages and dendritic cells (DCs) that mediate antigen presentation, co-stimulation and cytokine production within the immune synapse (FIG 1), which induce the adaptive immune response[8]. The adaptive arm involves B and T cells, and it is slower but more specific and long-lived[8]. Athero-inflammation involves activation of both innate and adaptive immune responses, with both inherently linked[10] (FIG 2). The immune cells within arteries are switched on[11] due to the persistence of inflammatory stimuli or a failure in resolution leading to chronic inflammation[1]. To understand its genesis, we must consider both the interplay between cellular immunity and lipid retention, and the complex crosstalk between and within immune and non-immune cells[1,12] (Box 1).

A unique aspect that sets aside atherogenesis from other chronic inflammatory diseases is the key role that lipid particles take in its induction[1]. Modified lipoproteins such as oxidized LDL (oxLDL) trigger the immune response through their unique property of acting both as antigens activating the adaptive arm, and adjuvant molecular patterns activating the innate arm of the immune response[13–15] In advanced atherosclerosis, the culmination of complex chronic inflammatory processes results in the generation of a plaque with a fibrous cap and necrotic core, with clinical vulnerability to rupture leading to ischemic events[5].

The complexity of athero-inflammation was recently emphasized by single-cell biology studies in human and mice showing high heterogeneity of vascular leukocytes[7,16–25] (Box 1). This heterogeneity

underscores the importance of targeting specific cellular subsets to maintain tissue homeostasis while inhibiting CVD progression. Moreover, atherogenesis has emerged as a multi-organ process to which organs such as the bone marrow and spleen contribute[26–28]. In particular, the presence of clonal haematopoiesis of unknown origin (CHIP), an age-related process whereby somatic mutations arise in progenitor cells in the bone marrow leading to the expansion of specific clones with competitive advantage without inducing haematological disease[89], has been proposed as a new risk factor of CVD[29,90–93],

The first proof of the benefits of targeting inflammation in CVD in humans arrived with the CANTOS trial, showing improved clinical outcome in patients with a history of myocardial infarction that received treatment with antibodies against IL-1β[31]. This was quickly followed up with evidence that the anti-inflammatory effects of colchicine could reduce the cardiovascular risk of patients with chronic coronary disease[32]. Proof for a role of inflammation in CVD come also from further afield. Patients with chronic inflammatory diseases, such as lupus and rheumatoid arthritis (RA) have a significant increase (respectively 10- and 2- fold) of CVD and the risk is significantly correlated to the magnitude of inflammation[33,34]. Moreover, checkpoint inhibitors used in several cancers to enhance tumour surveillance by the immune system are associated with enhanced cardiovascular risk, adding to the cardio-oncology challenge[35,36]. Together, these studies highlight immunotherapeutics as the next step in cardiovascular disease therapy, and provide an opportunity to surpass the ceiling reached by management of classical risk factors to address the resudual CV risk[37]. At present, the challenge stands to identify crucial perpetrators of atherosclerosis-specific inflammation amongst the plethora of inflammatory mediators, while sparing host defence. Such process requires BOX 2……

In this Review, we provide an overview of the therapeutic potential of targeting the immune system in atherosclerosis. We summarise the published and ongoing clinical trials targeting the immune system in atherosclerosis. We identify important challenges that need addressing in order to advance translation of novel immunotherapeutic targets into the clinic. Finally, we highlight selected new therapeutic targets emerging from pre-clinical studies with the biggest potential for translational payoff in the medium-term.

Immune-therapeutics in clinical trials

Over the past 5 years promising results from clinical trials targeting inflammation in CVD accumulated. Here, we summarise the positive phase III, promising phase II, ongoing and ineffective

trials, and the lessons learnt as a result (FIG 3).

Positive phase III trials

Two immune-therapeutics have been successful in improving the cardiovascular outcome of patients with CVD: canakinumab and colchicine (Table 1)[31,32,38,39].

Canakinumab. The Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) was a double-blind randomised controlled trial (RCT) investigating the effects of canakinumab, a monoclonal antibody inhibiting the pro-inflammatory cytokine IL-1β[31]. In total, 10,061 patients with a history of MI, receiving optimal cardiovascular risk management and high-sensitivity C-reactive protein (hsCRP) levels higher than 2 mg/L, were randomised to either receive canakinumab or placebo. Canakinumab was administered subcutaneously at dosages of 50, 150 or 300 mg quarterly and patients were followed up for a median period of 3.7 years. 150 mg canakinumab led to a significantly lower rate of recurrent CV events than placebo, independently of lipid-level lowering (HR 0.85; 95% CI: 0.74–0.98; *P* = 0.021)[31]. There was no effect on total mortality due to a small but significant risk of infection. Notably, patients with reduction of on-treatment levels to hsCRP <2 mg/L with canakinumab benefitted most and the effect of canakinumab at reducing hsCRP was dose-dependent[40]. Sub-analysis extended the scope of canakinumab beyond IL-1β and cardiovascular (CV) events, showing an important role for IL-6 in CV event risk and beneficial effects of canakinumab on cancer mortality[41,42]. The CANTOS trial demonstrated, for the first time, proof-of-principle that therapeutic targeting of the immune system can be beneficial for cardiovascular outcome.

Colchicine. Colchicine, widely used for the treatment of gout and pericarditis, acts on inflammation by inhibiting cytoskeletal microtubule formation[43,44]. Colchicine has broad cellular effects including reduction of monocyte and neutrophil motility and inhibition of inflammasome assembly *in vitro*[45,46]. The LoDoCo2 trial included 5522 patients with stable chronic CAD[32]. After 1 month of open-label use of colchicine 0.5 mg *per os* daily, patients were randomised to receiving either colchicine or placebo and were followed-up for a median period of 28.6 months. Patients using colchicine had a 31% reduction (HR 0.69; 95% CI: 0.57–0.83; *P* <0.001) in the incidence of the primary composite end points of cardiovascular death, MI, ischemic stroke and ischemia-driven coronary revascularization compared to placebo. Unfortunately, information on the effects of colchicine on inflammatory markers is missing. The results of this trial were consistent with the phase II LoDoCo trial[39] and the COLCOT trial[38] which assessed the effect of colchicine in patients with MI, and provided further support for the potential benefits of anti-inflammatory therapy in patients with acute coronary

disease.

Taken together, these trials demonstrated that anti-inflammatory therapeutics are efficacious to reduce CV events in patients with stable CVD. Although CANTOS and LoDoCo2 have not yet changed the treatment strategy in everyday cardiovascular risk management, they are a crucial milestone for clinical translation of immunomodulatory therapeutics in CVD. Both treatments share the ability to target innate immunity, offering proof in man of the importance of the innate arm of the immune system in athero-inflammation.

Promising phase II trials

Several cytokine blockers have shown promising results in phase II trials; summarised in Table 2. The biology of cytokines in atherosclerosis is reviewed in Ziad malla Annals Med and briefly in Box 1. Cytokine blockers are the first line of biologics for the treatment of chronic inflammatory diseases, including RA, inflammatory bowel disease (IBD) and psoriasis[47–49]. An arsenal of potential therapeutics are therefore available, some of which are soon available as generic drugs.

IL-1 blocking

There are several options of IL-1 blockade, including using canakinumab (selective targeting of IL-1β), anakinra (IL-1 receptor antagonist that targets IL-1α and β), and xilonix (monoclonal antibody specifically targeting IL-1α). Anakinra showed a significant reduction of hsCRP in the acute setting in patients with ACS compared to placebo in two separate studies[50,51]. Xilonix showed a trend towards reduction of restenosis and incidence of major adverse CV events in patients undergoing percutaneous femoral artery revascularization[52]. Whilst the CANTOS trial showed the relevance of IL-1 targeting in stable CAD, these studies illustrate the importance of IL-1 as a target in the acute setting of thrombotic events. Additional studies in larger patient groups should be performed to further assess the effect of these therapeutics on cardiovascular outcome.

IL-6 blocking

Tocilizumab, a monoclonal antibody targeting IL-6R, showed a promising reduction of hsCRP in patients with NSTEMI[53] and STEMI, and an increased myocardial salvage index (MSI) in the latter group[54]. Although the positive effect of tocilizumab on the MSI was significant, the absolute difference in MI size ? between treatment and placebo groups was 5.6%, which may be of limited clinical relevance. Moreover, IL-6 blocking using Ziltivekimab, recently showed reduction of hsCRP levels in the setting of chronic kidney disease[55]. These studies demonstrate the effectiveness of IL-6

blockade on inflammation reduction and follow-up studies will provide a more complete picture of the clinical relevance of IL-6 targeted therapies in CVD.

Alternatives to IL-1 and IL-6 blockade include TNF and IL-23 blockers, whereby preclinical and clinical research demonstrated a pro-atherogenic role for TNF and IL-23[56–58]. Observational studies in patients with arthritis showed that inflammation is a strong risk factor for CV events and TNF blockade resulted in reduced atherogenesis and lower incidence of CV events[59,60]. However, in clinical trials of patients with heart failure, TNF blocking resulted in no efficacy or worsening of the clinical outcome[61,62], thus TNF blockers may not be suitable for patients with significant deterioration of the left ventricular systolic function.

IL-23 is present in human atherosclerotic plaques[57], and high plasma levels of IL-23 are associated with mortality in patients with carotid artery stenosis[57]. Mouse studies showed that IL-23 drives T_H17 cell function, contributing to aggravation of atherosclerosis[63–65]. Despite this pro-atherogenic role of IL-23 in mice, several meta-analyses showed either no effect or possible worsening of cardiovascular outcome after treatment with IL-23 blockers (ustekinumab and briakinumab) in patients with psoriasis[66–68]. These studies were primarily designed to assess the effect on psoriasis, and therefore we cannot draw conclusions owing to the effect on inflammation in atherosclerosis. Other alternative therapeutic targets currently tested in trials, including hydroxychloroquine and low dose IL-2, are discussed in Box 3 and Table 3.

Challenges in clinical trials

Several clinical trials have attempted to target inflammation in CVD, but have not resulted in the reduction of inflammation markers and/or CV events (Supplemental Table 1). Notable examples are methotrexate and a p38MAPK inhibitors that failed to reduce CVD events [69,70]. The majority of the trials that failed to show efficacy were performed in unselected patient groups. The CANTOS trial was the first trial to take a step towards precision medicine by specifically selecting patients with an increased residual inflammatory risk (hsCRP >2 mg/L)[31]. A potential explanation for negative results might be the heterogeneity of the patient group included in the trials. However, colchicine trials were also performed in unselected patient groups and did show beneficial effects on cardiovascular outcome[32]. This illustrates that we have to be open to the possibility that trial failures might also be mechanism-based and that inhibiting inflammation in CVD is effective provided the correct inflammatory target or drug is chosen.

Variability of disease settings in clinical trials in CVD may explain failure of p38MAPK inhibitor studies. P38MAPK is an intracellular kinase that is activated in CVD by several stressors such as oxidized LDL-cholesterol and hypertension, and it is involved in the stabilization of mRNA encoding for several key inflammatory mediators[71,72]. The first study with the P38MAPK inhibitor losmapimod was performed in patients with stable atherosclerosis. When vascular inflammation was assessed using FDG-PET/CT imaging, the overall uptake of FDG in the index vessel was not significantly reduced, but losmapimod did show reduction of inflammation in the most inflamed regions[73]. However, follow-up trials were performed in larger patient groups with acute disease setting, where no effect on clinical outcome was observed, suggesting that P38MAPK might have a selective role in a chronic stable setting of CVD in keeping with its biological role in prolonging inflammatory responses via modulation of mRNA stability[70,74].

Other studies relied on FDG-PET/CT imaging. The GLACIER trial enrolled 147 patients with stable atherosclerotic disease and randomized to a single dose of MLDL1278A, multiple doses of MLDL1278A or placebo and assed vascular inflammation using FDG-PET/CT imaging. This and the p38MAPK study showed no significant effect on carotid plaque inflammation[75], possibly due to the concomitant use of lipid-lowering medication that could have masked the effect of passive vaccination. This study also raises questions about the use of imaging as surrogate endpoint for CV events. FDG-PET/CT imaging might need improved tracers to detect meaningful CV inflammation[76]. Recently developed imaging technique based on CT-angiography showed that changes in perivascular adipose tissue attenuation may be a sentinel marker for coronary perivascular inflammation associated with CV outcomes[77–79]. Further improvement of plaque imaging will give us the tools to build valid surrogate endpoints. Although surrogate endpoints may currently not be specific enough and therefore have not reached the benchmark of a clinical trial, developments in the field of machine learning could be employed to combine multiple surrogate endpoints for a more accurate prediction of clinical outcome[80,81].

Considering the recent successes involving therapeutic targeting of the immune system in atherosclerosis, the number of ongoing trials is surprisingly low. One reason could be the high costs of clinical trials in cardiovascular research that make it less attractive for investments by the industry. CVD trials are event-driven, rather than symptom-driven, and therefore require high patient numbers and long follow-up time. Therefore, it is crucial to identify reliable surrogate markers for vascular inflammation to facilitate the design of small proof-of-principle trials, allowing for rapid innovation and reduced risk. One key need is the prompt identification of patients that

respond to a specific treatment and to discriminate them early from patients who do not exhibit benefit from the interruption of a specific inflammatory pathway. This concept is well exemplified by the CANTOS trial where the clinical benefit of canakinumab is concentrated in patients that respond to treatment with significant reduction of the levels of hsCRP. Patients with hsCRP reduction above the median had a 27% reduction in CV events compared to only 5% for the lower half[40]. In the future, new surrogate endpoints based on immunophenotyping and/or imaging could be used, provided that an association to CV outcomes is demonstrable.

Looking towards the future, the secondary effects of anti-inflammatory therapy should be carefully considered. Canakinumab administration was associated with a spectacular reduction of the incidence of lung cancer in the CANTOS trial[42]. In CIRT, methotrexate was linked to an increase in the incidence of skin cancer, emphasizing the duality of cancer and CVD[93,106]. Immunosuppression and chronic inflammation both increase the risk of cancer[82]. Furthermore, preclinical studies have spotlighted the existence of a communication of the immune system between MI and breast cancer that can accelerate cancer progression[83]. Finally, there are increasing reports that immune checkpoint inhibitors might enhance CV risk in patients with cancer[35,36], while inhibition of adaptive immunity induces the risk of cancer due to disruption antitumor immunity[84]. Now that anti-inflammatory therapies in CVD are close to implementation in clinical practice, it is key to unravel the complex immunological relationship between cancer and cardiovascular disease.

Finally, the pathogenesis of CVD is multifactorial, and several types of culprit lesions lead to the same clinical presentation and syndromes[85]. Immune signatures in different disease settings are distinct, as illustrated by the different signatures in plaque erosion and rupture[86] (Box 1), and calls for identification of the disease setting in which a therapeutic is most successful. Implementing deep immunophenotyping strategies can enhance the selection of patients with the highest chance of benefit from a specific therapy, and facilitate rapid identification of responders and non-responders[87]. Immunophenotyping of patients with CVD is still in its infancy, however we have a handful of markers that could guide selection such as hs-CRP and IL-6[40,41,88]. The discovery of CHIP as a novel risk factor of atherosclerosis will potentially enable further risk stratification of patients. For example, reanalysis of CANTOS suggests that anti-inflammatory treatment might be more effective in patients carrying CHIP mutations[206]. Taken together, extensive immunophenotyping and immune-based risk stratification may facilitate patient selection, stratification and identification of responders, allowing efficient design of follow-up clinical trials and realise the potential of targeted immunomodulatory therapies with CVD.

In summary, the challenges of addressing the low-grade inflammation associated with CVD are manyfold and encompass: the need for careful risk/benefit assessment, the existence of several coronary syndromes with potentially different endotypes and pathogenesis, our current inability to identify responders early, our reliance on hard endpoints in trial design due to the limitations of our current imaging capabilities. Further understanding of the immune signature of CVD together with evolution of CV imaging technologies will accelerate the translation of targeting inflammation from the preclinical to the clinical arena.

New avenues for future translation

Recent advances in our understanding for the pathogenesis of atherosclerosis have highlighted several potential cellular and molecular therapeutic targets. Here, we focus on a selection of the most promising strategic areas supported by the convergence of several lines of evidence from the cardiovascular and other diseases, and as such, closer to translation to patients in the medium term (FIG. 3).

Therapeutic targets emerging from immunometabolism and trained immunity. Changes in the blood and bone marrow are regulated by immunometabolic events. Western diet feeding and hyperglycaemia were shown to induce epigenetic reprogramming in murine HSCs and myeloid progenitor subsets which resulted in sustained monocyte and macrophage pro-inflammatory priming, thereby driving tissue inflammation and disease[94–97]. These effects persisted even after reversing lipid and glucose levels owing to a phenomena of "trained immunity", whereby transcriptomic, epigenetic and metabolic rewiring of a macrophage or progenitor cell upon primary stimulation leads to enhanced inflammation[98,99].

Histone deacetylases (HDACs) are enzymes that repress gene expression by removing open chromatin acetylation marks. Broad HDAC inhibition in atherosclerotic mice showed mixed results[100–102], and more selective compounds are needed. Inhibition or genetic deletion of HDAC3 or HDAC9 reduced atherosclerosis in mice[103–105]. HDAC9 has been associated with abdominal aortic calcification and ischaemic stroke in genome wide association studies in humans[106,107], highlighting the clinical potential of specific HDAC targeting.

Targeting metabolic rewiring is an alternative avenue, as increased glucose metabolism in human and mouse HSPCs dictates myeloid lineage commitment[108]. GLUT1, a ubiquitously expressed glucose transporter, is a well-recognised target in other inflammatory conditions[109,110]. GLUT1 deficiency in bone marrow cells resulted in reduced HSPC proliferation, myelopoiesis and atherogenesis[111].

However, further investigation of the effects of GLUT1 inhibition in humans is necessary, as patients with GLUT1 deficiency syndrome show neurological symptoms, such as epilepsy[112]. Targeting immunometabolic processes represents another avenue to target inflammation and immunity (review https://doi.org/10.1038/s41422-020-0291-z).

Targeting clonal haematopoiesis of intermediate potential (CHIP). The discovery of CHIP has led to the identification of new potential targets. Most commonly occurring mutations are loss of function mutations in *Dntm3a*, *Asxl1*, *Tet2*, and gain of function mutations of *Jak2* (Jak2^{VF}), all resulting in growth and survival advantages of mutation bearing cells[113]. *Tet2* deficiency or the *Jak2*^{VF} mutation induced accelerated atherogenesis in mice[29,30,114–116] . *Dntma3* or *Tet2* deficiency induced IL-1β production and NLRP3 priming *in vitro*[30,117,118] and *in vivo* inflammasome inhibition by MCC950 prevented *Tet2*-dependent atherosclerosis progression[30]. This was further extended to mouse models of heart failure, cardiac dysfunction and obesity[119–121], highlighting the potential of targeting CHIP-driven inflammation using inflammasome inhibitors. The AIM2 inflammasome activation has been associated with *Jak2*^{VF}-driven atherosclerosis, as deletion of essential inflammasome components like caspase 1/caspase 11 or gasdermin D downstream of the AIM2 inflammasome in a mouse model of *Jak2*^{VF}-driven atherosclerosis induced a more stable plaque phenotype[116] (Box 4). JAK2 inhibitors provide another promising strategy to target inflammation in atherogenesis. Ruxolitinib and fedratinib are FDA approved drugs for myeloproliferative neoplasms and currently being tested for other inflammatory conditions, such as RA[122]. Both were effective in murine and leporine models of atherosclerosis[123,124]. Although ruxolitinib, a JAK1/JAK2 inhibitor, reduced plaque size in *Jak2*^{VF}-dependent atherosclerotic mice[114,116], treatment also increased necrotic core and reduced cap thickness, resulting in an instable plaque phenotype[116]. Therefore, a more specific JAK2 inhibitor, such as fedratinib, could be more beneficial in CVD.

***Targeting monocyte recruitment*.** Monocyte recruitment in atherosclerosis depends on CCR2, CCR5 and CX3CR1[125]. Genetic deletion of CCR2 or its ligand CCL2, reduced bone marrow monocytosis and atherosclerotic lesion size in mice[126–129]. Similarly, monocyte recruitment and disease severity were diminished in mouse models of MI treated with siRNA targeting CCR2 or an inactive CCL2 protein derivate that acts competitively[130,131]. In humans, genetic predisposition to elevated CCL2 levels is associated with stroke, MI and CAD and blood and plaque CCL2 protein levels were correlated with stroke and plaque destabilisation[132,133]. Finally, MLN1202, a CCR2 blocking antibody, reduced hsCRP in patients with risk of CVD[134].Genetic deletion of CCR5, its ligand CCL5 or pharmacological inhibition with the FDA approved CCR5 receptor antagonist maraviroc reduces atherosclerosis in LDLR-/-

mice[135,136]. Interestingly, HIV patients treated with maraviroc also show reduced atheroprogression[137,138]. Circulating monocytes, however, traffic into tissues during both homeostasis, inflammation, and inflammation resolution[139,140], and the effect of targeting recruitment on these aspects will need monitoring.

Reprogramming inflammatory macrophages. Macrophage polarisation is orchestrated by key master regulators, including NFκB, STAT protein family, PPARγ and the IRF family[141]. Reprogramming pro-inflammatory culprit cell populations that drive vascular inflammation towards homeostatic resolving macrophage phenotypes could reduce disease burden. Pioglitazone is an FDA approved PPARγ agonist that is known to induce a pro-resolving macrophage phenotype by inducing the gene expression of anti-inflammatory markers. In murine atherosclerosis administration of pioglitazone reduced macrophage content and increased plaque stability[143,144]. Clinical studies investigating the role of pioglitazone in patients with CVD and/or T2D[145–148] have observed atheroprotective effects and reduction of cardiovascular events, highlighting its therapeutic potential in CVD.
In murine models of CVD, global and myeloid IRF5 deficiency reduced atherosclerosis and improved plaque stability[149,150] and IRF5 inhibition with nanoparticles decreased myocardial infarct size[151]. The transcription factor IRF5 is known to induce a pro-inflammatory phenotype in macrophages[152,153] and therefore is a promising therapeutic target in CVD. Inhibitors of IRF5 have been proven therapeutically effective in *in vivo* mouse models of SLE[154,155]. We soon hope to see IRF5 inhibitors in clinical development.

Targeting the inflammasome. Selective NLRP3 inhibition with MCC950, for example, reduced atherosclerosis in hypercholesterolaemic or diabetic mice[156,157] and reduced infarct size in pig models of MI[158]. MCC950 has been tested in phase II trials in RA, but had to be discontinued due to liver toxicity[159]. NLRP3 inflammasome inhibitors in chronic inflammatory and neuroinflammatory diseases, are on the rise and are being tested in clinical settings thereby making it an attractive therapeutic target in CVD[160]. OLT1177, for example, has been assessed in phase I/II clinical trials on osteoarthritis[161], acute gout[160], heart failure[162], and is currently being tested in a study on COVID-19 infection[163] and shows high tolerability. Alternatively, prevention of inflammasome priming using TLR signalling inhibitors[164] or targeting the AIM2 inflammasome could be employed.
Inhibition of caspase 1 could be an alternative strategy for targeting the inflammasome in atherosclerosis, as NLRP3 and AIM2 inflammasomes depend on its catalytic activity to convert IL-1 cytokines into their active form (Box 4). VX-765 has been shown to reduce atherosclerosis in mice[165], however clinical phase II studies for psoriasis and epilepsy testing caspase I inhibitors (VX-740, VX-

765) showed hepatotoxicity and further development was stopped[166], showcasing the importance of developing more specific inhibitors.

Targeting the adaptive immune system

Auto-immune responses to oxLDL, including atheroprotective autoantigens and oxLDL reactive T cells[167–169], have led to the development of successful active immunisation strategies in murine and leporine atherosclerosis models (reviewed in[170]). Immunisation with key antigen apoB-derived peptides induces athero-protective effects, via diverse mechanisms including the induction of a humoral antibody response, T_{reg} cell activation, suppression of CD4+ T cells, and modulation of DCs[171–173]. However, passive immunisation with MLDL1278A, an anti-oxLDL antibody, was not successful in reducing cardiovascular events on top of lipid lowering therapies, as discussed above[75]. To improve translation from preclinical to clinical settings, Wolf *et al*. compiled a list of 30 apoB peptides binding various Human Leukocyte Antigen types using *in silico* prediction methods which successfully induced a response in human T cells[174].

Another approach to targeting adaptive immune cells would be the direct targeting of atherogenic B cell subsets[175]. B-cell depletion therapies, are already in clinical use for RA and multiple sclerosis (MS), and mouse studies have shown that preferential B2-cell depletion using an anti-CD20 antibody reduced atherosclerosis[176,177]. A single dose of rituximab, a B-cell depleting anti-CD20 antibody, was shown to be safe and efficiently deplete B-cells in patients with acute STEMI in a clinical setting[178]. Antibodies for B-cell depletion targeting CD19 (blinatumomab, inebilizumab), CD22 (inotuzumab, ozogamicin), and B-cell maturation antigen (GSK2857916, AMG420) have also been approved or are currently in clinical development for the treatment of MS and cancer, however B-cell subtype specificity has to be considered and pre-clinical studies are more inconclusive (reviewed in [179]). Other promising strategies targeting B-cells include the impairment of B-cell survival and proliferation (belimumab, blisibimod, ianalumab, atacicept), the modulation of B-cell receptor signalling (epratuzumab, ibrutinib, acalabrutinib), antibody neutralisation (omalizumab) and the modulation of B-cell costimulation (abatacept) (reviewed in[175,179]).

Targeting co-stimulation

Immune checkpoints are immune regulatory costimulatory molecules that provide stimulatory or inhibitory signals to adaptive and innate immune cells[180]. They modulate the immune response in CVD[180]. *In vivo* studies in mice deciphered key costimulatory axes in atherosclerosis using genetic deletion, agonistic and antagonist antibodies: activation of CD27-CD70, BTLA, CD200R-CD200L,

CD80/CD86-CTLA4 pathways or inhibition of CD40-CD40L, OX40-OX40L, CD30-CD30L pathways could be beneficial therapeutic strategies in atherosclerosis[181–189]. Multiple checkpoint inhibitors and agonists targeting the above pathways are in clinical development for cancer and RA[190,191]. In preclinical models, specific inhibition of TRAF6, downstream of the pro-inflammatory pathway of CD40 signalling with small molecule inhibitors resulted in plaque stabilization without inducing side effects and sparing host defence[192]. Similarly, CD200R expression is restricted to the myeloid compartment making the CD200–CD200R pathway amenable to selective targeting of the monocyte-macrophage axis locally and in the bone marrow in CVD[185].

Specific targeting of the atherosclerotic plaque

Long-term immunosuppression may disrupt cardiovascular homeostasis and host defence[193]. Local delivery of drugs has been employed in the vascular field for many years using effective drug-eluting-stents containing sirolimus or paclitaxel, both with anti-inflammatory properties[194–196]. Furthermore, microneedle injections of dexamethasone in the adventitia prevented restenosis in patients who underwent Percutaneous Transluminal Angioplasty[197].

An alternative strategy to minimise the systemic side effects of off-target cell activation and improve accessibility to the cell-type of interest, is the use of targeted delivery approaches. Nanoparticles have a high engagement with myeloid cells and can be modified to target specific subsets with ligand-decorated nanomaterials[198], and have been employed to target macrophages in several CVD trials[199,200]. Flores *et al*. used PEGylated single-wall carbon nanotubes to deliver a downstream inhibitor of the antiphagocytic CD47 pathway and the specific targeting of lesional phagocytes resulted in reduced plaque burden without inducing toxic side effects[201]. Specific macrophage targeting with siRNA targeting the *Camk2g* gene, a pro-efferocytotic stimulus, resulted in increased plaque stability due to improved efferocytosis leading to rebalancing of the immune system in atherosclerosis (Box 6)[202]. Nanoparticles that were decorated with collagen-IV have been shown to accumulate in the lesion shoulder, and targeted delivery of IL-10, or Ac2-26, a mimetic of the anti-inflammatory mediator annexin-A1, stabilised atherosclerotic lesions[203,204]. TRAF6 inhibitors or pioglitazone delivered with nanoparticles were also effective[143,192]. These studies highlight the potential of modulating the immune system in CVD when specifically targeting atherosclerotic plaques to avoid toxic side effects.

Conclusions

Although our understanding of the role of inflammation in atherosclerosis has improved significantly over the past decades, the nuanced balance between pro- and anti-inflammatory cells remains elusive. To identify new therapeutic targets, we need to better interpret the determinants of this equilibrium. Single-cell biology approaches can accelerate translation by examination of immune signatures in patients with CVD. Identification of culprit cell types using multiomics approaches could help identifying the most suitable population for clinical trials and support target selection and informed decisions in a clinical setting moving towards personalized medicine. This strategy will guide targeting maladaptive inflammation while sparing pro-resolving immune functions. In addition, it is imperative to determine the window of opportunity for anti-inflammatory therapy in atherosclerosis, where the benefits of immune system inhibition outweigh the inevitable systemic immunosuppressive effects. The development of mRNA vaccines has revolutionized the field of RNA therapeutics, extending the toolkit for atherosclerotic vaccines and previously "undruggable" targets[205]. The association of CHIP with cardiovascular risk exemplifies the importance of patient stratification beyond traditional risk factors in order to define the patient population that benefits from treatment.

We have a long way to go. Our understanding for how the immune mediators within the plaque and remote tissues causes CVD is incomplete. Cardiovascular research lags behind cancer and metabolism when translating the impact of chronic inflammation in man. It is time to take inflammation seriously as a pathogenic drive of CVD, and direct resources towards mechanistic and translational studies to find cause and remedy. There has never been a more exciting time for research in cardiovascular inflammation.

Figure legends

Fig. 1 | **History of research into the role of inflammation in atherosclerosis**. This timeline shows the main milestones covering the past four decades of research into the role of inflammation in atherosclerosis[31,32,69,113,120,207–238]. In the 1980s, the introduction of immunohistochemical techniques to study the atherosclerotic plaque provided evidence of HLA-DR expression in the human plaque, followed by identification of macrophages and T cells in the plaque[207,208,234–236,239]. In the 1990s, pro-inflammatory cytokines, such as TNF, were found at the plaque site[209,210,212,213,240]. Furthermore, the first murine models of hypercholesterolemia were developed[213–215], and anti-oxLDL antibody titres were able to predict cardiovascular outcome[233]. In the 2000s, associations between inflammatory markers and cardiovascular events were observed. Elevated levels of CRP, IL-6 and TNF were found to be associated with worse clinical outcome in patients with CVD[217–221]. This led to the introduction of inflammation as a therapeutic target in CVD[222]. In the past two decades proof for the beneficial

targeting of inflammation in CVD was provided in clinical trials[31,32,69]. Preclinical insights are displayed in blue boxes and clinical insights in grey boxes. Abbreviations: ACS = Acute Coronary Syndrome; CVD = Cardiovascular disease; IL = interleukin; HLA-DR = Human Leukocyte Antigen-DR; TNF = Tumor Necrosis Factor; VSMC = Vascular Smooth Muscle Cells.

Fig.2 | **Inflammation in atherosclerosis.** Haemodynamic forces within medium-large arteries create areas of low shear stress that are often predictors of atherosclerotic plaque location. As the plaque begins to form, circulating lipoproteins and ApoB peptides enter the sub-intimal space where they may become modified and recognised by immune cells as danger signals. This results in the activation of TLRs and the inflammasome, eliciting the innate response. The innate response drives inflammation through the production and secretion of cytokines, NETs and upregulation of co-stimulatory molecules. Macrophages ingest plaque lipoproteins, becoming lipid laden foam cells that lay the foundations for the necrotic core, and present lipid antigens to (iNK)T cells. Presentation occurs within the plaque in addition to secondary lymph organs, such as the lymph node where APCs present to naïve T cells that differentiate into T_{reg} or T_{eff} cells. While T_{reg} cells have atheroprotective functions, T_{eff} cells engage the B cell response to produce circulating antibodies, enhancing inflammation and immune cell recruitment to the plaque. Altogether this contributes to endothelial dysfunction leading to further aggravation of inflammation through monocyte recruitment, increased uptake of lipoproteins adding to the lipid burden and smooth muscle cell activation and proliferation contributing to fibrous cap formation. Abbreviations: APC = Antigen Presenting Cell; DC = Dendritic Cell; HDL = High Density Lipoprotein; IFN = Interferon; IL = Interleukin; iNKT = invariant Natural Killer T cell; NETs = Neutrophil Extracellular Traps; LDL = Low Density Lipoprotein; TNF = Tumor Necrosis Factor; ROS = Reactive Oxygen Species.

Fig. 3 | **Targeting the immune system in atherosclerosis. (A)** Therapeutics targeting innate immunity tested in clinical trials including IL-1 inhibitors, IL-6 inhibitors, TNFα blockers and p38 MAPK inhibitors. **(B)** Therapeutics targeting adaptive immunity in clinical trials including low dose IL-2 targeting T_{reg} cells and proliferation inhibitors used in drug-eluting stents. **(C)** Therapeutics tested in clinical trials targeting lipoproteins to reduce inflammation. **(D)** Therapeutics with broad immunosuppressive effects tested in clinical trials including colchicine, low dose methotrexate, glucocorticoids and hydroxychloroquine. (See Table 1-3) Colour coding: green = beneficial to reduce CVD, red = no benefit in clinical trials, orange = potential beneficial therapeutics, black = currently in clinical trial**. (E)** Overview of therapeutics targeting innate immunity in pre-clinical development. **(F)** Overview of therapeutics in pre-clinical development targeting adaptive immunity through blockade

of co-stimulatory molecules and B- and T cell regulation. Abbreviations: APC = Antigen Presenting Cell; IL = Interleukin; LDL = Low Density Lipoprotein; TLR = Toll Like Receptor.

Table 1 | **Immunotherapies proven to be successful in phase III clinical trials in cardiovascular disease**

Trial	Agent	Target	Design	Patients	Primary end point	Main outcomes	Refs
CANTOS (2017)	Canakinumab	Inhibition of the IL-1β pathway	Double-blind RCT	10,061 with previous MI and high CRP levels	Nonfatal MI, nonfatal stroke, or cardiovascular death	Canakinumab 150 mg↓ recurrent CV events than placebo, independent of lipid-level lowering	[31]
LoDoCo2 (2020)	Colchicine	Broad cellular effect including inhibition of tubulin polymerization, alteration of leukocyte responsiveness, inhibition of inflammasome assembly and IL-1 release	Double-blind RCT	5,522 with chronic coronary disease	Composite of cardiovascular death, spontaneous MI, ischemic stroke, ischemia-driven coronary revascularization	↓ Risk of composite end point with colchicine compared to placebo	[32]
COLCOT (2019)				4,745 within 30 days after MI	Composite of death from cardiovascular causes, resuscitated cardiac arrest, MI, stroke, or hospitalization for angina leading to coronary revascularization	↓ Risk of composite end point with colchicine compared to placebo	[38]

CRP, C-reactive protein; CV, cardiovascular; IL, interleukin; MI, myocardial infarction; RCT, randomized controlled trial.

Table 2 | **Potentially successful immunotherapies in phase II clinical trials in cardiovascular disease**

Study	Agent	Target	Design	Patients	Primary end point	Key outcomes	Refs

Sayed et al. (2016)	Xilonix	Monoclonal antibody specifically targeting IL-1α	RCT	43 patients undergoing percutaneous SFA revascularization	Clinically significant target vessel restenosis, time to restenosis, and incidence of major adverse CV events	At 12-months follow-up no difference between Xilonix and placebo. At 3-month trend towards ↓ restenosis 0% vs 10% and CV events 9% vs 24% in the Xilonix cohort vs. placebo	[52]
MRC-ILA Heart Study (2015)	Anakinra	IL-1 receptor antagonist	Double-blind RCT	182 patients with NSTE-ACS, presenting <48 h from onset of chest pain	AUC hsCRP over 7 days	↓ hsCRP following 14 days IL-1Ra. MACE at day 30 and 3 months was similar but at 1 year there was a significant excess of events in the Anakinra group.	[51]
VCU-ART3 (2020)				99 STEMI-ACS	AUC for hsCRP at baseline, 72 hrs and day 14	↓ hsCRP-AUC at 14 days. ↓ incidence of new-onset heart failure, death and hospitalization for heart failure.	[50]
DANCE (2018)	Adventitial dexamethasone	Broad anti-inflammatory effect	Prospective single-arm comparing to contemporary trials	262 patients with symptomatic PAD receiving PTA (n = 124) or atherectomy (n = 159)	12-month primary patency	↓ restenosis during 12 months follow-up	[197]
Klevelan d et al. (2016)	Tocilizumab	Monoclonal antibody targeting IL-6	Double-blind RCT	117 patients with NSTEMI randomized at a median of 2 days after symptom onset	AUC hsCRP day 1-3.	↓ hsCRP	[53]

ASSAIL-MI (2021)			Double-blind RCT	199 patients within 6h of STEMI undergoing PCI	Myocardial salvage index measure by MRI after 3-7 days	Tocilizumab ↑ myocardial salvage index, and ↓CRP	[54]
RESCUE (2021)	Ziltivekimab	Monoclonal antibody directed against the IL-6 ligand	Double-blind RCT	264 patients with chronic kidney disease and hsCRP>2 mg/L	hsCRP after 12 weeks	↓ hsCRP levels in all doses groups compared to placebo	[55]

ACS, acute coronary syndrome; AUC, area under the curve; hsCRP, high sensitivity C-reactive protein; MI, myocardial infarction; RCT, randomized controlled trial; SFA, superficial femoral artery; PAD, peripheral artery disease; PCI, percutaneous coronary intervention; PTA, percutaneous transluminal angioplasty.

527 Table 3 | **Ongoing randomized controlled clinical trials targeting the immune system in atherosclerosis**

528

Trial	Agent	Target	Design	Patients	Primary end point	Refs
OXI NCT02648464	Hydroxychloroquine	Broad immunosuppression	Phase IV	125 patients with MI.	Rate of cardiovascular adverse events: MI, mortality, hospitalization for unstable angina, and heart failure	241
CHANGAN NCT02874287			Phase IV	35 patients with CAD and hsCRP>1 mg/L	Change of fasting hsCRP	242
LILACS NCT03113773	Low dose IL-2	Regulatory T cells	Phase I/II	57 patients with a history of CAD	Safety, tolerability, and circulating T_{reg} cell levels	243
IVORY NCT04241601			Phase II	60 patients with ACS and hsCRP > 2 mg/L	Change in vascular inflammation as measured by FDG PET/CT	244
NCT04762472	Montelukast	Leukotriene receptor	Phase IV	200 asymptomatic adults with exposure to air pollution	Subclinical atherosclerosis (brachial flow mediated dilation; carotid intima media thickness; blood inflammatory markers)	245
NCT04616872	Methotrexate-associated to LDL like nanoparticles	Broad immunosuppression	Phase II/III	40 patients with multi-vessel CAD and hsCRP >2 mg/L	Attenuation plaque volume measured by CTA	246
SARIPET NCT04350216	Sarilumab	IL-6R	Phase IV	20 patients with active RA and CRP levels >1 mg/dL	Carotid atheroma plaque changes using ultrasound	247
PAC-MAN NCT04148833	Paclitaxel	Proliferation	Phase II/III	40 patients with CAD	Attenuation plaque volume measured by CTA	248
GOLDILOX NCT04610892	MEDI6570	LOX-1 receptor (Uptake oxLDL)	Phase IIb	792 patients with a history of MI	Non calcified plaque volume measured by CTA	249
CLEAR-Synergy NCT03048825	Colchicine	Broad immunosuppression	Phase III	7,000 patients with MI	MACE	250

CONVINCE NCT02898610		Broad immunosuppression	Phase III	2,623 with ischemic stroke or high risk TIA	Non-fatal ischemic stroke, non-fatal MACE, vascular death	[251]
ZEUS	Ziltivekimab	IL-6 blocking	Phase III	6,200 patients with chronic kidney disease and elevated CRP ≥ 2mg/L	Time to first MACE.	[252]

ACS, acute coronary syndrome; CAD, coronary artery disease; CTA, computed tomography; angiography; hsCRP, high sensitivity C-reactive protein; MACE, major adverse CV events; MI, myocardial infarction.

Supplemental material

S Table 1 | Immunotherapies without beneficial effect on inflammation or event rate in cardiovascular disease

Therapeutics proven to be unsuccessful							
Trial	**Drug**	**Target**	**Design**	**Patients**	**Primary outcome**	**Key outcomes**	**Ref**
CIRT	Methotrexate	Dihydrofolate reductase inhibitor	Phase III Double-blind RCT	4786 patients with previous MI or multivessel coronary disease who additionally had either T2D or the metabolic syndrome.	Composite of nonfatal myocardial infarction, nonfatal stroke, or cardiovascular death.	No effect cardiovascular events than placebo and did not reduce levels of IL-1β, IL-6, or CRP.	[69]
SILENCE	Liposomal nanoparticle encapsulating prednisolone (LN-PLP)	Delivery to macrophages atherosclerotic plaque	Phase II RCT	30 patients with documented history of CVD.	FDG-PET CT to identify patients with marked arterial wall inflammation.	Short-term LN-PLP treatment did not reduce arterial wall inflammation measured by FDG-PET CT.	[253]

Bissonnette et al.	Adalimumab	Tumor necrosis factor-antagonist.	Phase II Double-blind RCT	107 patients with psoriasis.	Arterial inflammation detected by positron emission tomography-CT.	No difference in vascular inflammation after 16 weeks.	[254]
GLACIER	MLDL1278A	Anti-oxLDL antibody	Phase II Double-blind RCT	147 patients with evidence of vascular inflammation, as quantified by FDG-PET/CT.	Change in vascular inflammation measured by FDG-PET CT and hsCRP at 12 weeks.	No difference in vascular inflammation on FDG-PET/CT and no significant differences in hsCRP in MLDL1278A administration groups vs placebo.	[75]
Elkhawad et al.	Losmapimod	p38 mitogen-activated protein kinase inhibition	Phase II RCT	99 patients with atherosclerosis on stable stain therapy.	Vascular inflammation measured by FDG-PET CT and hsCRP.	No significant changes vascular inflammation and hsCRP.	[73]
SOLSTICE	Losmapimod		Phase II RCT	526 patients with NSTEMI.	hsCRP and infarct size.	hsCRP concentrations at 72 h ↓ losmapimod group than in the placebo group but were similar at 12 weeks. No differences in infarct size.	[74]

LATITUDE-TIMI 60			Phase III Double-blind RCT	3505 patients with acute MI.	Cardiovascular death, MI, or severe recurrent ischemia requiring urgent coronary revascularization.	No reduction of the risk of major ischemic cardiovascular events.	[70]

Abbreviations: ACS = Acute Coronary Syndrome; FDG-PET CT = fluorodeoxyglucose-positron emission tomography; hsCRP = high sensitivity C- reactive protein; CVD = Cardiovascular disease; LDL-C = LDL-cholesterol; IL = Interleukin; MI = myocardial infarction; RCT = Randomised Controlled Trial; T2D= Type 2 Diabetes.

Box 1 | **Cells involved in atherosclerosis**

Here we introduce the vast array of immune cells involved in atherosclerosis, with cells originating from both innate and adaptive immune backgrounds. Key literature has been referenced for a deeper explanation of their role in atherosclerosis, and arguments for any controversy highlighted. Key non-immune cells have also been included for completeness.

Monocytes

Monocytes circulate in the blood, and reside in the bone marrow and spleen during homeostasis[255]. Two main populations of monocytes can be distinguished: classical (mouse-Ly6Chi, human CD14+CD16-) and nonclassical monocytes (mice-Ly6Clo, human-CD14loCd16+). In atherosclerosis, classical monocytes are recruited to atherosclerotic plaques upon engagement of the chemokine receptors CCR2, CCR5, and CX3CR1[125]. In the plaque, they differentiate into dendritic cells (DCs) and macrophages that show high functional and phenotypic heterogeneity. In both mouse[256] and humans[257,258], it has been shown that increased severity of atherosclerosis is associated with an increase in the blood monocyte pool, and murine preclinical studies have not only demonstrated Ly6Chi splenic monocytes to contribute to the growing atheroma, but also contribute toward plaque instability[230,259]. On the other hand, monocyte recruitment can also play an important role in atherosclerosis regression[140], and “patrolling” Ly6Clo monocytes, which derive from Ly6Chi monocytes, and are important for endothelial maintenance, but their role in atherosclerosis is not well defined[260].

When considering the role of monocytes in atherosclerosis, changes in the early stages of haemopoiesis of HSPCs, progenitor cells that will differentiate into, and renew the myeloid,

lymphoid and erythrocytic lineages of the bone marrow, are key[27]. More recently, focus has broadened, looking to extra-medullary anatomical sites; locations outside of the bone marrow where HPSCs can produce monocytes[261,262]. Similarly to those derived from the bone marrow, circulating monocytes produced within these sites have been demonstrated to infiltrate atherosclerotic lesions and further drive atherogenesis[28,230,263–266]. Hypercholesterolaemia, stress, inflammation and other risk factors of atherosclerosis can induce emergency haematopoiesis, including extramedullary haematopoiesis[28] in the spleen, and contribute to disease progression by skewing of hematopoietic progenitor stem cells (HSPCs) in the bone marrow to myeloid lineages[267].

The spleen is also critical in the relationship between myeloid cells and atherosclerosis. The two main splenic compartments; the red and white pulp, are separated by the marginal zone, a site for a wide variety of cell types, including fixed macrophage populations, while also providing a temporary home for T lymphocytes DCs and B cells[268,269]. Owing to its highly vascularised nature, the continual supply of blood-borne immunocompetent cells entering and leaving the spleen, together with those cells fixed within, provides a highly efficient and effective location for antigen recognition and response, in addition to lymphocyte trafficking[268,269]. The marginal zone hosts important interactions between T follicular cells and B cells that result in atheroprotection[270].

Macrophages

Within the artery there are two distinct macrophage populations, located either within the intima[271], or the adventitia[272]. However, while both populations originate from embryonic precursors and their survival depends on colony stimulating factor 1 (CSF1), the turnover of adventitial macrophages derives from bone marrow (BM)-derived monocytes post-birth[273] while monocytes replenish arterial resident macrophages during early atherosclerosis[271]. In health and disease, Lyve1+ adventitial macrophages prevent unfavourable arterial remodelling, largely through regulating collagen production within vascular smooth muscle cells, and are therefore seen to be pro-atherogenic. Alternatively, arterial resident macrophages are associated with malevolent cholesterol uptake and initiation of vascular inflammation through the production of tumour necrosis factor (TNF)α, and IL-1β and interleukin (IL)-6 thereby activating and shaping immune and non-immune cells within the artery[271,274].

TNFα is a pro-inflammatory cytokine produced by several cells involved in atherosclerosis, including macrophages, endothelial cells and smooth muscle cells[275,276]. In mice, TNF knockout has

consistently shown to reduce atherosclerosis[277,278]. In humans, TNFα is present in plaques and levels of TNFα in the peripheral blood predict future coronary events in post-MI patients[221,275].

IL-1 is a pro-inflammatory cytokine that drives inflammation in atherosclerosis (reviewed here[279]). Both isoforms of IL-1, IL-1α and IL-1β are involved in atherosclerosis. Recent studies in mice showed that IL-1α plays a role in the remodeling of arteries during early atherogenesis, whereas IL-1β is mainly drives vascular inflammation in later stages of atherosclerosis[280]. However, IL-1β showed to have a protective role in mice in advanced atherosclerosis as well, via promotion and maintenance of a smooth muscle cells and collagen in the fibrous cap[281]. Additionally, IL-1α forms a link between the immune system and coagulation through the activation of IL-1α by thrombin, underscoring its importance of the pathogenesis of CV events[282]. In humans, IL-1 is expressed in coronary arteries of patients with CAD and is considered therapeutically tractable[283,284].

IL-6 is a pro-inflammatory cytokine of innate immunity, that serves as a secondary downstream mediator of cytokines such as IL-1. It is a central stimulus for the acute phase response. In particular, IL-6 stimulates the production of CRP, amongst other acute phase reactants, in hepatocytes[285]. IL-6 signaling contributes to atherosclerosis and plaque destabilization in mice[286]. Human data showed that elevated IL-6 is associated with an increased risk of MI, and genetic studies provided evidence of a causal role for IL-6 receptor signalling in CVD[220,287,288].

Arterial macrophage plasticity within distinct functional and ontogenetic signatures reflects the heterogeneous environment of plaque tissue[289], which continues to be increasingly appreciated. Genetic linage tracing studies and monocyte fate mapping have drastically helped to explore how local cues contribute to the conversion of monocytes into the myriad of macrophage sub-populations during atherosclerosis[140,290], and has helped to unpack how the transdifferentiation of local progenitors and local proliferation of residential macrophages contributes to plaque progression[271,291].

Three main macrophage populations with different inflammatory properties were identified in human[24] and murine single cell studies[292], suggesting that macrophage heterogeneity within the plaques cannot be explained simply by the polarization paradigm. Strikingly, an inflammatory macrophage population found in mice and humans expresses high levels of IL-1β, a well-recognized immune-target in atherosclerosis, further highlighting its relevance for atherosclerosis progression[292]. Another population has a more resident-like phenotype and is enriched in genes

involved in antigen presentation and endocytosis[24,292]. Finally, Trem2 macrophages, a subset previously identified in obesity as a protective one, highly express genes associated with lipid handling and metabolism and mouse studies have confirmed that these cells might be foamy macrophages, a hallmark of atherosclerosis[293]. Of note, Trem2 macrophages in human and mouse do not express inflammatory genes, suggesting they may have, like in obesity a homeostatic lipid handling role within the plaque[24,292]. This discovery drew important parallelisms between the biology of CVD and obesity[294], highlighting a common blueprint between the most prevalent metabolic diseases in our century[295]. At the same time, it called into question the concept of lipid-driven inflammation and further studies are warranted to reconcile inflammatory and lipid drivers of the disease.

Another avenue to consider is the role macrophages play in plaque rupture and thrombosis, including the production of matrix metalloproteinase and tissue factors[274,296], and their coordination of intraplaque efferocytosis (Box 6); the removal of apoptotic cells, a crucial mechanism at play in atherogenesis to resolve inflammation[297]. The impairment of efferocytosis is associated with disease progression and leads to larger necrotic cores, thus resulting in plaque destabilisation[274,298].

Foam cells are the hallmark of atherosclerosis. They derive from macrophages, DCs and smooth muscle cells undergoing transdifferentiation[299]. Foam cells drive necrotic core formation owing to their uptake of intraplaque lipids leading to increased ER stress and cell death[299]. However, more recently, an anti-inflammatory role for foam cells has been proposed, whereby distinct differences between lipid laden macrophages (foam cells) and plaque macrophages driving lesional inflammation were described[293], adding to the idea macrophage function is highly heterogeneous and may vary dependent upon the micro-environment they are found[300,301].

Dendritic Cells

DCs are another key player driving atherosclerotic plaque inflammation that bridges the innate response with the adaptive. There are three main DC subsets; namely plasmacytoid DCs (pDCs), and the two conventional DC subsets, cDC1 and cDC2. pDCs are generally located in blood and lymphoid tissues. Upon encountering pathogens, they are capable of producing large amounts of type-1 IFN and may partake in antigen presentation[302,303]. On the other hand the conventional DCs are found in both lymphoid and non-lymphoid sites[304]. cDC1 is involved in cross presentation of antigens, and is known to drive cytotoxic immune responses, while cDC2 activates innate lymphoid cells and induces the ILC3 and T_H17 immune responses[304,305].

DCs can carry out pro-atherogenic and anti-atherogenic functions, and DC numbers are generally thought to correlate with plaque vulnerability (reviewed here[306,307]). Coordination of innate and adaptive arms occur largely through antigen-presentation of engulfed oxLDL to $CD4^+$ T cells within the plaque, resulting in their activation[10,307]. Atherosclerotic plaque DCs can also leave the lesion and migrate to the lymphatic tissue via CCL19 and CCL21 and their receptor CCR7[308]. Once within secondary lymphoid sites, DCs elicit an adaptive immune response, which encompasses T and B cells[10,307]. CCL17 expressing DCs have a proatherogenic role[309]. $CD103^+$ conventional DCs (cDC1) are capable of promoting T_{reg} responses in an atheroprotecive manner[310]. Myd88 loss in cells expressing CD11c leads to loss of T_{reg} and increased atherogenesis in murine models[311]. Alternatively, pDCs have been described as both pro-atherogenic and anti-atherogenic, largely due to subset heterogenity[312]. Specifically, they have been described to promote atherosclerosis through activation of T_H1 cell responses and secretion of interferon (IFN)-α[313,314], while an anti-atherogenic function has been described promoting the induction of T_{reg} cells, and down-regulation of pro-inflammatory T cell subsets, discussed further here[312,315].

Neutrophils

Neutrophils are involved in all stages of atherosclerosis (reviewed in[316]). In mice, neutrophil depletion reduces atherogenesis, and increased levels of circulating neutrophils exacerbate plaque formation, suggesting a role in lesion development[317]. Neutrophils promote vascular inflammation through secretion of reactive oxygen species (ROS) resulting in increased permeability of the endothelial barrier[318]. They attract monocytes via secretion of chemotactic molecules, and may activate macrophages via extrusion of their nuclear material as Neutrophil Extracellular Traps (NETs)[319]. NETs contain histone H4, which binds and lyses smooth muscle cells resulting in plaque destabilisation[320]. In addition, NETs induce plaque erosion and platelet aggregation leading to thrombosis[321]. Overall, neutrophils play a pro-atherogenic role. However, during thrombotic events, neutrophils have reparative functions as well through promotion of endothelial repair and angiogenesis[322,323].

T-Cells

T cells are not only important for CVD progression but also for atherosclerosis initiation, owing to their presence within the arterial wall (reviewed here[324]).

Once activated, T cells directly perform effector functions within the arterial wall or provide help to B cells to produce antibodies[324]. These dynamics may protect against, or promote atherogenesis

depending on the subset involved. Of the subtypes, T_H1 have been consistently revealed to have a pro-atherogenic role[325], while T_{regs} are thought to having an athero-protective role via IL-10 and TGF-β, and T_H2 and T_H17 hold more conflicting functions[326,327]. The most abundant T cell population in murine plaques are the $CD4^+$ T-lymphocytes[328], which are predominately polarised towards a pro-inflammatory phenotype (T_H1) owing to their role in the secretion of IFN-γ[327]. This was further highlighted when focusing on the role of oxLDL in atherosclerosis, whereby blocking the TCR-dependent ApoB100 peptide interaction conferred protection against atherosclerosis[329].

Phenotyping of CD4+ T cells in murine atherosclerosis using scRNASeq found a multi-lineage CD4+ T cell population[330], which shared transcriptional similarities with ApoB-reactive CD4+ T cells[174]. Interestingly, during atherosclerosis progression on these T cells undergo a transition from T regulatory to a pro-inflammatory phenotype. $CD8^+$ T cells found within the lesion also report dual functions, eliciting proatherogenic effects through IFN-γ production and macrophage activation, and athero-protective effects[331].

An increase in CD8+ T cells in blood is associated with CAD[332,333] and CD8+ T cells outnumber CD4+ T cells in advanced human plaques[24,334,335]. Murine studies have reported both atheropromotive and atheroprotective functions of CD8+ T cells[336]. CD8+ T cells have been identified as drivers of plaque inflammation and apoptosis promoting instable plaque phenotypes and plaque erosion[87,86,337,338]. scRNAseq combined with TCR sequencing of human plaques revealed clonal expansion of CD8+ T cells within the plaque, which is usually associated with T cell activation[24]. Indeed, compared to the blood, CD8+ T cells in the plaque show distinct activation subtypes[24,339]. Importantly, CD4+ and CD8+ T cells in symptomatic patients showed a more activated and exhausted gene expression phenotypes, reflecting the highly inflammatory lesion mileu[24,339].

Invariant Natural Killer T cells

Considering the central role of lipids in atherosclerosis, invariant Natural Killer T (iNKT) cells are an interesting player due to their restriction to lipid antigens presented on CD1d by antigen presenting cells. They represent a distinct subset of T cells expressing unique invariant TCRs and NK cell surface molecules such as NK1.1 and Ly49[340]. Numerous murine studies illustrated the pro-atherogenic role of iNKT cells in atherosclerosis via the production of pro-inflammatory cytokines such as IFN-γ, reviewed in[340]. In humans, iNKT cells have been detected in larger quantities in rupture-prone plaques compared to stable plaques[341]. Interactions between iNKT cells and VSMCs, inducing VSMC apoptosis and iNKT cell growth, suggested a contribution to plaque stability[342]. The exact

mechanisms by which iNKT cells promote atherosclerosis are not fully understood, especially the data in human atherosclerosis is limited. Future studies will aid our understanding of how these potent effector cells induce atherosclerosis and may reveal NKT cells as a new potential therapeutic target in atherosclerosis.

B-Cells

Similarly to T cells, different sub-populations of B cells display differences in their contribution to atherogenesis[343]. A comprehensive overview for the role of all subsets of B cells in atherosclerosis can be found here[343].

B cells are central to humoral immunity and mediate the production of antibodies against oxidation specific epitopes (OSE) to help dampen inflammation [343]. Two lineages of B cells exist, with those produced in the foetal liver denoted $B_{1,}$ and those originating from the bone marrow $B_{2.}$ These lineages are further divided into B_{1a} and B_{1B} subpopulations, and B_2 cells that become follicular, or marginal zone B cells[343], respectively. B cells are not always found within the plaque, and are more commonly located within the adventitia or in node-like structures referred to as tertiary lymphoid organs that form as a result of chronic inflammation[327]. B_1 cells are described as athero-protective, owing to their production of IgM antibodies that block the uptake of oxLDL by macrophages within lesions[344], while B_2 cells are overall pro-atherogenic, owing to their production of antibodies with high affinity that further drive adaptive immunity[177,343]. Subsets of B2 B cells with atheroprotective functions arise in secondary lymphoid organs, such as the lymph node[345] and the spleen[346].

Non Immune Cells; Platelets, Endothelial Cells and Smooth Muscle Cells

While considering the role of the immune cells in atherosclerosis, it is also important to explore the mechanisms by which non-immune cells contribute towards inflammation in atherosclerosis. Activated endothelial cells are prolific in nature, and besides their effect on immune cell recruitment, they induce vascular smooth muscle cell (VSMC) phenotypic switching[347–349]. Following switching, VSMCs change from quiescent contractile, to proliferative and migratory cells, with both atheroprotective and atheropromotive potential, ultimately determined by disease progression and the stimuli present[350,351]. Phenotypically switched VSMCs may migrate to, and stabilise the necrotic core through extracellular matrix and proteoglycan production, or, they may destabilise the plaque and increase necrotic core rupture risk by trapping inflammatory monocytes, (macrophages, foam cells and lipids), causing fibrous cap thinning through proatherogenic matrix metalloproteinases (MMPs) secretion, cytokine production and increased local inflammation[352–354].

Another non-immune cell heavily involved in shaping atherogenic landscape are platelets. Able to communicate with both innate and adaptive players, by secreting a vast array of inflammatory mediators, and enrich the inflammatory milieu, they remain critical from early to late stage disease owing to their unique crosstalk[355].

Taken together, virtually each cellular component of the immune system has been found to have a role in atherogenesis. The immune system provides host defence according to very precise check and balances. The same rules apply to CVD pathogenesis, with specific subsets of immune cells performing either pro-atherogenic or anti-atherogenic functions. The complexity of athero-inflammation was further emphasized by single-cell studies in human and mice showing high heterogeneity of vascular leukocytes[7,16–25]. This heterogeneity underscores the importance of targeting specific cellular subsets to maintain tissue homeostasis while inhibiting CVD progression. A deeper understanding of immune cell plasticity and transcriptomic heterogeneity in the progression of atherosclerosis will help identify new therapeutic avenues. In addition, superposing the murine and human leukocyte single-cell transcriptional landscape of the atherosclerotic plaque will help to pinpoint the different pathways, genes or cells that can be used in animal models to study human disease. This will help future functional studies using relevant murine knock-out/knock-in models to refine their relevance to human disease.

Box 2 **| Inflammation in atherogenesis: can we still learn from mouse models?**

Laboratory mice have provided invaluable insight into the mammalian immune system, human disease, and drug development. They are relatively economical, easy to breed, and straightforward to manipulate genetically, and for these reasons, they are here to stay. However, the translation of beneficial responses to therapeutics from mice to humans has not been always successful[356–359]. For example, in the cardiovascular field, the creation of ApoE[360] and LDLR[361] murine models have opened an era of scientific advancement in the field of lipoprotein metabolism, in addition to inflammation in atherosclerosis. These mouse models have also led to the discovery of PCSK9 as a novel therapeutic biomarker[362] as murine PCSK9 overexpression matched function in human[363]. Conversely, drugs targeting angiogenesis had a less successful translation from human to mouse[364] as mice are not susceptible to human pathogens such as HIV and CMV, and this complicates the study of the role of infection in CVD.

There are well-known differences between the cardiovascular system of mice and humans. One key difference is the level of shear stress within the vasculature[365]. Another is the higher degree of

fibrosis in human vasculature than mice[366], in addition to a higher content of T cells[24]. Furthermore, mouse models of atherosclerosis form lesions predominantly at the aortic root, a pattern observed in patients with familial hypercholesterolemia but not relevant to the general population[367]. Notably, the frequency of rupture in mice lesions is very low and when it happens spontaneously (brachiocephalic artery) it is not at the same site as a human disease[368]. As a result, we need to rely on several models to study the spectrum of human cardiovascular disease thoroughly. Moreover, a recent study demonstrated that putative atherosclerosis-causing genes identified in murine atherosclerosis models reveal little association with human CAD, large artery ischemic stroke, or atherosclerotic plaque characteristics[369]. Thus, casting doubt upon our reliance on murine models of atherosclerosis.

Several well-characterized differences between mouse and human immunity are known, and include an assortment of both innate and adaptive immune system differences. Compared to human blood, mouse blood has a large majority of lymphocytes and a scarcity of neutrophils (75–90% lymphocytes, 10–25% neutrophils)[370]. Unsurprisingly this has resulted in differences between murine and human immune systems, including differences in the balance of leukocyte subsets, Toll-like receptors, antibody subsets, defensins, and NO production; reviewed in[370]. For example, mice do not express TLR10[371], have non-functional or differently functional TLR8[372], and do not seem to produce functional TNIP3, an inhibitor of NF-kB[372]. In addition, there are many cytokines and chemokines in humans for which no known ortholog exists in mice and vice versa[372]. Moreover, myeloid differentiation factor (MyD)88 deficiency, leading to impaired Toll-like receptor (TLR)- and interleukin-1 receptor-mediated immunity, has less consequences for human than for mouse[373,374].

Differences between human and mouse are partly attributable to variations in protein expression, and signalling in the immune systems between mice and humans. However, while these were previously unknowns, the majority have now been mapped. Genomic comparisons of mice and humans revealed significant transcriptional overlap, in addition to raising noteworthy differences[375]. Moreover, the immune response may vary between strains as a result of genetic mutations and polymorphisms arising from genetic drift and/or intentional breeding[376].

The existence of these differences in cardiovascular and immune systems call for a deeper understanding for all the elements that drive variation between mice and humans. It also places emphasis on human immunology, rather than relying solely on animal models of disease[377]. Organoid systems and lab-on a chip technology are being devised to fill the gap in translation

between human and mouse[378]. In the meantime, we strive to improve murine models; In order to reduce variability, laboratory mice are kept in specific pathogen free conditions to control microbial exposures. However, the immune profiles of lab mice, are characterised by a low density of mature T cells, scarceness of neutrophils and low lipopolysaccharide (LPS) responsiveness, compared to mice in the wild[370,377,379,380]. Mice in the wild have immune systems more compatible with those of a human immune systems. Therefore, part of the problem is not inherent to the use of murine models, but of our use of them, including importantly how to better mimic the typical human immune system. Thus, humanised mice have been created as a powerful tool to better research into human cardiac disease[381].

On the other hand, porcine models share cardiovascular anatomical features with man, offering advantages for some cardiovascular research. Moreover, there is more genetic homology with man. For instance, pig innate immune systems have ten different TLRs (TLR1-10) and duplication of IL-1β gene just as is found in humans. Furthermore, in swine, cDNA analysis and sequencing discovered a significant homology of porcine TLRs with their human counterparts (~80% for TLR1, 3, and 10; 85% for TLR7; and 73% for TLR8)[382]. However, despite some similarities to humans, their immune system is less explored.

A major problem in the porcine immune system includes the indistinguishable nature of DCs from other cells, such as macrophages and B cells, particularly in lymphoid tissues. Most porcine 'DC' markers are also expressed on many other leukocyte populations. Additionally, differences in PMN morphology and function have been reported between pig breeds. Finally, there is a severe lack of reagents, compared to the variety available commercially in human and mouse. The histological structure of the lymph node is, also, is different; In the pig, the medullary tissue is in the periphery of the lymph node and the cortical part in the central area. Thus, T and B cells are mainly found in an atypical localization[383]. For all these reasons, the study of the immune system and its effect on CVD in porcine models still present challenges.

Nevertheless, mice are still the foundation of basic research and offer too many advantages to discard. Albeit useful, no organoid or lab-on-a-chip system can fully reproduce the advantage of a fully structured immune system. Therefore, several questions must be considered: (1) what aspect of human CVD is being addressed with the model? (2) does the model recapitulate the human immune response in the disease condition or a particular stage of disease? (3) is the species or strain

appropriate to model the immune question of interest? (4) are there reagents available to study the immune system (5) how the genetic background of the model could impact the study outcome?

The answer, for now, is to learn from a variety of biological systems, asking each one the appropriate question it can answer to.

Box 3 |**Ongoing clinical trials**

Hydroxychloroquine is an anti-malaria and disease-modifying anti-rheumatic drug (DMARD) used for the treatment of inflammatory rheumatic diseases, especially SLE and RA[384]. In lysosomes, hydroxychloroquine inhibits the degradation of cargo by increasing the pH and preventing the activity of lysosomal enzymes. This drug can inhibit nucleic acid sensors such as cyclic GMP-AMP synthase and prevent TLR7 and TLR9 from binding their ligands, reducing the production of pro-inflammatory cytokines, including type I interferons[384]. Hydroxychloroquine has been associated with reduced risk of CV events. Observational studies showed that the use of hydroxychloroquine was associated with 72% decrease in the risk of CV events in patients with RA, and a 68% reduction of thromboembolic events was seen in patients with SLE[385,386]. Hydroxychloroquine is currently being tested in two clinical trials with patients suffering from CAD[241,242] (Table 3).

The cytokine IL-2 is essential for growth and survival of T_{reg} cells, whose role is the control of inflammation. Low dose IL-2 has been trialled in patients with SLE, RA and psoriasis[387]. The principle of using low doses of IL-2 for treatment of inflammatory disease is based on differential IL-2 sensitivity of immune cell subsets. Among all T and NK cell subsets, T_{reg} cells typically respond to the lowest concentrations of IL-2 due to elevated surface expression of the IL-2R alpha chain (IL2RA; CD25) and the high affinity IL-2 receptor (IL-2R) complex. In the treatment of inflammatory diseases low dose IL-2 showed efficacy in increasing T_{reg} number and expression of functional markers such as CD25[388]. Furthermore, high dose IL-2 has shown side effects in cancer therapy which seem to be out of proportion in the setting of autoimmune diseases[387]. The ongoing phase II LILACS trial is testing low dose IL-2 in patients with stable ischemic heart disease and ACS, and the first results showed effective expansion of T_{reg} cells[243,389]. We look forward to the results of these and other exciting trials listed in Table 3.

Box 4 | **Eliciting an innate response: Toll-like receptors and inflammasomes**

Toll-like receptors

TLRs are a family of 10 proteins in humans that recognises pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs, respectively) when triggering an innate immune response[390]. While family members exhibit pathogenic duality, TLRs such as TLR2 and TLR4 are thought to initiate the immune response in the arterial wall by recognising modified lipoproteins in concert with scavenger receptors[15,391,392] leading to cellular activation and induction of pro-inflammatory cytokines, such as IL-1β, whilst also priming the inflammasome which regulates IL-1β production[393]. TLRs are significantly upregulated in human atherosclerotic tissue[24,394], and on circulating monocytes of patients with acute coronary syndrome (ACS)[395,396], linking TLRs to cardiovascular events. Although TLR4 is the most powerful TLR in pathogen-related situations, mutations and/or deletions of TLR4 in experimental models of atherosclerosis demonstrate mixed results, with a varying reduction or no effect on lesion size[397–399]. On the other hand, genetic deletion of TLR2 consistently reduced lipid deposition[400] and intralesional macrophage content[401,402]. Moreover, inflammation in human atherosclerosis has been shown to be driven by TLR2 via the IL-1R (Interleukin-1 receptor) and TLR signalling adaptor Myeloid differentiation primary response protein 88 (MyD88)[403]. Overall, this body of evidence strongly suggests TLR2 as being one of the most pro-atherogenic TLRs.

Inflammasomes

Inflammasomes are multimeric protein complexes that form in response to endogenous and exogenous danger signals (respectively DAMPs and PAMPs) and promote the production of pro-inflammatory cytokines, including IL-1β and IL-18, as well as pyroptosis mediated cell death[404,405]. The nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is an important driver of lipid-driven vascular inflammation and atherosclerosis. Triggers for the formation of the NLRP3 inflammasome include potassium efflux, mitochondrial reactive oxygen species (ROS) and cathepsin-B[405]. All of these triggers are likely present within the atheroma, however a key driver of NLRP3 inflammasome activation within the intima is cathepsin-B downstream of cholesterol crystal, oxLDL and calcium phosphate crystal accumulation within phagocytic cells[406,407].

In addition, a crucial role of the Absent in Melanoma 2 (AIM2) inflammasome is emerging in atherosclerosis. In atherosclerotic mice, AIM2 activation resulted in the production of IL-1β and IL-18 accompanied by an instable plaque phenotype, while deletion or pharmacologic inhibition with an Aim2-antagonizing synthetic oligonucleotide increased plaque stability. Interestingly, Aim2 activation was also shown to be driving CHIP driven atherosclerosis. AIM2 dependent inflammasome

formation is dependent on the detection of cytosolic double-stranded DNA (dsDNA), possibly downstream of atherosclerosis associated necrosis and apoptosis which leads to the release of cellular contents into the extracellular space driving inflammation.

Under normal conditions, there are minimal levels of NLRP3 or AIM2 inflammasome complexes within the cell[408], and the inflammasome remains in an inactive state through ubiquitination[409]. Both, NLRP3 and AIM2 require an initial priming signal which in an NFκB dependent fashion promotes the expression of proteins involved in inflammasome signalling, in addition to promoting the inflammasome's deubiquitylation[408,409]. Hereafter, activation signals promote the assembly and activation of the inflammasome, enabling the proteolytic function of caspase-1[405]. In addition to the processing of IL-1 cytokines, inflammasome activation facilitates the cleavage of gasdermin D, thus inducing plasma membrane pore formation and pyroptosis, which is critical for the release of IL-1β and IL-18 to further promote inflammation in atherosclerosis[404,405,410–412].

Box 5 **| The microbiome**

The human gut microbiome consists of >1,000 species of microorganisms including bacteria, viruses, protozoa, archaea, and fungi. Besides the obvious contribution of gut microbiota to digestion and maintenance of the gut barrier function, they play an important role in the development of the immune system, host defence and metabolic homeostasis and are key to maintaining human health[413]. Dysbiosis of the gut microbiome, a change in the microbiota composition, contributes to the pathogenesis of inflammatory diseases, including IBD, obesity and RA by inducing systemic inflammation[413]. Changes in the oral microbiome in periodontal disease have been associated with CVD[414]. Microbiome dysbiosis is associated with obesity and diabetes[415,416], major risk factors of CVD, but the link between gut microbiome dysbiosis and CVD in humans needs further clarification[417]. The microbiota contributes to CVD through alteration of cholesterol metabolism or the production of bacterial metabolites, such as trimethylamine-*N*-oxide (TMAO). Increased red meat or egg consumption increases TMAO levels[418,419], which are correlated with CV events[420], and coronary atherosclerosis[421,422]. Elevated TMAO levels are also associated with common risk factors of atherosclerosis, including metabolic syndrome[423] and T2D[424]. TMAO is associated with lipid metabolism, endothelial cell activation, inflammatory pathways and pro-thrombotic pathways thereby contributing to CVD risk[425].

Several cytokines play an important role in the regulation of atherosclerosis and disease associated systemic changes in these cytokines might affect the gut microbiome and host defence mechanisms, thereby contributing to atherosclerosis progression. Indeed, myeloid deficiency of IL-23 or IL-23R in *Ldlr*$^{-/-}$ mice exacerbates atherosclerosis[426], possibly due to an impairment of the

intestinal barrier by the expansion of pathogenic bacteria which cause a systemic increase in LPS and TMAO. However, the genetic depletion of IL-23R did not result in changes in atherosclerosis in mice[427], indicating additional contributing mechanisms affecting the host-microbiome relationship are in play. B cells respond to antigens present in the blood, including the LPS produced by commensal microbiota, and have been associated with modulating pro-atherogenic T cell responses[428,429]. Further studies on the role of the microbiome in human CVD, and its relationship with immune system development and activation are warranted.

Box 6**| Re-balancing the immune system in CVD**

The balance between pro- and anti-inflammatory immune processes is important for tissue homeostasis and inflammation. When acute inflammation cannot be resolved, chronic inflammation as seen in atherosclerosis develops. Key mechanisms in resolution of inflammation involve efferocytosis and rebalance from pro-inflammatory lipid mediators towards specialised pro-resolving mediators (SPMs). Unspecific targeting of inflammation in CVD might affect immune subsets with homeostatic functions and induce the inhibition endogenous plaque-resolving immune processes, such as efferocytosis.

Targeting efferocytosis. Defective efferocytosis and the lack of immunomodulation promote an inflammatory environment within the plaque as well as the formation of the necrotic core and plaque destabilisation due to secondary necrosis of apoptotic cells[430]. Efferocytosis is mediated through phagocytic receptors, such as MerTK or LRP1, and apoptotic cell ligands. In atherosclerosis, impaired efferocytosis can be attributed to the downregulation or cleavage of efferocytosis receptors[431–433], and the dysregulation of expression of “eat me” signals[434,435]. Atherosclerotic mice with increased MerTK expression show higher levels of efferocytosis and less necrotic core formation[431,432]. Loss of LRP1 in macrophages or haematopoietic cells in atheroprone mice leads to increased lesion area and necrotic core size[436], highlighting the potential of enhancing efferocytosis therapeutically. One avenue for enhancing efferocytosis is masking the “don’t eat me” signal CD47 on apoptotic cells. Blocking CD47 with a neutralising antibody improved efferocytosis and ameliorated atherosclerosis in *Apoe*$^{-/-}$ mice[437]. Drugs targeting CD47 (Hu5F9-G4,TTI-621) are currently being tested in clinical studies as cancer therapies[438,439] However, anti-CD47 in a clinical setting may induce various side effects owing to its role in the regulation of additional cellular processes[440], such as anaemia, due to high CD47 expression on HSPCs and erythrocytes[441] In addition, total loss of CD47 or its ligand thrombospondin, which is associated with the regulation of inflammatory responses rather than efferocytosis, increased size of the necrotic core[442,443].

Therefore, the pharmacological properties of the antibody and target accessibility should be considered, before advancing into human studies. Of note, concomitant inhibition of CD47 and TNFα using anti-CD47 antibody therapy and commercially available anti-TNFα antibodies such as infliximab or etanercept offers a synergistic benefit in the clearance of apoptotic cells[435].Given that the risk of future cardiovascular events can be reduced in patients with RA undergoing anti-TNFα therapy[444,445], there is a strong rationale for combining anti-inflammatory and pro-efferocytic therapies for the treatment of advanced atherosclerosis[430].

Specialized pro-resolving mediators. Mediators involved in the resolution of inflammation in atherosclerosis include IL-10, annexin A1, and SPMs, such as resolving D1, 15-epi lipoxin A4 and resolving E1[446]. Chronic inflammation in murine and human atherosclerotic plaques is characterised by a disbalance of pro-inflammatory mediators, e.g. leukotrienes, and SPMs[447]. In addition, a low resolvin D1:leukotriene ratio in saliva was proposed as a biomarker of non-resolving inflammation[448]. In the atherosclerotic plaque lipid and peptide pro-resolving mediators signal through G protein-coupled receptors (GPCRs): N- Formyl peptide receptor 2 (FPR2) and chemokine- like receptor 1 (CMKLR1). The systemic administration of pro-resolving mediators, including resolvin D1, 15-epi lipoxin A4, Ac2-26 (annexin A1-derived peptide), and resolvin E1 in advanced murine and leporine models of atherosclerosis reduced atherosclerosis[449–453]. These studies highlight that restoring the balance of pro-inflammatory and pro-resolving mediators to induce resolution of inflammation is an exciting therapeutic avenue, especially since atherosclerosis in a clinical setting is usually treated once plaques and non-resolving inflammation have been established. Resolvin E1 analogues have been tested clinically for ocular inflammation but have not progressed since[454,455]. However, before moving into a clinical setting in CVD the effect of activation of immunosuppressive mechanisms should be evaluated. Most of the above-mentioned mediators have systemic roles in maintaining tissue homeostasis. However, specific delivery of Ac2-26, a synthetic analogue of annexin A1, using monocyte/macrophage-targeting nanoparticles increased plaque stability[203]. Therefore, selectively targeting specific pro-resolving or pro-inflammatory cell types in atherosclerosis will most likely have the most beneficial outcomes in the clinic.

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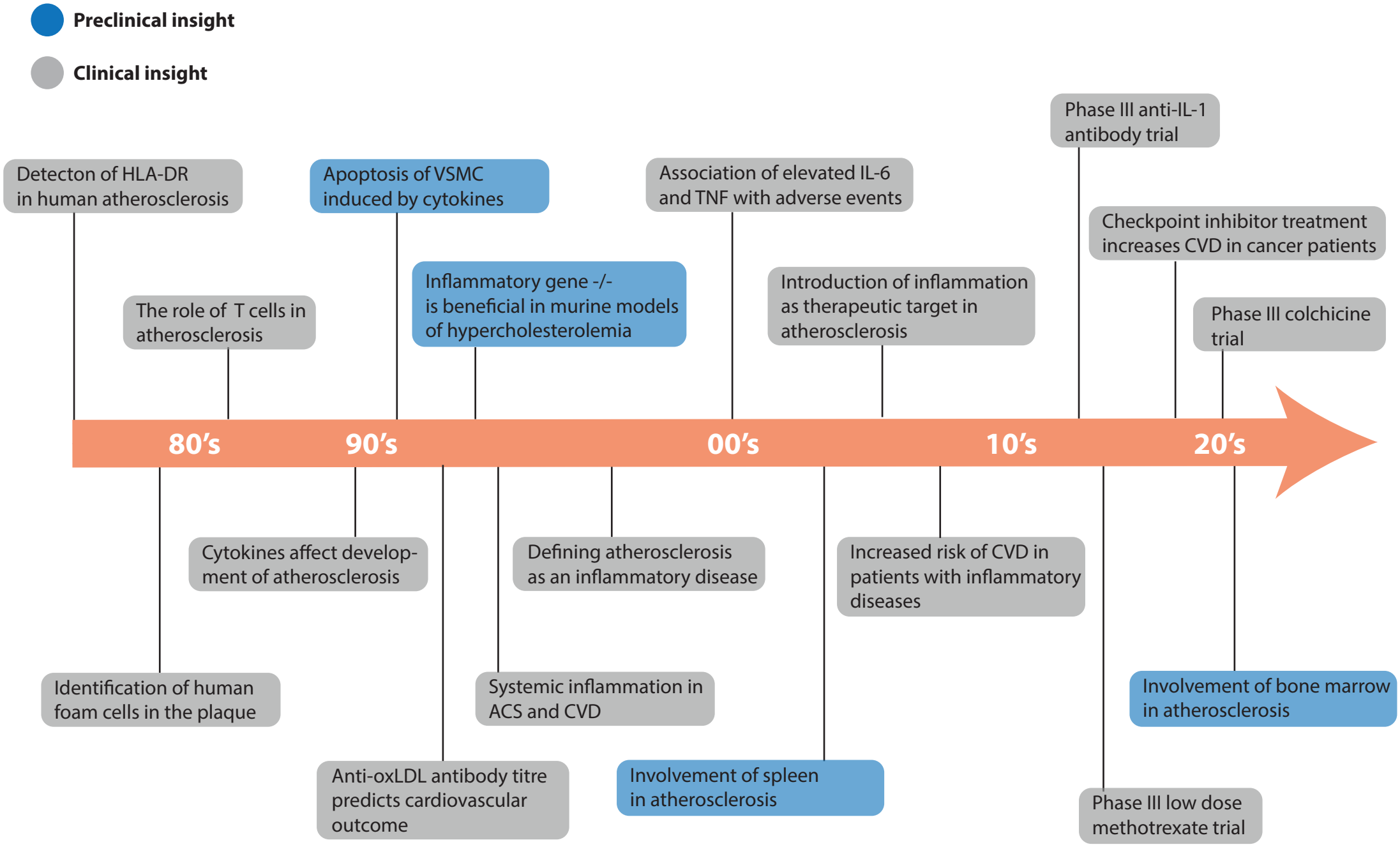
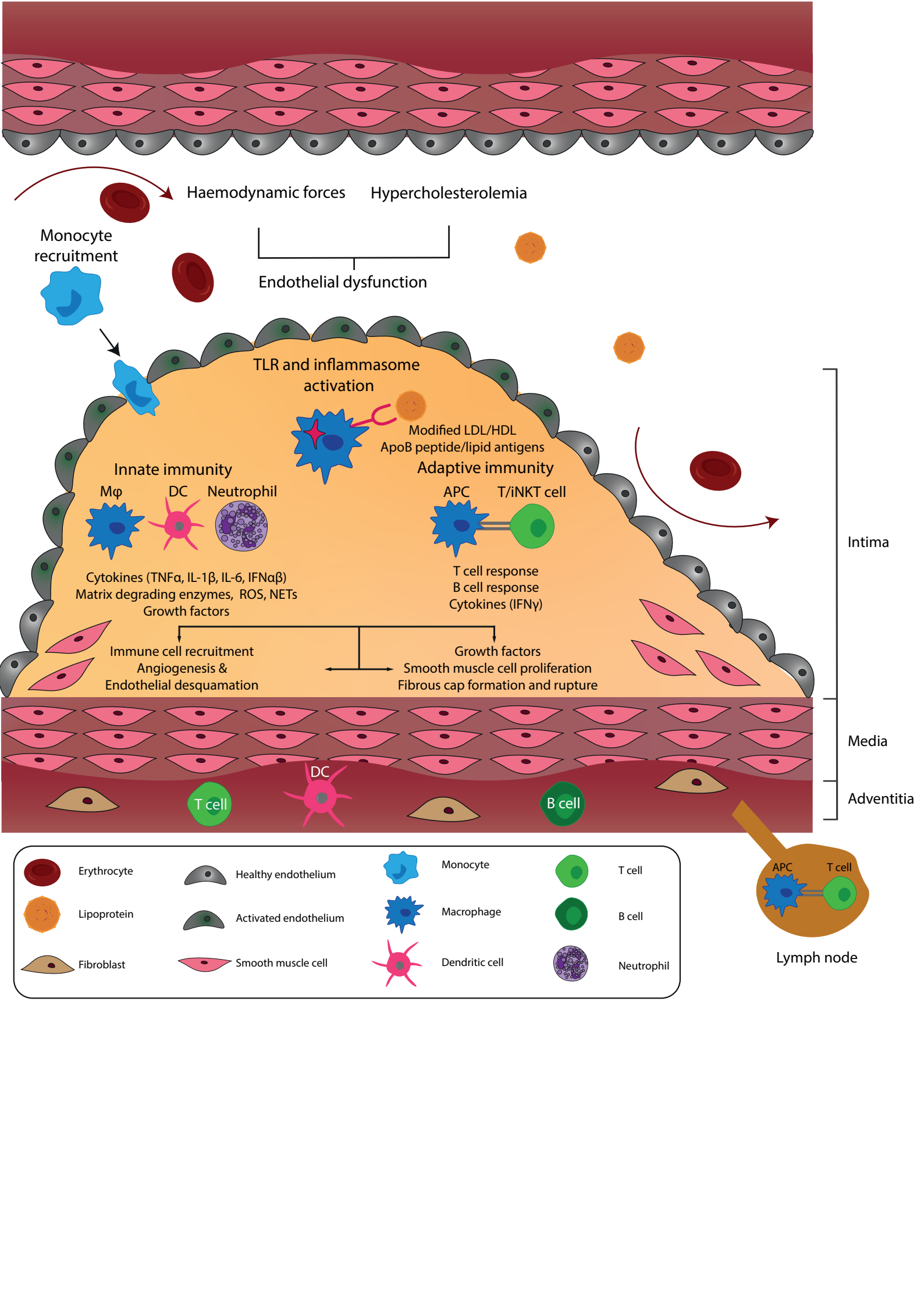
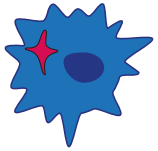

Preclinical insight
Clinical insight
Detecton of HLA-DR in human atherosclerosis
The role of T cells in atherosclerosis
Identification of human foam cells in the plaque
Cytokines affect development of atherosclerosis
Anti-oxLDL antibody titre predicts cardiovascular outcome
Apoptosis of VSMC induced by cytokines
Inflammatory gene -/- is beneficial in murine models of hypercholesterolemia
Systemic inflammation in ACS and CVD
Defining atherosclerosis as an inflammatory disease
Involvement of spleen in atherosclerosis
Association of elevated IL-6 and TNF with adverse events
Introduction of inflammation as therapeutic target in atherosclerosis
Increased risk of CVD in patients with inflammatory diseases
Phase III anti-IL-1 antibody trial
Checkpoint inhibitor treatment increases CVD in cancer patients
Phase III low dose methotrexate trial
Phase III colchicine trial
Involvement of bone marrow in atherosclerosis
80's
90's
00's
10's
20's


Haemodynamic forces
Hypercholesterolemia
Monocyte recruitment
Endothelial dysfunction
TLR and inflammasome activation
Modified LDL/HDL
ApoB peptide/lipid antigens
Innate immunity
Mφ
DC
Neutrophil
Adaptive immunity
APC
T/iNKT cell
Cytokines (TNFα, IL-1β, IL-6, IFNαβ)
Matrix degrading enzymes, ROS, NETs
Growth factors
T cell response
B cell response
Cytokines (IFNγ)
Immune cell recruitment
Angiogenesis &
Endothelial desquamation
Growth factors
Smooth muscle cell proliferation
Fibrous cap formation and rupture
Intima
Media
Adventitia
DC
T cell
B cell
APC
T cell
Lymph node
Erythrocyte
Lipoprotein
Fibroblast
Healthy endothelium
Activated endothelium
Smooth muscle cell
Monocyte
Macrophage
Dendritic cell
T cell
B cell
Neutrophil

Immunotherapeutics tested in clinical trials

A

Innate immunity



IL-1 inhibitors
Canakinumab
Xilonix
Anakinra

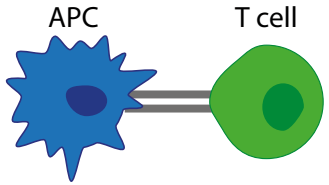
TNFα blockers
Adalimumab

IL-6 inhibitors
Tocilizumab
Ziltivekimab
Sarilumab

p38 MAPK inhibitors
Losmapimod

B

Adaptive immunity

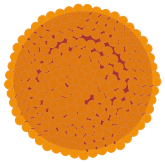


Treg targeting
Prolukin (Low dose IL-2)

Proliferation inhibitors
Sirolimus
Paclitaxel

C

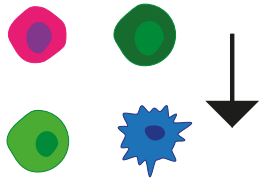
LDL-cholesterol



BI-204 (anti-oxLDL antibody)
Darapladib (Lp-PLA2 inhibitor)
Varespladib (sPLA2s inhibitor)
MEDI6570 (LOX-1 antibody)

D

Broad immunosuppression



Colchicine
Low dose methotrexate
Dexamethasone injections
Prednisolone nanoparticles
Hydroxychloroquine

Pre-clinical immunotherapeutic targets

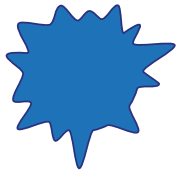
E

Innate immunity

Monocyte/HSPC

JAK2 inhibitors
GLUT1 inhibitors
Inflammasome inhibitors

Lesional inflammatory macrophage



TLR modulators
Inflammasome inhibitors
Traf6 inhibitor

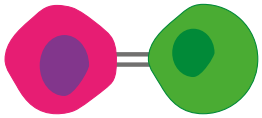
Efferocytotic macrophage

Anti-CD47
siCam2k
Ac2-26

F

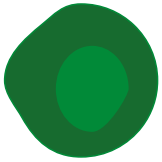
Adaptive immunity

APC T effector



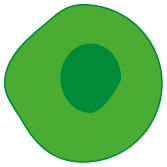
Anti-CD40L
Anti-Ox40L
Anti-CD30L
CD200

B cell



anti-CD20
anti-BTLA4

T reg



CD27 agonist
ApoB vaccinations