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Immune and autoimmune mechanisms in cardiovascular disease

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Published in
Frontiers in Cardiovascular Medicine

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ISSN 1664-8714
ISBN 978-2-83251-403-0
DOI 10.3389/978-2-83251-403-0

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Immune and autoimmune mechanisms in cardiovascular disease

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Citation

Wolf, D., Winkels, H., Gerdes, N., Kahles, F., Dutta, P., Giannarelli, C., eds. (2023). *Immune and autoimmune mechanisms in cardiovascular disease*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-403-0

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frontiers | Frontiers in Cardiovascular Medicine

TYPE Editorial
PUBLISHED 09 January 2023
DOI 10.3389/fcvm.2022.1125959

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

RECEIVED 16 December 2022
ACCEPTED 28 December 2022
PUBLISHED 09 January 2023

CITATION
Winkels H, Gerdes N, Kahles F, Dutta P, Giannarelli C and Wolf D (2023) Editorial: Immune and autoimmune mechanisms in cardiovascular disease.
Front. Cardiovasc. Med. 9:1125959.
doi: 10.3389/fcvm.2022.1125959

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Editorial: Immune and autoimmune mechanisms in cardiovascular disease

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KEYWORDS

inflammation, immunity, autoimmunity, atherosclerosis, cardiovascular, T cells, B cells

Editorial on the Research Topic
Immune and autoimmune mechanisms in cardiovascular disease

Atherosclerosis and its sequelae, myocardial infarction, ischemic heart disease, and stroke remain the leading cause of mortality worldwide. A large body of clinical and pre-clinical evidence has suggested that cardiovascular disease (CVD) is driven by a chronic inflammatory response in arteries and the ischemic heart that leads to an accumulation of leukocytes in affected tissues. Inflammation in the cardiovascular system is accompanied by an autoimmune response involving T- and B-lymphocytes recognizing atherosclerosis-relevant autoantigens in arteries and myocardium-specific autoantigens in the heart. Although it is clinically well-established that cardiometabolic conditions, such as hypertension, diabetes, and obesity, are linked to chronic low-grade inflammation and enhance the risk for CVD, the underlying precise mechanisms remain poorly defined. While inflammation in some CVD entities is already therapeutically modifiable, for instance by canakinumab, an antibody neutralizing the pro-inflammatory cytokine IL-1β or colchicine, autoimmunity remains an unresolved clinical problem. However, preclinical models suggest that cardiovascular autoimmunity may be addressable by novel immunomodulation or tolerogenic vaccination with autoantigens to boost the protective limbs of autoimmunity and limit inflammation. Such strategies promise specific and causal therapeutic interventions that may overcome side-effects of unspecific anti-inflammatory therapies. Exact triggers, antigens, and dynamics of this immune response as well as potential targets for future clinical therapies, however, remain only partially understood. Here, we present a series of articles highlighting novel aspects of the (auto-) immune and inflammatory response in CVD.

The recruitment of leukocytes into tissues represents a hallmark of inflammation. Leukocyte migration into atherosclerotic plaques or the heart is orchestrated by a complex interplay of chemokine-, integrin-, and selectin receptors and ligand pairs. Our series starts with Mauersberger et al. summarizing current concepts of leukocyte recruitment into the atherosclerotic aorta with a specific emphasis on cell types and therapeutically addressable recruitment pathways. Gerhardt et al. provide insights into how recruited leukocytes, particularly cells of the adaptive immune system, may mediate the instability of atherosclerotic plaques in humans. Single cell RNA sequencing (scRNA-seq) and other high-parametric methodologies have advanced our

understanding of cellular heterogeneity in tissues and made it possible to construct cell type atlases in an unsupervised manner. Slenders et al. describe and comment on the application of these novel tools for immunophenotyping in atherosclerosis and build associations to histo-pathological plaque features and clinical outcomes in humans. In contrast to vascular pathophysiology, Anto Michel et al. focus on the advancements in understanding cellular heterogeneity in the heart, in particular following ischemic injuries in mice and humans. Newer evidence from scRNA-seq confirms that atherosclerotic plaques are frequently populated by myeloid cells. Myeloid cell recruitment into atherosclerotic lesions is closely linked to clinical outcomes in humans. Adaptive immune cells have been shown to orchestrate and modulate myeloid cell functions and their polarization into specific macrophage subtypes with pro- and anti-inflammatory roles. von Ehr et al. provide an overview of macrophage subtypes and their roles during different stages of atherosclerotic plaque development. Platelets are crucial in the initiation of blood clots to restore vascular integrity and stop bleeding upon injury. Platelets also participate in atherothrombosis and thrombo-inflammation, two intertwined processes that ascribe these anucleate cells a central role in orchestrating acute and chronic pathological processes. Hamad et al. call attention to platelet subtypes that range from pro-coagulant and aggregatory platelets to those featuring a secretory phenotype. This report addresses how inflammatory conditions appear to differentially engage and modify distinct subpopulations of platelets.

Beyond the recruitment of specialized cell types into cardiovascular key organs, cardiometabolic risk factors and specific pro-inflammatory signaling pathways initiated by these factors represent another layer of CVD-associated inflammation addressed in our topic series. Ganesh et al. highlight how immune cells residing in the gut may impact on local and distant tissue homeostasis thus constituting a potential therapeutic target that is closely linked to metabolic alterations. Interestingly, gut-resident leukocytes are strongly affected by dietary pattern and metabolic dysregulation such as hyperglycemia or diabetes. Eventually, gut immune cells appear to relay disease-relevant signaling to promote CVD. Mammalian target of rapamycin (mTOR) signaling plays an important role in sensing and integrating the metabolic and inflammatory environment on a cellular level. The mTOR signaling complexes govern essential cellular activities including growth, proliferation, motility, energy consumption, and survival. Kaldirim et al. dissect cell-specific mTOR signaling in CVD and how it could be targeted to cease acute and chronic inflammation. Gissler et al. dissect the role of members of the Tumor Necrosis Factor associated Factors (TRAFs), which represent signaling mediators downstream of potent pro-inflammatory receptors of the TNF-superfamily. These have been postulated as potential therapeutic targets because of their potency to modulate and integrate metabolic, inflammatory, and immune signaling events.

Multiple articles of this series focus on the potential role of antigen-specific T- and B-lymphocyte responses in CVD. Recent evidence has highlighted that a multiplicity of autoreactive immune cells directed against lipids, oxidation-specific epitopes, and the myocardium may exist even in healthy individuals. Clinically, it is known that checkpoint-inhibition may re-activate some of these hibernating autoreactive T cells directed against myocardium-specific self-antigens with the capability to elicit cardiac autoimmunity and autoimmune myocarditis. Immunotherapies, particularly immune checkpoint blockade, have been a revolution in cancer treatment but their potentials in cardiovascular complications remain obscure. Nettersheim, Picard et al. highlight underlying pathways and raise attention on how immunotherapies in cancer might contribute to atheroprogression. Some of the recently described ApoB-reactive T-helper cells may originate from T_H17 and -regulatory cells (T_{reg})—a topic summarized by Wang et al. The transcription factor Autoimmune Regulator (AIRE) is important for thymic expression of many tissue antigens and the establishment of central tolerance toward them. In an original research article, Nettersheim, Braumann et al. demonstrate that plaque development and presence of Apolipoprotein B100-reactive CD4 T cells, however, is not affected in AIRE-deficient mice. Besides T cells, that may shape the inflammatory microenvironment in plaques by secreting cytokines and direct cell-cell interactions, B cells primarily act by secreting (auto-) antibodies. The current concepts of B cell functions and subtypes in atherosclerosis are summarized by Smeets et al. Interestingly, B cell subpopulations appear to mediate both pro- and antiatherogenic functions. Furthermore, not all of these effects depend on the antibody-expressing and -secreting capacity of the B cells. The review proposes to enhance function of antigen-specific B cell clones as a potential atheroprotective therapy. Building up on this knowledge, Douna et al. demonstrate that the long-considered pro-atherosclerotic cytokine IFN-g executes anti-atherosclerotic functions by re-wiring B cells that subsequently prevent T follicular helper cell differentiation and foster T_{reg} development, thereby ameliorating atherosclerosis. Following *ex vivo* IFN-g-stimulation, B cells express the co-inhibitory molecule PD-L1 and inhibit T follicular helper cell activity. Pattarabanjird et al. uncover that the chemokine receptors CCR6 and CXCR4 are highly expressed on memory B cells associated with IgE sensitization to alpha-gal. Likewise, the authors uncover a correlation between CCR6, CXCR4, IgE expression in switched memory B cells and CAD severity. ApoB autoantibodies are thought to exert pro- and anti-atherosclerotic functions dependent on the subtype. Marchini et al. demonstrate that these autoantibodies do not associate with atherosclerotic disease, but rather with cardiometabolic risk factors, such as hypertension and obesity sparking the interesting idea that formation of auto-reactive B cells is primarily driven by metabolic alterations and does not necessarily represent a prerequisite for CVD. Our series concludes with Zhu et al., who developed a new preclinical model of aortic aneurysm formation in rats.

Overall, our topic series covers several fundamental and novel aspects of cardiovascular immunity and inflammation, an emerging field that is at the promise to build the basis for the future development of tailored personalized treatment and preventative strategies with novel immunomodulation, vaccination, and CAR T cell therapies in the future.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Program (grant agreement No. 853425). DW is a member of SFB1425, funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

Acknowledgments

We thank all authors contributing to this Research Topic.

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frontiers in Cardiovascular Medicine

REVIEW
published: 11 January 2022
doi: 10.3389/fcvm.2021.797046

Immune Mechanisms of Plaque Instability

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Inflammation crucially drives atherosclerosis from disease initiation to the emergence of clinical complications. Targeting pivotal inflammatory pathways without compromising the host defense could compliment therapy with lipid-lowering agents, anti-hypertensive treatment, and lifestyle interventions to address the substantial residual cardiovascular risk that remains beyond classical risk factor control. Detailed understanding of the intricate immune mechanisms that propel plaque instability and disruption is indispensable for the development of novel therapeutic concepts. In this review, we provide an overview on the role of key immune cells in plaque inception and progression, and discuss recently identified maladaptive immune phenomena that contribute to plaque destabilization, including epigenetically programmed trained immunity in myeloid cells, pathogenic conversion of autoreactive regulatory T-cells and expansion of altered leukocytes due to clonal hematopoiesis. From a more global perspective, the article discusses how systemic crises such as acute mental stress or infection abruptly raise plaque vulnerability and summarizes recent advances in understanding the increased cardiovascular risk associated with COVID-19 disease. Stepping outside the box, we highlight the role of gut dysbiosis in atherosclerosis progression and plaque vulnerability. The emerging differential role of the immune system in plaque rupture and plaque erosion as well as the limitations of animal models in studying plaque disruption are reviewed.

Keywords: plaque erosion, trained immunity, clonal hematopoiesis, infection, COVID-19, stress, gut dysbiosis, atherosclerosis

OPEN ACCESS

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *18 October 2021*
Accepted: *15 December 2021*
Published: *11 January 2022*

Citation:
Gerhardt T, Haghikia A, Stapmanns P and Leistner DM (2022) Immune Mechanisms of Plaque Instability. Front. Cardiovasc. Med. 8:797046. doi: 10.3389/fcvm.2021.797046

CLINICAL RELEVANCE

Atherosclerotic plaques are characterized by hyperlipidemia and non-resolving inflammation, tightly linked by complex innate and adaptive immune processes (1, 2). Atherosclerosis is the underlying pathology of cardiovascular disease (CVD). Instability of atherosclerotic plaque and subsequent atherothrombosis are the most common causes of myocardial infarction (MI) (3). The 17.8 million deaths attributed to CVD in 2017 (4) mark it to being a persisting pre-eminent global health problem despite highly effective options available to control conventional cardiovascular risk factors (4, 5). Immunity and inflammation likely contribute substantially to this residual risk (5, 6). We currently witness an exciting series of trials that highlight immune pathways as a central target for cardiovascular secondary prevention and clinically affirm the inflammation-hypothesis of atherosclerosis.

Recently, the CANTOS trial substantiated an immunomodulatory therapy to improve cardiovascular outcomes. In this study, targeted inhibition of the inflammatory cytokine Interleukin-1β by canakinumab, a neutralizing antibody, markedly reduced adverse cardiovascular events as compared to placebo in a large past-MI study cohort with "residual inflammatory risk (RIR)" as defined by a high sensitivity C-reactive protein level >2 mg/L (7). Another clinical outcome study, the COLCOT trial, investigated a second preventive treatment strategy after recent MI using the broad anti-inflammatory agent colchicine or placebo. The outcome showed a 23% relative reduction in adverse cardiovascular events after a median follow up of 22.6 months in the treatment group, mainly driven by reductions in the rates of stroke and angina requiring revascularization (8). A similarly meaningful effect was recently confirmed for patients with chronic coronary disease in the LoDoCo2 trial demonstrating significant cardiovascular risk-reduction upon Colchicine treatment (HR 0.69 at 0.5 mg daily) (9), although this was not associated with a difference in all-cause mortality (10). IL-1 strongly induces Interleukin-6 (IL-6) (11) and has been attributed a causal role in human coronary heart disease (12). Current trials have shown beneficial effects of the receptor antagonists Tocilizumab and Ziltivekimab on myocardial salvage (13), biomarkers of inflammation in MI patients (13, 14), or patients at high atherothrombotic risk (15). However, a concomitant increase in triglyceride levels associated with tocilizumab has prevented its use in large clinical trials (11). The investigators of the recently published "CRP apheresis in Acute Myocardial Infarction (CAMI-1)" study moved downstream of IL-6 in the inflammatory cascade, directly targeting the inflammatory marker high-sensitivity C-reactive protein (hsCRP) (16). In this non-randomized pilot study, 66 patients with acute ST-elevation-MI were treated with repeated CRP-aphereses, reducing the mean hsCRP concentration by 53.0 ± 15.2%. On the one hand, the study demonstrates a correlation between the systemic CRP level, the extent of myocardial damage and the restriction of myocardial function. On the other hand, it introduces CRP-apheresis as an interesting and safe, but technically challenging method to target direct detrimental effects of CRP after acute MI. However, the study was insufficiently powered for outcome analyses. Thus, the therapeutic effect will need to be analyzed in a larger, controlled, randomized trial (16).

Beyond the IL1-IL6-CRP axis and colchicine, a plethora of other promising targets within the innate and adaptive arm of the immune system are being explored (11, 17, 18). While these landmark trials have launched a thrilling new era of treatment in CVD, a more precise and conclusive understanding of the underlying immune mechanisms is essential to routinely apply and personalize the new therapeutic concepts. Recent novel findings based on new techniques have expanded and refined our knowledge of atherosclerotic plaque instability. In the following, we integrate conventional paradigms with new discoveries, to showcase today's understanding how maladaptive immune responses drive plaque instability. We discuss different manifestations of instability, plaque rupture and plaque erosion, and summarize the accumulating evidence that they have little in common but their clinical appearance. We aim to provide a more global perspective by considering systemic influences that lead to acute plaque destabilization and highlight the limitations that animal models entail in the endeavor to explain how stable plaques become unstable.

ATHEROSCLEROTIC PLAQUE FORMATION AND FEATURES OF PLAQUE INSTABILITY

Preferably at sites of low endothelial shear stress, increased blood levels of low-density lipoprotein (LDL) and other cardiovascular risk factors, such as metabolic syndrome or cigarette smoking (19), favor loss of endothelial integrity and endothelial activation. This allows for accumulation of lipids in the arterial intima, where they are oxidized, aggregate and are engulfed by smooth muscle cells (SMCs) and macrophages, causing foam cell formation (20–23). Modified lipids act as chronic stimuli for innate and adaptive immune-responses that orchestrate a smoldering low-grade inflammation of the vessel wall (18). Subsequent cell apoptosis and necroptosis, complicated by failed efferocytosis (dead cell removal by phagocytes), cause formation of a lipid-rich necrotic core (NC) and production of thrombogenic tissue factor (21). NC components and inflammatory cells lead to degradation of plaque-stabilizing extracellular fibrous matrix (ECM) like collagen and proteoglycans and thinning of the fibrous cap (20). Hypoxia-inducible factors produced by cells contained in the NC, promote pathologic neoangiogenesis, which favors intraplaque hemorrhage and further expansion of the NC. Unresolved inflammation triggers plaque calcification, which further reduces mechanical stability of the plaque (20). Thus, features of plaque instability include large NCs (>24–50% of total lesion area), high amounts of inflammatory cells, thin fibrous caps (23 ± 19 μm), reduced ECM, abundant neovascularization and intraplaque hemorrhage and calcification (24).

Plaque Rupture and Plaque Erosion—The Concept of Plaque Instability Needs Revision

Instability of coronary atherosclerotic plaque culminates in abrupt vascular thrombus formation that impedes blood flow and leads to critical myocardial ischemia (25). Clinically, this often manifests as life-threatening Acute Coronary Syndromes (ACS) (4). Experts long equated coronary thrombosis with rupture of the typical "vulnerable plaque," the thin-capped fibroatheroma (TCFA) (26). Recent technological and conceptual breakthroughs have initiated a paradigm shift and diversified understanding of underlying pathomechanisms. Contemporary data from intravascular ultrasonographic imaging studies provided evidence that the traditional "vulnerable plaque" phenotype with TCFA does not correlate with the likelihood of clinical destabilization (6). This led to abandoning the classic "vulnerable plaque" concept (27, 28).

Besides the well-studied plaque rupture, *in vivo* high-resolution (10–15 μm) intravascular plaque imaging by optical

coherence tomography (OCT) identified superficial erosion with thrombus formation on an intact fibrous cap in 25–40% of ACS patients (29–34). Likely due to better control of traditional risk factors, this percentage seems to be ever increasing (35, 36) and superficial erosion may become the dominating plaque morphology in the foreseeable future. While discussing mechanisms of plaque instability, we must keep this novel dichotomy in mind, since, as discussed below, underlying pathomechanisms seem to differ substantially and could well lead to a fundamental shift in the management of ACS patients sooner rather than later. Other, less frequent substrates for coronary thrombosis are calcified nodules in 2–8% (29, 30, 32) and dissection of the vessel wall in 1–2% (30, 31) of ACS events. In this review, we focus on the two predominant mechanisms of ACS, plaque rupture and plaque erosion.

MOUSE MODELS OF PLAQUE INSTABILITY

Monogenetic knock out mice for the low-density lipoprotein (LDL) receptor ($Ldlr^{-/-}$)- or Apolipoprotein E ($Apoe^{-/-}$) are the two most widely used animal models of atherosclerosis (37). Both are suboptimal to study coronary plaque instability. First, atherosclerosis develops rarely in the coronary arteries, but rather in the aorta and other larger vessels (38) in which hemodynamics and vascular histology are naturally very different. Second, plaque disruption and thrombotic occlusion almost never occur spontaneously, which has in part been attributed to lower surface tension and smaller vessel diameter in mice than in humans (39). Double-KO (dKO) mice for the high-density lipoprotein receptor gene (scavenger receptor b1; $Scrb1^{-/-}$) and *Apoe* or *Ldlr* develop in many aspects human-like CA-lesions and spontaneous plaque disruption with occlusive coronary arterial atherothrombosis, but exhibit complex comorbidities and die prematurely at early age (40). $Apoe^{-/-}$ or $Ldlr^{-/-}$ dKO mice that carry an additional heterozygous function impairing mutation in the Fibrillin-1 gene ($Fbn1^{C1039G+/-}$) show features of plaque vulnerability including increased apoptosis of smooth muscle cells (SMCs), larger necrotic cores, increased macrophage- and T-cell infiltration, intraplaque hemorrhage and hypervascularization after 10 (41), 12/24 (42) or 35 (43) weeks of feeding with atherogenic high-fat diet. Remarkably, in one study, $Apoe^{-/-}$ $Fbn1^{C1039G+/-}$ mice had atherosclerosis also in the coronary arteries, frequently suffered strokes (64% of cases) and 70% died suddenly (43). However, rates of spontaneous plaque rupture varied greatly between studies, occurring in 5% (41) to 70% (43) of $Apoe^{-/-}$ $Fbn1^{C1039G+/-}$– and 20% of $Ldlr^{-/-}$ $Fbn1^{C1039G+/-}$(42) mice and mice showed no evidence of plaque erosion.

Besides genetic KO models, some surgical models have been tested. Tandem stenosis of the carotid artery of hypercholesterolemic $Apoe^{-/-}$ mice induced features of instability, including intraplaque hemorrhage (50%) and fibrous cap disruption (32%) 7 weeks postoperatively. Notably, no eroded plaque was identified (44). Based on studies that show endothelial denudation and flow disturbance as likely pathogenic triggers in plaque erosion (45, 46), electrical injury of the carotid artery adventitia of $Apoe^{-/-}$ mice, followed by flow perturbation by constrictive periadventitial cuff after an intermediate healing-period was used as a model to specifically study mechanisms that pertain to plaque erosion (47), but naturally, this cannot be used as a causal model for plaque erosion. In a promising recent study, pressure overload in hearts of chow diet fed $Apoe^{-/-}$ mice by minimally invasive transverse aortic constriction surgery induced coronary lesions in 93% of animals, most frequently in the LAD (54%). Histology identified MI in 74% of animals, in 34% of cases due to plaque rupture and in 13% due to formation of a thrombus on an intact fibrous cap, i.e. plaque erosion (48).

In sum, mouse models of acute plaque instability are scarce and, in most cases, unreliable or rather artificial. Experimental modeling of the rupture/erosion dichotomy has been impossible until recently, although it is urgently needed. A few novel surgical approaches that have recently shown to induce plaques that rupture or erode could soon yield seminal results.

IMMUNE CELLS CRUCIALLY DRIVE ATHEROSCLEROSIS

Plaque stability fundamentally depends on the level of inflammatory cell infiltration (49). Multidimensional single-cell RNA sequencing and mass cytometry of mouse and human atherosclerotic plaque have provided insights into the cellular landscape of atherosclerotic plaque at unprecedented granularity (50–55), comprehensively reviewed in Hill et al. (56). Although the relative frequency of immune cell populations varies between studies, macrophages and αβ T-cells consistently represent the most abundant cell types and overwhelming evidence supports their crucial role in atherosclerosis development and progression. We concentrate mainly on these two and neutrophils in the following, although a plethora of different innate and adaptive cells are relevant to atherosclerosis progression. For an in depth discussion of the role of dendritic cells, mast cells, B cells, natural killer (NK) cells, and unconventional T-cells in atherosclerosis, we refer the reader to other recent and up-to-date review articles, respectively. Briefly, dendritic cells accumulate in plaques and form foam cells, regulate T-cell activation and proliferation by antigen-presentation, mediate efferocytosis and secrete immune-modulating cytokines and chemokines (18, 57, 58). Mast cells accumulate in the arterial adventitia and are activated to degranulate, releasing various mediators, some of which destabilize the plaque (proteases, proinflammatory cytokines), but also others that promote plaque stability by inhibiting thrombus formation or providing oxygen to hypoxic areas of the plaque (59, 60). B-cells produce antibodies and may have proatherogenic or disease-limiting properties, in both, an antibody-mediated as well as an antibody-independent manner, depending on their sub-phenotype and antibody profile. Their complex role in atherogenesis and –progression is reviewed in Roy et al. (18) and Sage et al. (61). NK cells seem to play a subordinate role in atherosclerosis: although NK-cell derived perforin and granzyme B were demonstrated to aggravate disease (62), a later study using a selective NK-cell loss-of-function model did not confirm a direct effect of these cells on lesion size

or -stability (63). Similarly, depletion of γδ T-cells, an MHC-independent subset of T-cells that carry TCRs composed of γ- and δ-chains, had no effect on the development of atherosclerosis in high-fat diet fed TCRδ$^{-/-}$ApoE$^{-/-}$ mice (18, 64). NK T-cells are found in minor amounts in atherosclerosis and promote NC-growth and inflammation by production of cytotoxic and proinflammatory cytokines and chemokines (18, 65, 66).

Macrophages and Neutrophils

Atherosclerosis is initiated by risk factor- and flow-induced endothelial damage, which predisposes for focal retention of apolipoprotein B (apoB)-containing lipoproteins in the arterial intima. It was long thought to be a predominantly lipid-driven disease (67). Despite the indisputable causal role of lipid deposition, it is the ensuing smoldering inflammation and maladaptive immune responses that propel plaque progression and link traditional risk factors with atherosclerosis (1) (**Figure 1**). Smoking, hyperglycemia, and hypertension activate vascular smooth muscle cells (SMCs) and the vascular endothelium, especially at sites of disturbed, non-laminar flow (11, 68). This entails upregulation of adhesion molecules like ICAM-1, ICAM-2, VCAM-1, E-Selectin, and P-Selectin and the presentation of tethered CXC chemokines and cytokines on the luminal surface of the endothelium (2, 69, 70), in part mediated by increased activity of the sympathetic nervous system through locally released noradrenaline (70). Monocytes traffic to the site of injury, extravasate, and differentiate into macrophages, a process mainly dependent on monocyte chemoattractant protein-1 (MCP-1) and CC-Chemokin-Ligand-5 (CCL5) (11). Monocyte-derived macrophages overexpress scavenger receptors like CD36, LOX-1, SR-A and CXCL16 (71) and excessively engulf LDL, giving rise to dysfunctional lipid-laden foam cells (72) that accumulate cytoplasmic cholesterol crystals (73).

A self-amplifying vicious circle perpetuates the inflammatory response: in plaque macrophages, cholesterol crystals act as danger signals that prime formation and activation of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3)-containing inflammasome (73), a key signaling platform that activates caspase-1, which mediates proteolytic activation of the cytokines IL-1β and IL-18 (73). IL-1β up-regulates a plethora of proinflammatory cytokines, induces monocyte differentiation into macrophages (74), and triggers an endothelial cell response that further stimulates myeloid recruitment (1, 75) and leukocyte diapedesis and differentiation (76). Additionally, IL-1β-induced chemokines and activated SMCs recruit granulocytes to the plaque (74, 77, 78). Neutrophil exposure to cholesterol crystals induces formation of neutrophil extracellular traps (NETs) (77), extracellular networks of decondensed chromatin, histones, and antimicrobials that are part of the neutrophil host defense against pathogens (79). NETs act as crucial priming cue for proinflammatory cytokine production in macrophages through activation of the NLRP3 inflammasome. *AopE*$^{-/-}$ mice that lack key enzymes required for NETosis consistently displayed smaller lesions (77).

In sum, lipid-deposition and endothelial activation and dysfunction entail the accumulation of myeloid cells in the vascular wall. Potentiated by neutrophils, this creates a proinflammatory cytokine environment and gives rise to foam cells.

Of note, arterial macrophages do not follow the classical M1/M2 subset framework (80), but are very heterogeneous in polarization and function (51, 81, 82). Not all of them drive atherosclerosis progression and some even promote healing and resolution of inflammation (18). Macrophage heterogeneity increases with disease progression (82). Recent single-cell experiments have, at a transcriptional level, identified at least five distinct macrophage-subsets in murine atherosclerotic aortas (82), of which at least four (marked with *) have also been identified in human plaque (81): resident*, inflammatory*, interferon-inducible cell (IFNIC)*, aortic intima-resident (Mac^{AIR}) and Trem2^{+} (triggering receptor expressed on myeloid cells-2) foamy macrophages*. Resident macrophages, the only macrophage population in healthy arteries, populate the adventitia and have been suggested to limit arterial stiffness by inhibition of collagen production by SMCs (83). If they promote or alleviate atherosclerosis, is unknown (18). They mainly derive from embryonic CX3CR1^{+} precursors, and, especially in advanced plaques, are maintained through local proliferation (84). Inflammatory macrophages and IFNICs, which both differentiate from circulating monocytes and are only present during atherosclerosis, are clearly proinflammatory and drive lesion progression (18). Inflammatory macrophages highly express inflammatory chemokines and show increased expression of inflammasome components and proinflammatory cytokines such as IL-6, IL-1β and TNF (50, 51), while IFNICs produce proinflammatory interferon-I (51, 53). Another macrophage subset that drives lesion progression is the intima-resident Mac^{AIR} subset: in mouse models of atherosclerosis, Mac^{AIR} depletion reduced lipid deposition and fatty streak formation (85). This specialized resident macrophage subset differentiates from infiltrating monocytes shortly after birth, but, during inflammation progression may also be replenished from circulating monocytes (18, 85). Macrophage-derived Trem2^{+} foam cells, on the other hand, have been shown to suppress inflammatory gene expression and alleviate vascular inflammation (53, 86).

αβ T-Cells

The profound inflammatory myeloid response in atherosclerosis is accompanied by infiltration of adaptive immune cells, which, mainly through cytokines and antibodies, decisively regulate plaque inflammation (87) (**Figure 1**). Activated T-lymphocytes are present in all stages of atherosclerosis (88). CD4^{+} T cells comprise 25–38% of leukocytes in mouse and human plaques (1, 50–52). T cells home to the aorta in a manner partially dependent on the surface expressed chemokine receptors CCR5 (89), CXCR3 (90), and CXCR6 (91, 92). Single cell data shows that plaque T cells have gene signatures associated with activation, exhaustion, inflammation, and cytotoxicity (52). T cell responses in atherosclerosis are antigen specific and major histocompatibility complex (MHC)-dependent and -restricted (49, 87, 93, 94). Of note, the best-known atherosclerosis-antigens are peptides from apoB100, the core protein of LDL and its oxidized form oxLDL (93–95). Consequently, atherosclerosis

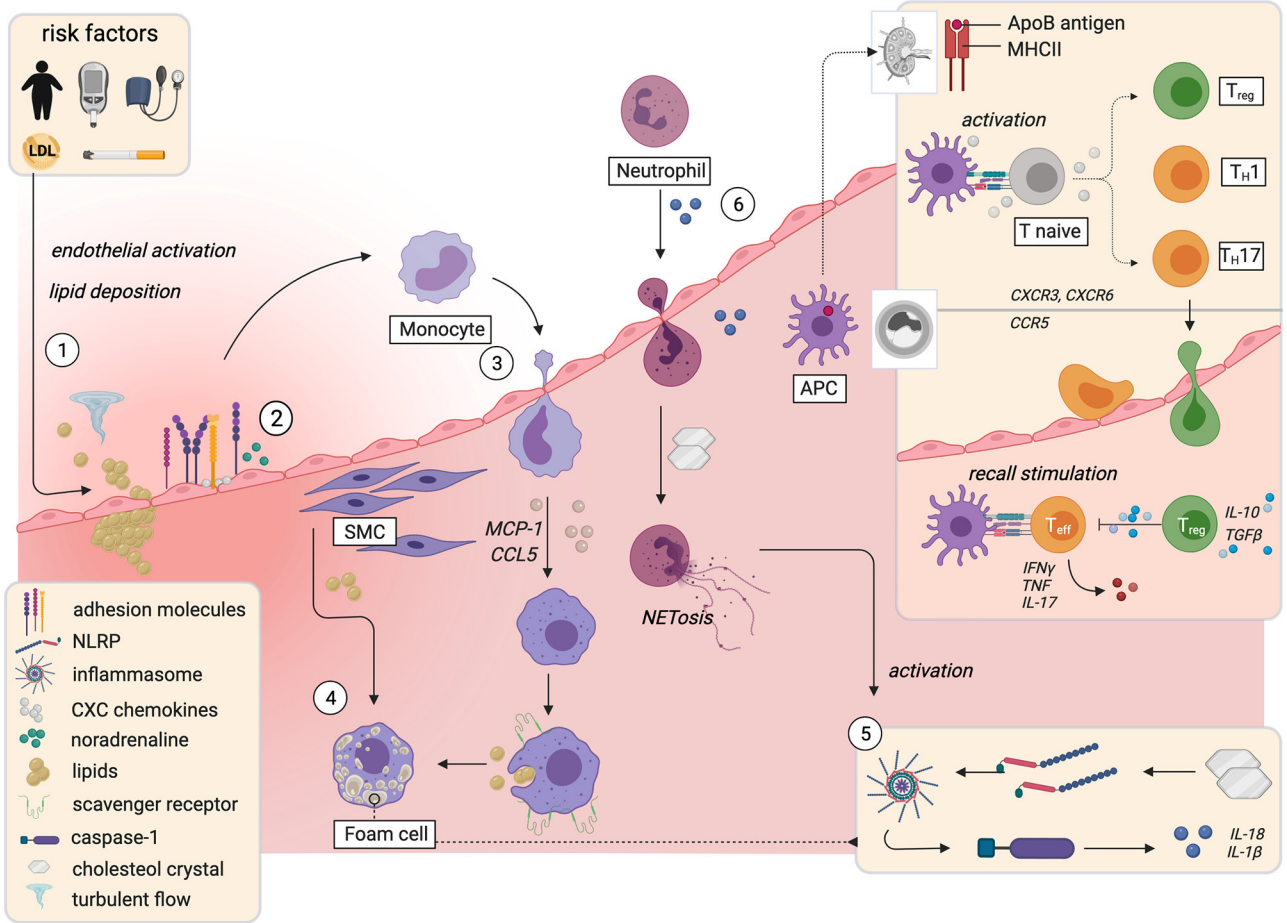

FIGURE 1 | Immune mechanisms implicated in initiation and progression of atherosclerosis. The influence of risk factors and turbulent flow conditions activate the endothelium and prime the endothelial sites for focal retention of apolipoprotein B-containing lipoproteins (1). This causes upregulation of leukocyte adhesion molecules and the presentation of chemokines and cytokines on the luminal surface, partially mediated by noradrenaline (2). Monocytes extravasate into the plaque and differentiate into macrophages that take up lipids and form foam cells (3). Smooth muscle cells acquire macrophage characteristics and also become foam cells (4). Cholesterol crystals that accumulate in foam cells prime formation of the NLRP3-inflammasome, which activates caspase-1. Caspase-1 triggers activation and release of IL-1β and IL-18 (5). The resulting proinflammatory milieu leads to neutrophil recruitment and NETosis, which further enhances the release of proinflammatory mediators (6). In lymph nodes, T-cells recognize fragments of apolipoproteinsB100 and other plaque antigens. Upon recall stimulation in the plaque, they differentiate into proinflammatory effector- or anti-inflammatory regulatory sub-phenotypes (box upper right corner). APC, antigen presenting cell; ApoB, apolipoproteinB100; LDL, low density lipoprotein; MHCII, major histocompatibility complex II; NET, neutrophil endothelial trap; NLRP; NACHT, LRR and PYD domains-containing protein 3; SMC, smooth muscle cell; T_{eff}, T-effector cell; T_H, T-helper cell; T_{reg}, regulatory T-cell. Created with BioRender.com.

involves a relevant autoimmune response (1, 87, 96), although it is not a classical autoimmune disease, initiated by detrimental immunity against itself (87).

Antigen-loaded dendritic cells (DCs) that have migrated to lymph nodes, typically initiate T-cell activation in atherosclerosis (97), while plaque-resident DCs and -macrophages as well as adventitial B cells display antigen for recall stimuli that induce antigen-experienced memory T-cells (1, 49, 98). Upon specific recognition of cognate MHC/antigen complex by the T cell receptor (TCR) and co-stimulatory molecules, T-cells respond by clonal expansion and differentiation. $CD4^+$ T-cells further differentiate into distinct regulatory or effector sublineages, namely T-helper 1 (T_H1),−2 (T_H2),−9 (T_H9),−17 (T_H17),−22 (T_H22), follicular helper T (T_{FH})-cells, or T_{reg} subtypes (Type 1 regulatory (Tr1) or *Forkhead Box P3* (*FOXP3*)$^+$ T_{reg} cells) (87, 98). Each subset displays a distinct transcriptional program and cytokine pattern that may promote or curb atherosclerosis (98). Cytokines and costimulatory signals from antigen presenting cells determine the microenvironment that polarizes T-cells toward immunogenic or tolerogenic responses (58).

The balance between pro-inflammatory effector T-cells (T_{eff}) and anti-inflammatory regulatory T-cells (T_{reg}) determines the fate of the plaque (98, 99). Briefly, T-helper 1 (T_H1) cells are proatherogenic (1, 100, 101) and dominate mouse and human plaques (52). They express proinflammatory interferon-γ (IFNγ), interleukin (IL)-2 and-3 and tumor necrosis factor (TNF), which activate macrophages and reduce plaque stability (1). Activation of the NLRP3 inflammasome in macrophages by cholesterol

crystals (73) is linked to T-helper 1 (T_H1) differentiation and IFNγ production via caspase-1 dependent proteolytic activation of the co-stimulatory cytokine IL-18 (102). Similarly, most MHC-I–dependent cytotoxic $CD8^+$ T cells, which secrete perforin, granzyme B, TNF, and IFNγ, and preferentially accumulate in the fibrous cap (103), drive plaque instability by enhancing inflammation and promoting necrotic core formation. In contrast, $FOXP3^+T_{reg}$ cells are atheroprotective and sustain immune system homeostasis, balancing out proatherogenic responses (104–106). They secrete inhibitory cytokines such as IL-10 (107) and plaque-stabilizing transforming growth factor (TGF)-β (108), induce anti-inflammatory macrophages (109), and regulate proliferation of proinflammatory effector cells (106, 109). T_H17 function in atherosclerosis remains controversial. T_H17 cells produce IL-17A and -F, both of which induce pro-inflammatory cytokines (87), and IL-17A blockade reduces experimental atherosclerosis (110). However, a T_H17 subtype induced by IL-6 and TGFβ produced atheroprotective IL-10 (107, 111) together with IL-17 (112). Some studies further attribute plaque-stabilizing function to T_H17 cells (113). Similarly, the function of the quantitatively less important T_H2, T_H9, T_H22, and T_{FH} subsets in atherosclerosis remains a matter of debate (1, 98).

MALADAPTIVE IMMUNE RESPONSES THAT DESTABILIZE THE PLAQUE

The crosstalk between innate and adaptive responses in the atherosclerotic plaque creates a delicate balance between pro- and anti-inflammatory mediators (18). Some recently discovered harmful immune mechanisms may help us understand why proinflammatory factors eventually prevail, causing plaque disruption and dramatic clinical sequelae.

Trained Immunity

Memory was long seen as an exclusive hallmark of the epitope-specific adaptive arm of the immune system. Recent findings teach us otherwise. Epigenetic changes are long-term alterations in gene expression through modification of DNA accessibility, e.g. through acetylation or methylation, without permanent genetic changes (114). KDM5 (lysine demethyltransferase 5) and Set7 (SET domain containing 7, histone lysine methyltransferase) are among important epigenetic enzymes (115). Exogenous insults such as the fungal ligand β-glucan or lipopolysaccharide (LPS) from microbes (116, 117), but also endogenous "sterile danger signals" (118) such as modified LDL and other atherogenic stimuli may induce sustained epigenetic, metabolic and functional reprogramming in myeloid cells, natural killer cells and innate lymphoid cells that enhance their cytokine production upon restimulation, even with a stimulus different from the initial "training-cue" (115, 116). This adaptive-like, but antigen-independent hyperresponsiveness of innate immune cells to recall stimulus is known as "trained immunity" (119). It is important to note that trained immunity does not refer to one particular regulatory program, but to different long-lasting (114), but reversible, epigenetic alterations of transcriptional pathways induced by different stimuli (120). Although beneficial in the setting of recurrent infection, trained immunity can lead to a maladaptive hyperinflammatory immune response in chronic inflammatory diseases like atherosclerosis (114, 120).

Compared to naïve controls, macrophages primed with modified LDL ("training") retained more intracellular cholesterol and released more TNF-α, IL-6 and matrix metalloproteinases (MMPs), Zn^{2+}-based destabilizing catalytic proteases that degrade extracellular matrix (ECM) (121), upon proinflammatory restimulation *in vitro* (23) (**Figure 2A**). This was based on epigenetic modification, since the "training effect" was prevented by the blockade of histone methyltransferases (23). A concomitant metabolic rewiring involved mTOR/HIF1α-dependent upregulation of glycolytic capacity and oxidative phosphorylation (122). LDL-trained macrophages (23) and monocytes from patients with familial hypercholesterolemia (123) showed enrichment of the transcriptionally permissive chromatin mark histone H3 trimethylated at lysine 4 (H3K4me3) in the promotor region of genes that encode proatherogenic and proinflammatory cytokines. In line with this, blood monocytes from patients with severe coronary artery atherosclerosis exhibit characteristics of a trained phenotype in terms of histone hypermethylation, cytokine production, and metabolic reprogramming (124, 125). Given the short half-life of blood monocytes, it is noteworthy that hypercholesterolemia-induced changes in the epigenome may occur not only in mature myeloid cells, but also at the level of myelopoiesis: high-fat-diet (HFD)-induced (="training") hypercholesterolemia in $Ldlr^{-/-}$ mice led to profound proinflammatory epigenetic reprogramming of long-lived bone marrow granulocyte-macrophage progenitor cells and a hyperinflammatory response of their progeny that persisted for several weeks after discontinuation of the HFD despite normalized cholesterol levels and systemic inflammatory markers (118). This was mediated by the NLRP3-inflammasome and IL-1β (118).

Although deeper mechanistic and clinical evidence of the direct detrimental effect of trained immunity in atherosclerosis is needed, it is tempting to speculate that the observed accelerated myelopoiesis, hyperresponsiveness, and increased production of proinflammatory mediators in response to repetitive stimulation with modified plaque lipids may promote plaque inflammation that ultimately fuel plaque instability and disruption. Given that innate immune memory is a relatively new discovery in the context of atherosclerosis, essential questions remain unanswered. Does the training effect and duration vary between the different innate immune cell types? Does it directly influence the players of the adaptive immune system that are omnipresent in the plaque? Is trained immunity thoroughly reversible and can we therapeutically exploit this? Could some of the "genetic" cardiovascular risk be due to training-induced epigenetic alterations being passed on in the germline?

Phenotypic Instability of LDL-Induced Protective Autoimmune Cells

As outlined above, T_{regs} are clearly atheroprotective, stabilize the plaque, and outbalance proinflammatory immunity. In

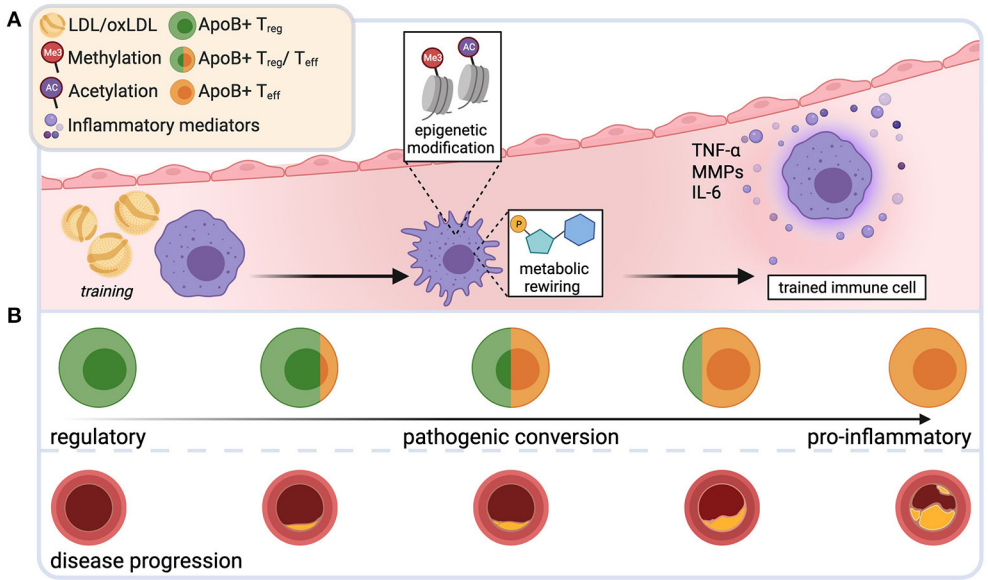

FIGURE 2 | Novel immune mechanisms that drive plaque instability. Repetitive exposure to low-density lipoprotein (LDL) and its oxidized form (oxLDL) during plaque progression may induce epigenetic changes and metabolic rewiring in innate immune cells. Such trained cells produce more proinflammatory mediators upon restimulation, aggravating the proinflammatory bias in the plaque milieu **(A)**. During early atherosclerosis, apolipoproteinB100-specific T-cells are mostly regulatory T cells (T_{reg}). During disease progression, these cells undergo a phenotypic switch and acquire phenotypic and functional characteristics of harmful effector T cells (T_{eff}) **(B)**. Created with BioRender.com.

atherosclerotic *Ldlr*$^{-/-}$ mice, hypercholesterolemia was shown to initially drive T_{reg} differentiation and homing to the inflamed aorta (126). T_{reg} numbers significantly correlated with LDL plasma levels in subclinical human atherosclerosis (127). This was likely antigen-driven, since hypercholesterolemia increased T-cell receptor (TCR) signaling and proliferation in CD4$^+$ T cells (128), supporting the role of LDL or its major protein component apolipoproteinB100 (apoB100) as autoantigens in atherosclerosis. During disease progression, numbers of circulating and lesional T_{regs} decline and CD4$^+$ T effector (T_{eff}) populations increase (129). The loss of regulatory T-cells can be attenuated by a switch to a normal diet (129).

At later stages of atherosclerosis, while retaining key features of regulatory T-cells, T_{regs} show mixed, multilineage phenotypes with characteristics of T_H1 and T_H17 cells, secrete proinflammatory cytokines, lose their suppressive function and fail to protect from atherosclerosis (89, 96, 129, 130). These data suggest the existence of an LDL-driven immunosuppressive regulatory response in atherosclerosis that undergoes pathogenic conversion during disease progression (**Figure 2B**). Indeed, studies directly demonstrated the existence of CD4$^+$ T-cells with specificity for apoB100-peptides in mice (96) and humans (94, 96), using MHCII multimers loaded with ApoB peptide oligomers (131). In mice, autoreactive T cells specific for $ApoB_{978-993}$ were oligoclonal and expanded in hypercholesterolemic conditions. In early disease, they exhibited a T_{reg}-like transcriptome. During disease progression, they converted into T_H1/T_H17-like proinflammatory T_{eff} cells that retained only a residual T_{reg} transcriptome. After adoptive transfer, they failed to protect from atherosclerosis, despite their regulatory properties (96). In individuals without atherosclerosis, two thirds of CD4$^+$ T cells reactive to another ApoB100 peptide, $ApoB_{3036-3050}$, expressed the T_{reg}-defining transcription factor *FoxP3*. In patients with manifest coronary artery disease, this proportion was reduced to 30%, whereas a large proportion showed a mixed, partially proinflammatory T_{reg}/T_{eff} phenotype (94). Loss and pathogenic conversion of a suppressive, stabilizing T cell population may tilt the delicate balance of pro- and anti-inflammatory immunity in the plaque and could thus be a key factor driving plaque progression and instability. Preventing the proinflammatory switch of T_{regs} or mobilizing T_{reg} cells to maintain or restore immune tolerance in atherosclerosis bears interesting therapeutic potential. Several promising tolerogenic vaccination approaches, largely focused on ApoB, LDL or oxLDL, are currently being explored (132).

Clonal Hematopoiesis

Sustained somatic mutations in bone marrow hematopoietic stem cells (HSCs), frequently loss-of function mutations of epigenetic transcriptional proliferation regulators commonly associated with myeloid cancers (*DNMT3A*, *TET2*, *ASXL1*, *JAK2*) (133) may impart selective advantages to HSCs and drive their clonal expansion (134, 135). Mutated clones differentiate into circulating granulocytes, monocytes, and lymphocytes (136–138) (**Figure 3A**). Witness that this is not typically accompanied by blood leukocytosis (137, 139).

If mutant clones are present in ≥2% of peripheral blood leukocytes (variant allele frequency, VAF) and no

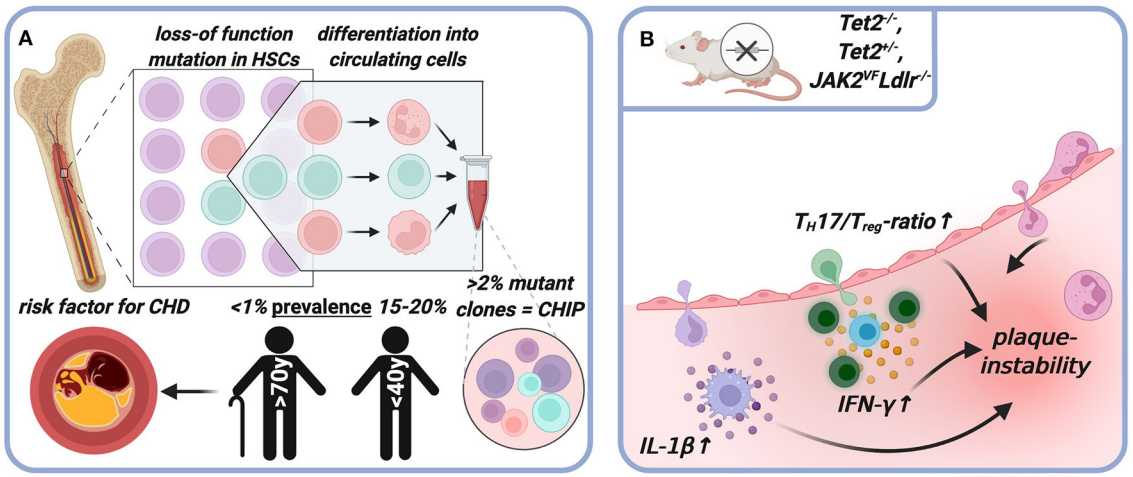

FIGURE 3 | Clonal hematopoiesis and atherosclerotic plaque progression. Sustained somatic mutations in bone marrow hematopoietic stem cells (HSCs) drive their clonal expansion and differentiation into circulating mutated leukocyte clones with myeloid bias **(A)**. Mutated leukocytes invade the plaque and overproduce inflammatory mediators, mutated regulatory T-cells are phenotypically unstable and switch toward inflammatory effector cells **(B)**. Created with BioRender.com.

hematologic malignancy has been diagnosed, this is called clonal hematopoiesis of indeterminate potential (CHIP) (135). Of note, CHIP may occur in the absence of known driver mutations (140, 141) and if present, >90% CHIP carriers harbor only one mutation (141). CHIP-prevalence is strongly age-related, ranging between <1% in those younger than 40 to 15–20% in those older than 70 years (142), which could in part explain the age-related increase in CVD (141). CHIP associates with a 40% increase of all-cause mortality (134) and doubles the risk of incident coronary heart disease (CHD), upstream and independent to traditional risk factors (134, 137). The risk of early-onset MI (<50 years old) quadruples in CHIP carriers as compared to non-carriers (138). If the VAF is 10% or more, CHIP-bearers without symptomatic CHD have dramatically higher coronary calcium scores, a radiological surrogate marker of coronary plaque burden (138).

In the light of these findings, CHIP has emerged as independent risk factor for plaque instability, likely in part due to effects of driver mutations on immune effector cells (141). For some mutations, mouse and human studies suggest causality (**Figure 3B**): monocytes from patients with DNMT3A mutation showed increased inflammatory gene expression, including those of the NLRP3 to IL-1β to IL-6 to CRP pathway (143). *Tet2*$^{-/-}$ or *Tet2*$^{+/-}$ (138, 139) -as well as *JAK2*^{VF}*Ldlr*$^{-/-}$ (144) mice had larger atherosclerotic lesions and this was still true when Tet2 was selectively knocked out in the myeloid compartment (138, 139). *Tet2*$^{-/-}$ macrophages accumulated more in the arterial wall (139), and *Tet2*$^{-/-}$ and *JAK2*^{VF}macrophages had a more proinflammatory phenotype and produced more IL-1β (139, 144). Neutrophils adhered and transmigrated more into larger *JAK2*^{VF}*Ldlr*$^{-/-}$ lesions and after 12 weeks of HFD plaques displayed signs of instability such as larger NCs (140). Although CHIP driver mutations induce myeloid bias (145), the destabilizing effect appears to extend to other immune compartments: T-cells from patients with *DNMT3A* mutations exhibit highly-inflamed transcriptomes and altered T_{H-}signatures (143). Patients with *DNMT3A* mutation showed an increased T_H17/T_{reg} ratio (146) and increased IFN-γ production in T-cells (147). Specific LysM^{Cre}-driven deletion of *Tet2* in T_{regs} led to deranged suppressor function, phenotypic dysregulation and eventually to a phenotypic switch toward a proinflammatory T_H17/T_{FH} effector phenotype (148), similar to what has been described for pathogenic ApoB-reactive autoimmunity in atherosclerosis (96).

Note the recent twist introduced by a new study, which suggests reverse atherosclerosis-CHIP causality (149, 150). HSCs proliferate more in the presence of atherosclerosis, likely due to enhanced inflammation and hypercholesterolemia, which accelerates somatic evolution and increases the risk of expansion of clones that carry CHIP driver mutations. It was demonstrated through a novel mathematical modeling approach that this increase in HSC proliferation alone would suffice to explain increased CHIP prevalence in CVD (149, 150). Taken together, accumulating evidence points to altered inflammatory signaling from expanded leukocyte clones that carry somatic mutations as key driver of plaque instability. Future experimentation will need to strengthen evidence of how CVD and clonal hematopoiesis are linked.

PLAQUE DISRUPTION

Immune Mechanisms of Plaque Rupture

Excessive accumulation of leukocytes, a highly proinflammatory cytokine milieu, necrotic core (NC)-enlargement by cell death, and protease-driven destruction of extracellular matrix (ECM) that results in cap-thinning are hallmarks of unstable plaque (151). Some of these characteristics are likely promoted by the mechanisms discussed above: LDL-trained "memory"-macrophages over-produce TNF-α, IL-6, and Matrix metalloproteinases (MMPs) (23). ApoB-specific

proinflammatory T_H1/T_{reg} and T_H17/T_{reg} cells that have lost their regulatory, immune-balancing properties (96) exacerbate plaque inflammation, likely mainly by IFN-γ production. The pathogenic proinflammatory switch of T-cells (148) as well as the production of inflammatory cytokines could be accelerated by a disproportionate influx of leukocyte clones that carry a CHIP-driver mutation (143, 146).

Moreover, activated plaque T-cells overexpress the co-stimulatory molecule CD40-ligand (CD40L) (152). Enhanced CD40L gene expression and other proinflammatory cues such as IFN-γ, TNF-α, and IL-1 upregulate its major ligand CD40 on lesional APCs and smooth muscle cells (SMCs) (152). Plaques from hypercholesteremic atherosclerotic mice treated with an anti-CD40L antibody are more matrix-rich and more stable and contain less macrophages and T-cells (153–155), mechanistically explained through decreased VCAM-1 expression (154) and enhanced TGF-β signaling (155); specifically, the binding of T-cell-expressed CD40L to surface-CD40 in DCs enhanced T_H1 polarization and increased T_H1-associated IFN-γ production (156). In a $CD4^+$ T-cell-specific conditional CD40L-KO model, plaques were less proinflammatory and had smaller NCs and thicker caps (156). Monocyte-to-macrophage differentiation, T-cell-CD40L interaction with macrophage-CD40 (157) and a proinflammatory cytokine-milieu all induce macrophages to overproduce a broad spectrum of highly destructive MMPs (121), which degrade ECM and promote thinning of the fibrous cap. IL-1β-induced chemokines and activated SMCs recruit neutrophils to advanced plaques, where they eject NETs (77, 78), mediators of an act of perfidious targeted killing: the NET-contained nuclear protein cytotoxic histone H4 was recently shown to bind to SMCs and lyse their membrane by pore formation, thereby causing their death (78). SMCs are major producers of elastin, collagen, and other ECM (151). Thus, SMC-death renders the plaque more volatile (158). Plaque stability was rescued by antibody-mediated neutralization H4 or shieling of its functional domain (78). Witness that SMCs are phenotypically highly plastic (**Figure 1**): In SMCs, cholesterol-uptake induces expression of typical macrophage markers (e.g. CD68 or Mac-2) and foam cell-like features, while SMC contractile genes are downregulated (159). Indeed, within advanced atherosclerotic plaques, several reports identify SMCs, not macrophages, in 30–70% of foam cells (22, 159–163). Using SMC lineage tracing and SMC-specific KO models in $ApoE^{-/-}$ mice, a recent elegant study demonstrated detrimental effects of this SMC-to-macrophage switch on plaque stability (163). Specifically, conditional deletion of the transcription factor Krueppel-like factor 4 (KLF4) prevented SMC switching, but also markedly decreased lesion size and increased fibrous-cap thickness and other indices of plaque stability. Excessive death of SMCs, macrophages and foam cells overcharge the plaque phagocytes' capacities for effective efferocytosis (disposal of dead cells through phagocytosis), leading to failed clearance of dead cells and NC enlargement (164). Caspase-1, activated by NLRP3-inflammasome assembly through, amongst others, intracellular cholesterol crystals (118) causes pyroptosis in the vessel wall, a proinflammatory form of lytic cell death that leads to rapid membrane destabilization and proinflammatory cytokine release (164). Similarly, necroptosis, another highly proinflammatory form of cell death, occurs in advanced plaque (164). Ligation of macrophage-expressed CD40 induces expression of procoagulant tissue factor in the NC (165). When finally, the thinned, weakened fibrous cap ruptures, this prothrombogenic material is exposed to the bloodstream and causes thrombin activation, platelet aggregation and thrombus formation, which leads to vessel occlusion and critical organ ischemia in the heart (35).

Immune Mechanisms of Plaque Erosion

Despite similar clinical presentation, postmortem studies report substantial histopathological differences between ruptured and eroded plaques that point to distinct underlying pathomechanisms (166). Macrophages, foam cells, and other inflammatory cells are rare in eroded plaques and surface SMCs are relatively abundant (166, 167). Necrotic cores are smaller, calcifications scarce, and extracellular matrix components, especially hyaluronan (HA), predominate (29, 167, 168). Thrombi that overlay erosion are platelet-rich and contain less fibrin and more myeloperoxidase-positive inflammatory cells as compared to thrombi that form on ruptured lesions (27, 28, 169).

Recent comparative studies have increased the granularity of knowledge regarding clinical, morphological and molecular differences between these two ACS-causing entities (29–34). They unanimously corroborate the early notion that the widely studied mechanism of plaque rupture does not pertain to plaque erosion, but that plaque erosion is likely a wholly different disease entity (28, 36). Detailed understanding of the unique pathobiology of plaque erosion is essential. First, the prevalence of plaque erosion is increasing and may, in part due to better control of traditional risk factors, become the dominating plaque morphology in ACS (28, 36, 170). Second, together with targeted immunomodulation, tailored therapy for plaque erosion may clear the path for precision management in ACS. Early pilot studies suggest that non-invasive pharmacological treatment without stenting may be sufficient in plaque erosion (170, 171).

The sparsity of macrophages in eroded plaque and the fact that endothelial denudation is a histological hallmark of plaque erosion (46, 166, 167) primed the early assumption that immune mechanisms and inflammation may not play a central role in the pathophysiology of erosion ACS (167, 168). Recent advances, however, suggest a complex interplay between local mechanical triggers and both an innate and an adaptive immune response as driving forces in plaque erosion.

Eroded plaques preferentially localize near coronary bifurcations (7), and many studies support a key role for turbulent local fluid dynamics as well as consequently altered endothelial shear stress as "first hit" initiators of the cascade that drives plaque erosion (29, 32, 172–175). During atherogenesis, disturbed flow primes lesion-specific overexpression of various toll-like receptors, including toll-like receptor 2 (TLR2) (47, 176), especially in endothelial cells and macrophages. (177–180) Exogenous, but also endogenous ligands such as

agonists released during tissue damage or apoptosis, cholesterol crystals or hyaluronic acid, activate TLRs (177–180). Through myeloid differentiation primary response gene 88 (MyD88) and other signaling adapters, TLR-ligation results in activation of interferon response factor (IRF) family members and nuclear factor-kB (NF-kB), leading to expression of leukocyte adhesion molecules and chemoattractants, activation of interferon- and proinflammatory cytokine pathways and generation of matrix metalloproteinases (MMPs). (45, 179, 180) General deficiency of MyD88, TLR2 or TLR4 significantly reduced atherosclerosis and vascular inflammation in mice and humans. (180) At sites of disturbed flow conditions predisposed for plaque erosion (32, 172–175), NF-kB activation through TLR2 has been proposed to facilitate disruption of endothelial cell (EC)-to-extracellular matrix contact, EC apoptosis, and the desquamation of the endothelial monolayer that is characteristic for eroded plaque. (45, 47, 177) Neutrophils adhere to areas of endothelial activation and -denudation, potentiate EC death (45, 47) and release NETs (28), which have been crucially implicated in the pathophysiology of plaque erosion (36, 45–47). NETs sustain a low-level proinflammatory response on the luminal endothelium (36, 77) and promote pathological thrombosis by induction of prothrombotic endothelial tissue factor (181) and by facilitating platelet adhesion, activation, and aggregation (182).

This interplay between endothelial dysfunction, TLR overexpression and neutrophil accumulation and activation is accompanied by a local enrichment of both $CD4^+$ and $CD8^+$ T-lymphocytes (29). Effector mediators secreted by cytotoxic T cells, such as granzyme A, granulysin, and perforin were shown to further promote EC death and $CD8^+$ T-cells, but not monocytes, adhered more to flow-primed endothelium in an integrin-β2 and integrin-α4 dependent manner *in vitro* (29). Adhesive interaction between HA and its major receptor CD44 (183) could provide a link between T-cell recruitment selectively to sites of endothelial erosion: CD44 is overexpressed on antigen-activated T-cells in areas of disturbed flow (184), and HA accumulates in eroded, but not in ruptured plaques (167, 168).

In sum, it seems that after endothelial activation and dysfunction as common initiator, plaque rupture is lipid-driven and kindled by macrophages, while endothelial damage, potentiated by neutrophils, underlies plaque erosion. However, both pathologies critically involve the adaptive arm of the immune system. In this context, interesting questions remain unanswered. If lipids do not play a substantial role in plaque erosion, can lipid lowering prevent this type of ACS? Furthermore, it is known that LDL-derived ApoB-peptides elicit antigen-specific responses and drive T cell activation and formation of memory pools in mouse and human atherosclerosis (94, 96). Since eroded plaques are lipid poor (166, 167), are different antigens involved in plaque erosion? If so, do antigen-specific cells persist in memory pools? And, since helper T-cell subsets may have stabilizing, anti-inflammatory or proinflammatory effects (98), how are helper T-cells differentiated?

SYSTEMIC INFLUENCES THAT THROW PLAQUES INTO CRISIS

Atherosclerosis is usually a silent, slowly developing, smoldering condition that leads to gradual build-up of plaques in the tunica intima of arteries. In most cases, its first clinical manifestation is a heart attack, which occurs when the plaque abruptly destabilizes, occluding a vessel necessary for the blood supply of the myocardium. Some systemic influences episodically accelerate plaque progression, massively raising the likelihood of destabilization.

Acute Infections and Cardiovascular Risk—Implications for the COVID-19 Pandemic?

Acute infections abruptly raise the incidence of MI. This risk culminates directly after infection and decreases over time (185–187). Notably, the risk is not pathogen-specific and therefore rather mediated by the host response than by certain elements of microbes. A recent study found a six times higher risk for acute MI during the first 7 days after a diagnosis of influenza A or B and to a smaller extent also for non-influenza respiratory viruses (185). In the very recent IAMI-trial (188, 189) and other smaller trials (190), patients hospitalized for acute MI who were subjected to influenza vaccination had a lower mortality risk and a better cardiovascular outcome than patients treated with a placebo shot. A severe increase in CV-risk was also reported in the context of community-acquired bacterial pneumonia (186). Risk peaked in the first week after infection but was still elevated up to two years after hospitalization (186). Non-respiratory infections like urinary tract infection implicate comparable risk (187). Emerging data point to a similar increase in CV-risk associated with COVID-19-disease (191, 192) and preexisting CVD independently associated with worse outcome in COVID-19 patients (193, 194).

Some experimental evidence from mice and humans mechanistically lines this clinical association (**Figure 4**). In $ApoE^{-/-}$ mice infected with influenza A virus, histological analyses showed increased inflammatory macrophage and T-cell accumulation in atherosclerotic plaques (195) and post-mortem analyses showed enrichment in myeloid- and T-cells in the coronary adventitia of patients who died from acute infection (196). Intra-abdominal sepsis accelerated atherosclerosis within only 24 h in *ApoE*-KO mice, in part mediated by increased lesional TNF, IL-6 and CCL2 (197). Endotoxemia upregulated the chemoattractant Leukotriene B4 (LTB4) in aortas of $ApoE^{-/-}$ mice, which caused neutrophil invasion and subsequent enhanced collagen digestion, necrosis, and rapid destabilization in plaques (198). Enhanced NET-release in the arterial lumen during endotoxemia increased monocyte adhesion and accumulation (199). Exposure to various microorganisms can induce epigenetic changes that mediate trained immunity (123, 124).

As discussed above, those trained cells are proinflammatory, proatherogenic and have destabilizing effects on the plaque (200). Plausibly, trained immunity has been proposed as a

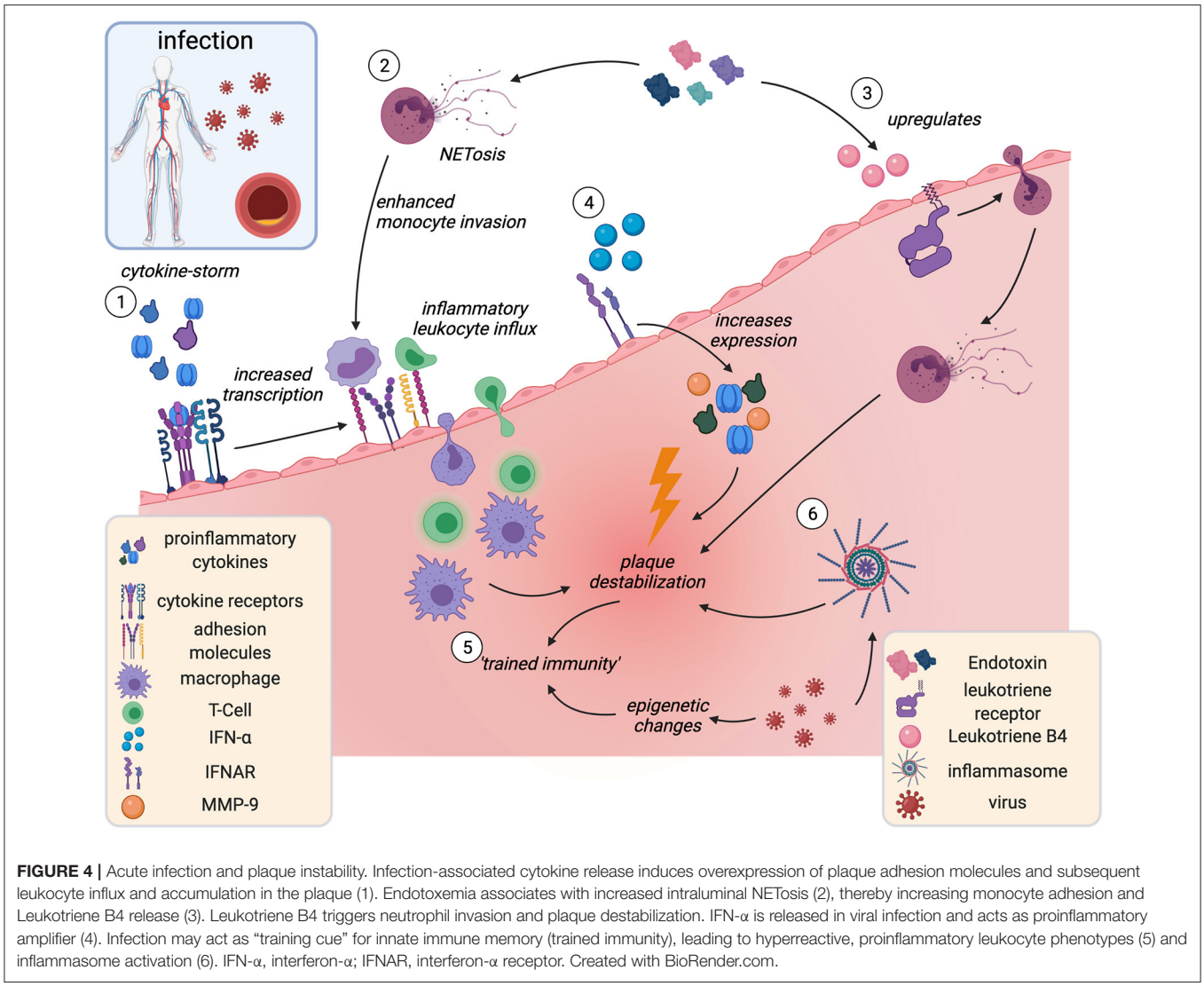

FIGURE 4 | Acute infection and plaque instability. Infection-associated cytokine release induces overexpression of plaque adhesion molecules and subsequent leukocyte influx and accumulation in the plaque (1). Endotoxemia associates with increased intraluminal NETosis (2), thereby increasing monocyte adhesion and Leukotriene B4 release (3). Leukotriene B4 triggers neutrophil invasion and plaque destabilization. IFN-α is released in viral infection and acts as proinflammatory amplifier (4). Infection may act as "training cue" for innate immune memory (trained immunity), leading to hyperreactive, proinflammatory leukocyte phenotypes (5) and inflammasome activation (6). IFN-α, interferon-α; IFNAR, interferon-α receptor. Created with BioRender.com.

mechanistic link for how infections augment CV risk (200). A breach of immune tolerance by molecular mimicry (201) as well as pathogen-induced inflammasome activation (202) in myeloid cells have been shown to contribute to accelerated atherosclerosis in the context of viral infection.

Although evidence is still scarce, some of the mechanisms by which infections cause plaque vulnerability could pertain to COVID-19: various pathogens upregulate expression of adhesion molecules on ECs, including vascular ECs in mice and humans (203–205). Similarly, COVID-19 patients have elevated plasma levels of leukocyte adhesion molecules VCAM-1, ICAM-1, VAP-1, and PECAM-1 (206, 207). By hyper-acute induction of adhesion molecules in the plaque, systemic infection could enhance inflammatory leukocyte influx and accelerate plaque destabilization (203). Circulating proinflammatory cytokines such as IL-1α, IL-1β, and TNF-α, released in a storm in severe COVID-19 (208), could potentiate this effect and activate lesional inflammatory cells (11). IFN-α, typically released in viral infection (209) and elevated in COVID-19 patients (210), is a proinflammatory amplifier in atherosclerosis plaque (209). It has been shown to increase levels of MMP-9, TNF-α and IL-12, which all contribute to plaque destabilization (209). Accumulating evidence points to endothelial activation and dysfunction as key elements of COVID-19 disease (208, 211). Given this pathophysiological similarity, it could be hypothesized that the COVID-19-associated increase in MI-risk be driven by an increase in the incidence of plaque erosion rather than plaque rupture, but only time will tell if this hypothesis holds.

Acute Mental Stress—An Increasingly Prevalent Modifiable Risk Factor

The incidence of sudden cardiac death and acute MI shot up after the Northridge earthquake in California (212) and the Great East Japan Earthquake (213) as well as during the first days of the Gulf war in Israel (214). Even everyday life acute stressors such as watching the national team play during the World Soccer Championship triggered a steep rise in ACS events (215). Those and other studies established acute stress as

independent risk factor for CHD and MI (216, 217), in some studies as strong as hypertension and diabetes (218). Chronic stress exposure as experienced through job loss (219) or marital strain (220) increases the risk for CVD by 40–50% (218) and MI is more common in individuals who experience repetitive or permanent stress (216). Recent studies have characterized a "stress-sensitive neuro-immune axis" (221) between the sympathoadrenal system and the bone marrow that links stress and atherosclerotic plaque instability (**Figure 5**): stress activates the amygdala and hypothalamus (222), triggering a transient rise in circulating catecholamines through the sympathetic nervous system. Their corresponding α- and β-adrenergic receptors are expressed on hematopoietic stromal cells and most leukocytes (221). Experimentally, surplus noradrenaline released during mild chronic stress triggered β3-adrenergic receptor-mediated decrease of the cytokine CXCL12 in stromal cells, which curtails excess hematopoiesis and mediates HSC quiescence. This accelerated hematopoiesis and, hence, promoted inflammatory blood monocytosis and neutrophilia (223). In $ApoE^{-/-}$ mice, blood leukocytosis through chronic stress-related acceleration of hematopoiesis led to inflammatory myeloid cell- and neutrophil accumulation as well as a more inflammatory cytokine profile in atherosclerotic lesions and promoted plaque instability (223). In line, blood leukocytosis was present in humans exposed to chronic stress (221).

Another plausible link between stress, inflammation, and accelerated atherosclerosis was interrogated by a recent study: catecholamine-induced trained immunity. (Nor)adrenalin-primed human monocytes showed a heightened TNF-α and IL-6 production in response to proinflammatory restimulation and upregulation of glycolysis and oxidative phosphorylation (224). Similarly, a hyperresponsive and proinflammatory phenotype was observed in monocytes of patients with chronic catecholamine-overstimulation due to pheochromocytoma, which persisted for 4 weeks after removal of the tumor. Epigenetic profiling of these cells showed a trend toward H3K4me3 enrichment at the promoter regions of proinflammatory genes (224).

A different study provides insight into how the immune response to acute and chronic stress differs. The researchers show that in mice and humans, *acute* mental stress (a key soccer game for humans, immobilization, or predator-prey stress through fox odor in mice) does not cause accumulation, but instead leads to rapid transient depletion of inflammatory monocytes and lymphocytes, although not neutrophils from the peripheral blood. Cell tracing experiments revealed recruitment of these cells to distinct tissues, including atherosclerotic aortas in $ApoE^{-/-}$ mice. This was driven by a noradrenaline-mediated upregulation of ICAM1 and VCAM1 on vascular endothelial cells (VECs) and increased levels of the chemoattractants CXCL1 and CCL7, released predominantly by VECs and macrophages. Besides intimal inflammatory myeloid accumulation, plaques showed decreased SMC numbers, more extracellular matrix breakdown and more likely ruptured under stress in a rupture-prone $ApoE^{-/-}$ pressure overload-model (218). In a multisystem 18F-fluorodeoxyglucose (FdG) positron-emission tomography (18F-FDG PET) imaging approach, evidence of a correlation of amygdala activation with enhanced bone marrow signal was shown, which associated with worse cardiovascular outcome in patients over a 3.7-year follow-up period (222). Building on this, multisystem 18F-FDG-PET imaging was employed to show concordant vascular inflammation, amygdala-, and bone marrow activation after acute MI, which concurrently returned to baseline signal after 6 months (225).

GUT MICROBIOTA-DEPENDENT MODULATION OF ATHEROGENESIS AND PLAQUE PHENOTYPE

Growing evidence suggests a link between the gut microbiome and the pathogenesis and progression of atherosclerosis (226) and plaque vulnerability (227). This link is based on changes in microbial diversity, compositions, and metabolism and intestinal barrier function affecting host physiology at the system level. Additionally, bacterial DNA has also been identified locally in coronary atherosclerotic plaques pointing to the presence of bacteria in atherosclerotic lesions (228), although their causal role for plaque development and vulnerability still remains a matter of debate (229). In this section we will focus on distinct gut bacterial signatures and microbial-related bioactive metabolites that have been increasingly recognized to shape atherosclerotic disease progression and plaque morphology: TMAO (230) and SCFA (231).

Contributory Role of Gut Dysbiosis in Atherosclerotic Plaque Development and Vulnerability

The human gut microbiota is mainly composed of the phyla *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria*, and *Verrucomicrobia*, *Firmicutes* and *Bacteroides,* although the large majority of the bacteria are members of the phyla *Firmicutes* and *Bacteroides* (232). Alteration of the microbial homeostasis into a so-called dysbiotic state critically affects human health (233), and may contribute to the progression of atherosclerotic vascular disease (230). Importantly, the contributory role of gut dysbiosis for atherosclerotic plaque development should not be confused with earlier studies postulating an atherogenic role of specific microbial pathogens such as *Chlamydia pneumoniae* and *Helicobacter pylori* by directly invading vascular cells and leukocytes and promote inflammatory processes within the vascular wall, which later could not be confirmed in clinical intervention studies (234). Within the gut microbiome, a negative correlation of gut bacterial diversity and plaque size has been observed in a mouse model of high fat diet-induced atherosclerosis (235). Moreover, increased abundance of *Firmicutes* along with an increase in bacterial lipopolysaccharides (LPS) causing endotoxemia has been associated with pro-atherogenic macrophage M1 activation in obesity (236). Importantly, beyond pro-atherosclerotic clinical conditions such as obesity and metabolic syndrome, antibiotic treatment may also lead to disturbance of microbial homeostatis and, thus, critically affect human health. In a recent study, it was shown that administration of a macrolide antibiotic causes

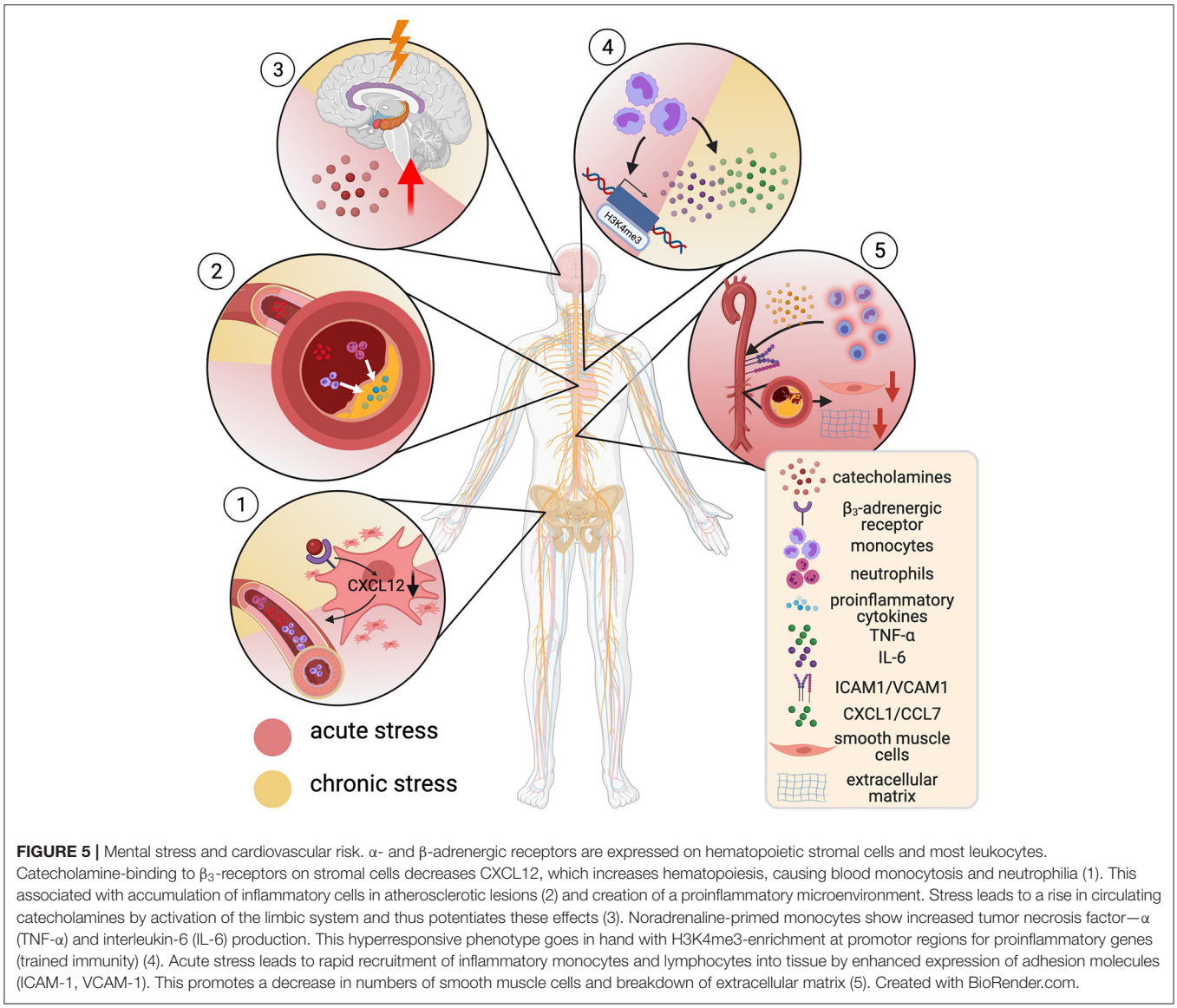

FIGURE 5 | Mental stress and cardiovascular risk. α- and β-adrenergic receptors are expressed on hematopoietic stromal cells and most leukocytes. Catecholamine-binding to β_3-receptors on stromal cells decreases CXCL12, which increases hematopoiesis, causing blood monocytosis and neutrophilia (1). This associated with accumulation of inflammatory cells in atherosclerotic lesions (2) and creation of a proinflammatory microenvironment. Stress leads to a rise in circulating catecholamines by activation of the limbic system and thus potentiates these effects (3). Noradrenaline-primed monocytes show increased tumor necrosis factor—α (TNF-α) and interleukin-6 (IL-6) production. This hyperresponsive phenotype goes in hand with H3K4me3-enrichment at promotor regions for proinflammatory genes (trained immunity) (4). Acute stress leads to rapid recruitment of inflammatory monocytes and lymphocytes into tissue by enhanced expression of adhesion molecules (ICAM-1, VCAM-1). This promotes a decrease in numbers of smooth muscle cells and breakdown of extracellular matrix (5). Created with BioRender.com.

microbial alterations characterized by loss of microbial diversity and higher *Firmicutes/Bacteriodetes* ratio (237). Consequently, enhanced CD68-expressing foam cells and increased M1 polarization was found in the plaque content of atherosclerosis-prone ApoE$^{-/-}$ mice. These findings suggest a pathway in which antibiotic administration impacts the inflammatory process within atherosclerotic plaques by affecting the composition and activity of the microbiome.

On the genus level, analysis of fecal samples from healthy individuals and patients with symptomatic atherosclerosis by means of shotgun sequencing has identified the genus *Collinsella* to be enriched in patients with symptomatic atherosclerosis, whereas the abundance of *Eubacterium* and *Roseburia* was higher in healthy controls (238).

This bacterial profile was associated with an altered metagenome marked by enriched genes in the peptidoglycan pathway in symptomatic patients. Notably, peptidoglycan is known to bind to CD14 on macrophages and induce proinflammatory cytokine production (e.g. IL-1, IL-6 and TNF-α) and metalloproteinase 9 in a TLR-2 dependent manner in atherosclerotic plaques and, thus, promotes a vulnerable plaque phenotype (239).

The Gut Microbial-Host TMA/TMAO Axis: Novel Driver of Atherosclerosis

Beyond changes in the composition of gut microbiota, its metabolic property has been increasingly acknowledged as a potential factor to contribute to atherosclerosis and to shape plaque development. One of the gut microbiota-dependent metabolites that has raised significant attention in recent years is trimethylamine-N-oxide (TMAO), the hepatic oxidation product of the microbial metabolite trimethylamine (TMA) (226, 230). TMA is generated by microbial metabolism of dietary nutrients containing a TMA moiety, such as choline and L-carnitine.

Following absorption by the host, TMA is oxidized in the liver by flavin monooxygenase into TMAO and then released into the circulation (226).

This microbial-host TMA/TMAO axis is considered to mediate proatherogenic actions, in particular by promoting endothelial cell dysfunction (240) and macrophage foam cell formation (230). We recently demonstrated that a choline-rich diet increases the differentiation of pro-inflammatory $Ly6c^{high}$ monocytes (241). Accordingly, we found a link between increased TMAO levels and high proportion of intermediate $CD14^{++}CD16^{+}$ monocytes subsets in high-risk patients.

Notably, in two clinical studies using optical coherence tomography to assess coronary plaque characteristics, increased plasma trimethylamine-N-oxide levels were linked to increased incidence of plaque rupture supporting a critical role of TMAO in vascular inflammation (227, 242). Beyond the pathomechanistic link between TMAO and atherosclerotic plaque development, TMAO has also been reported to promote atherothrombosis by increased platelet activation (243) and tissue factor expression in endothelial cells (244). In recent studies, microbial enzymes that process the generation of TMA have been targeted using small molecule inhibitors that can modulate TMA/TMAO levels and detrimental cardiovascular effects in experimental settings (245). These studies may open new avenues to develop novel strategies for atheroprotection by targeting specific gut microbial enzymes to modulate their metabolic activity.

Therapeutic Potential of Dietary Interventions to Modulate Short Chain Fatty Acids

Beyond metabolites with detrimental effects on the cardiovascular system, recent studies have placed a spotlight on microbially produced short chain fatty acids (SCFA), such as acetate, butyrate and propionate, from fiber-rich diet as potentially health promoting metabolites. Dietary intervention studies have highlighted the rapid increase in the levels of bacteria that metabolize dietary plant polysaccharides to produce SCFA including *Roseburia, Eubacterium rectale* and *Ruminococcus bromii* (246). In one of the largest metagenome-wide association studies to date, Jie et al. found markedly reduced butyrate-producing bacteria in patients with atherosclerotic cardiovascular disease as compared to healthy controls (247) highlighting the potential atheroprotective role of butyrate and possibly other short-chain fatty acids.

Subsequent experimental studies have demonstrated that colonizing germ-free $Apoe^{-/-}$ mice with butyrate *Roseburia intestinalis* mediates atheroprotecting effects by improving intestinal barrier function and reducing the amount of endotoxin in the bloodstream with subsequent reduction in mRNA levels of *Tnf-α* and *Vcam1* in the aortic wall. Notably, germ-free $Apoe^{-/-}$ mice colonized with the *R. intestinalis* showed reduced atherosclerotic lesion size with lower number of macrophages and increased levels of collagen, suggesting that colonization with *R. intestinalis* promotes the stability of atherosclerotic plaques (231).

Another SCFA with potential atheroprotective properties is propionate which has been studied in different models of atherosclerosis. Bartolomaeus et al. studied propionate in an hypertension induced atherosclerosis model by infusion of $ApoE^{-/-}$ mice with angiotensin II (Ang II) (248). While Ang II increased splenic effector memory T-cells (T_{EM}) and decreased splenic $CD4^{+}$ naive T-cells (T_N), these shifts in T-cell populations were prevented upon treatment with propionate. This was accompanied by reduced aortic $CD4^{+}$, $CD8^{+}$ T cell, and $F4/80^{+}$ macrophage numbers after propionate treatment. Similar to the effects on splenic immune cells, propionate decreased the frequencies of aortic $CD4^{+}$ T_{EM} and increased the frequencies of $CD4^{+}$ T_N cells. These effects translated into a reduction in atherosclerotic lesion burden. In addition to the systemic effects on T-cell immunity, our group very recently identified alteration of intestinal immune system with beneficial effects on lipid metabolism in an hypercholesterolemic atherosclerotic model (249). In particular, we found an increase of intestinal $CD25^{+}Foxp3^{+}T_{regs}$ and elevated IL-10 levels in the intestinal microenvironment, which in turn downregulated the expression of the intestinal cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) with subsequent decrease in intestinal cholesterol absorption. Consequently, propionate reduced pro-atherogenic lipoproteins resulting in reduced atherosclerotic plaque lesions.

Collectively, these findings suggest a significant potential of increasing SCFA bioavailability to prevent and treat atherosclerotic cardiovascular disease. However, their long-term metabolic effects and consequences on cardiovascular outcome need to be further explored in future studies.

CONCLUSION

Immune cells orchestrate a chronic smoldering inflammation in the vessel wall that drives atherosclerosis development, progression, and destabilization. Pathogenic conversion of autoreactive regulatory T-cells and smooth muscle cells disrupts the sensitive balance between proinflammatory and protective immunity in the plaque. Leukocyte clones that carry CHIP driver mutations and trained hyperreactive myeloid cells boost this inflammatory response. Systemic infection and acute mental stress escalate plaque inflammation partially by hyper-acute recruitment of proinflammatory leukocytes into the vessel wall. Inflammation likely provides the missing element responsible for the cardiovascular risk that remains despite aggressive lipid-lowering and traditional risk factor control. Recent investigations have demonstrated the potential of anti-inflammatory drugs to lower secondary adverse cardiovascular events, and this is certainly only the beginning. Advances in multi-omics, cell tracing, and machine learning have elucidated a plethora of new possible targets for anti-inflammatory therapy that await trial. Inflammation drives plaque disruption which dramatically manifests as myocardial infarction. This can be caused by rupture of the fibrous cap or superficial erosion, which involves thrombus formation on an intact fibrous cap. The pathomechanisms that underly these two entities seem to be fundamentally different and may require tailored personalized therapeutic strategies. If

this holds true in large scale clinical studies, it would implicate a fundamental shift in the management of ACS patients. The discovery of a reliable biomarker of plaque phenotype would entail the possibility that more than one third of ACS patients may not require invasive diagnostics, saving them any associated complications. Our joint future effort should be directed toward developing therapeutic concepts that individually stratify ACS patients according to plaque morphology and inflammatory profile, thus precisely targeting individual needs.

AUTHOR CONTRIBUTIONS

TG designed and structured the article and determined contents. TG and AH drafted the first version of the manuscript. TG and PS prepared the figures. DL provided critical revisions for important intellectual content of this article. All authors have read and given final approval of the current version of the article.

FUNDING

TG received funding support by grants from the German Cardiac Society (DGK) and the German Centre for Cardiovascular Research (DZHK). PS was supported by the DGK Otto-Hess-Fellowship. DL reports research grants from the DZHK and the BIH.

ACKNOWLEDGMENTS

TG is part of the Berlin Institute of Health (BIH) Junior Clinician Scientist Program. AH is participant in the BIH-Charité Advanced Clinician Scientist Pilotprogram funded by the Charité – Universitätsmedizin Berlin and the Berlin Institute of Health. We thank Dr. Marcel Knöchelmann for language editing and critical reading of the manuscript.

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Conflict of Interest: DL reports conflict of interest by research and educational grants as well as speaker fees from Abbott Vascular.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Cardiovascular Medicine

REVIEW
published: 11 January 2022
doi: 10.3389/fcvm.2021.813984

Where the Action Is—Leukocyte Recruitment in Atherosclerosis

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Atherosclerosis is the leading cause of death worldwide and leukocyte recruitment is a key element of this phenomenon, thus allowing immune cells to enter the arterial wall. There, in concert with accumulating lipids, the invading leukocytes trigger a plethora of inflammatory responses which promote the influx of additional leukocytes and lead to the continued growth of atherosclerotic plaques. The recruitment process follows a precise scheme of tethering, rolling, firm arrest, crawling and transmigration and involves multiple cellular and subcellular players. This review aims to provide a comprehensive up-to-date insight into the process of leukocyte recruitment relevant to atherosclerosis, each from the perspective of endothelial cells, monocytes and macrophages, neutrophils, T lymphocytes and platelets. In addition, therapeutic options targeting leukocyte recruitment into atherosclerotic lesions—or potentially arising from the growing body of insights into its precise mechanisms—are highlighted.

Keywords: atherosclerosis, vascular inflammation, leukocyte recruitment, adhesion molecules, integrin, transendothelial migration

OPEN ACCESS

Edited by:
Holger Winkels,
Universitätsklinikum Köln, Germany

Reviewed by:
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University of Minnesota, United States
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *12 November 2021*
Accepted: *15 December 2021*
Published: *11 January 2022*

Citation:
Mauersberger C, Hinterdobler J, Schunkert H, Kessler T and Sager HB (2022) Where the Action Is—Leukocyte Recruitment in Atherosclerosis. Front. Cardiovasc. Med. 8:813984. doi: 10.3389/fcvm.2021.813984

INTRODUCTION

Atherosclerosis is a chronic disease characterized by the accumulation of lipoprotein particles and inflammatory cells inside the arterial vessel wall of large- and medium-sized arteries. Atherosclerotic plaques may destabilize during the progression of the disease leading to plaque rupture/erosion ultimately resulting in partial or complete vessel obstruction which may cause cardiovascular events such as myocardial infarction (MI) or stroke (1). Altogether, cardiovascular diseases represent the leading cause of death worldwide (2). Mechanistically, atherosclerosis has long been considered a solely metabolic-driven disease; a result of high plasma lipid levels and passive uptake of cholesterol into the vessel wall at atherosclerosis-prone regions marked by disturbed blood flow patterns (3, 4). Over the past decades, however, evidence accumulated highlighting the contribution of immune cells in the etiology of atherosclerosis (4). Leukocytes, the effector cells of the immune system, contribute to all stages of the disease. Specifically, monocyte-derived macrophages, neutrophils and T lymphocytes are involved in inflammatory processes inside the vessel wall during lesion initiation, progression and rupture (5, 6). Thus, leukocyte recruitment to the vessel represents an essential early step in initiation and progression of atherosclerosis preceding local actions of intimal leukocytes. In this review, we aim to summarize basic concepts of leukocyte recruitment and highlight novel findings in the context of atherosclerosis.

BASIC CONCEPTS OF LEUKOCYTE RECRUITMENT IN VESSELS

Leukocyte recruitment from blood to distinct tissues is essential in both non-sterile and sterile inflammation but also under steady-state conditions (7). For simplicity, this review will focus on leukocyte recruitment to diseased vessels in the context of atherosclerosis. Classically, the major players in the recruitment process are the endothelium and different leukocyte subsets including monocytes, neutrophils and lymphocytes. To achieve selective recruitment, all players interact in a strictly orchestrated manner (8). The traditional model of the leukocyte recruitment cascade describes three major steps following tissue/endothelial cell (EC) activation: rolling, activation, and arrest (9). However, recent experimental evidence has expanded our knowledge on the leukocyte recruitment process, suggested additional steps, and refined the molecular principles underlying different stages (10). Within the next paragraph, we aim to outline basic and novel concepts of leukocyte recruitment from the circulation.

Inflammatory Tissue Activation

Inflammatory tissue activation is the initial step in the leukocyte recruitment cascade in both non-sterile (infectious) and sterile (non-infectious) diseases. It occurs as a physiological response of the immune system to various stimuli including tissue damage and cell death, pathogens, or toxic compounds (11). While some responses are shared between non-sterile and sterile diseases, some are specific to certain pathologies. In this context, atherosclerosis-specific mechanisms will be further outlined in later sections.

Classically, acute inflammation is triggered by conserved pathogen-associated molecular patterns (PAMPs) and endogenous stress signals [damage-associated molecular patterns (DAMPs)] that are recognized by respective receptors on tissue-resident immune cells and non-immune cells (12, 13). Activation of these receptors results in the release of pro-inflammatory cytokines and chemokines (14–16). ECs are one of the main targets of pro-inflammatory cytokines (17, 18). As a result, they upregulate adhesion molecule and chemokine expression. Some cytokines such as histamine activate ECs by binding to G-protein-coupled receptors (GPCRs; = type I activation) and induce intracellular signaling cascades that lead to rapid translocation of preformed molecules (19). In contrast, type II activation of ECs is slower but longer lasting (20). It can be triggered by various inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin (IL)1-β, and leads to *de novo* synthesis of adhesion molecules and chemokines (20, 21). The expression and extracellular secretion of various chemokines such as C-C motif chemokine ligand (CCL)2 and C-X-C motif chemokine ligand (CXCL)1 leads to—among other functions—attraction of leukocytes (=chemotaxis) (22–24). Moreover, activated tissue-resident leukocytes, specifically macrophages, can also secrete chemotactic molecules such as CCL3 (23). In addition, activated platelets can deposit chemokines such as CCL5 and CXCL4 on ECs contributing to the chemotaxis of leukocytes to sites of inflammation (25, 26). Of note, differential recruitment of leukocyte subsets is favored by the specificity of certain chemotactic molecules and their respective receptors on leukocytes (27, 28).

In summary, inflammatory tissue activation precedes actual leukocyte recruitment by priming ECs and inducing leukocyte chemotaxis.

Leukocyte Tethering and (Slow) Rolling

Leukocyte tethering (=capture) and subsequent rolling is the first interaction step between ECs and leukocytes. It is mainly mediated by platelet (P)-selectin, endothelial (E)-selectin and leukocyte (L)-selectin (29). Although first described in platelets, P-selectin is also expressed on activated ECs. Selectins consist of an extracellular N-terminal lectin domain, an epidermal growth factor-like domain, a series of repetitive complement control proteins, a transmembrane domain and a C-terminal intracellular domain (20). With their N-terminal lectin domain, they are able to bind to glycosylated ligands in a calcium-dependent manner on the cell surface of opposite cells (30). Of note, selectin binding is highly dependent on correct glycosylation involving modifications by several enzymes that link various types of saccharide molecules (10, 31).

In vessels, leukocyte rolling is predominantly achieved by the interaction of endothelial E- and P-selectin with P-selectin glycoprotein ligand-1 (PSGL-1) and other glycosylated ligands [e.g., CD44 and ESL-1 (E-selectin ligand 1, specifically binding to E-selectin)] on leukocytes (32–36). In ECs, P-selectin is prestored in vesicles called "Weibel-Palade bodies" and translocated to the luminal membrane as a response to inflammatory stimuli,

Abbreviations: AGE, advanced glycosylation end product; ApoB-100, apolipoprotein B 100; *Apoe*$^{-/-}$, apolipoprotein E knockout; CAD, coronary artery disease; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CD, cluster of differentiation; cGMP, cyclic guanosine monophosphate; CICADA, CoronAry heart DiseAse study; COPD, chronic obstructive pulmonary disease; CX3CL1, C-X3-C motif chemokine ligand 1; CX3CR1, C-X3-C motif chemokine receptor 1; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; ESL-1, endothelial selectin ligand 1; F-actin, filamentous actin; FLIPR, flow-induced protrusions; FOXP3, forkhead box protein P3; GPCR, G-protein-coupled receptor; GPIbα, glycoprotein Ibα; GWAS, genome-wide association study; ICAM-1/-2/-3, intercellular adhesion molecule 1/2/3; IL, interleukin; JAM-A/-C, junctional adhesion molecule; Klf2, Krüppel-like factor 2; LBRC, lateral border recycling compartments; LDL, low density lipoprotein; *Ldlr*$^{-/-}$, low density lipoprotein receptor knockout; LFA-1, lymphocyte function-associated antigen 1; LOX-1, lectin-like oxidized LDL receptor 1; Ly6C, lymphocyte antigen 6 complex, locus C; Mac-1, macrophage receptor 1; MMP, matrix metalloproteinase; NET, neutrophil extracellular traps; NF-κB, nuclear factor κB; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; NO, nitric oxide; *NOS3,* nitric oxide synthase 3 (= eNOS) gene; Nrp1, neuropilin 1; oxLDL, oxidized low density lipoprotein; PAMP, pathogen-associated molecular patterns; PECAM-1, platelet/endothelial cell-adhesion molecule 1; PCSK9, proprotein convertase subtilisin/kexin type 9; PSGL-1, platelet selectin glycoprotein ligand 1; Rac1, Ras-related C3 botulinum toxin substrate 1; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; S100A8/9, S100 calcium-binding protein A8; sLox-1, soluble lectin-like oxidized LDL receptor 1; sGC, soluble guanylyl cyclase; SGEF, SH3-containing guanine nucleotide exchange factor; SNP, single nucleotide polymorphism; TAZ, transcriptional coactivator with PDZ-binding motif; TCR, T cell receptor; Th1/2/17, T helper cell subtype 1, 2, 17; TLR, Toll-like receptor; TNFα, tumor necrosis factor alpha; Treg, regulatory T cell; Trio, triple functional domain protein; VCAM-1, vascular cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin; VEGFR, vascular endothelial growth factor receptor; VE-PTP, vascular endothelial protein tyrosine phosphatase; VLA-4, very late antigen 4; YAP, yes-associated protein.

while E-selectin is synthesized *de novo* upon cell activation (19). L-selectin also interacts with PSGL-1 but is mainly expressed on leukocytes and thus important specifically in secondary leukocyte capture (37, 38). L-selectin on leukocytes was also shown to interact with glycosylated ligands on the endothelial membrane (39–41). However, these experiments were mainly performed in the context of lymph node homing. Still, PSGL-1 is expressed on both leukocytes and vascular ECs (42), which suggests a relevant contribution of L-selectin-mediated rolling in vessels. Additional to leukocyte-leukocyte interactions, platelet-leukocyte interactions are involved in secondary leukocyte capture processes (43, 44). Platelet P-selectin can engage with both endothelial and leukocyte PSGL-1, thereby acting as a bridge between the two cell types. Recent studies have identified further relevant players, such as the interaction of leukocyte macrophage receptor 1 (Mac-1) with platelet CD147 (45) in platelet-mediated leukocyte recruitment. In addition to membrane-bound selectins, extracellular matrix proteins, such as galectins, are likely to be involved in the leukocyte adhesion cascade including rolling (46). Here, extracellular galectins might bind glycosylated ligands on both ECs and leukocytes thus facilitating further interactions. Indeed, slow rolling was impaired in Galectin-3 knockout mice (47). Taken together, leukocyte rolling on ECs is mediated by the interplay of molecules on both cell types. Regarding the important role of endothelial P- and E-selectin, prior EC activation and subsequent selectin expression is key to initiate the leukocyte rolling process.

Recent experimental evidence indicated so-called "integrin-mediated rolling" and "slow rolling" as intermediate steps between rolling and firm arrest (10, 48). Integrin-mediated rolling is achieved by a transient interaction of leukocyte integrins in an intermediate conformational state with their respective adhesion molecules on ECs (10). To some extent, integrin-mediated rolling is also selectin-dependent (49, 50). Although selectins do not bind to leukocyte integrins directly, they can induce integrin activation via an intracellular signaling cascade, for example via leukocyte PSGL-1 (51–53). As a consequence, leukocyte integrins undergo conformational changes that result in increased binding to respective endothelial adhesion molecules (54). Of note, also soluble selectins, known biomarkers for inflammation (55, 56), can induce integrin activation (57). Slow rolling can be viewed as a specific type of integrin-mediated rolling: It is induced by pro-inflammatory cytokine exposure, and mediated mainly by the subsequent upregulation of endothelial E-selectin which induces integrin activation (10, 58).

As described above, myeloid cells, namely monocytes/macrophages and neutrophils, exert key functions in atherosclerosis. Recent evidence suggests that neutrophils are among the first cells recruited to inflamed tissues, thereby facilitating subsequent monocyte uptake (59, 60). This suggests underlying differences in the recruitment cascade of neutrophils and monocytes. Indeed, differences in integrin-mediated rolling and slow rolling have been described. In monocytes, integrin-mediated rolling is mainly conveyed via ß1-integrins such as very late antigen 4 (VLA-4) (10, 61). In contrast, ß2-integrins such as lymphocyte function-associated antigen 1 (LFA-1) and Mac-1 seem to be crucial for integrin-mediated rolling and slow rolling in neutrophils (49, 58, 62).

Taken together, initial leukocyte tethering and subsequent rolling and slow rolling is essential to enable leukocyte contact with ECs.

Leukocyte Activation and Arrest

Following leukocyte rolling, leukocytes need to firmly adhere to the endothelium for further transmigration. This is achieved by stable interactions between leukocyte integrins and endothelial adhesion molecules (19). However, this process requires prior activation of leukocyte integrins (63). Integrin activation is mainly mediated via so-called "inside-out signaling"; that means the activation of intracellular signaling cascades in response to chemokine binding to dedicated receptors on leukocytes (19). Concretely, secreted chemokines that are present in the extracellular glycocalyx bind to GPCRs on leukocytes, which results in conformational (=affinity) and expression density (=avidity/valency) changes (8, 64). Traditionally, chemokines were supposed to be presented by EC-bound glycosaminoglycans (65, 66). This is contrasted by new experimental studies indicating only transient interactions between chemokines and glycosaminoglycans, which allows retention of chemokines in the glycocalyx space close to the endothelium. However, most likely mainly free chemokines have the ability to bind to GPCRs on leukocytes (67). Chemokines inducing integrin activation are often the same molecules relevant for chemotaxis but quantitative differences in receptor expression and ligand binding may explain the differences in chemoattractant and pro-adhesive response (68).

Upon chemokine binding to GPCRs, complex intracellular signaling cascades get activated which are reviewed in detail elsewhere and are still not deciphered completely (8, 69). One major downstream effect, as mentioned above, are conformational changes in the extracellular domains of leukocyte integrins (from a bent, low-affinity conformation to an extended, high-affinity conformation) (69). Recently, talin and kindlin-3 have been identified as two intracellular molecules that bind to the cytoplasmic tail of integrins and, independent of each other, increase integrin affinity via conformational changes (8, 70–73).

Following activation, heterodimeric leukocyte integrins engage with their counterreceptors on ECs and consequently, leukocytes firmly adhere to the endothelium. Classical integrin-adhesion molecule combinations include interactions between VLA-4 with vascular cell adhesion molecule 1 (VCAM-1), LFA-1 with intercellular adhesion molecule (ICAM)-1, ICAM-2 and ICAM-3, and Mac-1—which is specifically described in human neutrophil arrest (74)—with ICAM-1 (75–79). Additionally, Mac-1 binding to CD40 ligand (CD40L) has been recently identified to contribute to the leukocyte arrest process (80–82).

In contrast to "inside-out signaling," "outside-in signaling" describes the process in which signals are transduced into leukocytes upon integrin engagement (83). Several studies suggest that this mechanism is specifically important in post-arrest adhesion strengthening and further leukocyte activation (83–85).

Firm arrest is crucial for leukocyte emigration into inflamed tissues as it paves the way for final transmigration through the vascular endothelium.

Crawling and Leukocyte Transmigration

Compared to the preceding steps of the leukocyte recruitment cascade, detailed understanding of the final step, leukocyte transmigration, has been achieved fairly recently. Intraluminal crawling is essential to later leukocyte transmigration as cells can thereby migrate to preferred sites of transmigration (86). Leukocyte Mac-1 and LFA-1 binding to endothelial ICAM-1 play a key role in the process of crawling (87–90). Crawling and subsequent leukocyte transmigration is promoted by various stimuli (10). In particular, binding of leukocyte integrins to adhesion molecules was shown to induce EC activation (91), comparable to the outside-in-signaling as observed during firm arrest in leukocytes. Concretely, integrin-adhesion molecule interactions result in the clustering of adhesion molecules in specific EC regions yielding ICAM-1- and VCAM-1-rich domains (10, 92, 93).

Leukocyte transmigration can happen via two different routes: the classical, paracellular route (migration between two EC bodies) or a transcellular route (migration through thin parts of an EC body) (89). If migration happens via the paracellular route, EC junctions need to be modified transiently to reduce contact between adjacent ECs. This is achieved, for example, by active squeezing of leukocyte nuclei to disassemble endothelial actin filaments (94), by EC contraction mediated by cytoplasmic structural proteins and by reduced expression of EC adherens junctions such as vascular endothelial cadherin (VE-cadherin) (51, 95). Mechanistically, this is supposed to be triggered by intracellular signaling cascades as a response to LFA-1 and VLA-4 binding and subsequent ICAM-1 and VCAM-1 clustering on EC, which results in increased cytosolic calcium levels leading to enhanced myosin light chain kinase activity, dissociation of vascular endothelial protein tyrosine phosphatase (VE-PTP) from VE-Cadherin and phosphorylation as well as dephosphorylation of distinct tyrosine residues on VE-cadherin (96–98). As a result, VE-cadherin, which is linked to the actin-cytoskeleton via catenins, dissociates from this connection and is internalized, thus contributing to loosening of endothelial tight junctions (99). By contrast, other adherens junction molecules, such as platelet/endothelial cell-adhesion molecule 1 (PECAM-1/CD31) and CD99, but also tight junction molecules, such as junctional adhesion molecule (JAM)-A, are actively transported to the site of diapedesis in so-called lateral border recycling compartments (LBRC) (8, 100). By both homophilic (leukocytes express identical molecules) and heterophilic (leukocytes express non-identical ligands) interactions, endothelial adhesion and junctional proteins achieve shuffling of the migrating leukocyte through the lateral border (91, 101, 102).

A second route using transcellular migration has been described, specifically at thin parts of ECs (93, 103). In this case, tight junctions between ECs remain intact (103). Instead, leukocytes are transported through the EC body by caveolin 1-rich and adhesion molecule-rich (specifically ICAM-1) transcellular pores (95).

However, despite our increasing knowledge on the molecular processes of para- and transcellular leukocyte migration, the mechanisms that decide on the actual migration route have not yet been deciphered completely. The decision is most likely based on a combination of factors including vessel type (macrovascular vs. microvascular), leukocyte subset, tight junctional organization, inflammatory activation and other, yet unidentified aspects (89, 104).

LEUKOCYTE RECRUITMENT IN ATHEROSCLEROSIS

Whereas the molecular mechanisms of leukocyte recruitment are being elucidated in increasing detail, our knowledge of the specific processes that drive leukocyte migration into atherosclerotic plaques is still rather incomplete. It is likely that there are many shared steps, although some players appear to be more important in atherosclerosis than in recruitment cascades in other tissues. While the involvement of different leukocyte populations in atherosclerosis is known since long on an observational level from histological specimens, most of our current understanding in this field is derived from experiments with induced atherosclerosis in genetically altered mice. Important models for this are mouse lines lacking the genes for apolipoprotein E (*Apoe*) or low density lipoprotein receptor (*Ldlr*) fed a cholesterol-enriched diet (in the following referred to as $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice, respectively).

Increasingly, these findings may also be supported by the results of genetic or proteomic association studies in humans: Single nucleotide polymorphisms (SNPs), for example, are used to compare the individual genetic profile of coronary artery disease (CAD) patients and healthy controls, and the enrichment of certain SNPs in the patient cohort can accordingly be linked to CAD in so-called genome-wide association studies (GWAS). Thus, with increasing numbers of SNPs and individuals included, a large number of variants associated with CAD have already been identified (105, 106). Although many of these SNPs reside in areas of unknown function in the genome, some of the associated genes have already been linked to leukocyte recruitment processes (107). Bringing together knowledge derived from these different approaches, we aim to summarize and discuss the currently known important players in immune cell recruitment into atherosclerotic plaques below (**Figure 1**).

Endothelial Cell Priming

Keep It Flowing

The endothelium is permanently exposed to blood flow-induced shear stress and adapts to changes in flow by several immediate responses, e.g., conformational remodeling of the glycocalyx, opening of ion channels, and activation of different membrane receptors such as GPCRs and integrins (108). A central role in these mechanotransduced responses can be attributed to the mechanosensory PECAM-1, VE-cadherin and vascular endothelial growth factor receptor (VEGFR) complex: Acute onset of laminar flow promotes PECAM-1 phosphorylation followed by Src-dependent phosphorylation of VEGFR-2 and−3,

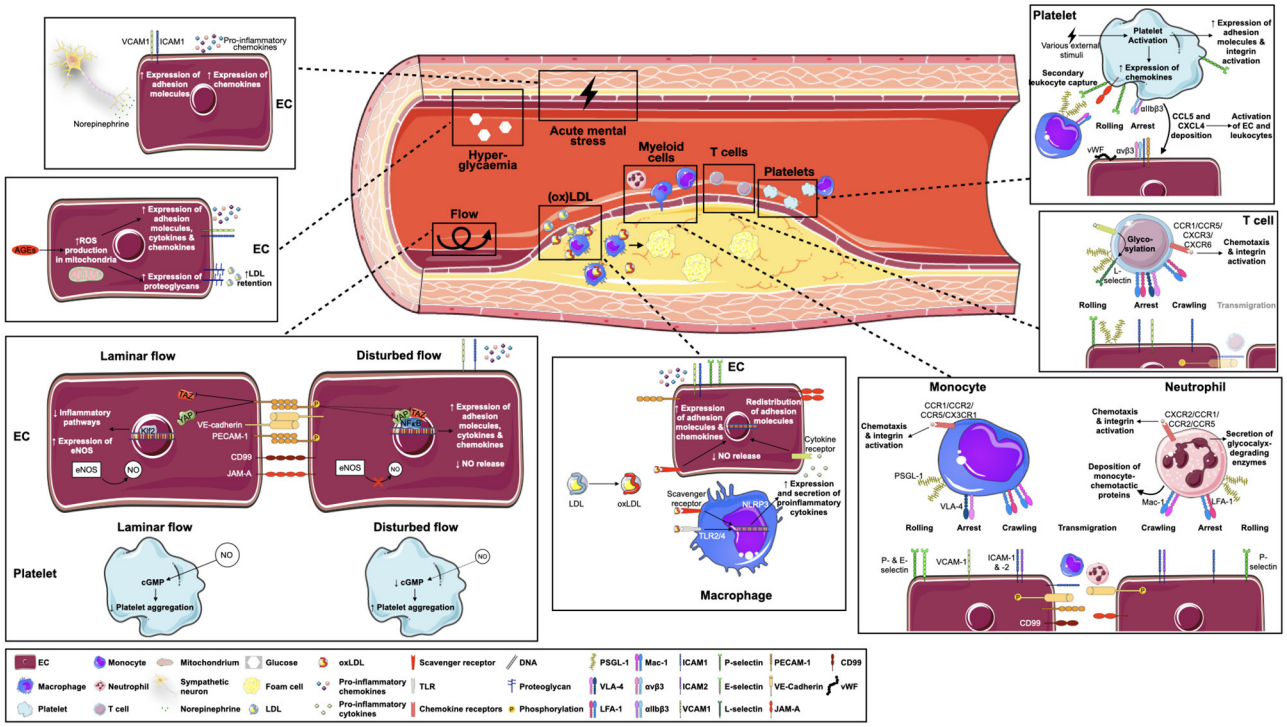

FIGURE 1 | Key factors in atherosclerosis-specific leukocyte recruitment. Leukocyte recruitment into atherosclerotic plaques is multifaceted and involves several players. Endothelial activation through disturbed flow patterns and oxidized lipoproteins, but possibly also via hyperglycemia or local sympathetic innervation, forms the basis for subsequent leukocyte-endothelial interactions. Of note, specific leukocyte populations respond differently to atherogenic triggers and use unique molecules and receptors to achieve leukocyte rolling, arrest, crawling and transmigration. Additionally, platelets strongly contribute to leukocyte recruitment by secondary leukocyte capture and activation of endothelial cells and leukocytes. AEG = advanced glycosylation end products; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CD99, cluster of differentiation 99; cGMP, cyclic guanosine monophosphate; CXCL, C-X-C motif chemokine ligand; CX(3)CR, C-X(3)-C motif chemokine receptor; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; E-selectin, endothelial selectin; ICAM-1/2, intracellular adhesion molecule 1/2; Klf2, Krüppel-like factor 2; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; L-selectin, leukocyte selectin; Mac-1, macrophage receptor 1; NO, nitric oxide; (ox)LDL, (oxidized) lipoprotein; PECAM-1, platelet endothelial cell adhesion molecule 1; P-selectin, platelet selectin; PSGL-1, P-selectin glycoprotein ligand 1; ROS, reactive oxygen species; TLR, toll-like receptor; VCAM-1, vascular adhesion molecule 1; VE-cadherin, vascular endothelial cadherin; VLA-4, very late antigen 4; vWF, von Willebrand factor, YAP, yes-associated protein.

proteins which are both linked to PECAM-1 via VE-cadherin and subsequently activate multiple intracellular pathways (109–111). Many downstream functions of this complex are transmitted via phosphatidylinositol 3-kinase/Akt signaling, e.g., leading to global activation of β1-integrins and the small GTPase RhoA which finally triggers promotion of focal adhesions, cytoskeletal adaption and alignment of the cell in the direction of flow (112, 113), or activation of endothelial nitric oxide (NO) synthase (eNOS) leading to NO-mediated vasodilation (114–116). Lastly, these processes stimulate the activation of transcription factors such as Krüppel-like factor 2 (Klf2) (117) and inhibit pro-inflammatory action of the Hippo pathway effectors yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which have been identified as key mechanotransducers in response to disturbed flow (118). In summary, initial pro-inflammatory activity triggered by flow onset is followed by alignment of the cell in the direction of flow, enhanced NO production and inhibition of inflammatory pathways, maintaining an anti-inflammatory status of the endothelium in response to laminar shear.

In contrast, disturbed flow, which preferentially occurs in branches, bifurcations and curvatures of the vessel—regions, where atherosclerotic plaques are mainly found—has been shown to lead to different responses: impaired NO release (119, 120), reactive oxygen species (ROS) production (121), deposition of fibronectin and fibrinogen to the subendothelial basement membrane (122, 123), and activation of pro-inflammatory transcription factors such as nuclear factor (NF)-κB and the aforementioned transcription cofactors YAP/TAZ, inducing the expression of several pro-inflammatory proteins such as VCAM-1, ICAM-1, CCL2, IL-6, and CXCL8 (111, 118, 124). Additionally, shear stress response regulatory elements have been found in the promoter of *NOS3*—the gene encoding eNOS—downregulating its expression in response to flow (125). Apart from that, evidence also suggests a flow-dependent expression of Toll-like receptor (TLR) 2 on EC (126, 127). Therefore, permanent changes in shear stress hinder the cell from adapting to the direction of flow and from overcoming the initial pro-inflammatory response phase, rendering these areas prone to leukocyte influx. This is also mirrored by the role of PECAM-1: While loss of *PECAM1* is

associated with reduced NO production in EC exposed to laminar flow (115, 128) and was shown to accelerate the onset of collagen-induced arthritis in mice (129, 130), in regions of disturbed flow, PECAM-1 elicits pro-inflammatory effects in the vasculature via increased NF-κB activation and VCAM-1 expression as shown in *Pecam1*-knockout mouse models (111, 131). Accordingly, more recent findings led to the conclusion that PECAM-1 exerts both pro- and anti-atherosclerotic properties in EC depending on the type of flow (132, 133). Moreover, common variants assigned to *PECAM1* in humans have been associated with CAD in a large GWAS (134), highlighting the role of PECAM-1 in atherosclerosis beyond its known effects in cell culture and mouse models.

Another striking role in the abovementioned process of flow-induced EC priming can be attributed to NO, a gas produced mainly by eNOS that diffuses across cell membranes and, via soluble guanylyl cyclase (sGC), promotes the formation of the second messenger cyclic guanosine monophosphate (cGMP) in various neighboring cells. Among its several important downstream functions are smooth muscle cell relaxation, thus regulating the vascular tone, and platelet aggregation (135). Recently, it was shown that not only NO production in EC but also cGMP formation in one of its effector cells, namely platelets, is strongly shear-dependent (136). Insufficient NO availability, as it may be caused by impaired blood flow, is the major cause of endothelial dysfunction and leads to several pro-atherogenic responses in the vasculature by directly impacting leukocyte recruitment, e.g., increasing NF-κB activation and enhancing endothelial expression of VCAM-1, E-Selectin, and ICAM-1 (137). Furthermore, common polymorphisms in *NOS3*, but strikingly also several other genes of this pathway—among them *GUCY1A1* encoding for the α_1-subunit of sGC and *PDE5A* for a cGMP degrading enzyme—have been linked to coronary artery disease susceptibility in GWAS (138–140) and once again highlight the role of this pathway in atherogenesis.

oxLDL and Other Evildoers

The second important pillar for atherosclerotic EC priming is attributed to inflammatory mediators. While the role of PAMPs in atherogenesis is not investigated to a large extent, although the role of viruses and bacteria on plaque progression or rupture has been increasingly recognized (141), most research refers to the sterile character of atherosclerotic inflammation. In this context, modified low density lipoprotein (LDL) plays a fundamental role.

LDL from the circulation can be incorporated by EC either by receptor-mediated endocytosis or caveolae mediated transcytosis (142, 143), whereby the latter one is generally regarded as the more relevant way of atherogenic LDL accumulation (144). In the subendothelial space, LDL is retained by extracellular matrix proteoglycans (145) and, catalyzed by enzymes such as lipoxygenases or myeloperoxidases, metal ions and free radicals, chemically modified to various degrees. This leads to the formation of strongly pro-atherogenic LDL variants such as oxidized LDL (oxLDL) (137). The exact process of LDL oxidation is not fully elucidated but is supposed to be linked to oxidative stress resulting from a disbalance between ROS production and antioxidant defense mechanisms (146), as triggered, for example, by aging (147) or smoking (148). As a consequence, LDL loses its ability to bind the LDL receptor but strongly enhances affinity for scavenger receptors such as CD36 and lectin-like oxidized LDL receptor-1 (LOX-1) on EC, vascular smooth muscle cells, and macrophages (149). Intriguingly, a degradation product of LOX-1 can also be found in plasma [soluble Lox-1 (sLox-1)] and has recently emerged as a potential biomarker for cardiovascular disease incidence (150).

Ultimately, among the myriad pro-atherogenic responses of EC to such modified LDL are inhibition of NO production (151, 152), regulation of microRNAs (153), enhanced expression of E- and P-selectin, VCAM-1, ICAM-1 (154, 155), CCL2, CXCL2, 3 and 8 (156), and redistribution of JAM-A to facilitate transmigration (157)—thus paving the way for leukocyte infiltration. Therefore, oxidation of LDL is without doubt a central aspect of atherogenesis (149). However, while oxLDL is the best-studied form of modified LDL, there are several other modifications of LDL with pro-atherogenic properties, such as desialylation (158) or sphingomyosinase-induced aggregation (159).

Strikingly, these processes appear to begin already very early in life in genetically predisposed individuals (160), highlighting the influence of heritable factors on atherogenesis, most of which remain unexplored (161). Among the known contributors to atherogenic EC priming, however, is also hyperglycaemia. For example, hyperglycaemia driven accumulation of advanced glycosylation end products (AGEs) in vessels promotes ROS formation and adhesion molecule expression in EC, and release of pro-inflammatory cytokines such as IL-1β, IL-6, CCL2 and CXCL8 from leukocytes (162). Moreover, it impairs eNOS function and promotes the expression of proteoglycans, associated with increased LDL retention in the vascular wall (137, 163). Another important driver of atherogenesis is psychological stress (164, 165). Just recently, we showed that acute mental stress promotes atherosclerosis-related recruitment of leukocytes in mice by increasing the expression of endothelial adhesion molecules and the release of chemokines (166), adding to the knowledge of neuroimmune linkages in atherosclerosis.

Stage Free for Leukocytes

In response to upregulated adhesion molecules on EC and an increased chemokine gradient, leukocyte adhesion is initiated. However, this is not a static "one after the other" process. Several inflammatory stimuli emanating from vascular cells, platelets and leukocytes—as a response to the flow and lipid-driven EC priming, but also interlocking from the very beginning—continuously contribute to EC activation. Therefore, this section will focus on the role of the second important player in the atherosclerotic recruitment process: leukocytes.

The Classic: Monocytes

Monocytes and macrophages are the central figures in the history of atherosclerosis research. Once migrated, monocytes differentiate into macrophages, the major leukocyte population within atherosclerotic plaques (167), which strongly engulf modified LDL and fulfill several proatherogenic functions. There

are three main subsets of monocyte populations in humans, that is classical $CD14^{++}$ $CD16^{-}$ and non-classical $CD14^{+}$ $CD16^{++}$ monocytes and a small, less investigated group of intermediate $CD14^{+}$ $CD16^{+}$ monocytes (168, 169). In mice, C-C motif chemokine receptor (CCR)2^{+} C-X3-C motif chemokine receptor (CX3CR)1^{+} lymphocyte antigen 6 complex, locus C $(Ly6C)^{high}$ monocytes are closely related to the classical cells in humans and $CCR2^{-}$ $CX3CR1^{++}$ $Ly6C^{low}$ monocytes to the non-classical population (170). Although fate mapping and adoptive transfer experiments suggest that $Ly6C^{low}$ monocytes are derived from $Ly6C^{high}$ cells, hypercholesterolaemia is associated with impaired $Ly6C^{low}$ formation, despite a strong expansion of the $Ly6C^{high}$ population leading to systemic monocytosis (171–173). Importantly, both monocyte subsets fulfill different roles in monocyte recruitment. In steady state, $Ly6C^{low}$ monocytes are dependent on CX3CL1 (fractalkine) stimulation via CX3CR1 and are constantly patrolling the vessel by communicating with endothelial ICAM-1 and−2 via LFA-1, but scarcely transmigrate (174). Although in the setting of tissue damage it has been suggested that they are among the first cells to extravasate and to promote recruitment of other leukocytes by release of inflammatory cytokines (175), $Ly6C^{low}$ monocytes were associated with markedly less recruitment to atherosclerotic plaques than $Ly6C^{high}$ monocytes (176). However, under atherosclerotic conditions their patrolling behavior is strongly upregulated in a CX3CR1-independent manner, and genetic depletion of $Ly6C^{low}$ monocytes was associated with pronounced endothelial apoptosis, suggesting an important role for endothelial maintenance in atherosclerosis (177). Nonetheless, in the following, we focus on the recruitment of inflammatory monocytes.

An initial trigger for leukocyte extravasation are local chemotactic gradients. For monocytes, CCL2 is a key chemokine that targets the CCR2 receptor highly expressed on classical/ $Ly6C^{high}$ monocytes (28). Both *Ccr2* and *Ccl2* knockout in atherosclerosis-prone mice (178, 179) as well as *Ccr2* targeted siRNA treatment (180) significantly reduced atherosclerotic plaque formation, whereas in contrast, leukocyte-specific overexpression of *Ccl2* in $Apoe^{-/-}$ mice promoted atherosclerosis progression (181). In humans, CCL2 levels in atherosclerotic lesions have only recently emerged as a potential indicator of plaque vulnerability (182). Interestingly, individuals with familiar hypercholesterolemia—a strong genetic predisposition to atherosclerosis—were found to have a 3-fold higher CCR2 expression on classical monocytes than healthy subjects, whereas cholesterol lowering therapy with a proprotein convertase subtilisin/kexin type 9 (PCSK9) antibody reduced monocyte CCR2 surface expression by 60% in these patients (183). This is further evidence that lipid-related and inflammatory processes strongly interact. Yet, despite the striking role of CCR2, also CCR5 is crucially involved in $Ly6C^{high}$ monocyte chemotaxis. While *Ccr5* deficiency resulted in reduced mononuclear cell infiltration and lesion formation as well as decreased neointima formation (184–186), combined inhibition of CCL2, CX3CR1, and CCR5 resulted in as much as a 90 % reduction in atherosclerotic plaque formation in $Apoe^{-/-}$ mice, in spite of persistent hypercholesterolaemia (187). However, rather than having chemotactic functions, the CX3CR1/CX3CL1 interaction may impact cell survival (188).

Following attraction to atherosclerosis-prone endothelium, pro-inflammatory monocytes initiate rolling particularly by interaction of PSGL-1 with P- and E-selectin expressed on activated EC. $Apoe^{-/-}$ mice with a functional knockout of the gene encoding for PSGL-1 were shown to develop smaller atherosclerotic plaques (189) and in a similar way, P-selectin deficiency was associated with less leukocyte recruitment in atherosclerosis (190–192). Plaque leukocyte recruitment and consequently plaque size were also decreased when atherosclerotic mice were treated with EC-avid nanoparticles inducing endothelial silencing of P- and E-selectin, in parallel with ICAM-1, ICAM-2 and VCAM-1 (193). While lack of E-selectin alone reduced the progression of atherosclerotic plaque formation only to a minor extent (194), the combined genetic silencing of P-and E-selectin in $Ldlr^{-/-}$ mice even led to an 80% reduction in lesion formation (195). An inhibitory peptide preventing monocyte binding to selectins was shown to decrease monocyte recruitment and subsequently atherosclerotic lesion size, particularly by inhibiting monocyte activation via NF-κB (196). Moreover, intravital microscopy experiments in inflamed cremasteric veins indicated that E-selectin selectively affects the rolling velocity of inflammatory monocytes, whereas the flux of rolling neutrophils is regulated by P- and L-selectin (197). However, it is open whether this is also true for atherosclerotic arteries. As described above, transition of rolling to slow rolling and eventually to firm arrest requires integrin activation on the surface of leukocytes (102). Intravital microscopy in carotid arteries revealed a crucial function of CCR1 and CCR5 in this step for classical monocytes, but not of CCR2 (198). Although monocytes, in common with neutrophils, express the integrins LFA-1 and Mac-1, monocyte arrest seems to depend particularly on the VLA-4-VCAM-1 interaction (199–201). In line, blocking VLA-4 decreased leukocyte recruitment in atherosclerotic mice (202, 203) and functional downregulation of *Vcam1* significantly reduced atherosclerotic lesion formation in mice in a gene-dose dependent manner (204, 205), while similarly, treatment with a VCAM-1 blocking antibody attenuated atherosclerosis in $Apoe^{-/-}$ mice (206). Of note, it has been shown that rolling of monocytes may also be mediated by platelets bound to the extracellular membrane, but rather in a ß2-integrin dependent way (207).

When firmly attached to the endothelium, monocytes engage in crawling behavior in search of ideal sites for extravasation. Thereby, using the intracellular actomyosin machinery for directed movement, they develop integrin-rich protrusions that scan the endothelial lumen for chemotactic directionality (102) mainly by interacting with ICAM-1 or ICAM-2 on EC via both LFA-1 and Mac-1 (208). Experiments in mice could partly confirm the involvement of these adhesion molecules in atherosclerosis: $Apoe^{-/-}$ $Icam1^{-/-}$ mice displayed reduced atherosclerotic lesions (209) and similarly, fatty streak lesion area was smaller in mice deficient for either ICAM-1 or ß2-integrin (the common subunit of LFA-1 and Mac-1), but most distinctly for mice with a double knockout of both encoding genes (210). Also, $Icam1^{-/-}$ $Apoe^{-/-}$ mice were shown to

have significantly reduced atherosclerotic lesions, and soluble levels of ICAM-1 paralleled atherosclerosis progression in *Apoe*$^{-/-}$ mice with significantly elevated plasma concentrations compared to experiment onset (211). However, other studies could not confirm a substantial involvement of ICAM-1 loss on atherogenesis (204, 212). While the participation of LFA-1, on the other hand, was associated with atherosclerosis progression in rats (213), a study in *Ldlr*$^{-/-}$ mice could not endorse significant influence of Mac-1 on atherosclerotic lesion formation (214). Interestingly, a recent publication proposes a divergent influence of ß2-integrin on different stages of atherosclerosis—being protective in the initial phase, but pro-atherogenic in later stages, which could be partly driven by chronic dyslipidaemia (215).

Transmigration, the final step of the recruitment cascade, is facilitated by the redistribution of adhesion molecules such as JAM-A and VE-cadherin and formation of transmigratory cups characterized by local clustering of ICAM-1 and VCAM-1, actin remodeling and the formation of endothelial protrusions developing around the penetrating leukocyte (86). Thereby, also several effectors of the Rho-GTPase family are activated in EC, such as triple functional domain protein (Trio), Ras-related C3 botulinum toxin substrate 1 (Rac1), RhoG and its exchange factor SH3-containing guanine nucleotide exchange factor (SGEF) (216), stimulating the formation of the cup-like structures during transmigration of leukocytes but also promoting ROS production and subsequent activation of matrix metalloproteinases (MMP). In a later step, also RhoA and its effector Rho-associated protein kinase (ROCK) are activated, enlarging the transmigratory gap through enhanced actin-myosin contractility (217). Indeed, SGEF-deficient mice displayed decreased atherosclerotic plaque formation supposedly via reduced formation of endothelial docking structures (218) and similarly, inhibition of ROCK reduces atherosclerosis in mice (219, 220). Strikingly, the genes encoding for RhoA, Rac1 and SGEF were also associated with CAD by GWAS (139, 221–223).

Further, VE-cadherin plays a crucial role in leukocyte transmigration. It is linked to the actin-cytoskeleton via catenins and constitutively associated with the phosphatase VE-PTP, which stabilizes VE-cadherin junctions both by dephosphorylation and inhibition of Rho GTPase signaling (96, 224, 225). Beyond its previously described effects, oxLDL was also shown to directly promote monocyte transmigration by down-regulating VE-cadherin and upregulating PECAM-1 (226). Besides, PECAM-1 deficiency or blocking antibodies have been shown to specifically inhibit transmigration *in vitro* and *in vivo* in various inflammatory disease models in mice (99). Together with CD99, but also JAM-A, PECAM-1 is actively transported to the transmigration site via LBRCs and likely required for leukocyte diapedesis, as blocking of this targeted process resulted in considerably lower monocyte transmigration *in vitro* (227). In line, impaired JAM-A expression in EC or blocking JAM-A by a peptide antagonist inhibited leukocyte recruitment and atherosclerotic plaque formation in hyperlipidemic mice (228, 229). Remarkably, also the expression and distribution of JAM-A appears to respond to changes in flow (230). Given that CD99 also exerts an important influence on monocyte transmigration (231), in an interesting experiment, vaccination directed against CD99 was shown to reduce leukocyte numbers in atherosclerotic plaques and attenuate atherosclerotic lesion formation in mice (232). However, the role of PECAM-1—although quite obviously promoting leukocyte transmigration *in vitro*—seems to be more complex in atherosclerosis in general, as it exerts different influences on atherogenesis partly depending on the hemodynamic environment (see section Keep it Flowing).

Following their recruitment into plaques, monocytes massively differentiate into macrophages and, to a lesser extent, presumably also into dendritic cells (233). However, accumulation of macrophages in atherosclerotic lesions is likely a complex interplay of monocyte recruitment and local macrophage proliferation which also involves tissue-resident macrophages (234, 235). Within plaques, macrophages strongly engulf modified LDL particularly via scavenger receptor A1 (SRA1), LOX-1 and CD36, which is supported by TLR2, 4 and 6 signaling and promoting their phenotypic change to cholesterol-rich foam cells (236). Subsequently, foam cells can induce the release of pro-inflammatory cytokines (237) and *Vcam1* expression in early aortic fatty streaks in mice (238). Excessive cholesterol accumulation may also lead to the formation of cholesterol crystals which trigger activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, a huge cytosolic oligomer inducing cleavage and secretion of IL-1ß and IL-18 (239). However, the inflammatory role of foam cells has been challenged by recent findings, which support a more diverse function in atherosclerosis (240) and suggest non-foamy macrophages to be the actual contributors of pro-inflammatory signaling in atherosclerotic plaques (241). As such, monocyte-derived macrophages again strongly promote endothelial activation and subsequent invasion of additional leukocytes, so permitting the plaque to grow and grow under continuous LDL supply (242).

The Newcomer: Neutrophils

Neutrophils are the most abundant leukocyte population in human blood and it has been just in the course of the last decades that they emerged from more or less neglected bystanders in atherogenesis to forerunners of monocyte infiltration (243). In infection, they are among the first cells to invade into inflamed tissues and promptly release cytotoxic ROS and proteases or form neutrophil extracellular traps (NET) targeted to rapidly eliminate pathogens (77). However, such behavior has also been observed in sterile inflammation as in atherosclerosis, making neutrophils furthermore an important contributor to atherosclerosis progression and complications such as stroke or acute coronary syndrome (244). While many steps of neutrophil recruitment into inflamed tissues are shared with monocytes and therefore close to the mechanisms described above, below, we focus on specific differences in the recruitment behavior of neutrophils.

Comparable to atherosclerotic monocytosis, also neutrophil levels in the blood are frequently increased in atherosclerosis (245) and related to future major adverse cardiovascular events in patients with acute coronary syndrome (246). Neutrophils circulating in the bloodstream are highly sensitive to various chemotactic signals. The traditional view that monocyte

chemotaxis rather depends on CC-chemokines and neutrophil chemotaxis rather on CXC-chemokines is supported by predominant expression of CXC-chemokine receptors in neutrophils (247, 248) and fueled by a recent study concluding that CCR1, CCR2, CCR3, and CCR5 are not involved in neutrophil recruitment in acute inflammation in mice (28). However, several other studies have also demonstrated an important role for CC-chemokines in neutrophil attraction (249), suggesting that a strict separation of monocyte and neutrophil relevant chemokines does likely not represent the whole picture. As follows, important chemokines to trigger neutrophil activation in mice are CXCL1, CXCL2, and CXCL5 (presumably also representing CXCL8 in humans) as well as CCL5. Evasin-3, a pharmacological inhibitor of CXCL1 and CXCL2, reduced intraplaque neutrophil and MMP9 content (250). Similarly, the nicotinamide phosphoribosyltransferase inhibitor FK866, which was shown to strongly inhibit CXCL1 production in EC *in vitro*, reduced neutrophil infiltration and MMP-9 content in atherosclerotic lesions (251). Moreover, neutrophil recruitment to large arteries was shown to depend on CCR1, CCR2, CCR5, and C-X-C motif chemokine receptor (CXCR)2 in early stages of atherosclerosis, thus being particularly dependent on platelet-derived CCL5 stimulation (243). Another important role in neutrophil chemotaxis was also shown for CCL3, as leukocyte-specific CCL3 depletion inhibited atherosclerotic lesion formation particularly by affecting neutrophil accumulation (252).

Importantly, neutrophils also contribute to monocyte recruitment by depositing chemotactic proteins on the endothelium (244). One such example is cathelicidin. Mice lacking the corresponding gene developed significantly smaller atherosclerotic lesions with lower numbers of plaque macrophages (253). Similarly, it was reported that neutrophil-derived α-defensin can complex with CCL5 and be presented on EC, causing enhanced monocyte adhesion and vascular inflammation (254); while cathepsin G—also released from granules of neutrophils—is specifically deposited on the arterial endothelium of arteries but not on venule EC, promoting adhesion and extravasation of myeloid cells specifically in atherosclerosis-susceptible areas of vessels (255).

Neutrophils can facilitate their way to the endothelium through the dense network of endothelial glycocalyx by releasing proteolytic proteins, MMPs and ROS, thus locally breaking this physical barrier down (256). At the endothelium, neutrophil tethering and rolling is mainly mediated by PSGL-1 binding to P-selectin and CD44, but not to E-selectin, which is thus assumed to be selectively used by inflammatory monocytes (197). This is an important observation, as preformed P-selectin is available much quicker upon activation of EC than E-selectin which requires *de novo* synthesis, and which could therefore partly explain the delayed secondary recruitment of inflammatory monocytes compared to neutrophils in inflammation. CD44 has been studied quite extensively for its involvement in atherosclerosis, but depletion in atherosclerotic mice tended to yield conflicting results (257). Depletion of L-selectin, which might be specifically important in secondary capture, was consensually shown to promote atherosclerosis, accompanied by a drop of aortic B cells (258, 259). *In vivo*, however, the rolling behavior of monocytes and neutrophils on carotid artery bifurcations of $Apoe^{-/-}$ mice appears to be quite different: While the number of rolling neutrophils, in contrast to monocytes, increased during high-fat diet, the rolling rate of monocytes decreased during the same period (260). Another striking observation is that under high shear stress, by cytoskeletal reorganization during rolling neutrophils can form slings out of their membrane which are characterized by surface expression of distinct sticky PSGL-1 clusters and LFA-1, thus facilitating contact with the endothelium (261). During rolling, neutrophils were also shown to secrete S100 calcium-binding protein (S100)A8 and S100A9, calcium-binding proteins constitutively expressed in myeloid cells that account for ∼45% of the cytoplasmic proteins in neutrophils (262). Interestingly, apart from their numerous functions in inflammation, these proteins were also associated with leukocyte chemotaxis, inducing VCAM-1 and ICAM-1 expression in EC while upregulating Mac-1 expression in leukocytes, subsequently resulting in increased TLR4-mediated Mac-1/ICAM-1 binding, decelerated leukocyte rolling and enhanced firm adhesion on the endothelium (263, 264). In line, blocking antibodies or genetic depletion of S100A9 reduced leukocyte recruitment in several murine inflammatory disease models (262), making these proteins a promising pharmacological target in inflammation-related diseases.

Transition from slow rolling to neutrophil arrest seems to particularly depend on LFA-1 binding to endothelial ICAM-1, which is in contrast to monocytes requiring VLA-4/VCAM-1 interactions. Hereby, CXCR2 is assumed to be of central importance for "inside-out" activation of LFA-1 (265). And while monocyte crawling is dependent on both Mac-1 and LFA-1, neutrophils seem to exclusively crawl via Mac-1, interacting with ICAM-1 and ICAM-2 (88, 208, 266). Remarkably, when Mac-1 is blocked, neutrophils can also crawl against the direction of flow *in vitro* by engaging LFA-1, while Mac-1 favors flow-directed crawling behavior (87). During crawling, neutrophils flatten and form protrusions reaching into the endothelial surface similar to monocytes, while on the front of the moving cell, filamentous actin (F-actin) and on the end, the so-called uropod, myosin filaments aid the cell in directed movement on the endothelium (267). Lack of the actin cytoskeleton transcription factor MKL1 in neutrophils almost completely abrogated migration *in vitro* (268). Importantly, neutrophils were recently found to actively scan for activated platelets via protruding PSGL-1 clusters at the uropod, and such interactions with platelets were crucial for intravascular migration of neutrophils (269). Also EC derived CXCL1 was shown to support neutrophil crawling, while CXCL2, mainly produced by neutrophils themselves, particularly aided in breaching of endothelial junctions together with its atypical chemokine receptor 1 which was found to be enriched in endothelial junctions (270).

Following firm arrest, neutrophil interaction with EC via β2-integrin/ICAM-1 triggers VE-cadherin phosphorylation and subsequent loosening of endothelial adherent junctions to promote the favored paracellular route for diapedesis (97). This is quite similar to the process described for monocytes above, and requires the formation of transmigratory cups which were

reported to form around neutrophils specifically in a "dome" shaped manner (271), involving RhoA and leukocyte-specific protein 1 mediated formation of contractile F-actin structures that tightly surround the invading cell in order to prevent vascular leakage during transmigration (272, 273). Strikingly, neutrophils were also observed to return to the circulation by reverse transendothelial migration in the context of low JAM-C expression in mice (274). Mechanistically, local proteolytic cleavage of JAM-C, driven by neutrophil-derived elastase, was shown to promote this behavior (275).

After infiltrating the plaque, neutrophils have a plethora of possibilities to further promote leukocyte recruitment and subsequent progression of atherosclerosis. They can release granula proteins such as cathelicidin, cathepsin G, elastases, MMPs and ROS, or activate NET formation, thus attracting further leukocytes, promoting oxidative stress, LDL modification, EC activation, activation of macrophages and cellular damage (244).

The Player With the Many Faces: T Cells

While myeloid cells form the first line of defense and respond rapidly but uniformly to a broad spectrum of identified threats, cells of the adaptive immune system perform highly specific tasks that are individually tailored to the particular profile of their target, resulting in a delayed but finely matched immune response. Thus, it is not surprising that T cells—a highly abundant population in human atherosclerotic plaques—are divided into multiple different subpopulations. This includes naïve, memory and effector CD4+ and CD8+ T cells, but also regulatory T cells (Treg) (276). CD4+ T cells are generally associated with increased atherosclerotic plaque growth (277–280). Following antigen-presentation, CD4+ T cells are activated and differentiate into T helper (Th)1, Th2, Th17 cells or other subsets and resemble, together with memory T cells, the major proportion of T cells to be found within atherosclerotic plaques (281). However, they can also give rise to Tregs in the periphery (282). Among the CD4+ T cells, Th1 cells are the most abundant T-subtype in human atherosclerotic lesions (283) and can generally be attributed as pro-atherosclerotic (284, 285), while the role of the other T cell subsets is still a matter of debate and discussed in detail elsewhere (286). Tregs, on the other hand, which express the characteristic transcription factor forkhead box protein P3 (FOXP3) and CD25, are clearly associated with an anti-inflammatory role by suppressing the proliferation of pro-inflammatory effector T cells and influencing macrophage function toward an anti-inflammatory phenotype (287, 288). Depletion of Treg cells in mice aggravates atherosclerosis (289–291) and Tregs express IL-10 and transforming growth factor (TGF)-β, both associated with anti-atherosclerotic effects in atherosclerosis (292). Interestingly, however, just recently an autoreactive phenotype in Tregs directed against apolipoprotein B-100 (ApoB-100), the core protein of LDL, in late-stage atherosclerosis was identified (293), questioning the classical view of Tregs as solely beneficial cells. Moreover, a recent study showed that dyslipidaemia reprograms the metabolic footprint of Tregs toward an effector-like migratory phenotype, challenging the classical hypothesis that Treg migration into plaques might be reduced (294). Finally, CD8+ T cells are more frequent in blood of CAD patients than in healthy individuals (295, 296) and generally associated with pro-atherosclerotic effects in preclinical studies (297–299), but, in contrast, inhibiting CD8+ cells in advanced lesions also resulted in less stable lesions (300).

T cells can use both classical myeloid cell like and antigen-dependent patterns for migration into tissues (256). While most of the T cells found within plaques are antigen-experienced T cells (286) and T cells targeting ApoB-100 were shown to circulate in human blood (301), a recent study suggests that naïve T cells can also be primed directly in the vessel wall (302). Of note, this was not related to tertiary lymphoid organs which can be found in later stages of atherosclerosis within the adventitia and promote Treg expansion (303). However, the antigens to which T cells respond in atherosclerosis are mostly unknown which renders it difficult to study antigen-dependent effects in T cell migration. Therefore, the precise mechanism of T cell recruitment to atherosclerotic plaques, albeit of great interest, is still subject of basic research, and many of the subsequent insights are derived from *in vitro* findings.

To migrate into murine atherosclerosis-prone vessels, circulating T cells roll on endothelial P-selectin using PSGL-1 *in vivo* (302), with a potential role for its co-factors CD44 and CD43 (304, 305). In contrast to monocytes, however, to be fully active, PSGL-1 in T cells requires prior glycosylation (35). Mac-1 has not been described to participate in T cell recruitment but L-selectin, which is important for lymphocyte trafficking into lymph nodes (256), was shown to play a role in T cell migration into peripheral tissues, especially in recruitment into the adventitia of healthy, non-inflamed arteries in mice (306). This again suggests a role for naïve T cell recruitment, as T cells generally lose L-selectin expression upon antigen-presentation. Notably, in a study using intravital microscopy, rolling of T cells on carotid artery bifurcations could not be observed in early atherosclerosis, but was induced on pronounced atherosclerotic lesions in mice (260), indicating a more pronounced role for T cells in late atherosclerosis.

Induction of firm adhesion requires integrin activation, which is accomplished by chemokines in a similar way as in myeloid cells. Different T cell subsets react to different cytokines, but CCR1 and CCR5 have been shown to be expressed on most atherosclerosis relevant T cells and thus are supposed to play a major role in T cell recruitment in response to CCL5 (307). However, CCR1 and CCR5 appear to exhibit opposing effects, as CCR1 seems to be anti-atherosclerotic in the context of T cell recruitment in murine atherosclerosis (308), whereas CCR5 rather has a pro-atherogenic role (section The Classic: Monocytes). Another chemokine receptor to be found on Th1 cells is CXCR3, which is required for Th1 differentiation (309). It requires binding of CXCL10, which, when inhibited, decreases atherosclerotic lesion size and specifically T cell accumulation in murine atherosclerotic lesions (310). Interestingly, CXCL10 levels were suggested to be higher in obese compared to non-obese subjects, functionally promoting adhesion capacity of leukocytes *in vitro* (311). Similarly, genetic depletion of *Cxcr3* or antagonizing CXCR3 pharmacologically in mice reduced

atherosclerosis progression and infiltration of inflammatory T cells, while Treg numbers rised (312, 313). CXCR6 has been described as a marker of polarized Th1 cells (314) important for T cell homing, as absence of CXCR6 inhibited recruitment of T cells, diminished IFNγ production and atherosclerotic lesion formation in *Apoe*$^{-/-}$ mice (315). CXCL16 is the ligand for CXCR6 and chemoattractive when expressed and deposited on EC, but also functions as a scavenger receptor for oxLDL on monocytes and macrophages (316). In contrast to other oxLDL scavenger receptors, CXCL16 depletion was associated with reduced plaque formation in *Ldlr*$^{-/-}$ mice (317), thus acting in both pro- and anti-atherosclerotic ways, depending on the context. Last but not least, CCR7, which mediates T cell homing to lymph nodes, and its ligands CCL19 and CCL21 have also been identified within atherosclerotic lesions of humans and mice (318). However, studies on atherosclerosis in *Ccr7*$^{-/-}$ mice achieved controversial findings (281).

To summarize, several chemokines and respective receptors are involved in T cell attraction and inside-out signaling. However, Th1 cells are also able to bypass extracellular chemokine signals by absorbing chemokines stored intraendothelially in vesicles via dense lymphocyte-endothelial synapses (319). Moreover, EC also seem to be able to act in an APC like manner, presenting antigens specifically to memory T cells via their T cell receptor (TCR) and thereby activating them toward tissue migration (320). Whether these scenarios are also relevant for recruitment into atherosclerotic vessels is thus far not known.

Adhesion of the now activated T lymphocytes occurs presumably via the β2-integrin LFA-1, whereby VLA-4 and CD47 are also thought to play a role (79, 321). Similar to monocytes, crawling T cells interact with ICAM-1 via LFA-1 (322), polarize into a leading edge and tailing uropod and probe the endothelium by invasive protrusions (323) which is controlled by RhoA and continuous actin reorganization (324). The transmigration process of T cells in atherosclerotic arteries to date is mostly unknown, but according to T cell migration into other peripheral inflamed tissues likely similar to other leukocytes, favoring paracellular migration involving ICAM-1-mediated signaling and VE-cadherin dissociation from VE-PTP (325, 326). In contrast, TCR-activated effector memory CD4+ cells can also transmigrate in an alternative way involving CX3CL1 and LBRC adhesion proteins *in vitro* (327). Again, if this also happens in transmigration through atherosclerotic arteries, is not known.

Platelets: Small but Effective Partners in Crime

Although far from giving a full picture, the role of platelets in atherosclerosis is becoming increasingly clear, revealing that their involvement extends well-beyond thrombus formation. In fact, thrombocytes also contribute to leukocyte recruitment in early atherogenesis, as will be elucidated in the following.

While shear stress can trigger endothelial activation, leading to upregulation of adhesion molecules and chemokines (see section Keep it Flowing), shear stress is also known to directly trigger platelet activation and aggregation in atherosclerotic vessels, thus promoting thrombotic arterial occlusion (328). However, shear stress or shear activated EC can stimulate platelets also in earlier phases of atherogenesis and promote platelet adhesion to the EC surface via enhanced adhesion molecule expression or decreased release of NO and prostacyclin (329), thus shifting the balance between inhibitory and activating pathways in platelets in favor of platelet activation. Of interest, impaired function of the ATP-binding cassette transporter G4 in bone marrow megakaryocyte progenitors giving rise to platelets has been shown to inhibit cholesterol efflux from these cells, thus promoting platelet production and accelerating atherosclerosis (330). Additionally, oxLDL was also shown to directly activate platelets via binding to CD36, thereby impairing cGMP mediated anti-inflammatory effects (331), while the traditional risk factors hyperlipidaemia, hyperglycaemia and hypertension were likewise associated with increased platelet reactivity (332).

Upon activation, platelets undergo shape change and increase surface expression of P-selectin and CD40L, while integrin αIIbβ3 adopts its active conformation (333). Such activated circulating platelets were shown to readily bind to EC and monocytes, deposit the chemokines CCL5 and CXCL4 (also known as PF4) on both EC and monocytes and subsequently promote leukocyte accumulation and atherosclerotic lesion formation in the arterial intima, notably already prior to the development of manifest atherosclerotic lesions (25, 334). Moreover, atherosclerotic plaques also suggest presence of macrophage-platelet aggregates, indicating that platelet-binding to monocytes persists beyond the recruitment process (335). In this interplay, platelets induce a pro-inflammatory phenotype in macrophages characterized by increased production of the cytokines IL-6 and IL-1β and impaired capability to phagocytose dying cells, which in turn results in increased necrotic core area in atherosclerotic plaques of *Ldlr*$^{-/-}$ mice. In line, the amount of circulating monocyte-platelet aggregates was significantly increased in CAD patients (336, 337). Mechanistically, platelet binding to EC is a two-step process initiated by platelet rolling on the glycoprotein von Willebrand factor (vWF) released and bound by the endothelium, which involves platelet glycoprotein (GP)Ibα and P-selectin. Subsequently, firm adhesion of platelets is mediated via integrin αIIbβ3 which binds to endothelial αvβ3 and ICAM-1 (338), and involves PECAM-1 signaling (339). The interaction between activated platelets and leukocytes is conveyed by P-selectin interaction with leukocyte PSGL-1, which is supported by platelet glycoprotein Ibα (GPIbα), JAM-A, and JAM-C binding to leukocyte Mac-1 (340–342). In this context, the role of JAM-A is striking, as it seems to act as a brake on platelet activation: not only do knockout mice deficient for JAM-A display enlarged thrombi (343) and accelerated early-stage neointima formation (342), but also a hyperreactive phenotype significantly aggravating atherosclerotic lesion formation (344). However, in sharp contrast to these previous findings, a peptide antagonist intended to inhibit JAM-A function was shown to exert beneficial effects in atherosclerotic *Apoe*$^{-/-}$ mice by inhibiting platelet adhesion to the endothelium (228). Therefore, further studies are warranted to clarify a possible correlation of these findings.

Strikingly, endothelium-adherent platelets were observed to form exceptionally long, flow-induced protrusions (FLIPR) from their membrane under high shear stress. Mediated by P-selectin/PSGL-1-interaction, such FLIPR can deliver platelet microvesicles to rolling monocytes and neutrophils which promotes their activation, as demonstrated by increased CD11b expression and L-selectin shedding (345). Another interesting observation is that, although platelets can adhere to EC at various shear rates *in vitro*, their ability to capture leukocytes may be limited to regions of disturbed flow (346). Moreover, the absence of platelets in mice markedly suppresses neutrophil crawling, whereas depletion of their neutrophil ligand PSGL-1 significantly alters neutrophil surface distribution of Mac-1 and CXCR2, thereby impairing directed intravascular motility and transmigration (269). On the other hand, platelets were also shown to recruit to atherosclerotic plaques by interacting with previously adhered monocytes and neutrophils in form of secondary capture (260).

Apart from favoring leukocyte recruitment by direct binding, several pro-inflammatory mediators released from activated platelets also promote leukocyte recruitment (332). One such platelet-derived chemokine of pivotal relevance to leukocyte recruitment is CCL5. It is deposited on activated EC in arteries mainly by platelets and significantly involved in monocyte and neutrophil adhesion to EC, as shown by *in vitro* and *in vivo* experiments (243, 347). Antagonizing CCL5, in turn, reduced neointima formation, leukocyte infiltration and atherosclerotic plaque formation in mice (348–350). CCL5 was also shown to form complexes with CXCL4 which synergistically enhances the capacity of CCL5 to recruit monocytes (351). Strikingly, this interaction could be selectively disrupted by the peptidic inhibitor CKEY2 and its mouse ortholog MKEY, thus decreasing monocyte recruitment and atherosclerotic plaque formation in mice (352). Another such liaison was found between CCL5 and the neutrophil-derived protein human neutrophil peptide 1 (HNP1), also facilitating monocyte recruitment to sites of inflammation. This could likewise be inhibited by application of the peptide antagonist RRYGTSKYQ (254).

A further important platelet derived chemokine is CXCL4 which is highly abundant in platelet α-granules and plays a critical role in coagulation. Importantly, CXCL4 could be localized in human atherosclerotic lesions and its presence on EC and macrophages positively correlated with clinical parameters for atherosclerosis (353). Moreover, CXCL4 was shown to directly bind oxLDL and to increase its uptake in vascular cells and macrophages (354), which is supported by histological findings in human atherosclerotic lesions (355). Also, upon stimulation with CXCL4, macrophages abolish expression of the atheroprotective scavenger receptor CD163 (356) and, as suggested by a recent study, give rise to a new macrophage phenotype called M4, characterized by the simultaneous expression of MMP7 and S100A8 (357). Additionally, CXCL4 appears to prompt differentiation of monocytes to macrophages (358). In line with these observations, depletion of CXCL4 in mice significantly reduced atherosclerotic lesion formation (359).

Another platelet derived factor is CXCL12. Interestingly, polymorphisms within the gene encoding for *CXCL12* in humans have been genome-wide significantly associated with CAD (360, 361). CXCL12 can signal both via CXCR4—relevant for neutrophil retention in the bone marrow—and CXCR7. While CXCR7 is barely detectable on blood leukocytes, it appears to be upregulated during monocyte-to-macrophage differentiation. This was accompanied by a switch in intracellular signaling in response to CXCL12 toward more pro-inflammatory pathways and subsequently enhanced phagocytotic activity of macrophages (362). While CXCL12 promotes monocyte chemotaxis in a CXCR4-dependent manner, monocyte adhesion to platelet-bound CXCL12 is rather mediated by CXCR7 (363). Furthermore, in a paracrine manner, CXCL12 was also shown to regulate platelet activation (364, 365). In line, *Cxcl12* overexpression increased, while endothelial-cell specific *Cxcl12* depletion reduced atherosclerotic lesion area in mice (366, 367) and platelet surface CXCL12 expression correlated with the risk of adverse cardiac events in symptomatic CAD patients undergoing percutaneous coronary intervention (PCI) (368). To the several other mediators released from platelets upon activation belong CXCL3, CXCL5, CXCL7, CXCL16, CCL3, and macrophage migration inhibitory factor (MIF). Noteworthy, platelets also release several angiogenesis-related proteins such as vascular endothelial growth factor (VEGF) or platelet derived growth factor (PDGF) with important influence on atherosclerosis. While angiogenic factors do not necessarily appear to influence leukocyte recruitment directly, neovascularization within atherosclerotic plaques is a hallmark of progressive atherosclerosis exponentially expanding the area over which leukocytes can penetrate, hence further promoting leukocyte infiltration and plaque destabilization (369).

Lessons Learned: Clinical Implications and Therapeutic Options

Plasma levels of cholesterol, which circulates in the blood via LDL and strongly stimulates atherogenesis, can be successfully lowered by treatment with statins—an approach representing the mainstay of atherosclerosis therapy today. Further pharmacological strategies to prevent cardiovascular events include antihypertensive and antihyperglycemic agents, if applicable (370). However, according to a 2010 meta-analysis, average statin therapy in randomized, controlled trials (RCT) still leaves a mean residual risk of over 75% for major cardiovascular events in these patients (371). Intensified statin treatment and additional use of ezetimibe or PCSK9 inhibitors, which reduce levels of circulating LDL by a different mechanism, further strongly reduces this risk but, again, far from completely abolishing it (372, 373). Therefore, it becomes clear that fighting only the traditional risk factors is not sufficient to eliminate atherosclerosis.

At this point, anti-inflammatory treatment strategies come to play (**Figure 2**). The IL-1β neutralizing antibody canakinumab was one of the first solely anti-inflammatory drugs shown to reduce the recurrence of cardiovascular events in high-risk CAD patients (374), and as suggested from mouse experiments, particularly does so by reducing leukocyte production in the bone marrow and deactivating EC toward less leukocyte recruitment

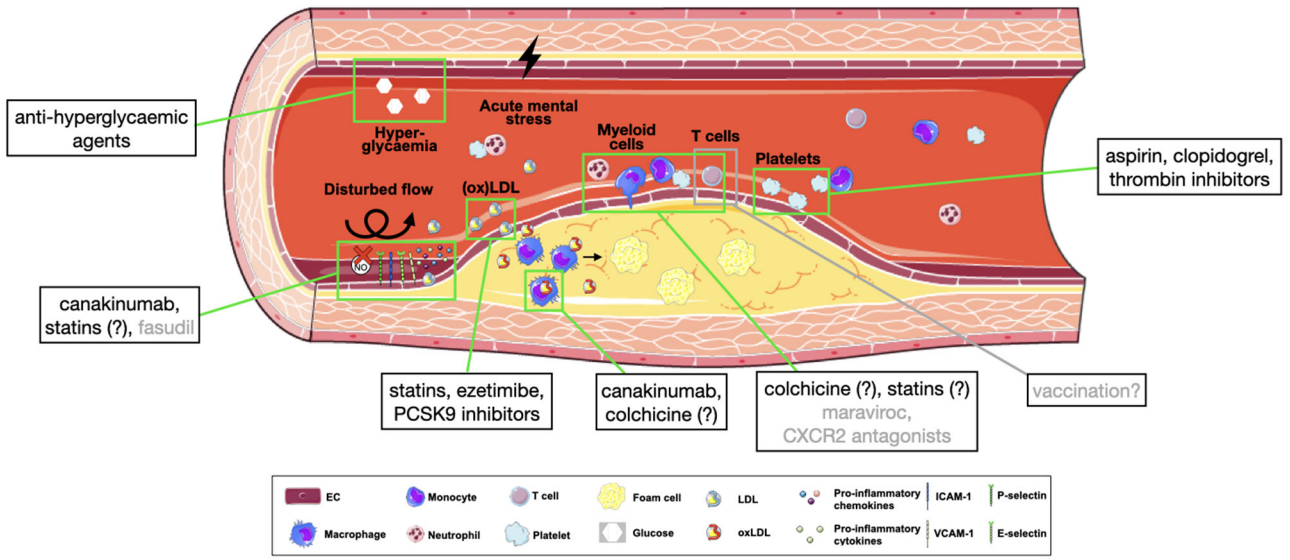

FIGURE 2 | Pharmacological targets in atherosclerotic leukocyte recruitment. Simplified overview of approved (black) or potential (gray) targets in the process of leukocyte recruitment into atherosclerotic plaques. Substances with an incompletely clarified mechanism of action are marked with (?). CXCL2, C-X-C motif chemokine ligand 2; ICAM-1, intracellular adhesion molecule 1; ICAM-2, intracellular adhesion molecule 2; NO, nitric oxide; (ox)LDL, (oxidized) lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9; VCAM-1, vascular adhesion molecule 1.

(375). Moreover, another such highly anti-inflammatory drug, colchicine, was proven to reduce the risk of myocardial infarction, ischemic stroke, or cardiovascular death by over 25%, of note in addition to baseline treatment with lipid-lowering agents in 97% of enrolled patients (376). Colchicine's mechanism of action is not fully understood, but suggested to inhibit inflammasome activation, neutrophil recruitment and leukocyte-platelet interactions (377), thus directly affecting leukocyte migration into atherosclerotic plaques.

Of great interest, statins exert anti-inflammatory effects beyond their action on LDL (378), which may partly explain their superiority in cardiovascular risk reduction compared with other lipid-lowering agents. Mechanistically, in absence of hypercholesterolaemia, statins were shown to improve endothelial function, particularly by improving NO availability, stability of adherens junctions and reducing ROS formation (379–381), to inhibit neovascularization (382, 383), and, importantly, to selectively block LFA-1 and subsequent lymphocyte adhesion (384). Similarly, antithrombotic agents—a major pillar of secondary prevention—do not only reduce aggregation, but also platelet activation. Aspirin, for example, inhibits GPIIb/IIIa and P-selectin expression and release of chemokines (385, 386), clopidogrel was shown to improve systemic NO bioavailability and reduce soluble CD40L and CCL5 release (387) and thrombin inhibitors reduce formation of platelet-leukocyte aggregates and atherosclerotic plaques in mice (388, 389). Also some anti-hyperglycaemic agents have shown to exert beneficial effects on cardiovascular outcomes, both in diabetic and non-diabetic patients with heart failure (390, 391). Of note, the mechanism of action of some of these agents may affect NLRP3 inflammasome function in macrophages (392).

Up until now, strategies aimed at reducing vascular oxidative stress have not been shown beneficial in atherosclerosis patients (137). Interestingly, however, the Rho kinase inhibitor fasudil was associated with enhanced NO bioavailability, thus improving endothelial function in atherosclerotic patients (393). When regarding chemokine receptors, CXCR2 antagonists have been or are currently investigated in pilot or phase II studies in inflammatory diseases and COPD, while the CoronAry heart DiseAse (CICADA) study is specifically testing the cardiovascular effects of such agent (394). The CCR5 inhibitor maraviroc was shown to reduce aortic plaque size when treating atherosclerotic mice (395) but also to decrease atherosclerosis progression in HIV patients (396) and thus represents another interesting target for further studies.

A major fly in the ointment, however, is that large-scale inhibition of adhesion molecules or chemokines is often a double-edged sword, as several key players in leukocyte recruitment act differently in different tissues or have different, sometimes conflicting effects on atherosclerosis, as is the case with PECAM-1 (397). Another drawback is the importance of leukocyte recruitment for fighting infections. This limits therapeutical benefits of canakinumab, e.g., (374). Therefore, in contrast to targeting LDL, ubiquitous inhibition of adhesion molecules or chemokines is often not feasible. However, chrono-pharmacological treatment is one example for a more targeted approach: In mice, CCL2-dependent myeloid cell recruitment to atherosclerotic plaques peaks in the early morning and

could be effectively targeted by time-adjusted treatment—importantly, without affecting cell adhesion in the cremasteric microcirculation at the same time (398). Another nascent concept is vaccination in atherosclerosis, aiming at inducing antigen-specific regulatory T cells to suppress deleterious effector T cell expansion and thus inhibit atherosclerosis. However, it is left open whether this strategy is feasible and beneficial (286).

Thus, it remains exciting to see what new therapeutics or therapeutic concepts will emerge in the future that prove helpful in curbing the inflammatory aspect of atherosclerosis and, in particular, leukocyte recruitment into the vessel walls.

CONCLUSIONS

By following leukocytes step by step on their way into atherosclerotic plaques, it became clear that all leukocytes, although differing in their affinity for specific adhesion molecules or chemokines, use the same overall concept of tethering and rolling, adhesion, crawling and transmigration. Rolling is mainly mediated by PSGL-1 and during rolling, chemokine-chemokine receptor interactions activate the high affinity conformation of leukocyte integrins in a process called inside-out signaling, paving the way for firm adhesion via VLA-4 or LFA-1. Many chemokines have different affinities for different leukocytes, e.g., CCL2 rather favors classical monocytes and CXCL1 and 2 neutrophils, which may allow for targeted recruitment of a particular cell type. Other chemokines such as CCL5 are similarly important for monocytes, neutrophils and T cells, and are partially derived from platelets, which also directly contribute to leukocyte migration by binding to ECs and leukocytes. The concept of crawling and transmigration, however, appears to be very similar between leukocytes, involving cup formation and breaking of endothelial junctions. An important part of the recruitment process is EC priming, which enables expression or upregulation of adhesion factors such as P-selectin, ICAM-1 or VCAM-1 on ECs as binding partners for leukocyte ligands. ECs are mainly activated by two mechanisms: Disturbed flow and inflammatory mediators, most importantly oxLDL. Recent research shows important and divergent contributions of the different leukocyte subsets on plaque formation, with neutrophils being among the first cells to invade, paving the way for monocyte migration which, inside the plaque, differentiate to macrophages, ingest oxLDL, and promote further recruitment of leukocytes. T cells, on the other side, might contribute to atherosclerosis by targeting specific—mainly unidentified—antigens within the plaque.

Therapeutically targeting recruitment related processes is mainly drawn back by the multitude of important functions that most involved factors have in the immune system. Nevertheless, canakinumab and colchicine, two anti-inflammatory agents contributing to recruitment-related processes, were already proven beneficial in CAD patients. And surprisingly, statins, the mainstay of current atherosclerosis therapy, appear to not only lower LDL levels but also to inhibit leukocyte migration by affecting endothelial and leukocyte function. The same holds true for antithrombotic agents, which affect leukocyte recruitment in multiple ways. Many ongoing studies are investigating the effect of potential additional treatment strategies that mainly target the inflammatory nature of atherosclerosis. However, several open questions show that there is still a long way to go in basic research and on the road from bench to bedside, before we can control the excess inflammatory recruitment of leukocytes in atherosclerotic plaques in patients.

AUTHOR CONTRIBUTIONS

CM, JH, and HBS conceptualized the content, reviewed literature, wrote the manuscript, and generated figures. HS and TK discussed and edited the review. All authors contributed to the article and approved the submitted version.

FUNDING

HBS has received funding from the European Research Council under the European Union's Horizon 2020 Research and Innovation Programme (STRATO, Grant Agreement No. 759272), the Else-Kröner-Fresenius-Stiftung (2020_EKSE.07), Else-Kröner-Forschungskolleg München, Technical University Munich, the Deutsche Herzstiftung (F/28/17), and the Deutsche Forschungsgemeinschaft (DFG) (SA 1668/5-1). TK was funded by the Corona Foundation as part of the Junior Research Group Translational Cardiovascular Genomics (S199/10070/2017) and the German Research Foundation (DFG) as part of the collaborative research center SFB 1123 (B02).

ACKNOWLEDGMENTS

We thank Servier Medical Art (https://smart.servier.com) for providing free illustrations for figures.

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frontiers in Cardiovascular Medicine

BRIEF RESEARCH REPORT
published: 13 January 2022
doi: 10.3389/fcvm.2021.812769

Autoimmune Regulator (AIRE) Deficiency Does Not Affect Atherosclerosis and CD4 T Cell Immune Tolerance to Apolipoprotein B

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Edited by:
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Reviewed by:
Amanda Foks, Leiden University, Netherlands
Patrick Murphy, UCONN Health, United States

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *10 November 2021*
Accepted: *21 December 2021*
Published: *13 January 2022*

Citation:
Nettersheim FS, Braumann S, Kobiyama K, Orecchioni M, Vassallo M, Miller J, Ali A, Roy P, Saigusa R, Wolf D, Ley K and Winkels H (2022) Autoimmune Regulator (AIRE) Deficiency Does Not Affect Atherosclerosis and CD4 T Cell Immune Tolerance to Apolipoprotein B. Front. Cardiovasc. Med. 8:812769. doi: 10.3389/fcvm.2021.812769

Atherosclerosis is a chronic, lipid-driven disease of medium sized arteries which causes myocardial infarction and stroke. Recently, an adaptive immune response against the plaque-associated autoantigen Apolipoprotein B100 (ApoB), the structural protein component of low-density lipoprotein, has been implicated in atherogenesis. In healthy individuals, $CD4^+$ T cells responding to ApoB mainly comprised regulatory T cells, which confer immune tolerance and atheroprotection. Mice and patients with atherosclerosis harbor increased numbers of proatherogenic ApoB-reactive T-helper cell subsets. Given the lack of therapies targeting proatherogenic immunity, clarification of the underlying mechanisms is of high clinical relevance. T cells develop in the thymus, where strong autoreactive T cells are eliminated in the process of negative selection. Herein, we investigated whether the transcription factor autoimmune regulator (AIRE), which controls expression of numerous tissue-restricted self-antigens in the thymus, is involved in mediating tolerance to ApoB and whether *Aire* deficiency might contribute to atherogenesis. Mice deficient for *Aire* were crossbred to apolipoprotein E-deficient mice to obtain atherosclerosis-prone $Aire^{-/-}$ $Apoe^{-/-}$ mice, which were fed a regular chow diet (CD) or western-type diet (WD). $CD4^+$ T cells responding to the ApoB peptide p6 were analyzed by flow cytometry. We demonstrate that *Aire* deficiency influences neither generation nor activation of ApoB-reactive T cells and has only minor and overall inconsistent impacts on their phenotype. Furthermore, we show that atherosclerotic plaque size is not affected in $Aire^{-/-}$ $Apoe^{-/-}$ compared to $Aire^{+/+}$ $Apoe^{-/-}$, irrespective of diet and gender. In conclusion, our data suggests that AIRE is not involved in regulating thymic expression of ApoB or atherosclerosis. Alternative mechanisms how ApoB-reactive CD4 T cells are selected in the thymus will have to be investigated.

Keywords: autoimmune regulator, thymic selection, immune tolerance, atherosclerosis, adaptive immunity, antigen-specific, T cells, dextramer

INTRODUCTION

Atherosclerosis is a chronic condition of large- and medium-sized arteries that involves formation of leukocyte- and lipid-rich plaques in the arterial wall (1). Atherogenesis is accompanied and modulated by $CD4^+$ T cells responding to plaque-associated autoantigens (2, 3). One of these autoantigens is Apolipoprotein B (ApoB), the core protein of low-density lipoprotein (LDL) and other lipoprotein particles (2). ApoB-reactive $CD4^+$ T cells ($ApoB^+$ T cells) can be detected in peripheral blood mononuclear cells (PBMCs) and lymph nodes from healthy and atherosclerotic humans and mice, respectively (4, 5). In healthy individuals these cells are mainly regulatory T cells (T_{regs}) (4), which confer atheroprotection in mice (6, 7) and predict lower cardiovascular event rates in humans (8). T_{regs} express the transcription factor (TF) forkhead box protein P3 (FOXP3) and maintain peripheral immune tolerance by attenuating pathogenic (auto-)immune responses through multiple mechanisms including secretion of the anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) (9). In atherosclerotic individuals, $ApoB^+$ T cells frequently coexpress TFs typical found in pro-atherogenic T helper 1 (T_H1) or T helper 17 (T_H17) cells (4).

Central T cell tolerance develops in the thymus, where bone-marrow-derived progenitors undergo a complex maturation and selection process, of which in the end only few single $CD4^+$ or $CD8^+$ T cell receptor (TCR)-αβ expressing T cells will leave into the periphery (10, 11). TCR gene segment rearrangement catalyzed by recombinase activating gene (RAG) 1 and 2 is stochastic and yields a unique combination of a TCR-α and -β chain. At this stage, so-called double-positive (DP) thymocytes express the TCR co-receptors CD4 and CD8, which aid in binding of the TCR to peptide-loaded major histocompatibility complexes (MHC) I and II, respectively. The newly formed TCR will be functionally tested in the process of positive and negative selection. DP cells with a functional TCR that recognize ubiquitous self-antigens presented on MHC class I or II molecules with sufficient affinity pass this selection process, downregulate either CD8 (when binding to MHC II) or CD4 (when binding to MHC I), and mature into CD4 or CD8 single positive (SP) cells (12). Too strong interactions of DP cells with ubiquitous self-antigens lead to apoptosis (cortical negative selection) (12). Surviving SP cells migrate into the thymic medulla, where they undergo negative selection (12). Only SP cells with low affinity to tissue-restricted antigens (TRAs; antigens only expressed by one or a few anatomical sites), which are mainly presented by medullary thymic epithelial cells (mTECs) and to a lesser extent by other cell types such as thymic B cells or dendritic cells (DCs), survive this process and mature into effector T cells (T_{eff} cells) (12). SP cells with high affinity to TRAs undergo apoptosis and a subgroup of CD4 SP cells with intermediate affinity develop into invariant natural killer T or natural regulatory T_{regs} (nT_{regs}) which are also termed thymic T_{regs} (tT_{regs}) (13, 14). The expression of many - but not all - peripheral tissue genes in mTECS is regulated by the two transcription factors AIRE and FEZF2 (15–17). Mutations in the *AIRE* gene cause a rare autoimmune disorder in humans, which is termed autoimmune polyglandular syndrome type I (APS 1) or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) and involves chronic mucocutaneous candidiasis as well as dysfunction of the adrenal, parathyroid and other endocrine glands (18, 19). Mice deficient for AIRE show increased numbers of antigen-specific effector T cells and develop multi-organ autoimmunity (15, 20–22). Yet, it is unclear whether AIRE controls the expression of ApoB and the autoimmune $CD4^+$ T cell response in atherosclerosis. ApoB expression is high in mature mTECs, but almost absent in immature mTECs, cortical TECs (cTECs), thymic DCs, and macrophages (23–25). Whereas Sansom and colleagues found highly increased ApoB mRNA levels in mature AIRE-positive mTECs compared to AIRE-negative mTECs or mTECS from *Aire*-deficient mice (**Supplementary Figure 1**) (25), others reported similar ApoB expression in mature mTECs isolated from *Aire*-deficient mice and wildtype controls (15, 24).

Herein we crossed *Aire*- and *apolipoprotein E* (*Apoe*)-deficient ($Aire^{-/-}$ $Apoe^{-/-}$) mice to test whether lack of AIRE would increase peripheral ApoB-reactive effector T cell numbers and functions and increase atherosclerosis. We demonstrate that AIRE-deficiency does neither affect generation of $ApoB^+$ T cells nor their phenotypes and has no impact on atherogenesis, pointing toward a dominant role of an alternative central or peripheral mechanism in enabling immune tolerance to ApoB.

METHODS

Mice

Autoimmune regulator–deficient ($Aire^{-/-}$) mice on C57BL/6J background were purchased from Jackson Laboratories (cat. #004743 Bar Harbor, ME) and crossbred with apolipoprotein E-deficient ($Apoe^{-/-}$) mice to obtain $Aire^{-/-}Apoe^{-/-}$ mice. Mice were housed in a specific pathogen–free environment and fed chow diet (CD) until 10 weeks of age. At 8 weeks of age, mice were either fed CD or western-type diet (WD), adjusted calories diet with 42% from fat (Harlan Labs Cat #: TD.88137, CA, USA) and remained on CD or WD for 12 weeks until organ collection. All animal experiments were conducted in accordance with the institutional guideline for the La Jolla Institute for Immunology animal facility.

Atherosclerosis Quantification

The whole aorta (thoracic and abdominal) was excised, cleaned *in situ*, and pinned out after paraformaldehyde incubation at room temperature for at least 2 h. Atherosclerotic lesions were visualized by Sudan-IV staining and quantified as the percentage Sudan-IV-positive area of the size of the whole aorta. Quantification was performed using ImagePro software (Media Cybernetics, Rockville, MD, USA).

Flow Cytometry

Cell suspensions were prepared from thymus, blood, spleen, or pooled lymph nodes (axillary, cervical, inguinal, para-aortic, mesenteric) and incubated with fluorochrome-coupled antibodies against the indicated antigens for 20 min at RT in RPMI-1640 containing 10% rat serum and 10 μg/ml

antiCD16/CD32 antibodies to block unspecific Fc-receptor interactions. Cells were washed in PBS and fixed in 2% Paraformaldehyde (PFA) for 10 min. If not otherwise indicated, T-helper cells were identified as $CD4^+$ TCR-β^+ Lin^- L/D (live/dead dye, Tonbo Biosciences, San Diego, Ca, USA)$^-$. Lin contained antibodies against CD11b, F4/80, CD19, B220, CD11c, Nk1.1, TER-119, and CD8. Transcription factors were stained with a permeabilization/fixation protocol according to the manufacture's recommendations (Thermo Fisher Scientific, Waltham, MA, USA). T_{regs} were identified as $CD4^+$ $Foxp3^+$ $CD25^+$, T_H1 cells were identified as $CD4^+$ $Foxp3^-$ T-bet^+, T_H17 cells were identified as $CD4^+$ $Foxp3^-$ RORγt^+, T_H2 cells were identified as $CD4^+$ $Foxp3^-$ T-bet^- RORγt^- $GATA3^+$, and T follicular helper (T_{FH}) cells were identified as $CD4^+$ T-bet^- RORγt^- Bcl-6^+. T-effector memory subsets were identified as T_{EM} ($CD44^+$ $CD62L^-$), T-central memory cells as T_{CM} ($CD44^+$, $CD62L^+$), and naïve T cells as $T_{naïve}$ ($CD44^-$ $CD62L^+$). To stain intracellular cytokines (IL-17A, IL-12p40, TNF-α, IFN-γ, IL-10, and IL-4), single cell suspensions were prepared from pooled lymph nodes and stained with anti-CD45 (30-F11: Thermo Fisher Scientific), anti-CD4 (RM4–5: Biolegend; San Diego, CA), and anti-TCRβ (H57–597: Thermo Fisher Scientific) antibodies. Subsequently, the cells were stimulated with cell stimulation cocktail (Thermo Fisher Scientific) and monensin (Thermo Fisher Scientific) for 5 h. Dead cells were identified by staining with Ghost Dye UV450 (Tonbo Biosciences) and IC Fixation Buffer (Thermo Fisher Scientific) was used for fixation and permeabilization. Samples were acquired with a FACS LSR-II or FACS Fortessa (BD Biosciences, San Diego, CA, USA). If not stated otherwise, all anti-mouse antibodies were purchased from Biolegend (San Diego, CA, USA) and used in a final dilution of 1:50 (cytokines/transcription factors/cytoplasmatic proteins) and 1:200 (extracellular markers). Data were analyzed with FlowJo software (Treestar, San Diego, CA, USA).

ApoB:MHC-II Multimers

Biotinylated ApoB:MHC monomers, in which the peptide $ApoB_{978-993}$ was fused to $I\text{-}A^b$ (the MHC-II allele expressed by C57BL/6J mice), were coupled to streptavidin-phycoerythrin or streptavidin-allophycocyanin labeled dextran backbones by Immudex (Copenhagen, Denmark) for the identification of ApoB-reactive $CD4^+$ T cells. ApoB:MHC dextramers were generated as described previously (26) and had the following specifications: ApoB:MHC dextramer-PE carried ∼20 ApoB:MHC monomers and ∼4 PE fluorochromes per dextran, while ApoB:MHC dextramer-APC carried ∼12 7 ApoB:MHC monomers and ∼9 APC fluorochromes per dextran.

Peptide:MHC Multimer Staining

Cell suspensions were prepared from pooled lymph nodes. Before incubation with multimers, $CD4^+$ T cells were enriched by a negative magnetic bead separation with biotinylated anti-CD11b, -CD11c, -TER119, -CD8, -F4/80, -Nk1.1, -B220, -CD19 antibodies (Tonbo Biosciences) and streptavidin-coupled magnetic microbeads (MagniSort, Thermo Fisher Scientific). Enriched $CD4^+$ T cells were > 90% pure, based on surface expression of TCR-β and CD4 as measured in flow cytometry. ApoB:MHC-streptavidin-PE and ApoB:MHC-streptavidin-APC dextramers were incubated for 1 h at room temperature in the dark in RPMI supplemented with 10% rat serum and 10 μg/ml anti-CD16/32 (Tonbo Biosciences). The final concentration for ApoB-MHC was 2.5 nM. Cells were subsequently stained for the indicated surface markers and live-dead dye (eF450 viability dye, Thermo Fisher Scientific). Dextramer staining was validated by a fluorescence-minus-two (FMT) control (APC and PE; **Supplementary Figure 2**). Non-T cells were identified by fluorochrome-labeled antibodies against CD11b, CD11c, F4/80, CD19, CD8, B220 (Lineage, all from Biolegend,). For extracellular staining, cell suspensions were fixed in 2% PFA. As indicated, intracellular staining for transcription factors was performed according to the manufacturer's instructions (Thermo Fisher Scientific). Data were analyzed with FlowJo software (Treestar). For calculation of the absolute numbers of $ApoB^+$ T cells, leukocyte numbers of enriched $CD4^+$ lymph node T cells were quantified (Hemavet, DrewScientific, Miami Lakes, USA) before multimer staining.

Statistics

Data are presented as mean ± SD. Differences between the groups were evaluated using one-way repeated measures analysis of variance (ANOVA) with a *post-hoc* Tukey's test. *P*-values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.4.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

AIRE Deficiency Has No Relevant Impact on Thymopoiesis in *Apoe*$^{-/-}$ Mice

Despite its central role in mediating negative selection of $CD4^+$ T cells, AIRE does not globally influence frequencies of maturing thymocytes (15, 22). To determine whether AIRE impacts thymopoiesis in *Apoe*$^{-/-}$ mice, we quantified different stages of T cell development by flow cytometry (**Figure 1A**). The earliest developing thymocytes do neither express CD4 nor CD8 and are thus called double negative (DN) cells. According to expression of the surface markers CD44 and CD25, DN cells are subdivided into four stages of maturity: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) cells. The DN4 population (DN4) further develops into double positive $CD4^+CD8^+$ cells, which mature into single positive $CD4^+$ or $CD8^+$ T cells (10). Aire deficiency was associated with a reduction of DN1 cells, but did not affect subsequent developmental stages (**Figures 1B,C**). We thus confirm Aire does overall not exert relevant influences on thymopoiesis in *Apoe*$^{-/-}$ mice, which is in accord with findings obtained in wildtypes (22). Considering that Aire affects thymocytes at the SP stage, causes and significance of the observed decrease in DN1 cell counts remain elusive and await confirmation in future studies.

Besides inducing elimination of specific autoreactive thymocytes, Aire was implicated in directing these cells into the T_{reg} line (27). Aire-deficient mice were repeatedly reported to harbor less thymic T_{regs} than wildtype controls (28, 29).

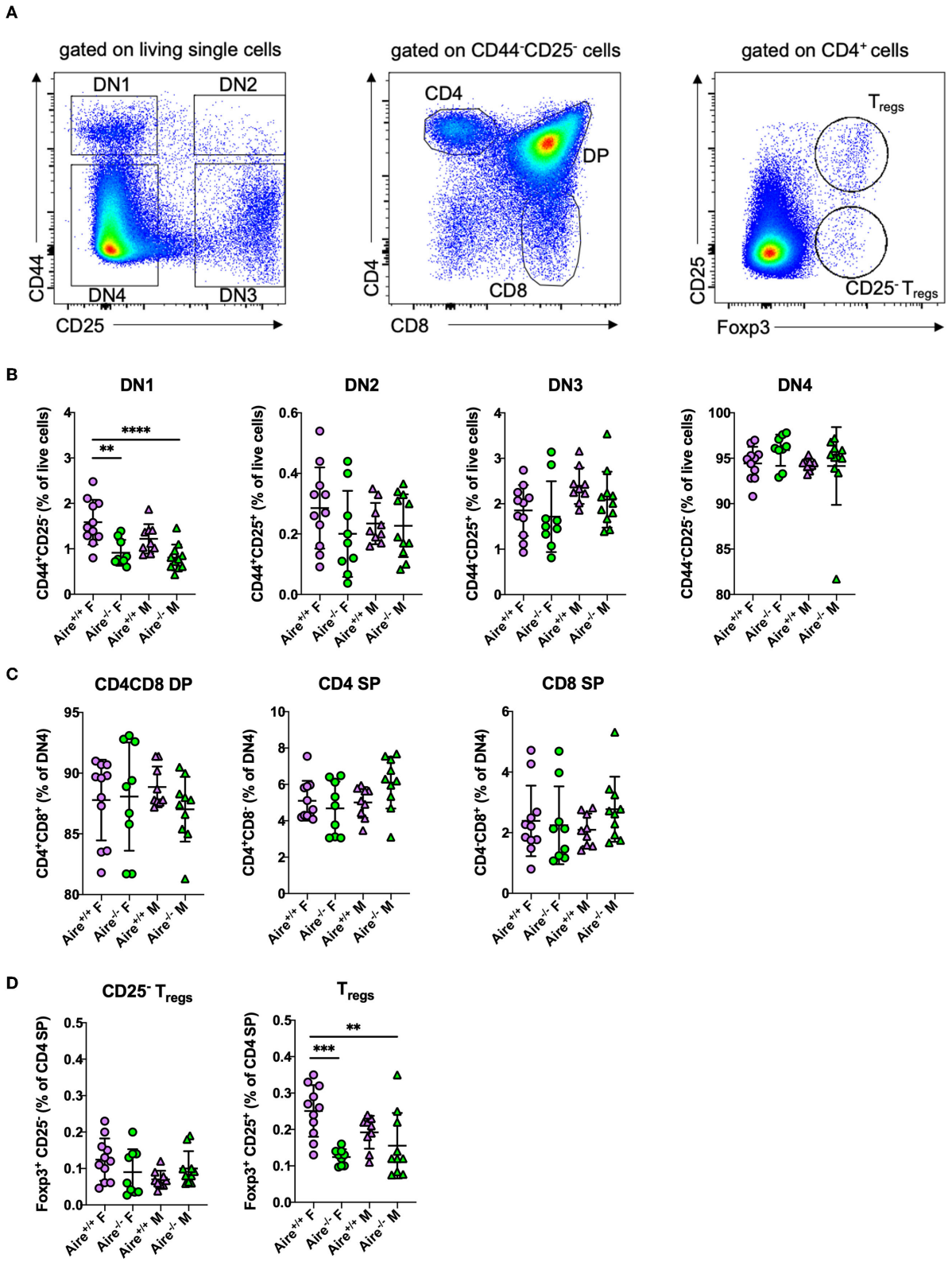

FIGURE 1 | Thymopoiesis is largely unaffected by AIRE deficiency in male and female *Apoe*$^{-/-}$ mice. **(A)** Gating scheme for flow-cytometric detection of maturating thymocytes: Double negative (DN) 1 (CD44^{+}CD25^{-}), DN2 (CD44^{+}CD25^{+}), DN3 (CD44^{-}CD25^{+}), and DN4 (CD44^{-}CD25^{-}) cells were identified by expression of the surface markers CD44 and CD25. CD4 and CD8 single positive (SP) or double positive (DP) cells were identified as CD44^{-}CD25^{-} cells with expression of CD4 and/or CD8. Regulatory T cells were identified as Foxp3^{+} CD25^{+} CD4^{+} cells and CD25^{-} T$_{reg}$ precursors were identified as Foxp3^{+} CD25^{-} CD4^{+} cells. **(B,C)** Quantification

(Continued)

FIGURE 1 | of different stages in thymic development in 20-week-old male and female *Aire*$^{-/-}$*Apoe*$^{-/-}$ and *Aire*$^{+/+}$*Apoe*$^{-/-}$ fed western-type diet for 12 weeks. **(D)** Quantification of thymic CD25^{-} and CD25^{+} regulatory T_{regs}. **(B–D)** $n = 9$–11 per group. Data are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Thymic T_{regs} classically develop from CD4^{+} T cells through subsequent induction of CD25 and Foxp3 (30). Besides that, an alternative developmental route has been described, in which Foxp3 is expressed before CD25 (30). Frequencies of CD25^{-}Foxp3^{+} T_{reg} precursors did not differ between the groups (**Figure 1D**). We observed a reduced number of CD25^{+} Foxp3^{+} T_{regs} in the thymus of female—but not male—*Aire*$^{-/-}$ *Apoe*$^{-/-}$ mice (**Figure 1D**), which is in contrast to observations made in male wildtype mice (29). Future studies will have to address, whether the gender effect might be overshadowed by the atherosclerotic environment.

Aire-Deficient *Apoe*$^{-/-}$ Mice Do Not Display Broad Immunological Abnormalities

AIRE deficiency did not affect leukocyte composition in the blood, spleen and lymph nodes in male and female *Apoe*$^{-/-}$ mice fed a WD (**Supplementary Figure 3**). In comparison to male *Aire*$^{+/+}$*Apoe*$^{-/-}$ mice, *Aire*$^{+/+}$*Apoe*$^{-/-}$ females had a modest yet statistically significant increases in CD4^{+}, CD8^{+}, and γδ T cell counts in lymph nodes, whereas female *Aire*$^{-/-}$*Apoe*$^{-/-}$ mice exhibited higher levels of γδ T cells in the spleen than *Aire*$^{-/-}$*Apoe*$^{-/-}$ males. We did not detect relevant differences in body weight or hematological parameters between *Aire*$^{+/+}$*Apoe*$^{-/-}$ and *Aire*$^{-/-}$*Apoe*$^{-/-}$ mice, (**Supplementary Table 1**). Aire deficiency was associated with an increased neutrophil count in female *Apoe*$^{-/-}$ mice exposed to a WD and with an increase in eosinophils and decrease in platelets in CD-fed male *Apoe*$^{-/-}$ mice. Furthermore, WD-fed males had a lower proportion of monocytes and CD-fed males had higher relative neutrophil and lower relative lymphocyte counts compared to females. Collectively, these data indicates that Aire deficiency does not induce broad alterations of innate and adaptive immunity.

Frequency and Activation of ApoB^{+} T Cells Are Not Influenced by Aire

We have recently identified ApoB-reactive CD4^{+} T cells in lymph nodes of healthy and atherosclerotic mice (5). Herein, we sought to evaluate whether Aire affects generation and activation of atherosclerosis-relevant ApoB^{+} T cells in *Apoe*$^{-/-}$ mice. We utilized a previously published fluorochrome-labeled multimer (5) consisting of recombinant I-A^{b} (the MHC-II allele expressed by C57Bl/6 mice) complexed with the ApoB peptide p6 to identify ApoB^{+} T cells in lymph nodes by flow cytometry (**Figure 2A**). We detected ∼500–1,000 ApoB^{+} cells (0.02–0.05% of all CD4^{+} T cells) in male and female *Aire*$^{-/-}$ and *Aire*$^{+/+}$ mice, without any apparent differences between the four groups (**Figure 2B**). To evaluate the impact of *Aire* deficiency on activation of ApoB^{+} and ApoB^{-} T cells, surface expression of CD44 and CD62L in lymph node CD4^{+} T cells was analyzed (**Figure 2A**). In line with previous data (5), the majority of ApoB^{+} cells consisted of antigen-experienced T-effector memory (T_{EM}, CD44^{+}CD62L^{-}) and T central memory (T_{CM}, CD44^{+}CD62L^{+}) cells, whereas only few naive ApoB^{+} T cells (CD44^{-}CD62L^{+}) were detectable (**Figure 2C**). In contrast, ApoB^{-} cells were predominantly antigen-unexperienced. AIRE-deficiency did not change antigen-experienced T_{CM}/T_{EM} ApoB^{+} and ApoB^{-} cells (**Figure 2C**, **Supplementary Figures 4A,B**). Previous studies have reported conflicting findings regarding the impact of AIRE on CD4^{+} T cell activation: Whereas Anderson et al. observed a near-doubling of CD4^{+} T_{EM} cells in lymph nodes of *Aire*$^{-/-}$ compared to *Aire*$^{+/+}$ mice (15), Ramsey and colleagues did not detect such a difference (20). Here we found that ApoB^{-} T_{EM} cells were 25% of all CD4^{+} T cells, regardless of AIRE expression, and thus twice as high as previously detected by Anderson et al. in *Aire*$^{-/-}$ mice (12%) (15). We therefore suggest that the inflammatory environment in WD-fed *Apoe*$^{-/-}$ mice largely overlayed any potential AIRE-deficiency caused effects on T cell activation. Female Aire-competent mice exhibited a significantly lower frequency of ApoB^{+} T_{CM} cells compared to males. Additionally, AIRE-deficient females had significantly fewer ApoB^{-} T_{CM} cells than male *Aire*$^{-/-}$ mice. However, overall CD44^{+} cells did not differ between males and females, arguing against a relevant gender dependent T cell activation (**Supplementary Figure 4C**). Collectively, these data indicate that AIRE exerts an insignificant effect on the generation and activation of ApoB^{+} cells in male and female *Apoe*$^{-/-}$ mice.

Aire Has Minor Impacts on Phenotypes of Antigen-Experienced ApoB^{+} T Cells

We have recently shown that ApoB^{+} T cells acquire a proinflammatory phenotype during atherogenesis. Antigen-experienced (CD44^{+}) ApoB^{+} T cells comprised a significantly lower proportion of Foxp3^{+} T_{regs} and more RORγt (the lineage defining TF of T_H17 cells) expressing cells compared to CD44^{+}ApoB^{-} T cells in atherosclerotic mice (5). To evaluate whether AIRE influences the phenotypes of antigen-experienced and -unexperienced ApoB^{+} and ApoB^{-} T cells we quantified the expression of Foxp3, RORγt (**Figure 3A**) and other lineage-defining TFs in these cells. In line with our previous findings (5), we detected significantly fewer Foxp3^{+} and more RORγt^{+} cells within the pool of CD44^{+} ApoB^{+} cells compared to CD44^{+} ApoB^{-} cells (**Figures 3B,C**). Foxp3 and RORγt expression in CD44^{+} and CD44^{-} ApoB^{-} T cells was not affected by AIRE deficiency (**Figure 3C**, **Supplementary Figure 5A**). Irrespective of AIRE expression, CD44^{+} ApoB^{+} T cells mainly comprised proinflammatory T-bet^{+} T_H1 cells, RORγt $^{+}$ T_H17 cells, and Bcl-6^{+} T_{FH} cells, which all did not express Foxp3 (**Figure 3D**). In atherosclerotic male and female *Aire*$^{-/-}$ mice, the frequency of CD25^{+} Foxp3^{+} T_{regs} was modestly decreased within CD44^{-} ApoB^{-} cells. AIRE-deficiency did not alter the phenotypes of CD44^{+}

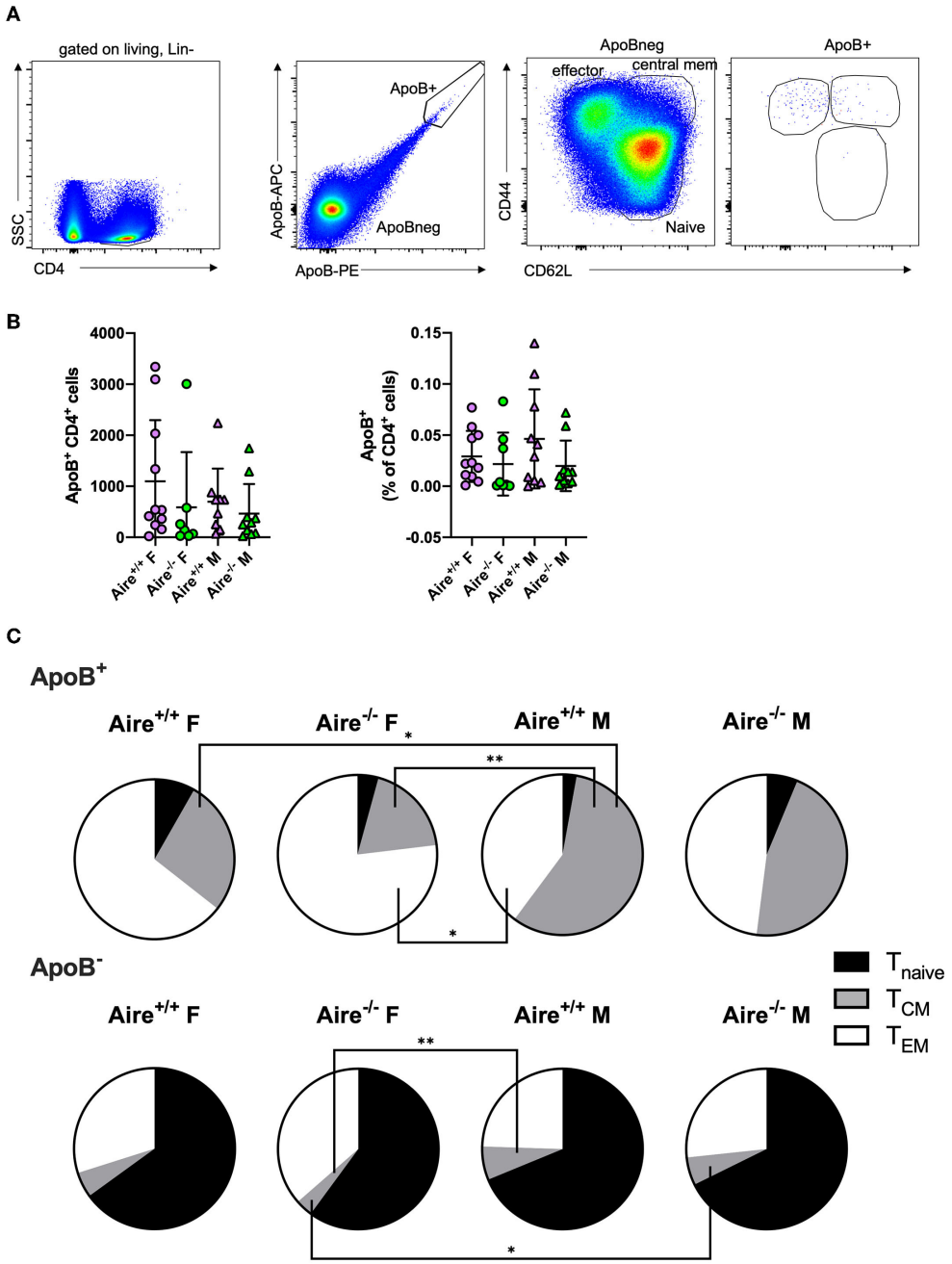

FIGURE 2 | Aire deficiency does not affect generation and activation of ApoB-reactive $CD4^+$ T cells. **(A)** Gating scheme for identification of $ApoB^+$ and $ApoB^-$ $CD4^+$ T effector memory (T_{EM}, $CD44^+CD62L^-$), T central memory (T_{CM}, $CD44^+CD62L^+$) and naïve T cells (T_{naive}, $CD44^-CD62L^+$) by flow-cytometry. **(B)** Absolute counts (left) and frequency (% of $CD4^+$ T cells, right) of $ApoB^+$ T cells isolated from lymph nodes of 20-week-old male and female *Aire*$^{-/-}$*Apoe*$^{-/-}$ and *Aire*$^{+/+}$*Apoe*$^{-/-}$ fed western-type diet for 12 weeks. Data are presented as mean ± SD. **(C)** Quantification of T_{EM}, T_{CM}, and T_{naive} $ApoB^+$ and $ApoB^-$ T cells. Pie charts show the group means of indicated cell types. **(B,C)** $n = 7$–11 per group. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. $^*p < 0.05$, $^{**}p < 0.01$.

$ApoB^-$ and $CD44^-$ cells (**Supplementary Figure 5B**). The observed reduction of $CD44^+$ $ApoB^+$ T_{regs} in atherosclerotic *Aire*$^{-/-}$ mice is in line with impaired thymic generation of T_{regs} in female *Aire*$^{-/-}$ *Apoe*$^{-/-}$ mice (**Figure 1D**). The consistency and relevance of these gender-specific observations are yet unclear and require confirmation in future studies.

Aire deficiency did not substantially affect expression of hallmark pro- and anti-inflammatory cytokines in activated $CD4^+$ T cells (**Supplementary Figure 6**). Whereas, no significant

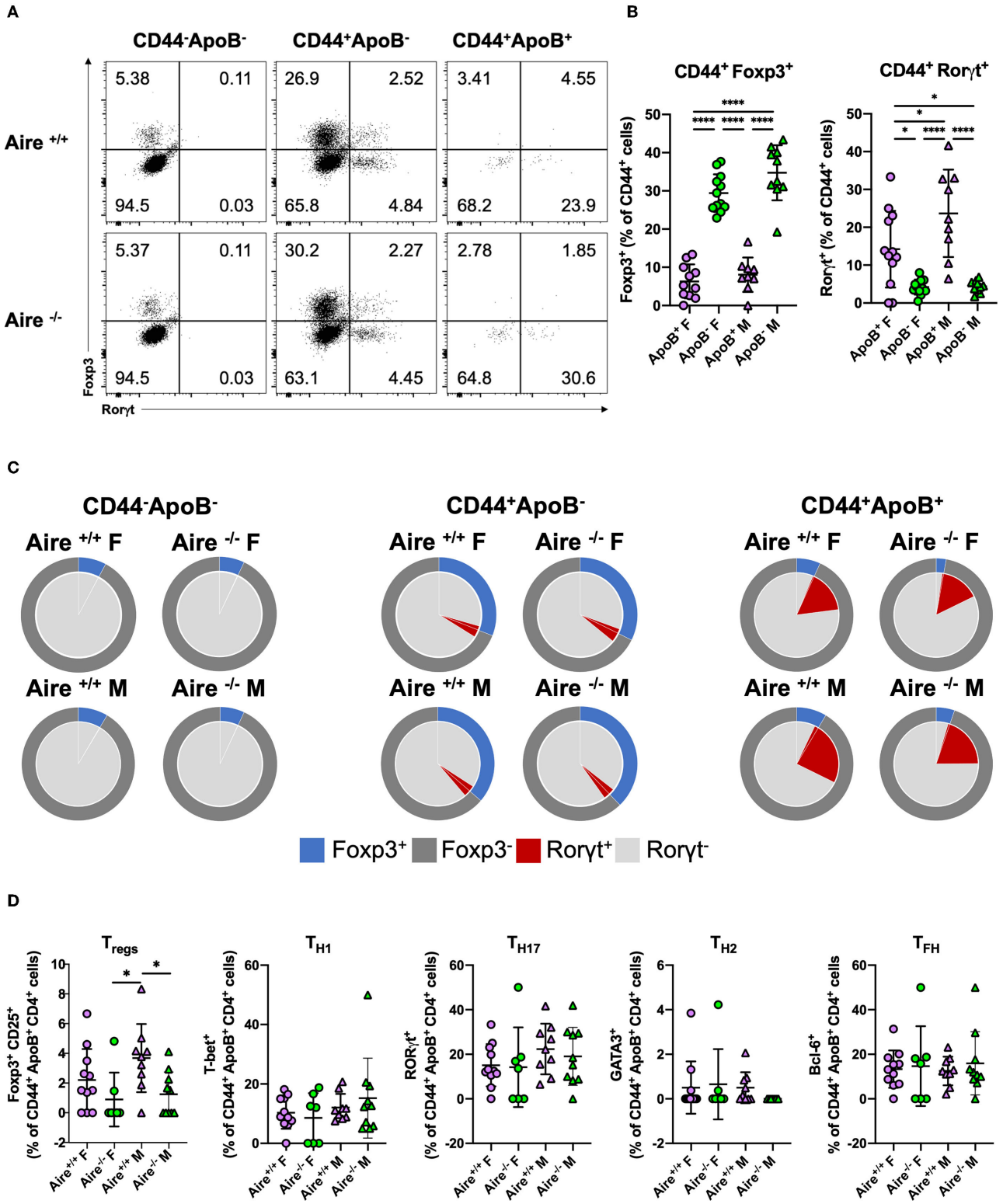

FIGURE 3 | Phenotypes of antigen-experienced ApoB$^+$ T cells are marginally influenced by AIRE deficiency. **(A)** Expression of the transcription factors Foxp3 and RORγt in CD44$^-$ ApoB$^-$, CD44$^-$ ApoB$^+$, and CD44$^+$ ApoB$^+$ cells analyzed by flow-cytometry. **(B,C)** Quantification of Foxp3$^+$ and RORγt$^+$ among CD44$^+$ ApoB$^+$ and ApoB$^-$ cells isolated from lymph nodes of 20-week-old male and female *Aire*$^{-/-}$*Apoe*$^{-/-}$ and *Aire*$^{+/+}$*Apoe*$^{-/-}$ fed western-type diet for 12 weeks. **(D)** Quantification of CD4 T cell lineage transcription factors T-bet (T_H1), GATA3 (T_H2), BCL6 (T_{FH}), Foxp3 (T_{regs}) and RORγt (T_H17). **(B,D)** Data are expressed as mean ± SD. **(C)** Pie charts show the group means of indicated cell types. **(B–D)** n = 7–11 per group. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. $^*p < 0.05$, $^{****}p < 0.0001$.

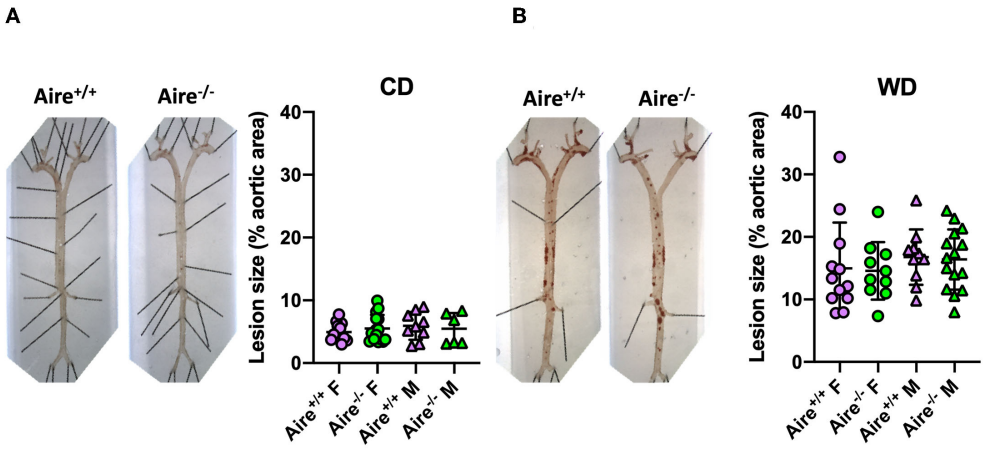

FIGURE 4 | Atherosclerotic lesion size is not influenced by Aire deficiency. Representative images quantification of Sudan IV-stained atherosclerotic plaques within the aorta of 20-week-old male and female *Aire*$^{-/-}$*Apoe*$^{-/-}$ and *Aire*$^{+/+}$*Apoe*$^{-/-}$ mice **(A)** fed chow diet (n = 6–15 per group) and **(B)** western-type diet *Apoe*$^{-/-}$ mice (n = 10–15 per group). Plaque size was calculated as percentage of whole aortic area. Data are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test.

differences in lineage hallmark cytokines were detected between the groups, CD44$^+$ CD4$^+$ T cells of female *Aire*$^{-/-}$ mice produced less TNF-α (which is mainly produced by T_H1 cells) compared to *Aire*$^{+/+}$ mice. Activated CD4$^+$ T cells of female *Aire*$^{+/+}$ (and to a lesser extent *Aire*$^{-/-}$) mice expressed higher amounts of IL-4 (typical for T_H2 cells) compared to males. The role of IL-4 in atherosclerosis is controversial (2) and gender-specific effects have not yet been described.

Aire Deficiency Does Not Affect Atherogenesis in *Apoe*$^{-/-}$ Mice

To determine whether AIRE deficiency affects atherogenesis, we quantified en face atherosclerotic lesions in the aorta. In line with previous data (31), lesion size in *Apoe*$^{-/-}$ mice fed a WD was higher compared to those fed a CD (**Figures 4A,B**). However, irrespective of diet and gender, Aire deficiency did not influence plaque growth.

DISCUSSION

We herein demonstrate that deficiency of the TF AIRE, which regulates expression of many TRAs, does not affect generation and activation of CD4$^+$ T cells responding to the ApoB peptide p6 in *Apoe*$^{-/-}$ mice exposed to a WD. Irrespective of Aire, ApoB$^+$ T cells exhibited a mixed proinflammatory T_H1 T_H17 T_{FH} phenotype, which is in line with previous data (5). AIRE deficiency associated with a reduction in thymic T_{regs} in female *Apoe*$^{-/-}$ mice and with a slight decrease in the frequency of lymph node ApoB$^+$ T_{regs} (which was only significant when compared with male but not female *Aire*$^{+/+}$ mice). Given that no relevant alterations of other lymph node ApoB$^+$ T cell subtypes could be detected in *Aire*$^{-/-}$ mice, the influence of AIRE on phenotypes of ApoB$^+$ T cells was overall modest and inconsistent. Alterations in the frequencies of T_{regs} or T_H1 cells have been shown to substantially impact atherogenesis: Depletion of T_{regs} induced a 1.5–2 fold increase in lesion size (6, 7), whereas expansion of T_{regs} through active immunization strategies reduced atherosclerosis by more than 50% in mice (32). Likewise, genetic or antibody-mediated depletion of T_H1 cells conferred significant atheroprotection (33, 34). However, in the present study, AIRE-deficiency influenced atherogenesis neither in CD- nor in WD-fed *Apoe*$^{-/-}$ mice. We thus conclude that the minor phenotypic alterations of ApoB$^+$ T cells in Aire-deficient mice do not reflect a clinically relevant effect. Rather, our findings collectively suggest that AIRE does not regulate thymic expression of ApoB peptides or other autoantigens triggering proatherogenic adaptive immune responses. Conflicting findings whether AIRE affects thymic ApoB expression have been reported (15, 24, 25). ApoB is a large protein and these inconsistencies might be attributable to the technology and sequences used to detect ApoB (microarray vs. bulk transcriptomics) (15, 24). It can thus not be excluded that thymic expression of some ApoB peptides might be AIRE-dependent. Yet, we show here that the CD4$^+$ T cell response toward ApoB p6 and atherosclerotic burden was unaffected by AIRE-deficiency.

Our study has several limitations. We analyzed atherosclerotic plaque are exclusively in the aorta and not in the aortic root. As aortic atherosclerotic plaque area was unchanged by Aire-deficiency in all groups, we also refrained from extensive phenotyping of the atherosclerotic plaque environment for cellular composition and structural alterations. We thus cannot exclude, that Aire-deficiency might affect these parameters. It should be noted, that we only analyzed CD4$^+$ T cells responding to the ApoB peptide, p6, which limits the generalizability of our findings. The choice of the p6 peptide was motivated by recent studies, in which p6-reactive CD4$^+$ T cells were conclusively identified as important modulators of atherogenesis (5, 35, 36). The restriction to p6 does not allow direct inferences on the role of Aire in modulating generation, activation, or phenotypic

alterations of $CD4^+$ T cells responding to other ApoB peptides. However, a clinically relevant effect can almost certainly be excluded, since Aire-deficiency had absolutely no influences on atherogenesis.

Whereas, ApoB has been shown to be expressed by mTECs (23–25), we herein observed no impact of AIRE on atherogenesis and on ApoB-reactive CD4 T cells. We thus suggest that other mediators of central tolerance might be involved in modulating proatherogenic immune responses. Besides AIRE, FEZF2 was identified to regulate TRA gene expression in mTECs (16). Additionally, DCs and B cells are implicated in thymic presentation of circulating self-antigens and negative selection of autoreactive T cells (12). Recent evidence suggested that peripheral mechanisms might play a dominant role in mediating proatherogenic immunity. Particularly, polyclonal and, more importantly, $ApoB^+$ T_{regs} undergo a phenotypic transition into proatherogenic T_H1/T_{reg}, T_{FH} (37, 38), or T_H1/T_H17 subtypes (5). Such phenotype switching also applied to adoptively transferred $ApoB^+$-T_{regs} that failed to confer atheroprotection to *Apoe*$^{-/-}$ mice (5). Adaptive immunity against ApoB is centrally involved in atherogenesis and specific therapies to modulate this proatherogenic immune responses do not yet exist. Hence, it is of high clinical relevance to clarify mechanisms of central and/or peripheral immune tolerance to ApoB. Particularly, the roles of FEZF2, thymic DCs and B cells in enabling central tolerance to ApoB and the underlying mechanisms of ApoB T cell phenotype switching need to explored in future studies.

Women are much more susceptible to several autoimmune diseases than men (39). AIRE expression was shown to be induced by androgen (40) but attenuated by estrogen (41), which might contribute to gender differences in prevalence of autoimmune diseases. Herein, we only detected minor and overall inconsistent immunological differences between male and female mice, whereas the overall phenotype regarding atherosclerotic lesion size and immunological parameters did not differ. Thymic generation of bulk T_{regs} was modestly increased in female compared to male *Aire*$^{+/+}$*Apoe*$^{-/-}$ mice. Considering the role of Aire in inducing thymic generation of some T_{regs} (28, 29) and the above-mentioned modulation of *Aire* expression by sex hormones, an increased T_{reg} frequency in the thymi of male compared to female *Aire*$^{+/+}$ mice would have been expected. Why the opposite was the case in our study, remains unclear and warrants exploration in future studies. Secondly, in comparison to male *Aire*$^{+/+}$*Apoe*$^{-/-}$ mice, females harbored increased $CD4^+$, $CD8^+$, and γδ T cell counts in the lymph nodes. Additionally, some inconsistent variations of blood monocytes, neutrophils and lymphocytes between Aire-competent males and females were observed, which exclusively appeared either in CD- or WD-fed mice. Increased $CD4^+$ T cells frequencies have been reported in female *Apoe*$^{-/-}$ mice (42) and humans (43). Yet, contrary to our findings, previous studies detected higher numbers of $CD8^+$ T cells in male *Apoe*$^{-/-}$ mice and humans compared to females and women, respectively (42, 43). We recently reported on increased IL-17, IFNg, and IL-10 production in p6-$ApoB^+$ $CD4^+$ T cells in comparison to ApoB-negative $CD4^+$ T cells (5). We here focused on the analysis of $CD4^+$ T cell lineage transcription factors in p6-reactive $CD4^+$ T cells, which was largely unaffected by Aire-deficiency. We do not report on the production of intracellular cytokines in p6-reactive $CD4^+$ T cells and cannot exclude any effects that Aire-deficiency cause in these cells. Bulk $CD4^+$ T cells of female mice exhibited increased IL-4 production. Also women exhibit a predominant T_H2 cytokine profile compared to men (44), which may be explained by elevated IL-4 production in progesterone-treated $CD4^+$ T cells (45). It may thus be interesting to investigate whether IL-4 harbors specific atheroprotective properties in females. Further work will be needed to elucidate the confirmability and biological importance of herein-revealed sex-related differences in immune cell composition during atherogenesis.

In conclusion, this study excludes AIRE as a mediator of central immune tolerance to ApoB and player in atherogenesis. Clarification of mechanisms underlying proatherogenic auto-immunity represents a fundamental requirement for development of anti-atherosclerotic immune therapies, which are currently lacking.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All animal experiments were conducted in accordance with the institutional guideline for the La Jolla Institute for Immunology animal facility.

AUTHOR CONTRIBUTIONS

FN and HW wrote the manuscript, prepared the figures, and analyzed the data. FN, SB, KK, MO, MV, JM, RS, AA, DW, and HW performed experiments. HW and KL conceptualized and supervised the work and provided funding. All authors substantially contributed to data research, critically discussed the content, reviewed the manuscript before submission, and read and agreed to the published version of the manuscript.

FUNDING

This research was funded by the Deutsche Forschungsgemeinschaft SFB TRR259 (397484323) and CCRC GRK2407 (360043781 to HW), the Neven-DuMont foundation to HW, the Koeln Fortune Program (363/2020 to FN), and HL115232, HL88093, and HL121697 from the National Heart, Lung and Blood Institute to KL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.812769/full#supplementary-material

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frontiers in Cardiovascular Medicine

REVIEW
published: 13 January 2022
doi: 10.3389/fcvm.2021.812702

Immunotherapeutic Strategies in Cancer and Atherosclerosis—Two Sides of the Same Coin

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The development and clinical approval of immunotherapies has revolutionized cancer therapy. Although the role of adaptive immunity in atherogenesis is now well-established and several immunomodulatory strategies have proven beneficial in preclinical studies, anti-atherosclerotic immunotherapies available for clinical application are not available. Considering that adaptive immune responses are critically involved in both carcinogenesis and atherogenesis, immunotherapeutic approaches for the treatment of cancer and atherosclerosis may exert undesirable but also desirable side effects on the other condition, respectively. For example, the high antineoplastic efficacy of immune checkpoint inhibitors, which enhance effector immune responses against tumor cells by blocking co-inhibitory molecules, was recently shown to be constrained by substantial proatherogenic properties. In this review, we outline the specific role of immune responses in the development of cancer and atherosclerosis. Furthermore, we delineate how current cancer immunotherapies affect atherogenesis and discuss whether anti-atherosclerotic immunotherapies may similarly have an impact on carcinogenesis.

Keywords: tumor, atherogenesis, cardiovascular disease, immunotherapy, immunity, T cell, checkpoint inhibition, co-stimulatory molecule

OPEN ACCESS

Edited by:
Emiel Van Der Vorst, Institute for Molecular Cardiovascular Research (IMCAR), Germany

Reviewed by:
Maria Jose Forteza, Karolinska Institutet (KI), Sweden
Rick I. Meijer, Radboud University Nijmegen Medical Centre, Netherlands
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *10 November 2021*
Accepted: *20 December 2021*
Published: *13 January 2022*

Citation:
Nettersheim FS, Picard FSR, Hoyer FF and Winkels H (2022) Immunotherapeutic Strategies in Cancer and Atherosclerosis—Two Sides of the Same Coin. Front. Cardiovasc. Med. 8:812702. doi: 10.3389/fcvm.2021.812702

INTRODUCTION

Although prevention strategies and therapeutic opportunities have been significantly improved during the past decades, atherosclerotic cardiovascular diseases (CVD) and cancer still represent the two most common causes of death worldwide (1). As already recognized by Rudolph Virchow in the nineteenth century (2, 3), the critical role of inflammatory processes in atherogenesis and carcinogenesis is now well-established and has prompted investigation of strategies to combat these deadly diseases by modulating underlying immune responses (4–10). Several anti-cancer immunotherapies, such as cytokines, antibodies targeting immune cell receptors, or immune checkpoints, dendritic cell therapy, and chimeric antigen receptor (CAR) T cell therapy, already found their way into clinical practice and thereby revolutionized cancer treatment (9, 11). In stark contrast, clinically approved immunotherapies for CVD are still not available [except for antibodies targeting proprotein convertase subtilisin/kexin 9 (PCSK9) to lower low-density lipoprotein (LDL) cholesterol, representing an immunotherapeutic approach in a broader sense (12, 13)]. In 2017, the CANTOS trial demonstrated that administration of an antibody directed

against the pro-inflammatory cytokine interleukin-1β (IL-1β) reduced cardiovascular events in patients with coronary artery disease (CAD), thereby providing first evidence for effectiveness of an immunotherapy in CVD (14). Yet, this therapy increased the risk of fatal infections and did not reduce mortality, which consequently prevented its approval for treatment of CVD (14). CANTOS illustrated the central dilemma of many immunomodulatory strategies: Broad interventions in the immune system can have detrimental side effects. In general, anti-atherosclerotic strategies are geared toward suppression of vascular inflammation (5, 8, 15), whereas immune-based cancer treatments aim at enhancing immune responses against tumor cells (7, 9). The therapeutic efficacy of several anti-cancer immunotherapies is constrained by their proimmunogenic (and thus proatherogenic) properties, increasing the risk to develop CVD in patients (16). Particularly, immune checkpoint inhibitors directly aggravate atherosclerotic plaque growth in patients (17). Whereas, cancer survival has dramatically improved over the past few decades (18), the exposure of cancer survivors to therapy-induced cardiovascular risk represents an emerging problem, which leads to excess cardiovascular mortality and thus significantly affects long-term prognosis (19–22). This problem is relevant, as the global cancer burden is expected to increase by ∼47% within the next 20 years and to reach more than 28 million cases in 2040 (23).

In recent years, vaccination strategies aiming to either induce immune responses against tumor-specific neoantigens (4) or to suppress immunity against atherosclerosis-related autoantigens (6) have emerged. Immunization strategies are promising as they enable specific immunomodulation without impairing host defense responses or accelerating progression of atherosclerosis. Whereas, anti-atherosclerotic vaccination strategies are still in their infancy (6), therapeutic cancer vaccines are already being investigated in clinical trials (4).

In this review, we will provide an overview of current immunomodulatory concepts for treatment of cancer and atherosclerosis with a focus on their reciprocal interactions and consequences. Finally, we will highlight the potential of immunization strategies against cancer and CVD that enable targeted, antigen-specific immunity without affecting the immune system.

INFLAMMATION AND ADAPTIVE IMMUNITY IN ATHEROGENESIS

Atherosclerosis involves formation of lipid-laden plaques in large and medium-sized arteries (24), which may rupture or erode and give rise to acute thrombotic vessel occlusion (25). Plaque formation primarily occurs in regions with disturbed blood flow and low endothelial shear stress (26). Such hemodynamic alterations induce a cascade of endothelial dysfunction, subendothelial accumulation and subsequent oxidation of lipoproteins, and finally an inflammatory response that is characterized by monocyte infiltration and foam cell formation (25, 26). Extensive research during the past decades has indicated that plaque-related inflammation is not simply a passive process but is rather orchestrated by an adaptive immune response involving T cells and humoral immunity (27).

T cells derive from hematopoietic progenitor cells and undergo a complex maturation and selection process in the thymus, which is characterized by development of a unique, antigen-specific T cell receptor (TCR) through random genetic recombination (28) and elimination of cells that are either non-functional or bind self-antigens with too high-affinity, which are potentially dangerous for the host (29). The high prevalence of autoimmune disorders indicates the insufficiency of this process. Eventually, the TCR and one of its co-receptors CD4 or CD8 are expressed on the T cell surface, which is released into the periphery and circulates through the body to encounter its cognate antigen (30). Activation of a naïve T cell requires two signals. First, the TCR must be bound by its cognate antigen: $CD4^+$ T cell activation requires presentation of an antigenic peptide-sequence, the so-called epitope, on major histocompatibility complex class II (MHC-II) molecules, which are exclusively expressed by professional antigen-presenting cells (APCs), such as dendritic cells, macrophages and B cells. $CD8^+$ T cells recognize antigens presented on MHC-I molecules, which are expressed by all nucleated cells (31). Second, the T cell must simultaneously receive a proper co-stimulatory signal, that is binding of a specific receptor (such as CD28) by its ligand expressed on the APC (32). Once activated, T cells proliferate and $CD8^+$ T cells become cytotoxic, whereas $CD4^+$ T cells can differentiate into a variety of different subtypes, which are characterized by expression of specific surface markers, transcription factors (TFs) and cytokines (33). For example, T helper 1 (T_H1) cells, which are characterized by expression of the TF T-box expressed in T cells (T-bet), exert pro-inflammatory effects through production of interferon gamma (IFN-γ) (**Figure 1**). In contrast, regulatory T cells (T_{regs}), which are characterized by expression of the TF forkhead box protein P3 (FoxP3), produce the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β) and thus ensure immune tolerance. For a thorough overview of different T cell subtypes and their role in atherosclerosis the interested reader is referred to Saigusa et al. (34).

Presence of T cells in atherosclerotic plaques was firstly described by Hansson and colleagues more than 30 years ago (35). T cells within the plaques were activated (36) and *in vitro* work showed lesional $CD4^+$ T cells responding to oxidized low-density lipoprotein (oxLDL), which established the theory of T cells contributing to plaque formation (37). By now, $CD4^+$ and $CD8^+$ T cell responses against plaque-associated autoantigens have been identified to modulate atherogenesis (34, 38, 39). Whereas, T cell reactivity against LDL was originally thought to be induced by oxidation-dependent generation of neoepitopes representing "altered self" (37), more recent work has identified $CD4^+$ T cells responding to peptides of native Apolipoprotein B (ApoB), the core protein of LDL, chylomicrons, and other lipoprotein particles. Several unmodified ApoB-peptides have been found to bind murine and human MHC-II molecules with high affinity and thereby evoke a $CD4^+$ T cell response (40–43). Such ApoB-reactive ($ApoB^+$) $CD4^+$ T cells mainly comprise T_{regs}, which confer atheroprotective properties in

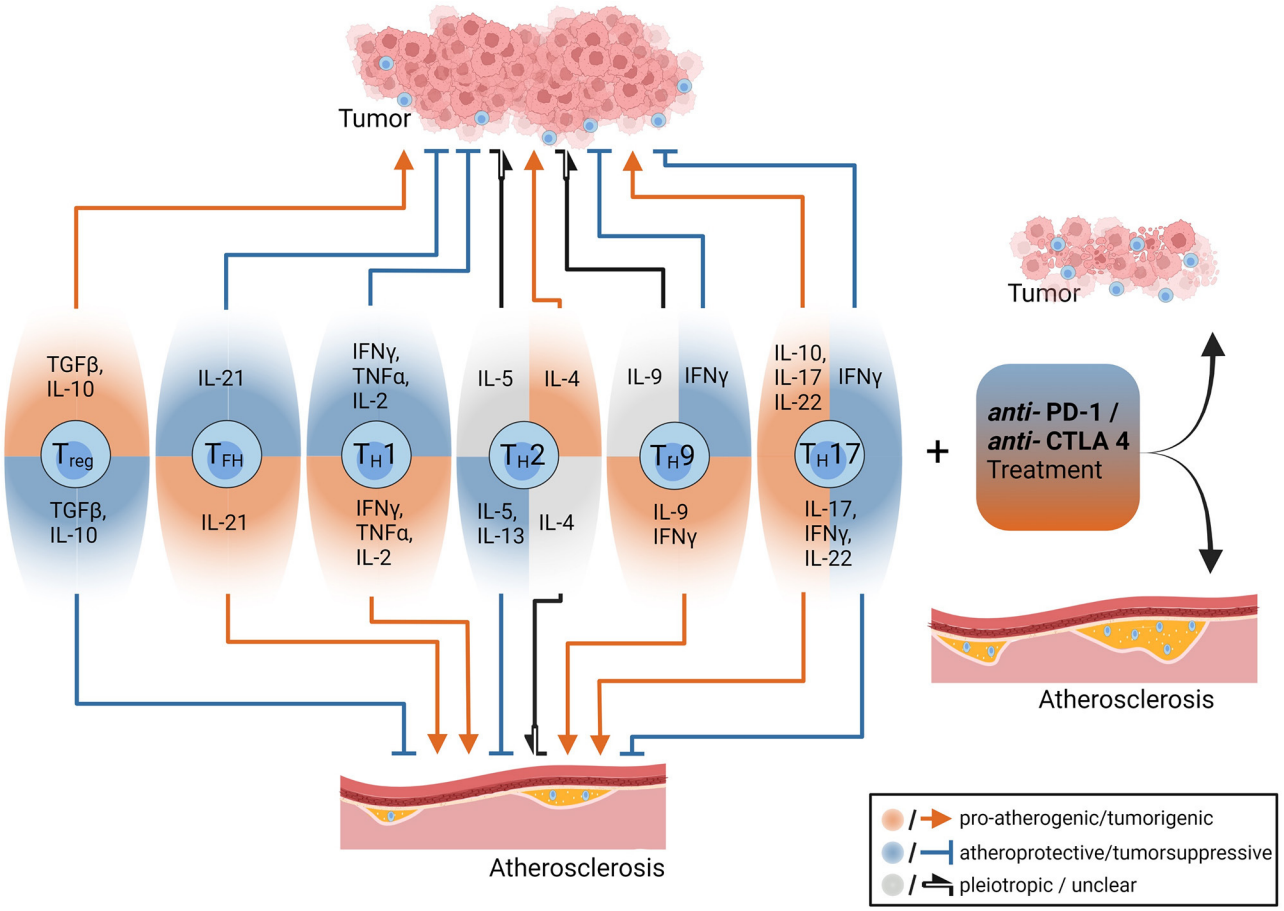

FIGURE 1 | The opposing roles of CD4$^+$ T-cells and anti-PD-1/anti CTL4 treatments in the pathogenesis of tumors and atherosclerosis. CD4$^+$ T cell subsets and their effector cytokines have different roles in tumors and atherosclerosis. Some cytokines such as interferon gamma have pro-atherogenic (red arrow) and tumor-suppressive effects (blue arrow with blunt end), while Interleukin-10 (IL-10) depending on its cellular source exerts anti-atherogenic and tumor-progressive functions. Treatment with immune checkpoint inhibitors (anti-PD1 and anti-CTLA-4 antibodies) reduces tumor burden but drives atherosclerosis among others by enhancing pro-inflammatory T cell effector functions. The figure was created with Biorender.com.

healthy humans, but coexpress TFs typical of proatherogenic T_H1 or T_H17 cells in individuals with subclinical atherosclerosis as determined by carotid ultrasound (42). Preclinical studies further elucidated that CD4$^+$ T_{regs}, particularly those reactive to ApoB, gradually acquire proatherogenic T_H1/T_H17, T_H1/T_{reg} or T follicular helper (T_{FH}) phenotypes during atherogenesis (43–45). Whereas, therapeutic interventions that aim to stabilize and/or expand ApoB$^+$ T_{regs} hold promise for atherosclerosis prevention and treatment, immunomodulatory therapies causing destabilization of T_{regs} naturally aggravate progression of atherosclerotic lesions (46).

Besides T cells, humoral immune responses against plaque-associated autoantigens have been implicated in atherogenesis (38, 47). Antibodies directed against oxLDL are detectable in human plaques (48) and in plasma samples of humans with our without atherosclerotic CVD (49, 50). Accordingly, B cells can be found in healthy and atherosclerotic vessels, especially in arterial tertiary lymphoid organs located in the adventitia (51). Autoantibodies against oxLDL were shown to block uptake of oxLDL by macrophages (52, 53) and to confer atheroprotection (54). Genetic B-cell depletion aggravated atherosclerosis in LDL-receptor-deficient (*Ldlr*$^{-/-}$) mice (55). Yet, depletion of mature B cells through administration of a CD20 monoclonal antibody was unexpectedly atheroprotective in Apolipoprotein E-deficient (*Apoe*$^{-/-}$) and *Ldlr*$^{-/-}$ mice (56, 57). This treatment preserved the production of natural IgM antibodies directed against oxLDL but reduced anti-oxLDL IgG antibodies (56). Adoptive transfer of B2 B cells, but not B1 B cells, to lymphocyte or B cell deficient *Apoe*$^{-/-}$ mice was proatherogenic (57). B cells mainly consist of B2 B cells (which are thus termed conventional B cells) that undergo maturation in the spleen and can produce high-affinity IgG antibodies after receiving T cell help (58). B1 B cells represent a specialized B cell subpopulation: They develop in the fetal and neonatal period, harbor the capability of self-renewal, mainly reside in body cavities and are characterized by the production of so called "natural" IgM antibodies (59).

Taken together, different B cell subsets and antibody subtypes may exert diametral functions in atherogenesis and therapeutic modulation of humoral immune responses could represent an attractive anti-atherosclerotic strategy but also promote atherosclerosis progression.

INFLAMMATION AND ADAPTIVE IMMUNITY IN CANCER

In the nineteenth century, the German physicians Rudolph Virchow, Wilhelm Busch and Friedrich Fehleisen independently hypothesized that inflammation may affect carcinogenesis (2, 60). Whereas, Virchow assumed that leukocyte infiltrates represented an underlying cause of cancer (2), Busch and Fehleisen suggested that inflammation may reverse tumorigenesis (60). After independently observing involution of malignancies in patients with erysipelas, they demonstrated tumor regression in cancer patients upon intentional infection with bacteria isolated from erysipelas (61, 62). Later, the American surgeon William Coley reported disappearance of tumors in patients with inoperable sarcoma or other types of cancer after treatment with heat-inactivated bacteria which was termed "Coley's Toxin" (63). In 1909, Paul Ehrlich suggested that cellular immunity may recognize neoplastic cells and protect from tumor development, although he was not able to experimentally substantiate this hypothesis (64). First experimental proof for anti-tumor immune responses was provided by Gross and Foley around 1950 (65, 66) and Paul Ehrlich's concept was adopted by Lewis Thomas and Sir Frank Macfarlane Burnet who proposed that lymphocytes recognize and target cancer cells through their expression of tumor-specific antigens, similar to homograft rejection (known as immunosurveillance) (7, 67). Yet, this theory was abandoned after immunologically impaired animals, such as athymic nude mice, showed similar susceptibility to experimentally induced tumors (68, 69). Several limitations of these experiments became evident: Nude mice—despite lack of T and B cells—are not completely immunocompromised and especially susceptible to 3-methylcholanthrene, the chemical carcinogen which was used for tumor induction (due to expression of highly active enzyme isoforms involved in biotransformation of the chemical) (68). Novel immunocompromised mouse models with pure genetic backgrounds demonstrated that lymphocyte deficiency (70), lack of perforin (an important component of cytotoxic T lymphocyte granules) (71, 72), and ablation of proinflammatory cytokine signaling (70–73) increased tumor susceptibility in mice, which led to the renaissance of the immunosurveillance theory (68). This preclinical evidence was supported by studies reporting an increased cancer risk in immunocompromised patients (74–76) and that tumor lymphocyte infiltration predicts better outcome (77, 78). Despite overall proof in support of the immunosurveillance theory was provided, subsequent work demonstrated that immunity may also exert tumor-sculpting effects (68): Tumors derived from immunocompromised mice were rejected more frequently when transplanted into immunocompetent recipients than tumors derived from wild-type controls (70, 72, 79, 80). Thus, the immune system of the wild-type donors must have shaped tumors to become less immunogenic and more resistant to the hosts (uncompromised) immune response.

To account for the dual role of the immune system in tumor development, G.P Dunn and R.D. Schreiber proposed the groundbreaking "immunoediting" or "three E's" theory in 2002 (68), which is the current explanation of tumor-related immune responses (81). The theory involves three processes: (1) In the elimination phase, which conforms to the original immunosurveillance theory, tumor cells are targeted by innate and, subsequently, adaptive immune cells including tumor antigen-specific $CD4^+$ and $CD8^+$ T cells. If the immune system is successful in destroying all tumor cells, progression to subsequent phases is prevented. (2) In the equilibrium phase, that may last for years, tumor cells that have survived the initial elimination process and the ongoing immune response are in balance. Although tumor growth is still under immunological control, the immune system fails in eliminating all tumor cells, and thus causes selection pressure on the surviving variants. (3) In the escape phase, tumor cells that have undergone extensive immunoediting, evade immunological control, and expand rapidly, resulting in development of clinically apparent disease (68).

More recently, immunological processes underlying the three phases of immunoediting have been characterized in greater detail, which has led to development of immunotherapies that efficiently enhance anti-tumor immune responses (82, 83). Modern technologies have enabled identification of tumor-specific, MHC-I and -II restricted neoantigens and detection of $CD8^+$ and $CD4^+$ T cells responding to such neoantigens (82, 83). These technologies include deep-sequencing approaches to determine the "mutanome," that is the entirety of tumor-specific mutations, followed by *in-silico* prediction algorithms to identify mutation-specific epitopes capable of binding to MHC-I or -II molecules (84–88). In a second step, immunogenicity of identified epitopes is verified through T cell restimulation assays of peripheral blood mononuclear cells from the sequenced patient (84, 86–89) or MHC-I (85, 90) and -II (91, 92) tetramers or multimers detecting tumor-neoantigen-specific $CD8^+$ and $CD4^+$ T cells, respectively. Finally, multimer-selected neoepitope-specific $CD8^+$ and $CD4^+$ T cells can be phenotyped by flow-cytometry or (single-cell) RNA-sequencing approaches (90–92). Whereas, initial studies were focused on the role of $CD8^+$ T cells in mediating anti-tumor immunity (85, 87, 93), subsequent work established that the immunogenic mutanome—against former expectations- predominantly induced a $CD4^+$ T cell response in mice and humans (86, 89–91, 94). Tumor neoepitope-reactive $CD4^+$ T cells were crucially involved in generation of potent anti-tumor $CD8^+$ T cell responses (92, 95). This T cell help is mainly mediated by interactions of CD40 ligand (CD40L), which is expressed on the surface of activated $CD4^+$ T cells, and CD40 on the surface of APCs (96). Additionally, $CD4^+$ T cells may exert direct anti-neoplastic activity through production of pro-inflammatory cytokines or execution of cytotoxic signals on tumor cells and aid in B cell mediated humoral anti-tumor responses through CD40L signaling (97).

Neoepitope-specific $CD4^+$ T cells of the T_H1 subtype are involved in anti-tumor responses (92). Adoptive transfer of neoepitope-specific $CD4^+$ T_H1 cells led to tumor regression in a patient with metastatic cholangiocarcinoma (98). In line with this, high levels of circulating tumor-antigen-specific T_H1 $CD4^+$ T cells and low levels of $CD4^+$ cells co-expressing the immune-checkpoints programmed cell death protein 1 (PD-1) and T cell immunoglobulin and mucin-domain containing-3 (Tim-3) predict better survival in lung cancer patients (99). In contrast, high levels of tumor-infiltrating T_{regs}, which can be found in various cancer types, are associated with poor prognosis (100, 101). Animal studies identified tumor-induced conversion of $CD4^+$ non-T_{regs} into T_{regs} as an important mechanism of immune escape (102) and, accordingly, circulating tumor-antigen-specific T_{regs} can be detected in cancer patients but not in healthy individuals (103). Other T helper cell subsets, such as T_H2 and T_H17 cells, can also be found in the tumor microenvironment, but their specific role in tumor immunity and prognostic importance are still under debate (104, 105).

In conclusion, $CD4^+$ T cells responding to tumor-specific neoepitopes play an important role in mediating anti-tumor immune responses. Yet, tumor cells may engage various escape mechanisms to acquire resistance to this response, which include induction of $CD4^+$ T cell phenotype switching from proinflammatory anti-neoplastic T_H1 cells into immunosuppressive and thus tumor growth-promoting T_{regs} (105).

EFFECTS OF CLINICALLY APPROVED CANCER IMMUNOTHERAPIES ON ATHEROGENESIS

Several immunotherapeutic strategies aim at preserving or restoring anti-tumor immune responses. Yet, the opposing roles of adaptive immunity in atherosclerosis and cancer development (**Figure 1**) implicate that such therapeutic approaches might involve proatherogenic side effects (17), especially if they are not antigen-specific but affect the immune system as a whole. In contrast, B cell depleting antibodies and antibodies targeting growth factor receptors overexpressed by tumor cells may confer atheroprotection. In the following section we will discuss effects of clinically approved cancer immunotherapies on atherogenesis and delineate their mechanistic background (an overview of these effects is given in **Table 1**).

Immune Checkpoint Inhibitors (ICIs)

Immune checkpoints refer to a variety of regulatory pathways that exert inhibitory actions on adaptive immune cells and beyond and are thus critical for preservation self-tolerance and prevention of exaggerated immune responses (130). The Nobel prize winning discoveries of James P. Allison and Tasuku Honjo, who unraveled that tumor cells may engage immune-checkpoint pathways to escape from anti-tumor immune responses, have paved the way for the development of monoclonal antibodies against these molecules—immune checkpoint inhibitors (ICIs) (131, 132). Ipilimumab inhibits the cytotoxic T lymphocyte antigen 4 (CTLA-4) and was shown to improve overall survival in patients with metastatic melanoma (133), which made it the first ICI approved by the Food and Drug Administration (FDA) in 2011 (134). Subsequently, four antibodies (pembrolizumab, nivolumab, cemiplimab, and dostarlimab) targeting the co-inhibitory programmed cell death protein 1 (PD-1) and three antibodies (atezolizumab, durvalumab, and avelumab) directed against the programmed cell death ligand 1 (PD-L1) were demonstrated to effectively improve survival in several malignancies (134, 135) which led to the FDA-approval for treatment of 19 different cancer types and two tissue-agnostic conditions [that is a tumor with a specific genetic alteration regardless of the cancer type and location (136)]. ICIs have become a cornerstone of modern cancer therapy and nowadays more than 40% of cancer patients are eligible for ICI treatment (137).

Given that immune checkpoints represent important regulators of physiological immune responses, ICI therapy can naturally involve inflammatory side effects, which are referred to as immune-related adverse events (IRAEs) (138). Although the precise pathomechanisms of such IRAEs are not yet fully clear, unconstrained activation of autoreactive T cells is suggested to play a dominant role (138). CTLA-4 and PD-1 are co-inhibitory molecules expressed on the cell surface of $CD4^+$ and $CD8^+$ T cells (139, 140). When bound by their ligands—CD80/CD86 and PD-L1/PD-L2—CTLA-4- and PD-1 suppress activation of T cells (140). As mentioned above, T cell activation requires simultaneous engagement of the TCR by its cognate antigen and proper costimulatory signals (32). Activation of CD28, the prototype co-stimulatory molecule, by its ligands CD80 or CD86 induces high T cell surface expression of the co-inhibitory molecule CTLA-4 (141). CTLA-4 binds CD80/CD86 with much higher affinity than CD28. However, in contrast to CD28, CTLA-4 does not exert stimulatory but inhibitory signals and thus attenuates T cell activation (141). Given that CD80/CD86 are expressed on the surface of APCs, CTLA-4 inhibits T cell activation mainly in the priming phase. Prolonged TCR stimulation during an ongoing immune response induces PD-1 expression on the cell surface of T cells (141). When bound by its ligands PD-L1 or PD-L2, which can be expressed by tumor cells, PD-1 attenuates TCR-signaling and thus reduces T cell proliferation and cytokine production. Thus, PD-1 mediates T cell inhibition in the effector phase and is used as a marker of T cell exhaustion (141). Consequently, antibody-mediated inhibition of CTLA-4, PD-1 and PD-L1 enhances T cell activation. IRAEs can affect almost every organ and mostly occur within 2–16 weeks after treatment initiation (138, 142). According to a recent meta-analysis including 36 phase II and III randomized controlled trials (RCTs), the pooled incidence of all IRAEs ranges between 54 and 76% (143). Whereas, the incidences of specific IRAEs depend on the ICI used and several other factors, the integumentary, gastrointestinal, endocrine, hepatic, and pulmonary systems are overall most commonly affected (143, 144). In a meta-analysis of 112 trials including 19,217 patients, IRAE-associated fatality rates ranged between 0.36% for anti-PD-1 mono-therapy and 1.23% for PD1/PD-L1

TABLE 1 | Pro-atherogenic and athero-protective effects of current cancer immunotherapies.

Type of immunotherapy	Specific approach/substance	Compounds	Effect on atherosclerosis in clinical trials	Effect on atherosclerosis in animal studies	Potential mechanisms
Immune checkpoint inhibitors	Anti-CTLA4-Abs	Ipilimumab	↑ (17, 106)	↑ (107–111)	- Increased plaque-infiltration by $CD4^+$ and $CD8^+$ T cells - Higher expression of proinflammatory cytokines (IFN-γ and TNF-α) by T cells - Enhanced T cell activation
	Anti-PD1-Abs	Pembrolizumab, Nivolumab, Cemiplimab, Dostarlimab			
	Anti-PD-L1-Abs	Atezolizumab, Durvalumab, Avelumab			
Monoclonal antibodies	Anti-CD20-Abs	Rituximab, Obinutuzumab, Ofatumumab	↓ (112–114)	↓ (56, 57)	- Depletion of mature B cells and reduction of anti-oxLDL IgG antibodies
	VEGF inhibitors	Bevacizumab, Ramucirumab	↑ (115–117)	↑ (118)	- Induction of an inflammatory endothelial cell phenotype and impairment of endothelial function - Reduction and functional impairment of T_{regs} and induction of proinflammatory T_H1 cells
	EGFR targeting Abs	Cetuximab, Necitumumab, Panitumumab	–	↓ (119–121)	- Reduced accumulation of macrophages in plaques - Reduced lipid uptake by macrophages and reduced foam cell formation - Reduced $CD4^+$ T cell activation, proliferation and plaque infiltration - Reduced pro-inflammatory cytokine production Reduced SMC proliferation
Cytokines	IFN-α	Interferon alfa	↑ (122, 123)*	↑ (124)	- Increased plasma cholesterol and triglyceride levels - Induction of lipid uptake by macrophages and increased foam cell formation - Inhibition of T_{reg} activation and proliferation - Direct stimulation of cytotoxic $CD4^+$ T cell function - Sensitization of antigen-presenting cells toward pathogen-derived TLR4 ligands
	IL-2	Aldesleukin	High-dose: ↑ (125) Low-dose: ?**	High-dose: ↑ (126) Low-dose***: ↓ (127)	- High-dose: unspecific expansion of T cells - Low-dose: selective expansion of functional T_{regs}
Antifolate	DHFR inhibition	Methotrexate	- (128)	↓ (129)****	- Attenuation of monocyte maturation and recruitment - Modulation of lipoprotein transcellular transport - Reduction of pro-inflammatory cytokine production

*↑ = proatherogenic effect. ↓ = atheroprotective effect. *These studies demonstrated an association between plasma IFN-α levels and atherosclerosis. A direct effect of IFN-α administration on atherosclerosis has not yet been shown in clinical trials. **Currently investigated in the "Low-dose interleukin-2 in patients with stable ischemic heart disease and acute coronary syndromes (LILACS)" trial. ***and complexed with a specific anti-IL2-mAB (JES6-1A12).****delivered via nanoparticles.*

Abs, monoclonal antibodies; CTLA4, cytotoxic T lymphocyte antigen 4; DHFR, dihydrofolate reductase; EGFR, epidermal growth factor receptor; IFN-α, interferon alpha; IFN-γ, interferon gamma; IL2, interleukin 2; mAB, monoclonal antibody; oxLDL, oxidized low density lipoprotein; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; SMC, smooth muscle cell; TLR4, toll-like-receptor 4; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

plus CTLA-4 combinational therapy and were most commonly caused by colitis, pneumonitis, hepatitis, myocarditis and neurotoxic effects (145). Cardiovascular IRAEs, which include myocarditis, pericardial diseases, heart failure, dyslipidemia, myocardial infarction, and cerebral arterial ischemia, are, overall, relatively rare with an incidence ranging between ∼3 and 20 per 1,000 patients (146). Yet, cardiovascular toxicities are severe in over 80% of cases (147) and myocarditis, which carries the

highest fatality risk of all IRAEs (40–50%), is of particular prognostic relevance (145, 147).

Besides acutely occurring cardiovascular IRAEs, recent evidence has suggested that ICI therapy may promote atherogenesis (148, 149). In a retrospective analysis of 1,215 patients treated with ICIs, atherosclerotic cardiovascular events (CVE) occurred in 1% within a follow-up period of 6 months (150). In three meta-analyses, the ICI-related incidence of myocardial infarction and stroke ranged from 0.4 to 1.0% and 1.1 to 2.0%, respectively (149). Yet, the majority of studies included in these meta-analyses were not specifically designed to assess CVE and may thus underestimate incidences (149). To evaluate the ICI-related risk of atherosclerotic CVE (defined as the composite of myocardial infarction, coronary revascularization, and ischemic stroke), Drobni et al. analyzed event-rates in 2,842 patients treated with ICIs and matched controls (17). Additionally, a case-crossover analysis was performed, in which event rates within the 2 years before (control period) and the 2 years after (at-risk period) initiation of ICI therapy were compared. ICI therapy was associated with a 3-fold and almost 5-fold higher risk of atherosclerotic CVE in the matched-control study and case-crossover analysis, respectively (17). In a nested imaging substudy including 40 patients, a 3-fold increase in aortic atherosclerotic plaque volume progression from 2.1%/year before to 6.7%/year after ICI initiation could be detected (17). Another recent study retrospectively analyzed 2-[^{18}F]fluorodeoxyglucose (FDG) positron emission tomography/computed tomography scans, which had been performed in 20 melanoma patients before and during ICI treatment (mean time interval: 4.4 months) (106). A significantly increased FDG uptake in large arteries after ICI treatment initiation could be detected, pointing toward an ICI-related induction of arterial inflammation (106). In accordance to these clinical findings, a series of animal studies reported enhanced plaque inflammation and accelerated atherogenesis in *LDLr*$^{-/-}$ mice genetically deficient for or treated with inhibitory antibodies against PD-1, PD-L1 and CTLA-4 (148, 149). This was accompanied by an increased number of plaque-infiltrating CD4^{+} and CD8^{+} T cells (107–111), higher expression of proinflammatory cytokines [IFN-γ and tumor necrosis factor alpha (TNF-α)] by T cells (107, 111), and enhanced T cell activation (108, 110, 111).

Collectively these data emphasize that ICI therapy promotes atherogenesis and substantially increases the risk of atherosclerotic CVE. Presumably, ICIs exert their proatherogenic effects—at least in part—through disinhibition of T cells responding to plaque-associated autoantigens. Atherosclerosis is a slowly progressing disease and all above-mentioned clinical studies were limited by relatively short follow-up periods. As indications for ICI therapy are rapidly expanding and cancer-survival has dramatically improved in recent years, the detrimental impact of ICIs on atherogenesis will, therefore, likely become a more relevant health issue in the future.

Antibody Therapy

Since the FDA approval of muromonab-CD3, a monoclonal antibody targeting the T cell co-receptor CD3, for the prevention of transplant rejection in 1986, more than 100 therapeutic antibodies have been included in clinical practice (135). Rituximab, a monoclonal antibody targeting the B cell receptor CD20, was approved for treatment of follicular lymphoma in 1997, which opened the door for the use of antibodies in cancer therapy (151). Cancer has emerged as the most common condition for antibody therapy with currently over 40 FDA-approved antibodies (including ICIs) for treatment of several cancer types (135). Antibodies can target cancer through several mechanisms, including direct tumor cell killing, immune-mediated tumor cell-killing, and inhibition of neovascularization or stroma cells (152, 153). Direct tumor cell killing can be achieved through eliciting agonistic activity to apoptosis-promoting receptors, inhibiting growth factor receptor signaling, neutralizing key enzymes, or delivering cytotoxic agents into the cell (152, 153). Mechanisms of immune-mediated tumor cell killing include induction of phagocytosis, complement-activation and cellular toxicity (152, 153). Besides ICIs, the use of several other monoclonal antibodies in cancer therapy is constrained by their cardiovascular side effects, which include myocarditis, heart failure, arrhythmia, orthostatic dysregulation and atherosclerotic cardiovascular events (16). Fortunately, the latter complication is rare and some antibodies can even confer atheroprotective effects.

Antibody-Mediated B Cell Depletion

During the past two decades, B cell depleting strategies have been used for treatment of B cell lymphoma and several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis (MS) (151, 154). In addition to rituximab and other antibodies targeting CD20 (e.g., obinutuzumab and ofatumumab), antibodies directed against the B cell surface proteins CD19 (blinatumomab), CD22 (inotuzumab ozogamicin and moxetumomab pasudotox), CD38 (daratumumab and isatuximab) and CD319 (elotuzumab) have been approved to treat these conditions (153).

As mentioned above, depletion of B cells through administration of a CD20-specific antibody ameliorated atherogenesis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (56, 57). In line with this, treatment of *Apoe*$^{-/-}$ mice with a monoclonal antibody targeting B cell activating factor-receptor (BAFFR) to selectively deplete mature B2 cells while sparing B1 cells conferred atheroprotection (155). Similarly, antibody-mediated inhibition of the cytokine B cell–activating factor (BAFF) reduced atherosclerosis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (156). These findings were recently confirmed in a clinical study: Patients who received rituximab therapy after kidney transplantation had a significantly lower rate of atherosclerotic CVE during 8 years of follow-up as compared to propensity-matched controls (112). Accordingly, rituximab therapy was shown to reduce carotid intima media thickness (113) and to improve flow mediated dilation of the brachial artery, a non-invasive marker of endothelial function (114). The effect of other B-cell–depleting antibodies (including those targeting receptors predominantly expressed on antibody-secreting plasma cells, such as CD39 and CD319) on atherosclerosis has not yet been investigated and the role of plasma cells in atherogenesis is not yet clear (157). Depletion of IgG-producing plasma cells reduced atherosclerotic

plaque development in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (158, 159) but associated with plaque instability, which may have deleterious consequences in patients with preexisting atherosclerosis (158). Evidence from preclinical and clinical studies indicate that antibodies targeting CD20 may confer atheroprotection, but these early findings will have to be confirmed in larger clinical trials.

Antibodies Targeting Vascular Endothelial Growth Factor

Bevacizumab was the first clinically approved monoclonal antibody targeting vascular endothelial growth factor (VEGF) (160). Originally thought to exert antineoplastic actions exclusively via inhibition of tumor angiogenesis, VEGF-targeted therapies have been demonstrated to arrest tumor growth through a variety of mechanisms, which are not yet fully understood (160). In addition to bevacizumab, an antibody targeting VEGF receptor 2 (ramucirumab) and small-molecules inhibiting VEGF receptor tyrosine kinases (sorafenib and sunitinib) have been FDA-approved (153). A major drawback of VEGF inhibitors is their tendency to induce atherosclerotic CVE. Recent meta-analyses including up to 22 studies reported a ≈ 1.4- to 2.5-fold higher risk of arterial ischemia in patients treated with bevacizumab (115–117). High-dose bevacizumab therapy was even associated with a 4.4- and 6.7-fold higher risk of cardiac and cerebral ischemia, respectively (115). A preclinical study confirmed and mechanistically substantiated these findings by demonstrating that administration of a VEGF-targeting antibody impaired endothelial function and increased atherosclerotic lesions by 33% in *Apoe*$^{-/-}$ mice (118). Accordingly, VEGF inhibitors were shown to induce an inflammatory phenotype in cultured human coronary artery endothelial cells (161). Besides affecting endothelial function, VEGF inhibitors may decrease the number of T_{regs} and impair their suppressive capacity, reduce expression of co-inhibitory T cell molecules, and thus induce proinflammatory T_H1 responses (162). Although experimental proof is missing, these immunological effects might contribute to the proatherogenic properties of VEGF-inhibiting antibodies (162).

Antibodies Directed Against Epidermal Growth Factor Receptors

Receptors of the epidermal growth factor receptor family, such as epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2/neu), may be overexpressed by tumor cells of several cancer types which can thus acquire the capability of autonomous and uncontrolled proliferation (163, 164). Overexpression of EGFR or HER2/neu is a strong predictor of a negative prognosis in a variety of malignancies (165, 166) and the development of monoclonal antibodies targeting such receptors has advanced cancer treatment. Early clinical studies and large-scale phase 3 trials showed improved outcome in patients with metastatic breast cancer and gastric cancer treated with trastuzumab (targeting HER2/neu) and patients with metastatic colorectal cancer and head and neck cancer treated with cetuximab (directed against EGFR) (167–170). Further HER2/neu and EGFR targeting antibodies have been clinically approved (153). A major drawback of growth factor receptor targeting antibodies (especially trastuzumab) is their potential to induce heart failure, which occurs in up to 20% of all cases (167, 171) and is 1.7 to 4 times more frequently compared to standard chemotherapy (172–174). Accordingly, mice lacking Her2/neu were demonstrated to develop dilated cardiomyopathy (175).

Direct effects of antibodies targeting growth factor receptors on atherogenesis have not yet been reported in clinical trials. Nevertheless, EGFR was detected in human atherosclerotic plaques (176) and increased HER2/neu plasma levels were shown to be associated with a higher risk of CAD (177). In line with this, evidence from preclinical studies indicated that inhibition of growth factor signaling may confer atheroprotection (119–121). In two elegant studies, Zeboudj, Ait-Oufella and colleagues demonstrated that cell-specific depletion of EGFR either in myeloid cells (119) or in $CD4^+$ T cells (120) protected *Ldlr*$^{-/-}$ mice from atherosclerosis. EGFR deficiency in myeloid cells limited macrophage accumulation within plaques and lipid uptake by macrophages, whereas $CD4^+$ T cell-specific depletion of EGFR reduced $CD4^+$ T cell activation, proliferation and infiltration in atherosclerotic lesions. Both cell-specific EGFR deletions were accompanied by reduced pro-inflammatory cytokine production (119, 120). Despite these promising findings, some uncertainties regarding the mechanistic implication of EGFR and its ligands in atherogenesis remain (178), beyond EGFR's profound immunomodulatory role systems-wide must be taken into account (179). Whether atheroprotective effects of growth factor receptor targeting antibodies also apply to humans is still unclear.

Cytokine Therapy

A variety of cytokines may exert significant anti-neoplastic effects either by directly inhibiting proliferation and inducing apoptosis of tumor cells or by stimulating anti-tumor immune responses (180, 181). Despite promising findings in early preclinical studies, utilization of cytokines as cancer therapeutics was later demonstrated to involve several limitations which hindered broad translation of this treatment approach into clinical practice (180, 181). Nevertheless, IFN-α and IL-2 were clinically approved for the treatment of different malignancies such as hairy cell leukemia, follicular non-Hodgkin lymphoma, melanoma, and Kaposi's sarcoma (IFN-α) or renal cell carcinoma and melanoma (IL-2) (180, 181).

Interferon Alpha (IFN-α)

Clinical application of IFN-α is particularly limited by its proatherogenic properties (182, 183). *Ldlr*$^{-/-}$ mice treated with IFN-α had accelerated atherosclerosis and increased plasma cholesterol and triglyceride levels (124). Several other proatherogenic effects of IFN-α have been reported, such as induction of lipid uptake by macrophages and foam cell formation (184, 185), inhibition of T_{reg} activation and proliferation (186, 187), direct stimulation of cytotoxic $CD4^+$ T cell function (188), and sensitization of antigen-presenting cells toward pathogen-derived toll-like receptor 4 (TLR4) ligands (189). Clinical studies demonstrated that plasma type I IFN (IFN-α and -β) levels are associated with atherosclerosis development

in patients with SLE (122) and human immunodeficiency virus-1 (HIV-1) infection (123). Experimental evidence suggested that IFN-α directly promotes atherogenesis by impairing vascular repair (190, 191) or inducing endothelial dysfunction (192) and may thus causally contribute to the highly increased risk of atherosclerotic CVE in SLE patients, which is not adequately explained by traditional risk factors (193). For a thorough review on the impact of IFN-α on different atherosclerosis-associated cell types and clinical implications the interested reader is referred to Chen et al. (182).

Interleukin 2 (IL-2)

IL-2 was originally termed T cell growth factor as it was first identified as a component of T cell culture fluids that induced proliferation of antigen-activated T cells (194, 195). It was thought to act as a crucial mediator in T cell immune responses and to play an important role in host response and tumor control, which led to test high-dose IL-2 as a novel cancer treatment in the mid 1980s (196). Although limited by toxicities such as capillary leak syndrome, fever, chills, malaise and arthralgias, this approach facilitated significant tumor regression and emerged as the first effective immunotherapy for human cancer (196). Yet, IL-2 deficient mice developed severe lymphoproliferation and autoimmunity which pointed toward an additional important role of the cytokine in maintaining self-tolerance (197, 198). Subsequent studies revealed that T_{reg} generation is dependent on IL-2 (195). T_{regs} express increased levels of the high-affinity IL-2 receptor alpha chain (IL-2Rα, also known as CD25) compared to effector T cells (T_{eff} cells) and are thus more sensitive for IL-2 (199). Accordingly, daily low-dose IL-2 therapy stimulated selective expansion of functional T_{regs} through increased proliferation, thymic export and resistance to apoptosis (while only minimally affecting conventional $CD4^+$ T cells) and thus led to a substantial clinical improvement in patients with active chronic graft-vs.-host disease (200, 201).

The specific role of IL-2 in atherogenesis has not yet been fully clarified. Increased IL-2 serum levels were shown to be associated with carotid artery intima-media thickness (202), a sonographic marker of atherosclerosis, and CAD (203). An early clinical study reported atherosclerotic CVE in 3.8% (angina or ischemic changes in 2.6% and myocardial infarction in 1.2%) of patients who received IL-2 for cancer therapy (125). Accordingly, IL-2 administration (2×10^4 units twice weekly for a period of 6 weeks) accelerated atherogenesis in $Apoe^{-/-}$ mice, whereas administration of an antibody targeting IL-2 was atheroprotective (126). Treatment of $Ldlr^{-/-}$ (204) and $Apoe^{-/-}$ mice (127) with low-dose IL-2 complexed with a specific IL-2 monoclonal antibody (JES6-1A12) conferred significant atheroprotection by inducing a substantial expansion of T_{regs} in atherosclerotic lesions and several other tissues. Neither IL-2 nor the anti-IL2 antibody alone affected atherogenesis (127) and the observed anti-atherogenic efficacy depended on the antibody clone used. Administration of IL-2 complexed with another IL-2 antibody clone (SAB6) induced expansion of natural killer (NK) and $CD8^+$ T cells (205). A subsequent study unraveled the mechanism by which the two different antibody complexes selectively induce expansion of T_{regs} or T_{eff} cells: (1) JES6-1 sterically blocks the interaction of IL-2 with IL-2Rβ and IL-2Rγ and allosterically disrupts binding of IL2 to IL-2Rα, thereby favoring activation of T_{regs} with high IL-2Rα expression; (2) S4B6 sterically hinders IL-2/IL-2Rα interaction and enhances IL-2/IL-2Rβ interaction, thus stimulating all IL-2-responsive T cells (206). More recently, a human anti-IL2-antibody (F5111.2) was developed that selectively promotes T_{reg} expansion when complexed with human IL-2 by inducing similar conformational changes (207). Administration of IL2-F5111.2 complexes yielded substantial therapeutic efficacy in humanized animal models of different autoimmune diseases, such as type 1 diabetes, autoimmune encephalomyelitis or xenogeneic graft-vs.-host disease (207). Whether such approach might be translatable into clinical practice has not yet been determined. The randomized, double-blind, placebo-controlled LILACS trial (NCT03113773) examined whether solely administering low-dose IL-2 is safe and effective in patients with stable ischemic heart disease and acute coronary syndrome (208). The study has been completed and its results are awaited for publication.

In conclusion, current evidence suggests that high-dose IL-2 therapy promotes atherogenesis through induction of pro-inflammatory T_{eff} cell responses, whereas administration of low-dose IL-2 might confer atheroprotection by selectively stimulating expansion of T_{regs}. Complexing IL-2 with specific anti-IL-2 antibodies might even enhance the latter effect through augmenting the selectivity to IL-2Rα, which is highly expressed on T_{regs}.

Methotrexate

Methotrexate (MTX) is a structural analog of folic acid (Vitamin B9) that inhibits enzymes involved in folate metabolism, such as dihydrofolate reductase (DHFR), and thus limits cellular division (209). DHFR catalyzes conversion of dihydrofolate to tetrahydrofolate, which acts as an important coenzyme in synthesis of pyrimidine and purine. In 1948 Farber et al. firstly reported that treatment with the folate analog aminopterin enabled temporary remission in childhood leukemia (210). Subsequently, MTX (initially termed amethopterin) was found to have better pharmacological properties than aminopterin (211) and thus emerged as one of the most extensively used chemotherapy agents for a variety of cancer types (209, 212). Besides anti-neoplastic properties, MTX exerts potent anti-inflammatory actions (213). Several studies reported efficacy of low-dose MTX in the treatment of rheumatoid arthritis (RA) (214, 215). The compound has become a mainstay in therapy of RA and other autoimmune diseases, although its immunosuppressive mechanisms of action have not yet been fully clarified (213). Observational data revealed that low-dose MTX therapy associated with a lower risk for CVD and cardiovascular mortality (216, 217). The Cardiovascular Inflammation Reduction Trial (CIRT) included 4,786 patients with CAD and additional metabolic risk factors (type 2 diabetes or metabolic syndrome) randomly assigned to receive low-dose MTX or placebo (128). After a median follow-up of 2.3 years, MTX neither reduced pro-inflammatory biomarkers [IL-1β, IL-6, and C-reactive protein (CRP)] nor CVE. MTX was associated with modest elevations in liver enzymes, reductions

in leukocyte counts and hematocrit levels, and an increased incidence of non-basal-cell skin cancers. Although the study had some methodical limitations (e.g., patients were not screened for an increased inflammatory risk), the data overall discouraged further investigations on MTX therapy for CVD prevention. In a recent murine study, nanoparticle-formulated MTX conferred substantial atheroprotection through modulating lipoprotein transcellular transport, reducing expression of pro-inflammatory cytokines and attenuating monocyte maturation and recruitment (129).

Although several immunotherapeutic approaches have yielded promising results in preclinical CVD models, only few of these strategies have proven beneficial in clinical studies (8, 218). Canakinumab (14), a monoclonal antibody targeting IL-1β, and colchicine (219, 220), an ancient drug traditionally used for gout therapy which exerts anti-inflammatory effects (among other potential mechanisms) through inhibition of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome in leukocytes (221), were demonstrated to reduce cardiovascular events in large-scale clinical trials. Although not targeting inflammatory pathways, monoclonal antibodies [evolocumab (13) and alirocumab (12)] or a siRNA-based compound [inclisiran (222, 223)] targeting PCSK9 to lower LDL cholesterol levels have proven clinically beneficial.

Considering that a pro-inflammatory immune response is critically involved in early elimination of mutated cells (68), immunotherapies exerting systemic anti-inflammatory effects may mitigate anti-tumor immunity and thus increase the risk of cancer development. In the following section we discuss recent evidence on the impact of above-mentioned immunotherapeutic strategies for CVD on cancer incidence (an overview is given in **Table 2**).

Canakinumab

Although canakinumab reduced CVE in patients with CAD, several limitations, such as a significant impairment of host defense and high costs, prevented its clinical approval for secondary prevention of CVD (14). Recent evidence suggests that IL-1β has a dual role in cancer development: On the one hand, it initiated pro-inflammatory anti-tumor immune responses by activating tumor antigen-specific T_H1 and T_H17 cells and facilitated tumor regression (233). On the other hand, IL-1β can promote carcinogenesis by inducing chronic inflammation, endothelial cell activation, angiogenesis, or development of immunosuppressive cells, such as tumor-associated macrophages and myeloid-derived suppressor cells (233). In contrast to anti-tumor immunity, which is critically involved in cancer elimination, chronic inflammation may drive tumorigenesis through several mechanisms including inhibition of antineoplastic immune responses, modulation of the tumor microenvironment to become more tumor-permissive, and direct tumor-promoting actions on epithelial and cancer cells (234). In line with this, IL-1β-deficient mice transplanted with melanoma cells were protected from development of local tumor and metastases (225) and canakinumab significantly reduced incidence of fatal cancer in the CANTOS trial (14). An exploratory analysis further revealed that canakinumab treatment especially reduced the incidence of lung cancer and lung cancer mortality (224). These findings motivated the initiation of three randomized phase III trials, CANOPY-A (NCT03447769), CANOPY-1 (NCT03631199), and CANOPY-2 (NCT03626545) (235), and a phase II trial, CANOPY-N (NCT03968419) (236) to investigate the potential of canakinumab in the treatment of non-small cell lung cancer (NSCLC). Although most trials are still ongoing, the CANOPY-2 study, which evaluated canakinumab in a second- or third-line treatment setting (that is in patients with locally advanced or metastatic NSCLC and tumor progression after or during platinum-based chemotherapy and PD-(L)1 inhibitor therapy) failed to meet the primary endpoint of overall survival (237).

Colchicine

Colchicine is derived from the autumn crocus, which has been used for gout therapy since ancient times (238). Until today, colchicine represents a first-line drug for gout treatment (239). More recently, the compound has emerged as a promising candidate for secondary prevention of CVD. In two large-scale RCTs, the COLCOT (219) and LoDoCo 2 (220) trials, collectively enrolling more than 10,000 patients with recent myocardial infarction or chronic coronary syndrome, low-dose colchicine therapy significantly reduced the incidence of CVE. In contrast to canakinumab, colchicine did not increase the risk of fatal infections, although in COLCOT non-fatal pneumonia occurred more often in colchicine-treated patients. Nevertheless, colchicine therapy did not significantly reduce cardiovascular mortality in these trials and was associated with an almost significant increase in non-cardiovascular mortality in LoDoCo2 (220). A smaller RCT, enrolling 795 patients, reported a significantly higher rate of all-cause mortality (mainly due to non-cardiovascular deaths) in patients with acute coronary syndrome when colchicine was added to standard therapy (240). Recent meta-analyses confirmed that low-dose colchicine therapy in patients with CAD was associated with a significantly reduced risk of CVE (myocardial infarction, stroke, and the need for coronary revascularization) and a non-significant reduction of cardiovascular deaths, which was counterbalanced by a non-significant increase in non-cardiovascular deaths (241, 242). Considering the robust reduction of CVE observed in these studies as well as the low price and wide availability of the compound, low-dose colchicine might become an option for secondary prevention in high-risk CVD patients, but the increased non-cardiovascular death rates need further investigation.

Despite the increase in non-cardiovascular mortality, colchicine was not associated with higher rates of cancer in any of the above-mentioned studies (219, 220, 240). Preclinical evidence showed that colchicine may reduce tumor growth of several cancer types, such as prostate (229), hypopharyngeal (230) and liver cancer (231) as well as tumor implantation of pressure-activated colon carcinoma cells (232). The incidence of all-cause cancers was significantly reduced in 13,679 male gout patients (at least temporarily) treated with colchicine compared with 10,371 control gout patients (228). Besides reducing tumor cell proliferation and inducing apoptosis (243),

TABLE 2 | Effects of anti-atherosclerotic immunotherapies (with clinically proven efficacy) on cancer.

Type of immunotherapy	Target	Compounds	Effect on cancer in clinical trials	Effect on cancer in animal studies	Potential mechanisms
Monoclonal antibodies	IL-1β	Canakinumab	↓ (224)	↓ (225)*	- Reduction of tumor-promoting chronic inflammation
	PCSK9	Alirocumab	–	↓ (226)	- Increased MHC I expression on tumor cell surface and enhanced tumor infiltration by cytotoxic T cells
		Evolocumab			
	CD3	Teplizumab	–	↓ (227)	- Induction of leukemic cell apoptosis
		Foralumab			
Natural anti-tubulin agent	NLRP3 inflammasome**	Colchicine	↓ (228)	↓ (229–232)	- Direct antiproliferative effects on tumor cells and induction of apoptosis - Enhancement of $CD4^+$ and $CD8^+$ T-cell-mediated anti-tumor immunity

*↑ = pro-carcinogenic effect. ↓ = anti-carcinogenic effect. * In this study IL-1β knockout mice were shown to be protected from tumor development, but antibody-mediated IL-1β depletion was not investigated. **besides other anti-inflammatory mechanisms.*
IL-1β, interleukin 1 beta; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; PCSK9, proprotein convertase subtilisin/kexin 9.

colchicine enhances $CD4^+$ and $CD8^+$ T-cell-mediated anti-tumor immunity by promoting dendritic cell maturation and antigen presentation (244). Colchicine toxicity at high doses prevents its application as anti-tumor drug (243). Whether low-dose colchicine might confer clinically relevant anti-cancer effects has yet to be determined.

PCSK9 Inhibitors

PCSK9, a protein which is primarily expressed in the liver, counteracts clearance of LDL cholesterol by inducing degradation of internalized LDL receptor in hepatocytes leading to increased plasma LDL cholesterol levels (245). Inhibition of PCSK9 has emerged as a highly effective second-line cholesterol lowering strategy, which has received class I recommendations by current guidelines (246). Two monoclonal antibodies targeting PCSK9, evolocumab (13) and alirocumab (12), and a siRNA-based compound, inclisiran (222, 223), are approved for clinical use in selected high-risk patients.

Cholesterol is a driving force in atherogenesis, yet its role in cancer is less clear: Both positive and negative correlations as well as absence of any associations between cholesterol levels and cancer development have been reported by clinical studies (247). Likewise, several meta-analyses concluded that statin therapy reduces cancer incidence or improves prognosis (248–250), whereas others found no such correlations (251, 252). PCSK9 inhibitors have not been reported to affect cancer incidence in clinical trials (12, 13). However, in a recent study PCSK9 inhibition could potentiate anti-tumor immune responses and thus substantially reduced tumor growth in murine cancer models (226), which was independent of cholesterol-lowering. PCSK9 induced lysosomal degradation of MHC-I and disrupted its recycling to the cell surface. PCSK9 inhibition, either through genetic deletion or administration of monoclonal antibodies, increased MHC-I expression on the tumor cell surface and thus enhanced tumor infiltration by cytotoxic T cells. Besides limiting tumor growth when administered alone, anti-PCSK9 antibodies significantly enhanced anti-tumor efficacy of ICI therapy (anti-PD-1) (226). Another recent study demonstrated that a nanoliposomal anti-PCSK9 vaccine limited tumor progression and improved survival in a murine model of colon carcinoma (253). Concluding, these preclinical data warrant further exploration of PCSK9 inhibitors as cancer therapeutics in clinical trials.

Anti-CD3 Antibody Therapy

Anti-CD3 monoclonal antibodies bind to the CD3-TCR complex on the surface of T cells and thus induce disappearance by shedding or internalization of the receptor complex (254). This process, which is termed antigenic modulation, renders T cells temporarily blind to their cognate antigen and leads to anergy or apoptosis of activated T cells (254). Anti-CD3 antibody therapy induced long-lasting T_{reg}-mediated immune tolerance through increased TGF-β production by apoptotic T cells and phagocytes involved in clearance of apoptotic T cells (255, 256). Clinical application of the murine muromonab-CD3 is limited by high immunogenicity and resulting side effects (e.g., nausea, fever, headaches) (254). Humanized anti-CD3 antibodies (e.g., teplizumab, and otelixizumab) and a fully human anti-CD3 antibody (foralumab) have been developed, that were well-tolerated in initial clinical studies (254). Early clinical evidence suggests reasonable efficacy of such novel anti-CD3 antibodies in the treatment or prevention of autoimmune diseases, such as multiple sclerosis (257), type 1 diabetes (258–261), and inflammatory bowel disease (262). In several preclinical studies, intravenous or oral administration of anti-CD3 antibodies conferred substantial atheroprotection by enhancing TGF-β production and thereby inducing anti-atherogenic T_{regs} (263–265). Muromonab-CD3 was reported to significantly decrease $CD3^+$ tumor cells in a patient with refractory T cell acute lymphoblastic leukemia (266), but was ineffective in enhancing immune activation in patients with solid tumors when administered in combination with high- or low-dose IL-2 (267, 268). However, a recent preclinical study demonstrated high anti-tumor efficacy of teplizumab and

foralumab in murine models of T cell acute lymphoblastic leukemia (227). To date, the potential of humanized or fully human anti-CD3 antibodies in the treatment of CVD or cancer has not yet been investigated in clinical trials. Considering the promising data from animal studies these compounds merit further investigation into their clinical application.

ADOPTIVE T CELL TRANSFER IN CANCER AND ATHEROSCLEROSIS

Chimeric antigen receptor (CAR) T cell therapy represents an innovative cancer treatment strategy, in which circulating T cells are isolated and genetically modified *in vitro* to express a synthetic tumor-antigen-specific receptor (269), which are subsequently expanded and infused back into the patient to attack tumor cells (269). In 2017, CAR T cells directed against CD19 (tisagenlecleucel and axicabtagene ciloleucel) showed substantial anti-tumor activity in patients with refractory large B cell lymphoma and follicular lymphoma (270, 271), which led to their FDA approval. Subsequently, anti-CD19 CAR T cells brexucabtagene autoleucel and lisocabtagene maraleucel were approved for treatment of mantle cell lymphoma and diffuse large B cell lymphoma, respectively (272, 273). Idecabtagene vicleucel targets B cell maturation antigen (BCMA) and is the first clinically approved CAR T cell therapy for multiple myeloma treatment (274). A major drawback of CAR T cell therapy is its association with severe and potentially fatal side effects (275). Cytokine-release syndrome (CRS), that can potentially develop into fulminant haemophagocytic lymphohistiocytosis (HLH), and CAR-T-cell-related encephalopathy syndrome (CRES), are the two most common adverse reactions (275). A recent study reported that CVE, such as new onset of heart failure or arrhythmias, occurred in 12% of 137 patients who received CAR T cell therapy (all events were associated with CRS) (276). Whether CAR T cells affect atherogenesis and increase the cardiovascular risk in the long-term, is unknown.

Adoptive transfer of autologous *ex vivo* expanded polyclonal T_{regs} has emerged as a promising strategy to treat autoimmune diseases and is currently investigated in clinical trials (277). First evidence suggested safety and efficacy of this approach for the treatment of type 1 diabetes (278, 279), prevention of graft-vs.-host-disease (280, 281), or transplant rejection (282). Therapeutic potency may be enhanced by utilization of antigen-specific rather than polyclonal T_{regs}. Administration of ovalbumin-specific T_{regs}, which respond to a major component of chicken egg white, was demonstrated to be safe and effective in patients with refractory Crohn's disease (283). Adoptive transfer of both polyclonal (284) and antigen-specific T_{regs} (responding to heat-shock protein 60) (285) conferred atheroprotection in *Apoe*$^{-/-}$ mice fed with a WD for 6–8 weeks. Yet, administration of ApoB^{+} T_{regs} to WD-fed *Apoe*$^{-/-}$ mice failed to limit plaque progression during a more extended period of observation (12 weeks) (43). In this study, more than half of all transferred cells lost expression of T_{reg} markers and converted into conventional T cells, which likely accounted for treatment failure. Clarification of the mechanisms underlying such phenotypic conversion and development of strategies to ensure T_{reg} stability are essential for clinical translation of this approach.

VACCINATION STRATEGIES IN THE TREATMENT OF CANCER AND ATHEROSCLEROSIS

Vaccination strategies aiming to induce pro-inflammatory immune responses against tumor-specific antigens or immune tolerance to plaque-associated autoantigens hold great promise for the treatment of cancer and atherosclerosis, respectively (4, 6). Through eliciting antigen-specific immunity, such approaches are highly effective without impairing the host defense against infectious agents and cancer cells or enhancing atherogenesis.

A series of animal studies have indicated the great potential of immunization against ApoB-related antigens for the treatment of atherosclerosis (6). The underlying idea originates from the observation that administration of oxLDL to hypercholesterolemic rabbits reduced atherosclerotic lesions (286). Subsequent studies identified an expansion of ApoB^{+} T_{regs} (42, 287, 288) and a humoral immune response against LDL (289) to account for the observed atheroprotection. Despite these promising preclinical data, several unknowns, such as optimal epitopes, adjuvants, administration route and vaccination scheme, stability of the atheroprotective immune response, and criteria for patient selection, have hitherto hindered translation of anti-atherosclerotic vaccination strategies into clinical practice (6). Recently, utilization of nanoparticle-formulated, nucleoside-modified messenger RNA (mRNA) without addition of adjuvants was demonstrated to enable sustained immune tolerance to MS-related autoantigens through induction of functional T_{regs} in mice (290). Considering that mRNA-based vaccines are already in clinical use, this approach has high translational potential for development of a vaccine against atherosclerosis.

Therapeutic cancer vaccines aim to induce a sustained effector immune-response against tumor-specific antigens (291). Initial approaches used self-antigens, which exist in non-malignant tissues, but are abnormally expressed or overexpressed by tumor cells, or applied non-self-antigens of viral origin (4, 291). Technological advances, such as next-generation sequencing, enabled identification of tumor-specific epitopes resulting from mutations (4, 291). These so-called neoepitopes or neoantigens are then evaluated for their capacity to bind human MHC-I or -II molecules. Candidates with high binding capacity can finally be utilized for development of personalized cancer vaccines or *in vitro* expansion of tumor-antigen specific CD4^{+} and CD8^{+} T cells, which are then transferred into the patient (4, 291). Vaccination with neoepitopes predicted to bind MHC-II or -I was shown to elicit strong CD4^{+} and/or CD8^{+} responses and thereby facilitate tumor rejection in animal models (292). In 2015, Carreno et al. firstly demonstrated the capability of a neoantigen-based vaccine strategy using dendritic cells as vaccine platform to induce a tumor-specific CD8^{+} T cell response in three patients with advanced melanoma (293). Subsequently peptide- and mRNA-based neoantigen vaccines were shown to induce strong CD4^{+} and CD8^{+} T cell responses

alongside with significant tumor regression in melanoma patients (90, 91). Despite the promising results of these early clinical trials, several open questions on how to improve efficacy and feasibility of neoantigen-based tumor vaccines remain, that include identification of optimal antigens, delivery platforms, adjuvants, and routes of administration (4). Several clinical trials investigating the efficacy of neoantigen-based vaccine approaches in different cancer types are underway and will likely help to find answers to these questions (4, 291).

CONCLUSION

Adaptive immunity is critically involved in the pathogenesis of atherosclerotic cardiovascular diseases and cancer, which represent the two most common causes of death worldwide. During the past few decades, novel treatment strategies enhancing anti-tumor immune responses have already found their way into clinical practice, whereas successful translation of strategies targeting atherogenesis-related immune responses into the clinic has not yet been accomplished. Given that some immunotherapies (e.g., CD20-, EGFR-, IL-1β- or PCSK9-targeting antibodies) were shown to protect from both cancer and atherosclerosis, inflammatory processes and immunity underlying carcinogenesis and atherogenesis may be closely interconnected. Further characterization of tumor-promoting and proatherogenic immune responses may help to identify novel pharmacological targets that allow simultaneous treatment of both disease entities. Further characterization of tumor-promoting and proatherogenic immune responses may help to identify novel pharmacological targets that allow simultaneous treatment of both disease entities. In that regard, multimodal sequencing approaches, such as Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq), which allow combined measurement of gene and surface-protein expression on a single-cell level, will be valuable tools. Active immunization represents a novel, promising approach for the treatment of cancer and atherosclerosis. Preclinical studies have underscored the great anti-atherosclerotic potential of vaccination against plaque-related autoantigens. Further research is required to optimize this promising approach. Major objectives in this context include identification of optimal vaccine delivery platforms, adjuvants and administration routes. Furthermore, development of clinically feasible approaches to identify eligible patients, to determine expression of target antigens within an individual patient, and to monitor treatment responses will be crucial for broad implementation of this approach into clinical practice. If these obstacles can be overcome, active immunization may prospectively take cancer and atherosclerosis therapy to the next level.

AUTHOR CONTRIBUTIONS

FN wrote the manuscript. FP prepared the figure. FN and HW conceptualized the work. HW and FH supervised the work and provided funding. All authors substantially contributed to data research, critically discussed the content, reviewed the manuscript before submission, and have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by the Deutsche Forschungsgemeinschaft [SFB TRR259 (397484323) and CCRC GRK2407 (360043781) to HW and HO 5279/2-1 to FH], the Neven-DuMont foundation (to HW), and the Koeln Fortune Program (363/2020 to FN).

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frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 13 January 2022
doi: 10.3389/fcvm.2021.791028

Chemokine Receptor Activation Enhances Memory B Cell Class Switching Linked to IgE Sensitization to Alpha Gal and Cardiovascular Disease

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to
Atherosclerosis and Vascular
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 07 October 2021
Accepted: 20 December 2021
Published: 13 January 2022

Citation:
Pattarabanjird T, Wilson JM,
Erickson LD, Workman LJ, Qiao H,
Ghosheh Y, Gulati R, Durant C,
Vallejo J, Saigusa R, Platts-Mills TAE,
Taylor AM, Ley K and McNamara CA
(2022) Chemokine Receptor
Activation Enhances Memory B Cell
Class Switching Linked to IgE
Sensitization to Alpha Gal and
Cardiovascular Disease.
Front. Cardiovasc. Med. 8:791028.
doi: 10.3389/fcvm.2021.791028

Background: Recent studies have suggested that IgE sensitization to α-gal is associated with coronary artery disease (CAD). However, the B cell subtype(s) responsible for production of IgE to α-gal and mechanisms mediating this production remain elusive.

Methods: Single cell multi-omics sequencing, was utilized to phenotype B cells obtained from 60 subjects that had undergone coronary angiography in whom serum IgE was evaluated by ImmunoCAP. Bioinformatics approaches were used to identify B cell subtype(s) and transcriptomic signatures associated with α-gal sensitization. *In vitro* characterization of chemokine/chemokine receptor pairs on switched memory B cells associated with IgE to α-gal was performed.

Results: Of the 60 patients, 17 (28%) were positive for IgE to α-gal. CITESeq identified CCR6+ class-switched memory (SWM) B cells and CXCR4 expresssion on these CCR6+ SWM B cells as significantly associated with IgE sensitization to α-gal but not to other common allergens (peanut or inhalants). *In vitro* studies of enriched human B cells revealed significantly greater IgE on SWM B cells with high CCR6 and CXCR4 expression 10 days after cells were treated with IL-4 and CD40 to stimulate class switch recombination. Both CCL20 (CCR6 ligand) and CXCL12 (ligand for CXCR4) increased the expression of IgE on SWM B cells expressing their receptors. However, they appeared to have unique pathways mediating this effect as only CCL20 increased activation-induced cytidine deaminase (AID), while CXCL12 drove proliferation of CXCR4+ SWM B cells. Lastly, correlation analysis indicated an association between CAD severity and the frequency of both CCR6+ SWM and CXCR4+ SWM B cells.

Conclusions: CCR6+ SWM B cells were identified as potential producers of IgE to α-gal in CAD patients. Additionally, our findings highlighted non-chemotaxis roles of CCL20/CCR6 and CXCL12/CXCR4 signaling in mediating IgE class switching and cell proliferation of SWM B cells respectively. Results may have important implications for a better understanding and better therapeutic approaches for subjects with IgE sensitization to α-gal.

Keywords: alpha-gal, B cells, coronary artery disease, IgE class switching, CITESeq

INTRODUCTION

The oligosaccharide galactose-α-1,3-galactose (α-gal) is a blood group-like oligosaccharide of non-primate mammals and the target of IgM, IgG, and IgA in all immunocompetent humans (1, 2). A subset of the population produce IgE to α-gal, and as a consequence are at risk for a syndrome of delayed anaphylaxis to mammalian meat, often called the α-gal syndrome (3). Tick bites and other parasitic exposures have been associated with α-gal IgE sensitization, however the B cell population(s) that produce this IgE and the mechanisms that lead to IgE class switch are not well-understood (4, 5). Recently our group reported that IgE sensitization to α-gal was associated with increased atheroma burden and unstable plaque characteristics in subjects undergoing evaluation for CAD (6, 7). Despite the high prevalence of α-gal sensitization in some areas and the putative connection with cardiovascular disease, the human B cell subtype that contributes to production of IgE specific for α-gal remains elusive.

To investigate human B cell subtypes that were associated with α-gal IgE sensitization, we utilized a novel single cell multi-omics sequencing platform to phenotype B cells obtained from a cohort of CAD patients with a high rate of sensitization to α-gal. CITEseq is a novel technology utilizing antibodies barcoded by DNA oligos to convert surface protein expression to sequencing readouts coupled with simultaneous mRNA sequencing (8). In conjunction with unsupervised bioinformatics pipelines, this platform allowed us to explore transcriptomic profiles and signaling signatures of human B cell subtypes associated with α-gal sensitization. Further, *in vitro* functional studies of specific B cell subtypes were performed to characterize roles of chemokine receptors in mediating IgE class switching. Identification of B cells subtypes and intrinsic signaling that contribute to α-gal sensitization will improve our understanding IgE class switch and may be helpful for future development of disease-modifying immunotherapies.

MATERIALS AND METHODS

Human Subjects

Subjects (age range 40–80 years old) presenting to the Cardiac Catheterization laboratory at the University of Virginia (UVA) Health System, Charlottesville, Virginia, USA for a medically-indicated coronary angiogram (Coronary Assessment in Virginia cohort (CAVA) were enrolled. All participants provided written informed consent before enrollment, and the study was approved by the Human Institutional Review Board (IRB No. 15328). Peripheral blood was obtained from these participants prior to catheterization. Peripheral blood from seven volunteers was also obtained after written informed consent as part of an IRB-approved study (UVA IRB No. 16017).

Quantitative Coronary Angiography

Patients underwent standard cardiac catheterization with two orthogonal views of the right coronary artery and four of the left coronary artery according to accepted standards. QCA was performed using automatic edge detection at an end diastolic frame. For each lesion, the frame was selected based on demonstration of the most severe stenosis with minimal foreshortening and branch overlap. Computer software was used to calculate the minimum lumen diameter, reference diameter, percent diameter stenosis, and stenosis length. Analysis was performed by blinded, experienced investigators. The Gensini score was used to assign a score of disease burden to each patient. Briefly, each artery segment is assigned a score ranging from 0 to 32 based on the percent stenosis. The severity score for each segment was multiplied by 0.5–5, depending on the location of the stenosis. Scores for all segments were then added together to given a final score of angiographic disease burden. Score adjustment for collateral was not performed for this study. Subjects with Gensini score >32 were classified as high CAD severity subjects and subjects with Gensini score ≤6 were classified as low CAD severity subjects. Clinical characteristics of the CAVA cohort subjects used in this study were provided in **Supplementary Table 1**.

Quantification of Serum IgEs in Humans

Serum IgE to α-Gal, dust mite (*Dermatophagoides pteronyssinus*), oak, timothy grass, and peanut were assayed using ImmunoCAP 250 (Thermo-Fisher/Phadia, Kalamazoo, MI). Of note, the α-gal IgE assay used here was the commercial ImmunoCAP which uses beef thyroglobulin on the solid phase. Subjects with IgE to α-Gal >0.1 kU_A/L were classified as α-Gal sensitized subjects. Subjects with IgE to inhalants and peanuts >0.35 kU_A/L were classified as inhalants or peanuts sensitized subjects.

PBMC Isolation

Blood from enrollees was drawn into BD K2 EDTA vacutainer tubes and processed at room temperature (RT) within one hour of collection. Whole blood in vacutainers were centrifuged at 400 x g for 10 min at RT to remove platelet rich plasma. Plasma was cryopreserved at −80°C freezer. PBMCs were isolated by

Ficoll-Paque PLUS (GE Healthcare Biosciences AB) gradient centrifugation using SepMate-50 tubes (Stemcell Technologies Inc.) following the manufacturer's protocol. Trypan blue staining of PBMCs was performed to quantify live cell counts. PBMCs were cryopreserved in freezing solution (90% FBS/10% DMSO), or used fresh in assays. PBMC vials were stored in Mr. Frosty (Thermo Fisher) for 48 h at −80°C and were then stored in liquid nitrogen until used.

CITESeq Optimization and Staining

PBMCs obtained from 60 CAVA subjects were labeled with the BD Single-Cell Multiplexing Kit (BD Biosciences) and CITESeq antibody-oligomers (Ab-Oligos) reagents following the protocol outlined by Vallejo et al. (9). Briefly, PBMC vials were thawed at 37°C and then washed with complete RPMI-1640 solution. Cells were aliquoted at 1 million cells per tube and incubated on ice with Fc Block (BD Biosciences) at a 1:20 dilution in super bright staining buffer (SB, eBioscience) and subsequently transferred to multiplexing kit tubes (BD Biosciences) and incubated for 20 min at RT. Cells were then washed 3 times and centrifuged at 400 x g for 5 min. DRAQ7 and Calcein AM were used to quantify cell viability. Tube contents were pooled in equal proportions with total cell counts not to exceed 1 million cells, and resuspended in 20 μL of SB with 50 unique CITESeq Ab-Oligos diluted at 2 μL each as listed in **Supplementary Table 2**. The pooled samples were then incubated on ice for 30–60 min, washed with 2 mL of SB, and centrifuged at 400 x g for 5 min per manufacturer's recommendations. This step was repeated two more times for a total of three washes. The cells were then counted again using BD Rhapsody Scanner.

Library Preparation

Cells were loaded at 800–1,000 cells/μL into the primed plate. Reverse Transcription was performed at 37°C on a thermomixer at 1,200 rpm for 20 min following with addition of Exonuclease I with 30 min incubation on a thermomixer and then transferred to heat block to incubate at 80°C for 20 min. BD's protocol was used to prepare the cDNA library as described in Vallejo et al. (9). The final QC and quantification check were performed using TapeStation and Qubit kits and reagents.

Sequencing

The samples were pooled and sequenced to the following depth recommended by BD: Ab-Oligos sequencing: 40,000 reads per cell; mRNA: 20,000 reads per cell; Sample Tags: 600 reads per cell. A total of 60,600 reads per cell were obtained for sequencing on the NovaSeq. The samples and specifications for pooling and sequencing depth, along with number of cells loaded onto each plate was optimized for S1 and S2 100 cycle kits (Illumina). Once sequencing was complete, the FASTA file and FASTQ files generated by the NovaSeq were uploaded to Seven Bridged Genomics pipeline, where the data was filtered in matrices and csv files. Doublet Finder package on R (https://github.com/chris-mcginnis-ucsf/DoubletFinder) was used to remove the doublets and cells with <128 antibody molecules sequenced were removed. All antibody sequencing data were CLR (centered log-ratio) normalized and converted to log2 scale. All transcripts were normalized by total UMIs in each cell and scaled up to 1,000.

CITEseq Data Pre-processing and Analysis

B cells within PBMCs obtained from CAVA subjects were first identified by manual gating determined by $CD19^{+}CD3^{-}$ antibody sequencing and thresholding of each antibody expression was determined based on expression on negative cells. UMAP dimensionality reduction and Louvain clustering on 26 antibodies B cell markers using the python scanpy package (https://github.com/theislab/scanpy/issues/350) were performed to subtype B cells. Expression of the 488 genes within B cell subtypes were compared between subjects with and without α-gal sensitization by performing *t*-test with Bonferonni corrected *p*-values to obtain false discovery rate (FDR) values and calculating fold change in gene expression by using R software. Differentially expressed genes were defined as genes with FDR < 0.05 and $Log_2FC < 1$ or > 1. Volcano plots of differentially expressed genes were visualized by using the python bioinfokit package. Ingenuity Pathway Analysis was performed on all the annotated RNA to analyze for differentially regulated cellular processes and canonical pathways.

Flow Cytometry Characterization of B Cells Obtained From CAVA Subjects

Cryopreserved PBMCs from 10 CAVA subjects (with and without α-gal sensitization) were thawed, washed twice with warm complete media (RPMI with L-Glutamine supplemented with 5% heat-inactivated human AB serum (Sigma), 1 mM sodium pyruvate, 0.01 M HEPES, 1x MEM non-essential amino acids, 50 μM 2-Mercaptoethanol, and 1 mM Pen-Strep (all from Gibco). Total B cells were enriched using the STEMCELL human B cell enrichment kit (StemCell Technologies). IgE-expressing B cells were identified and characterized using flow cytometry with mAbs as listed in **Supplementary Table 3**. Cells were acquired on Cytek Aurora and the analysis was performed using FCS Express 7. The gating strategy is shown in **Supplementary Figure 1**.

In vitro Stimulation of B Cells

Cryopreserved PBMCs from volunteers were thawed, washed twice and enriched for total B cells as described in section Flow Cytometry Characterization of B cells obtained from CAVA subjects. Enriched B cells were cultured in 200 μL of complete RPMI with 20 ng/mL human IL-4 (R&D system), ± 10 μg/mL agonistic anti-human CD40 mAb (ThermoFisher; clone 5C3), ± 20 ng/mL human CCL20 (R&D system), ± 100 ng/mL human CXCL12 (R&D system) ± 10 nM CCR6 inhibitor 1 (MedChemExpress) ± 5 uM AMD3100 (Sigma) for 10 days in Corning Costar 96-well flat bottom plates. Levels of proliferation and immunoglobulin class switching to IgG and IgE produced by human B cells were determined, before and up to 10 days after stimulation, by using flow cytometry with mAbs as listed in **Supplementary Table 3**. Cells were acquired on Cytek Aurora and the analysis was performed using FCS Express 7. The gating strategy is presented in **Supplementary Figure 1**.

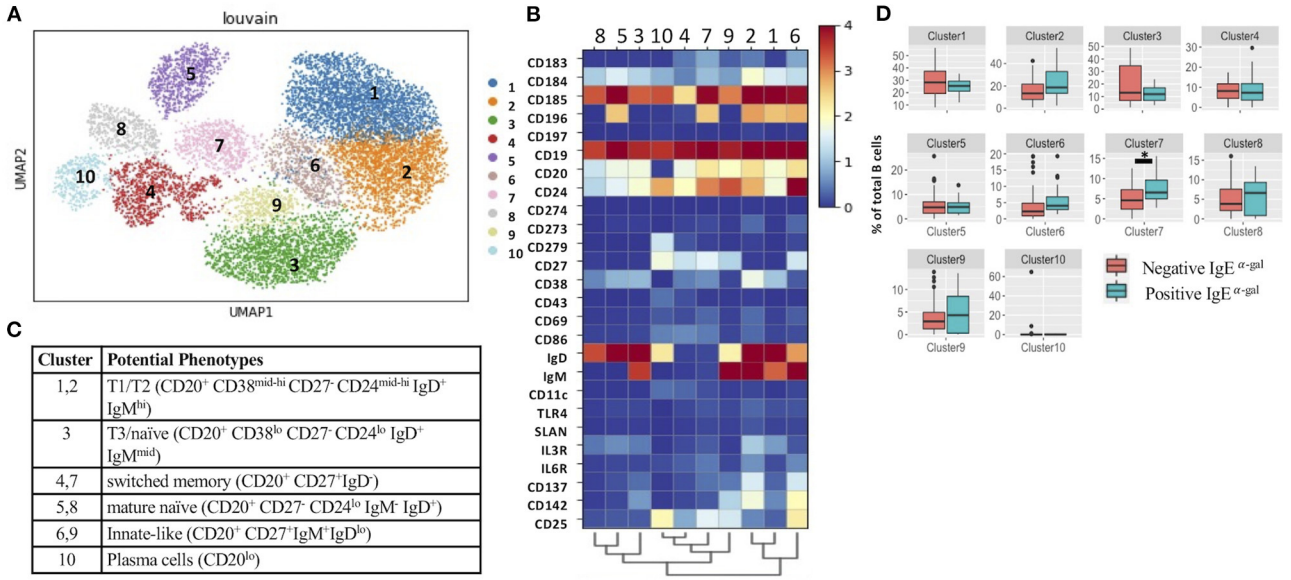


FIGURE 1 | Single cell multi-omics platform identified an association between circulating CCR6+ switched memory B cells and α-gal sensitization. **(A)** Representative metacluster UMAP showing 10 distinguished B cell subsets by using Louvain clustering. **(B,C)** Heatmap showing median expression of surface markers from metaclustering **(B)**, table showing potential phenotypes of each B cell subset resulting from Louvain clustering **(C)**. **(D)** Biaxial plots to compare frequency of each cluster as a percentage of total B cells in subjects with and without α-gal sensitization. *$p < 0.05$ by Mann-Whitney test.

Statistics

Statistics were calculated using GraphPad Prism Version 7.0a (GraphPad Software, Inc.), Python 3.0, R 3.6.1 or SAS 9.4. Results from all replicated experiments are displayed, and bar graphs display mean ± SEM.

RESULTS

Single Cell Multi-Omics Platform Identified an Association Between Circulating CCR6+ Switched Memory B Cells and α-gal IgE Sensitization

Consistent with a prior investigation that showed a high prevalence of α-gal IgE sensitization in the CAVA cohort (6), α-gal IgE was detected in 17 of the 60 patients (28%). Demographics of these patients are provided in **Supplementary Table 1**. To explore associations between α-gal sensitization and B cell subsets, we applied CITEseq with a 50-antibody panel on B cells obtained from 60 subjects in CAVA cohort. Among CD19+ B cells, 26-B cell antibodies were used in combination with metalouvain clustering to reveal 10 distinct B cell clusters within the PBMCs (**Figure 1A**). Potential phenotypes for each cluster were assigned based on expression of distinguishing B cell markers as represented in the heatmap (**Figures 1B,C**). Of all the clusters, only cluster 7 had elevated frequency in subjects with α-gal sensitization (**Figure 1D**). This cluster had features of a switched memory (SWM) B cell based on the expression profile of CD20+/CD27+/IgD-. Interestingly, IgE to other representative food (peanut) and aero-allergens (dust mite, oak, and timothy grass), did not have a significant relationship with frequency of any circulating B cell clusters (**Supplementary Figure 2**). Cluster 4 and 7 shared several markers, but CCR6 was highly expressed in cluster 7 but not in cluster 4 (**Figures 1B,C**). This result suggested that α-gal sensitization was specifically associated with SWM B cells that expressed CCR6. To further explore relationship between CCR6+ SWM B cells and IgE α-gal sensitization, we obtained B cells from CAVA subjects to compare percentages of IgE expressing SWM B cells in subjects with and without α-gal IgE sensitization. We found higher percentage of IgE+ SWM in subjects positive for α-gal IgE compared to those that were negative (**Supplementary Figure 3A**). In addition, higher percentage of IgE expressing CCR6+ SWM but not CCR6- SWM was observed in α-gal sensitized subjects (**Supplementary Figure 3B**).

CXCR4 Is Enriched in CCR6+ Switched Memory B Cells of α-gal Sensitized Subjects

Next, we evaluated cluster 7 using a 488 immune-relevant CITEseq transcriptomic sequencing to identify differentially expressed genes (DEGs) between α-gal sensitized and non-sensitized patients. Using an FDR of <0.05, CXCR4, STAT6, IL4R, CD79B and FCER1G were enriched in cluster 7 of subjects with detectable levels of IgE to α-gal (**Figure 2A**). Ingenuity pathway analysis of DEGs with an FDR cutoff of 0.1 indicated cellular processes such as B cell activation, proliferation, class switching, chemotaxis and IgE production enriched in subjects with α-gal IgE sensitization (**Figure 2B**). Canonical pathways dominated by activating pathways such as JAK/STAT, BCR, CXCR4, PI3K, NFKB, and IL-4 signaling (**Figure 2C**) were

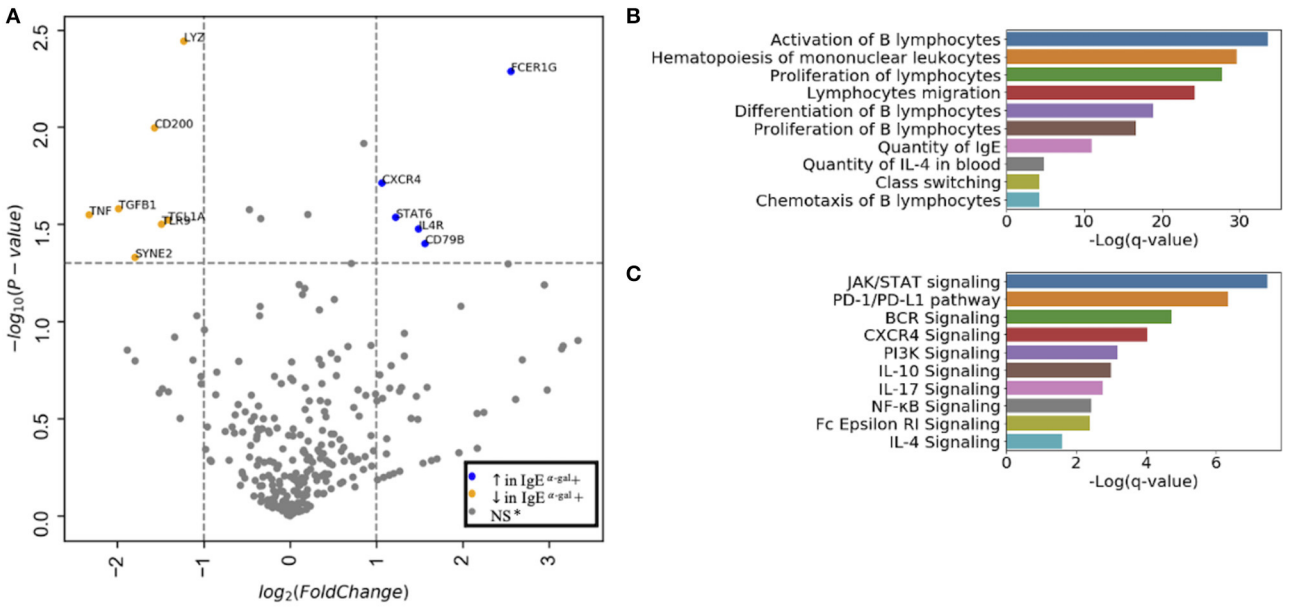


FIGURE 2 | CXCR4 signaling is enriched in CCR6+ SWM B cells of α-gal sensitized subjects. **(A)** Volcano plot of 488 gene array demonstrating DEGs of cluster 7 upregulated (blue) and downregulated (orange) in IgE α-gal + subjects. (**B,C**) Significant cellular processes **(B)** and canonical pathways **(C)** enriched in IgE α-gal + in cluster 7 cells. ns, non-significant.

enriched in subjects with α-gal sensitization. Of note, a known inhibitory pathway, PD1/PDL1 was also enriched in cluster 7 of subjects with α-gal sensitization.

A role for BCR activation, class switch recombination machinery and IL-4 signaling are well-documented in IgE class switching (10–12). Here, we further explored roles of chemokine receptors in contributing to B cell IgE class switching. As CXCR4 transcript expression in cluster 7 was enriched in α-gal sensitized subjects (**Figure 2A**), surface protein expression of CXCR4 on all 10 B cell clusters was investigated. Using the 26-surface antibody CITEseq panel, CXCR4 levels were evaluated using geometric mean (GM) and compared between α-gal sensitized and non-sensitized subjects. Of all the 10 clusters, enrichment of CXCR4 surface expression in α-gal sensitized subjects was only observed in cluster 6 and 7 (**Figure 3**). No difference in CXCR4 surface expression was observed in relationship to peanut or inhalant allergens sensitization (**Supplementary Figure 4**).

CCR6 and CXCR4 Are Important for IgE Isotype Switching

The combination of IL-4 and CD40 signaling are known to induce immunoglobulin class switching (13, 14). Here we performed *in vitro* culture of human B cells obtained from volunteers to further investigate IgE class switching in relation to CCR6 and CXCR4 expression. B cells treated for 10 days with IL-4/anti-CD40 had a higher percentage of SWM B cells compared to baseline and as compared to day 10 cells treated with IL-4 stimulation alone (**Figure 4A**). We subsequently evaluated expression of IgG, IgE, and the plasma cell marker CD138, with flow cytometry at day 0, 3 and 10 of culture (**Figures 4B–D**). Percentages of SWM B cells expressing IgG, IgE, and CD138 were low regardless of CCR6 expression at day 0 (**Figure 4B**) and increased at day 3 and 10 (**Figures 4C,D**). IgG and CD138, but not IgE, were expressed at higher levels on CCR6+ compared to CCR6- SWM B cells at day 3 (**Figure 4C**). However, at day 10 percentages of CCR6+ SWM B cells expressing IgE were higher when compared to the earlier time point and to CCR6- SWM B cells (**Figure 4D**). Interestingly, percentages of CCR6+ SWM B cells expressing IgG were lower compared to CCR6- SWM B cells at day 10 after stimulation (**Figure 4D**). Next, we continued our analysis of CXCR4- and CXCR4+ SWM B cells. We found low percentages of SWM B cells expressing surface IgG, IgE, and CD138 regardless of CXCR4 expression before stimulation (**Figure 4E**), which increased after stimulation for 3 days (**Figure 4F**). However, no significant differences among these populations were observed between CXCR4- and CXCR4+ SWM B cells. After 10 days, the CXCR4+ SWM B cells showed a significant increase in surface IgE and CD138 as compared to earlier timepoints and as compared to CXCR4- SWM B cells (**Figure 4G**). The results altogether suggested that greater class switching to IgE was observed in CCR6+ and CXCR4+ SWM B cells.

CCL20/CCR6 Intrinsic Signaling Augments IgE Isotype Switching Through Induction of AID Expression

Further, we investigated the roles of CCL20/CCR6 intrinsic signaling in mediating immunoglobulin isotype switching by using CCL20 along with IL-4 and anti-CD40 to stimulate enriched human B cells from volunteers. After 10 days of culture,

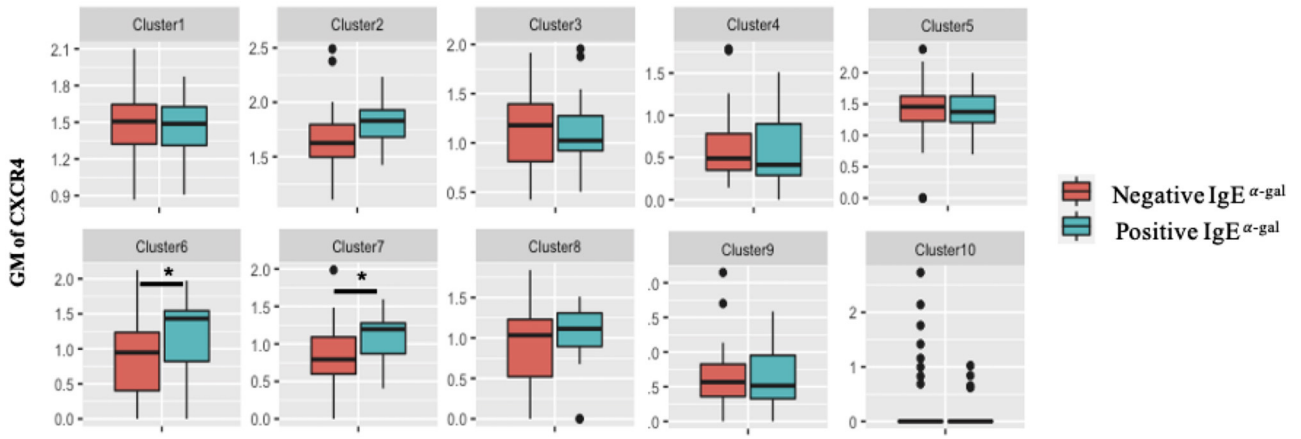


FIGURE 3 | CXCR4 surface expression increases in CCR6+ SWM B cells of α-gal sensitized subjects. Biaxial plots to compare geometric means (GM) of CXCR4 across 10 B cell clusters between subjects with and without α-gal sensitization. *$p < 0.05$ by Mann-Whitney test.

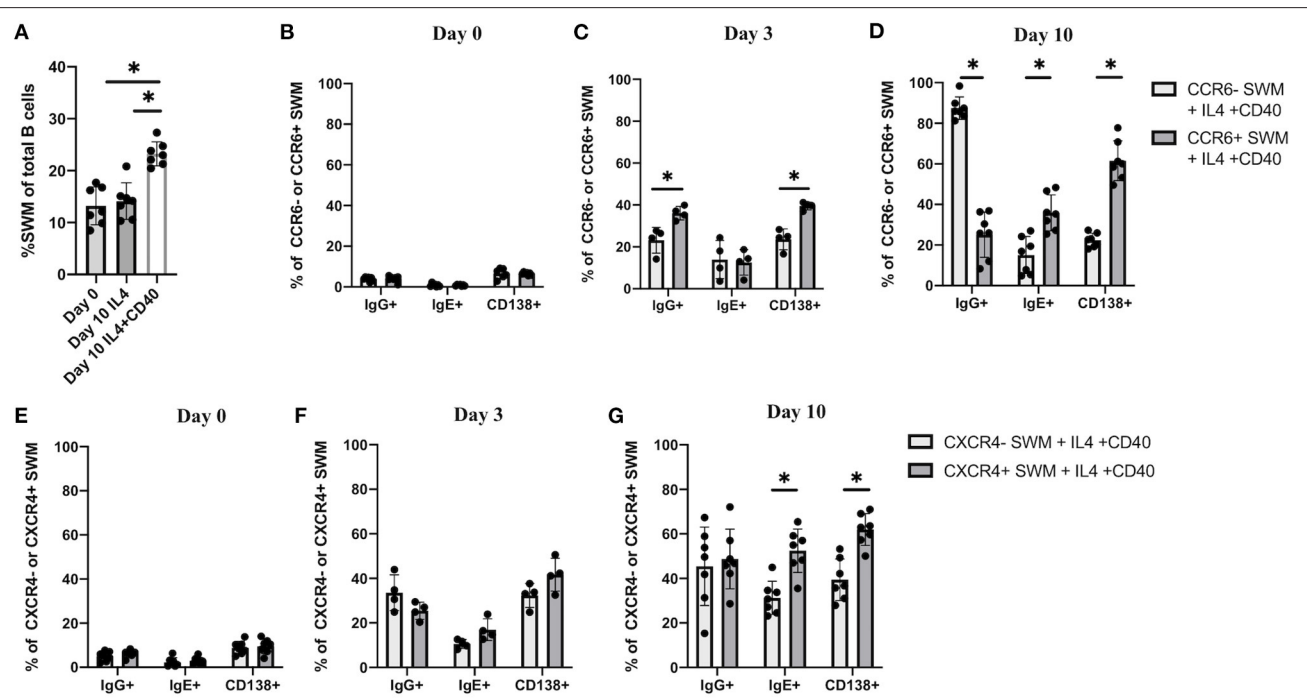


FIGURE 4 | CCR6 and CXCR4 are important for immunoglobulin isotype switching. **(A)** Percentage of SWM of total B cells at day 0 before stimulation and at day 10 post with 20 ng/mL IL-4 and ± 10 ug/mL anti-CD40 stimulation. **(B–D)** Percentage of IgG+, IgE+, and CD138+ of CCR6- SWM or CCR6+ SWM at day 0 **(B)**, day 3 **(C)**, and day 10 **(D)** after culturing with 20 ng/mL IL-4 and 10 ug/mL anti-CD40. **(E–G)** Percentage of IgG+, IgE+, and CD138+ of CXCR4- SWM or CXCR4+ SWM at day 0 **(E)**, day 3 **(F)**, and day 10 **(G)** after with 20 ng/mL IL-4 and 10 ug/mL anti-CD40. *$p < 0.05$ by Mann-Whitney test.

IgE expression was enhanced in CCL20 treated CCR6+, but not CCR6- SWM B cells (**Figure 5A**). No differences in IgG surface expression were observed with CCL20 stimulation in either of CCR6- or CCR6+ SWM (**Figure 5B**). These results suggested that intrinsic CCL20/CCR6 signaling in SWM B cells contributed to IgE class switching. As AID enzyme is known to induce immunoglobulin isotype switching (15), expression of AID was measured both in CCR6- and CCR6+ SWM under conditions of IL4 and anti-CD40 treatment ± CCL20. After 3 days of culture, CCL20 augmented AID expression in the CCR6+ SWM but not the CCR6- SWM B cells (**Figure 5C**; **Supplementary Figure 5**), while no change in cell proliferation was observed (**Figure 5D**). These findings suggested that CCL20/CCR6 intrinsic signaling induced AID expression.

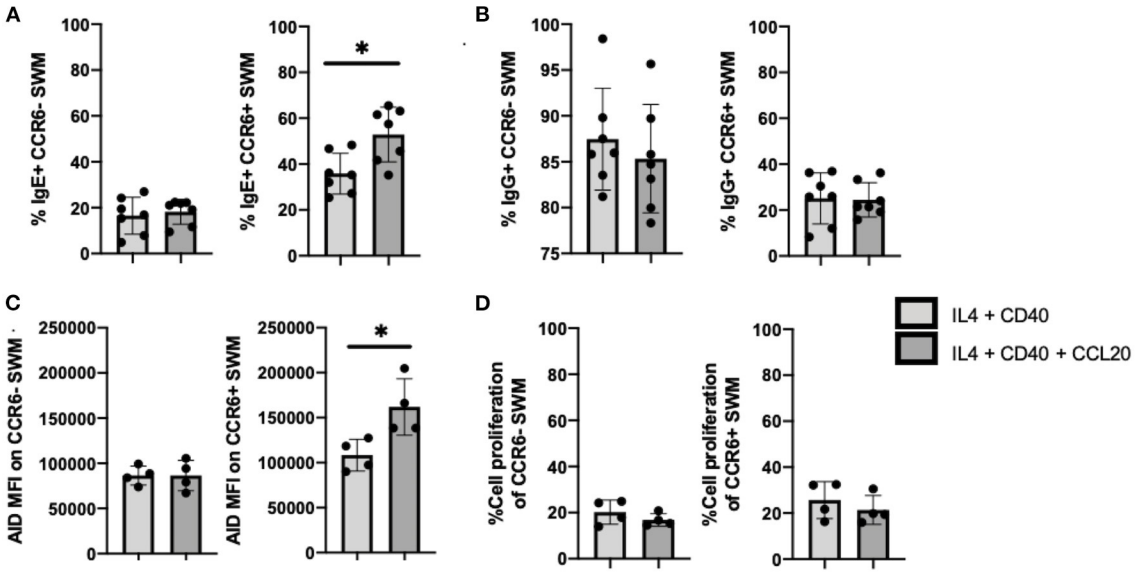


FIGURE 5 | CCL20/CCR6 intrinsic signaling augments IgE isotype switching through induction of AID expression. **(A)** Percentage of IgE+ of CCR6- SWM and CCR6+ SWM after 10 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 20 ng/mL CCL20. **(B)** Percentage of IgG+ of CCR6- SWM and CCR6+ SWM after 10 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 20 ng/mL CCL20. **(C)** Expression of AID measured by MFI on CCR6- SWM and CCR6+ SWM after 3 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 20 ng/mL CCL20. **(D)** Percentage of cell proliferation measured by cell-trace violet of CCR6- SWM and CCR6+ SWM after 3 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 20 ng/mL CCL20. *$p < 0.05$ by Mann-Whitney test.

CXCL12/CXCR4 Signaling Drives Proliferation of SWM B Cells

To follow up on our observation that IgE expression occurred preferentially in CXCR4+ SWM B cells (**Figure 4G**), we further investigated intrinsic roles of CXCL12/CXCR4 in mediating immunoglobulin class switching. CXCL12 stimulation did not affect IgE surface expression of CXCR4- SWM, however CXCR4+ SWM cells had a greater frequency of IgE expression after 10 days culture (**Figure 6A**). Similarly, CXCL12 did not alter IgG surface expression of CXCR4- SWM yet did enhance the percentages of IgG expression in CXCR4+ SWM B cells (**Figure 6B**). Effects of CXCL12 on AID expression and cell proliferation of CXCR4- and CXCR4+ SWM B cells were also evaluated. The result indicated no change in AID expression (**Figure 6C**; **Supplementary Figure 6**), but we did observe an increase in cell proliferation in CXCR4+ SWM cells after CXCL12 stimulation (**Figure 6D**). These results suggest that CXCL12/CXCR4 intrinsic signaling drove proliferation of SWM B cells, not class switching, leading to higher percentages CXCR4+ SWM with IgG and IgE expression.

Chemokine Receptor Inhibitors Reduce IgE Isotype Switching

CCR6 inhibitor 1 (CCR6i1) (16) and AMD3100 (17) were previously shown to be selective inhibitors for blocking CCL20/CCR6 and CXCL12/CXCR4 signaling, respectively. Here, we utilized these inhibitors to evaluate the impacts of blocking these chemokine receptor pathways on immunoglobulin isotype switching. After 10 days of culture, we found that the percentage of CCL20-stimulated CCR6+ SWM B cells expressing IgE trended to be lower with CCR6i1 treatment, whereas CCR6i1 had no impact on the percentage of IgG SWM B cells (**Figure 7A**). AMD3100 treatment of CXCR4+ SWM B cells stimulated with CXCL12 yielded fewer IgE and IgG expressing SWM cells (**Figure 7B**).

CXCR4 and CCR6 Expression on SWM B Cells Directly Associates With CAD Severity

Because CCR6 and CXCR4 on SWM B cells potentially play a role in mediating α-gal sensitization (**Figures 1D**, **2A**) and α-gal sensitization has previously been associated with CAD (6), here we explored the relationship between CCR6/CXCR4 expression on SWM B cells and atherosclerosis. In this study CAD severity was evaluated by quantitative coronary angiography (QCA) and quantified by the well-established Gensini scoring system (18). While the frequency of SWM B cells did not associate with CAD (data not shown), the expression of both CCR6 ($R^2 = 0.35$, $P < 0.001$) and CXCR4 ($R^2 = 0.24$, $p = 0.001$) on SWM B cells demonstrated significant correlations with CAD severity in linear regression analysis (**Figures 8A,B**). The correlations between CCR6 and CXCR4 on SWM B cells and CAD severity

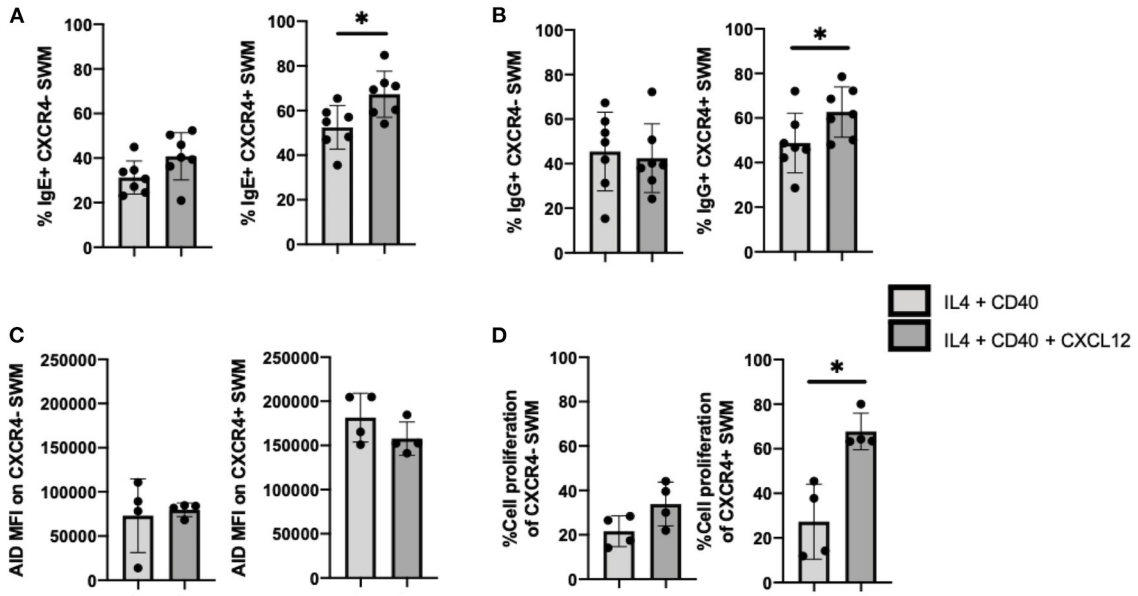


FIGURE 6 | CXCL12/CXCR4 intrinsic signaling drives proliferation of SWM B cells. **(A)** Percentage of IgE+ of CXCR4- SWM and CXCR4+ SWM after 10 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 100 ng/mL CXCL12. **(B)** Percentage of IgG+ of CXCR4- SWM and CXCR4+ SWM after 10 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 100 ng/mL CXCL12. **(C)** Expression of AID measured by MFI on CXCR4- SWM and CXCR4+ SWM after 3 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 100 ng/mL CXCL12. **(D)** Percentage of cell proliferation measured by cell-trace violet of CXCR4- SWM and CXCR4+ SWM after 3 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 100 ng/mL CXCL12. *$p < 0.05$ by Mann-Whitney test.

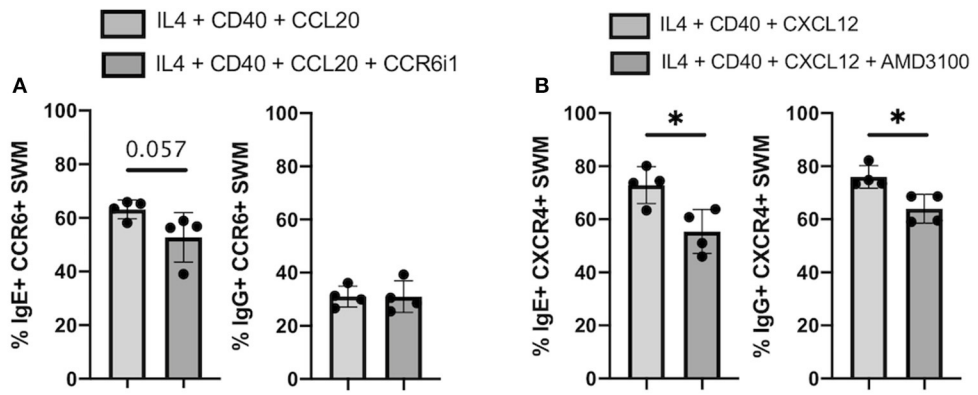
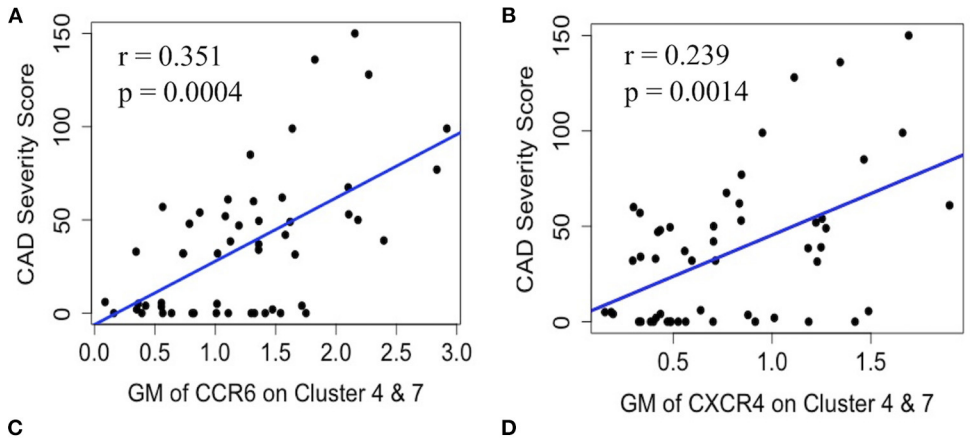


FIGURE 7 | Chemokine receptor inhibitors reduce IgE isotype switching. **(A)** Percentage of IgE+ and IgG+ of CCR6+ SWM after 10 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 20 ng/mL CCL20 ± 5uM CCR6i1. **(B)** Percentage of IgE+ and IgG+ of CXCR4+ SWM after 10 days culture with 20 ng/mL IL-4, 10 ug/mL anti-CD40 ± 100 ng/mL CXCL12 ± 5 uM AMD3100. *$p < 0.05$ by Mann-Whitney test.

remained significant when using cardiac risk factors as co-variates (**Figures 8C,D**).

DISCUSSION

Through utilization of CITEseq, here we identified a B cell cluster uniquely marked by CCR6+ CD20+/CD27+/IgD-/IgM- to associate with α-gal sensitization in CAVA subjects. CD27+/IgD- B cells can be traditionally classified as SWM B cells (19). CD27+ memory B cells harness somatically mutated BCR (20–22) and represent heterogeneous populations that differ in expression of the immunoglobulin isotypes IgM, IgG, IgA and IgE. Human IgE+ B cells have been long thought to mainly develop indirectly from IgG1+ B cell intermediates and rarely directly from IgM+ or IgD+ B cells (23, 24). This premise has been supported by recent studies which used deep sequencing of immunoglobulin somatic hypermutations (25). Roles of chemokine receptors in contributing to IgE production have also been studied in murine models. CCR6 knockout mice were previously shown to have lower serum IgE in hypersensitivity models (26). A



C

Predictors	β	Std. Error	p-value
GM of CCR6 on Cluster 4& 7	34.33	12.173	0.0005*
Age	0.49	1.714	0.093
Sex	-6.01	10.42	0.421
Diabetes	-9.34	9.71	0.801
Hypertension	-2.59	8.62	0.790
Statin use	0.77	12.41	0.811

D

Predictors	β	Std. Error	p-value
GM of CXCR4 on Cluster 4& 7	41.24	12.173	0.0046*
Age	0.53	0.99	0.354
Sex	-9.25	11.41	0.421
Diabetes	-2.66	10.53	0.801
Hypertension	-1.09	5.89	0.669
Statin use	-3.27	12.61	0.811

FIGURE 8 | CXCR4 and CCR6 expression on SWM B cells directly associates with CAD severity. **(A,B)** Linear regression between GM of CCR6 **(A)** and CXCR4 **(B)** on cluster 4 and 7 and CAD Gensini severity score. **(C,D)** Multivariate regression between GM of CCR6 **(C)** and CXCR4 **(D)** on cluster 4 and 7 and CAD Gensini severity score with age, sex, diabetes status, hypertension and statin use as covariates. *$p < 0.05$ by Mann-Whitney test.

study of patients with red meat allergy by Cox et al. reported that a CCR6+ B cell population produced IgE to α-gal (27). Additionally, inflamed skin at the site of tick antigen exposure also demonstrated the accumulation of high levels of CCR6^{high} B cells (28). Our study is the first to show that individuals with IgE sensitization to α-gal without diagnosed red meat allergy (α-gal syndrome) have high expression of CCR6 on SWM B cells. Taken together, our current findings and prior literature support the premise that CCR6+ class-switched memory B cells are a source of B cells that produce allergen-specific IgE, including IgE to α-gal. Moreover, we demonstrate that CCR6 and CXCR4 expression on SWM B cells is higher on those with severe CAD.

Within CCR6+ SWM, transcriptome analysis also revealed that high levels of CXCR4, STAT6, IL-4R, and CD79B (BCR co-receptor) expression associated with α-gal sensitization. IL-4 signaling in B cells has been recognized as a significant driver for IgE class switching through phosphorylation of STAT6 (11, 12). In addition, in agreement with our data indicating an increase in CD79B expression in alpha-gal sensitized subjects, IgE expression on B cells has been previously shown to be dependent on CD79 (10). To our knowledge, a role for CXCR4 in promoting B cell production of IgE has not been reported, but here we found that CXCR4 transcripts and protein expression were associated with α-gal sensitization.

More importantly, this study suggests that CCR6 and CXCR4 contribute to IgE class switch via mechanisms unrelated to chemotaxis. Traditionally, CCR6 and CXCR4 are known to promote migration and entry of B cells to germinal center (GC) regions where B cells class-switch and differentiate (29, 30). Here, we demonstrate for the first time that intrinsic signaling of CCL20/CCR6 can augment AID expression and facilitate IgE isotype switching. This IgE class switching potentially occurs indirectly via an IgG+ B cell given the frequency and kinetics observed over the 10-day experiment. However, future studies will be required to further characterize the mechanism whereby IgE is augmented.

Additionally, we also describe a novel role for CXCL12/CXCR4 signaling in contributing to the proliferation of SWM B cells. Non-chemotaxis roles for CXCR4 are not unprecedented. CXCR4 has been previously shown to promote the survival of a human B lymphoblast cell line in the setting of oxidative stress (31). The current study emphasizes that CXCR4 promotes proliferation of B cells in addition to regulating B cell survival as previously shown in the literatures (31, 32). However, signaling pathways that mediate CXCL12/CXCR4-induced proliferation are still unexplored and investigation to show whether CXCR4 can promote proliferation of other B cell subtypes apart from SWM B cells is still required.

As α-gal IgE sensitization has been shown to associate with CAD (6, 7), we further explored roles of CCR6 and CXCR4 in cardiovascular disease. Upadhye et al. and Srikakulapu et al. had previously investigated roles of CCR6 and CXCR4 on B cells in contributing to atherosclerosis (33, 34). However, those studies focused on atheroprotective B-1 cells and the role of CCR6 and CXCR4 in boosting anti-inflammatory IgM production. This study for the first time suggests a role for CCR6 and CXCR4 on SWM B cells that could be atherogenic. The SWM B cell is a subtype of B-2 cells which have been shown in murine studies to be atherogenic mainly through differentiation into plasma cells and production of pro-inflammatory IgG antibodies (35). In humans, nevertheless, the relationship between SWM B cells, IgG production, and CAD remains controversial (35–38). Our study underscores that the effects of CCR6 and CXCR4 on CAD might be B cell subtype dependent. Particularly in α-gal sensitized subjects, these chemokine receptors may aggravate CAD through promoting SWM B cells to produce IgE to α-gal. However, further studies with larger cohorts will be needed to validate the link between alpha-gal sensitization and CAD and also to further explore the role of CCR6 and CXCR4 in contributing to class switch and CAD. More detailed studies will also be required to confirm whether implicated pathways are specific for alpha-gal IgE class switch, or could be generalizable to IgE switching to other antigens.

In summary, here we identified that human CCR6+ class-switched memory B cells, but not other B cell subtypes, were associated with α-gal IgE sensitization. Further transcriptome analysis and *in vitro* characterization also revealed that CCR6 and CXCR4, acting via non-chemotactic mechanisms, contributed to IgE isotype switching and cell proliferation of SWM B cells, respectively. In addition, we demonstrated that relatively higher expression of CCR6 and CXCR4 in SWM B cells were associated with CAD severity, independent of traditional CAD risk factors. These findings suggest that therapies that modulate SWM B cells, potentially by targeting CCR6 or CXCR4, hold promise for reducing α-gal IgE production and cardiovascular disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GSE190570.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Virginia Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TP and CM conceived the study. TP designed, performed and analyzed experiments. CM, JW, and LE supervised experiments and provided insights on study designs. YG and RG designed the bioinformatics pipeline for CITEseq analysis. CD performed CITEseq experiments. JV and RS helped with CITEseq data thresholding. TP-M provided ImmunoCAP assays. AT provided patient cohorts. KL supervised CITEseq experiments. HQ performed *in vitro* B cell stimulation experiments. CM supervised the entire study. TP, JW, LE, and CM prepared the manuscript. All authors contributed to and approved the final manuscript.

FUNDING

This work was supported by National Institute of Health NIH R01HL 136098 (CM) R01-HL148109 (CM), P01 HL136275 (CM and KL), R21-AI-152447 (LE), R37-AI-20565 (TP-M), and AAAAI Faculty Development Award (JW). This study received assay support from Thermo-Fisher/Phadia. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

ACKNOWLEDGMENTS

We thank Mike Solga, Claude Chew, Alex Wendling, and Taylor Harper from the UVa flow cytometry core for their excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.791028/full#supplementary-material

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frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 02 February 2022
doi: 10.3389/fcvm.2022.781436

IFNγ-Stimulated B Cells Inhibit T Follicular Helper Cells and Protect Against Atherosclerosis

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OPEN ACCESS

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: 22 September 2021
Accepted: 10 January 2022
Published: 02 February 2022

Citation:
Douna H, de Mol J, Amersfoort J, Schaftenaar FH, Kiss MG, Suur BE, Kroner MJ, Binder CJ, Bot I, Van Puijvelde GHM, Kuiper J and Foks AC (2022) IFNγ-Stimulated B Cells Inhibit T Follicular Helper Cells and Protect Against Atherosclerosis. Front. Cardiovasc. Med. 9:781436. doi: 10.3389/fcvm.2022.781436

B and T cells are interconnected in the T follicular helper—germinal center B cell (T_{FH}-GC B cell) axis, which is hyperactive during atherosclerosis development and loss of control along this axis results in exacerbated atherosclerosis. Inhibition of the T_{FH}–GC B cell axis can be achieved by providing negative co-stimulation to T_{FH} cells through the PD-1/PD-L1 pathway. Therefore, we investigated a novel therapeutic strategy using PD-L1-expressing B cells to inhibit atherosclerosis. We found that IFNγ-stimulated B cells significantly enhanced PD-L1 expression and limited T_{FH} cell development. To determine whether IFNγ-B cells can reduce collar-induced atherosclerosis, *apoE*$^{-/-}$ mice fed a Western-type diet were treated with PBS, B cells or IFNγ-B cells for a total of 5 weeks following collar placement. IFNγ-B cells significantly increased PD-L1^{hi} GC B cells and reduced plasmablasts. Interestingly, IFNγ-B cells–treated mice show increased atheroprotective Tregs and T cell-derived IL-10. In line with these findings, we observed a significant reduction in total lesion volume in carotid arteries of IFNγ-B cells-treated mice compared to PBS-treated mice and a similar trend was observed compared to B cell-treated mice. In conclusion, our data show that IFNγ-stimulated B cells strongly upregulate PD-L1, inhibit T_{FH} cell responses and protect against atherosclerosis.

Keywords: cardiovascular disease, atherosclerosis, B cells, PD-L1, interferon-gamma

INTRODUCTION

Cardiovascular disease (CVD) remains a major global health problem despite great developments in diagnosis and treatment. The underlying cause for CVD is atherosclerosis, which is a chronic autoimmune-like disease and is characterized by the formation of lipid-rich lesions in the arteries. The current available treatments are aimed at lipid lowering and lead to a 25–30% relative risk reduction, indicative of an urgent need for novel disease-modifying drugs. In the last decade, accumulating evidence identified the immune system as a major contributor to the pathology of atherosclerosis (1). For this reason, considerable effort has been devoted to restore the dysregulation of the immune system and inflammatory pathways in atherosclerosis.

B and T-lymphocyte dependent immune responses play a key role in the pathophysiology of atherosclerosis and the role of most B and T cell subsets in atherosclerosis is now well-defined. For instance, B1 cells have shown a consistent atheroprotective effect (2–5), while in multiple studies B2 cells were seen to contribute to atherosclerosis (6–9). In addition, the involvement of Th1, Th2 and Treg cells in atherosclerosis has also been examined comprehensively as reviewed in (10). In contrast, only recently the contribution of other leukocyte subpopulations such as follicular (FO) B cells, marginal zone (MZ) B cells and follicular helper T cells (T_{FH}) to atherosclerosis have been identified. Although FO B cells, MZ B cells and T_{FH} cells are radically different cell types, they appear to be interconnected in the T_{FH}–germinal center (GC) B cell axis. FO B cells enter germinal centers, subsequently undergo proliferation and isotype switching and can differentiate in short-lived plasmablasts, which can further differentiate into long-lived plasma cells or memory cells. FO B cells promote the recruitment of T_{FH} cells and the generation of germinal centers through expression of inducible co-stimulator ligand (11). Previously, it has been shown that the T_{FH}–GC B cell axis is hyperactivated and promotes lesion formation in both apolipoprotein E-deficient ($apoE^{-/-}$) mice (12) and low-density lipoprotein receptor-deficient ($ldlr^{-/-}$) mice fed a hypercholesterolemia-inducing diet (13). In addition, both T_{FH} cells and FO B cells are proatherogenic and can aggravate atherosclerosis (14, 15).

The T_{FH}–GC B cell axis can be regulated by the co-inhibitory programmed death-1 (PD-1)/PD-L1 pathway. T_{FH} cells highly express PD-1 and their accumulation can be controlled by MZ B cells which express PD-L1. In response to high cholesterol levels, MZ B cells upregulate the expression of PD-L1 and thereby regulate T_{FH} cell accumulation which limits an exacerbated adaptive immune response (13). However, this mechanism fails to completely arrest disease development. Interestingly, in autoimmune encephalomyelitis, it has been shown that adoptive transfer of B cells expressing high levels of PD-L1 limited disease severity (16). Whether PD-L1 expressing B cells can also be used therapeutically to inhibit atherosclerosis development has not yet been reported. In this study, we therefore induced PD-L1-expressing B cells and investigated whether adoptive transfer of these cells could inhibit atherosclerosis development.

MATERIALS AND METHODS

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Female C57BL/6, B6.SJL-PtprcaPepcb/BoyCrl (also known as CD45.1) and apolipoprotein E-deficient ($apoE^{-/-}$) mice were bred in house and kept under standard laboratory conditions. Diet and water were provided *ad libitum*. All injections were administered i.v. to the lateral tail vein in a total volume of 100 μl. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/ml), atropine (0.1 mg/ml), and xylazine (8 mg/ml). Mice were bled and perfused with phosphate-buffered saline (PBS) through the left cardiac ventricle.

Cell Culture

B cells were isolated from splenocytes of C57BL/6 or $apoE^{-/-}$ mice using $CD19^+$ microbeads (Miltenyi Biotec) and cultured in complete RPMI medium. Isolated B cells were cultured with different concentrations of heat killed *Staphylococcus aureus* (Invivogen), B-cell activating factor (BAFF; R&D systems) or interferon-gamma (IFNγ; ThermoFisher) for 24 h. For co-culture experiments, $CD4^+$ T cells were isolated from splenocytes of C57BL/6 mice using a $CD4^+$ T cell isolation kit (Miltenyi Biotec). For some experiments, the $CD4^+$ T cells were labeled using a CellTrace Violet Cell Proliferation kit (ThermoFisher) according to the instructions of the manufacturer. B and T cells were co-cultured and stimulated with an agonistic plate-bound CD3 antibody (5 μg/ml) for 72 h. For adoptive transfer experiments, B cells were isolated from splenocytes of $apoE^{-/-}$ or CD45.1 mice, cultured in RPMI medium and stimulated for 24 h with 20 ng/ml of IFNγ. Subsequently, cells were washed, checked for purity using flow cytometry and resuspended for injections with PBS. For the injection of untouched B cells, B cells were freshly isolated from splenocytes of $apoE^{-/-}$ or CD45.1 mice and directly used for adoptive transfer experiments.

Real-Time Quantitative PCR

RNA was extracted from cultured B cells by using Trizol reagent according to manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the instructions of the manufacturer (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to housekeeping genes. Primer sequences are available in **Supplementary Table 1**.

In vivo Experiments

For all *in vivo* experiments, $apoE^{-/-}$ mice were used. $ApoE^{-/-}$ mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK). For the pilot study, mice were pre-fed a WTD for 2 weeks and were subsequently treated with 2×10^6 freshly isolated or cultured B cells and sacrificed after 3 days. To assess the impact of WTD on the percentage of PD-$L1^{hi}$ B cells, $apoE^{-/-}$ mice were fed a chow diet or a WTD for 2 or 7 weeks. For atherosclerosis experiments, carotid artery plaque formation was induced after 2 weeks of WTD feeding by perivascular collar placement in these mice as described previously (17). Mice continued to be fed a WTD for 5 weeks and during this period received 3 injections with either freshly isolated B cells (2×10^6 cells/injection), B cells stimulated with 20 ng/ml IFNγ for 24 h (2×10^6 cells/injection) or PBS. To discriminate between adoptively transferred cells and endogenous cells, B cells were isolated from CD45.1 mice for the last injection. Time between injections was 2 weeks. At the end

of experiments, mice were sacrificed and relevant organs were harvested for analysis.

Cytokine Analysis

Isolated splenocytes from PBS, B cell or IFNγ-B cell treated mice were cultured in complete RPMI medium and stimulated for 72 h with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml). The levels of cytokines in culture supernatants were measured using a Luminex bead-based multiplex assay (ProcartaPlex, Thermo Fisher Scientific) on a Luminex Instrument (MAGPIX). Recombinant cytokine standards (Thermo Fisher Scientific) were used to calculate cytokine concentrations and data were analyzed using Bio-Rad software.

Flow Cytometry

For flow cytometry analysis, Fc receptors of single cell suspensions were blocked with an unconjugated antibody against CD16/CD32. Samples were then stained with a fixable viability marker (ThermoScientific) to select live cells. Next, cells were stained with anti-mouse fluorochrome-conjugated antibodies (**Supplementary Table 2**). Regular flow cytometry was performed on a Cytoflex S (Beckman Coulter) and the acquired data were analyzed using FlowJo software. Gates were set according to isotype or fluorescence minus one controls.

Serum Measurements

Serum was acquired by centrifugation and stored at −20°C until further use. Total serum titers of IgM, IgG1, IgG2c and oxidized LDL-specific antibodies were quantified by ELISA as previously described (5).

Histology

Carotid arteries and hearts were frozen in OCT compound (TissueTek) and stored at −80°C until further use. Transverse cryosections proximal to the collar were collected and mounted on Superfrost adhesion slides (ThermoFisher). To determine lesion size, cryosections were stained with hematoxylin and eosin (Sigma-Aldrich). Quantification of lesion size was assessed every 100 μm from the first section with visible lesion proximal to the collar until no lesion could be observed. Plaque volume was determined with lesion size and the total distance of the lesions in the carotid artery. Phenotypic analysis of the lesion was performed on sections containing the largest three lesions. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Necrotic core size was determined manually by selecting acellular areas in the Masson's trichrome stained sections and shown as absolute area and percentage of the total plaque area. Corresponding sections on separate slides were also stained for monocyte/macrophage content using a monoclonal rat IgG2b antibody (MOMA-2, 1:1000, AbD Serotec) followed by a goat anti-rat IgG-horseradish peroxidase antibody (1:100, Sigma-Aldrich) and color development using the ImmPACT NovaRED substrate (Vector Laboratories). For the detection of vascular smooth muscle cells, cryosections were stained with a monoclonal rat alpha-smooth muscle actin antibody conjugated to Alexa fluor 647 (1:1500, Novus Biologicals). Furthermore, cryosections were stained with a monoclonal rat CD4 antibody conjugated to FITC (1:150, eBioscience) and a monoclonal rat IgG2b isotype control conjugated to FITC (1:150, MBL) to determine $CD4^+$ T cell infiltration. For all fluorescent images, cryosections were blocked with αCD16/32 Fc block (1:250, Biolegend) and nuclei were stained with DAPI. All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's *t*-test for two normally distributed groups. Data from three groups or more were analyzed by an ordinary one-way ANOVA test followed by Holm-Sidak *post-hoc* test. Probability values of $p < 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism.

RESULTS

IFNγ-Stimulated B Cells Express High Levels of PD-L1

Previously, it has been shown that hypercholesterolemia promotes PD-L1 expression on B cells in $ldlr^{-/-}$ mice, which can regulate T_{FH} cell accumulation, limiting an exacerbated adaptive immune response (13). In order to investigate if the administration of a Western-type diet (WTD) also affects PD-L1-expressing B cells in $apoE^{-/-}$ mice, we compared PD-L1 expression on B cells from chow fed $apoE^{-/-}$ mice and $apoE^{-/-}$ mice fed a WTD for 2 and 7 weeks using flow cytometry (**Figure 1A**; **Supplementary Figure 1A**). Whereas we observed no significant differences in $PD\text{-}L1^{hi}$ B cells between chow fed $apoE^{-/-}$ mice and $apoE^{-/-}$ mice fed a WTD for 2 weeks, longer administration of WTD increases the percentage of $PD\text{-}L1^{hi}$ B cells, in line with previous findings. Nonetheless, this elevation in PD-L1 expressing B cells upon hypercholesterolemia is not sufficient to halt disease development (13), indicating the need to further stimulate the regulation of the T_{FH}–GC B cell axis through the PD-1/PD-L1 pathway. We therefore aimed to generate a population of $PD\text{-}L1^{hi}$ B cells *ex vivo,* to adoptively transfer in WTD fed $apoE^{-/-}$ mice to halt atherosclerosis. We explored several stimuli that previously have been shown to control PD-L1 expression (18, 19) (**Supplementary Figures 1B,C**; **Figure 1**) and found that IFNγ dose-dependently increased the number of $PD\text{-}L1^+$ B cells, with 20.0 ng/ml of IFNγ leading to an almost pure population of PD-L1-expressing B cells (**Figure 1B**). Using qPCR, we also found an almost eight-fold induction of PD-L1 on mRNA level after IFNγ stimulation (**Figure 1C**). Furthermore, we observed that the majority of IFNγ-stimulated B cells expressed very high levels of PD-L1 (**Figure 1D**). Previously, it has been shown that IFNγ-signaling in B cells drives STAT-1 dependent expression of T-bet and BCL-6 and switches them toward a GC B cell phenotype with increased IFNγ and IL-6 production (20, 21). In line with this, our *ex vivo* IFNγ-stimulated B cells (IFNγ-B cells) showed a similar increase in STAT1, T-bet and BCL-6 (**Figure 1E**) gene expression. In contrast, we observed a trend toward less IL-6 and no difference in IFNγ expression (**Supplementary Figure 1D**).

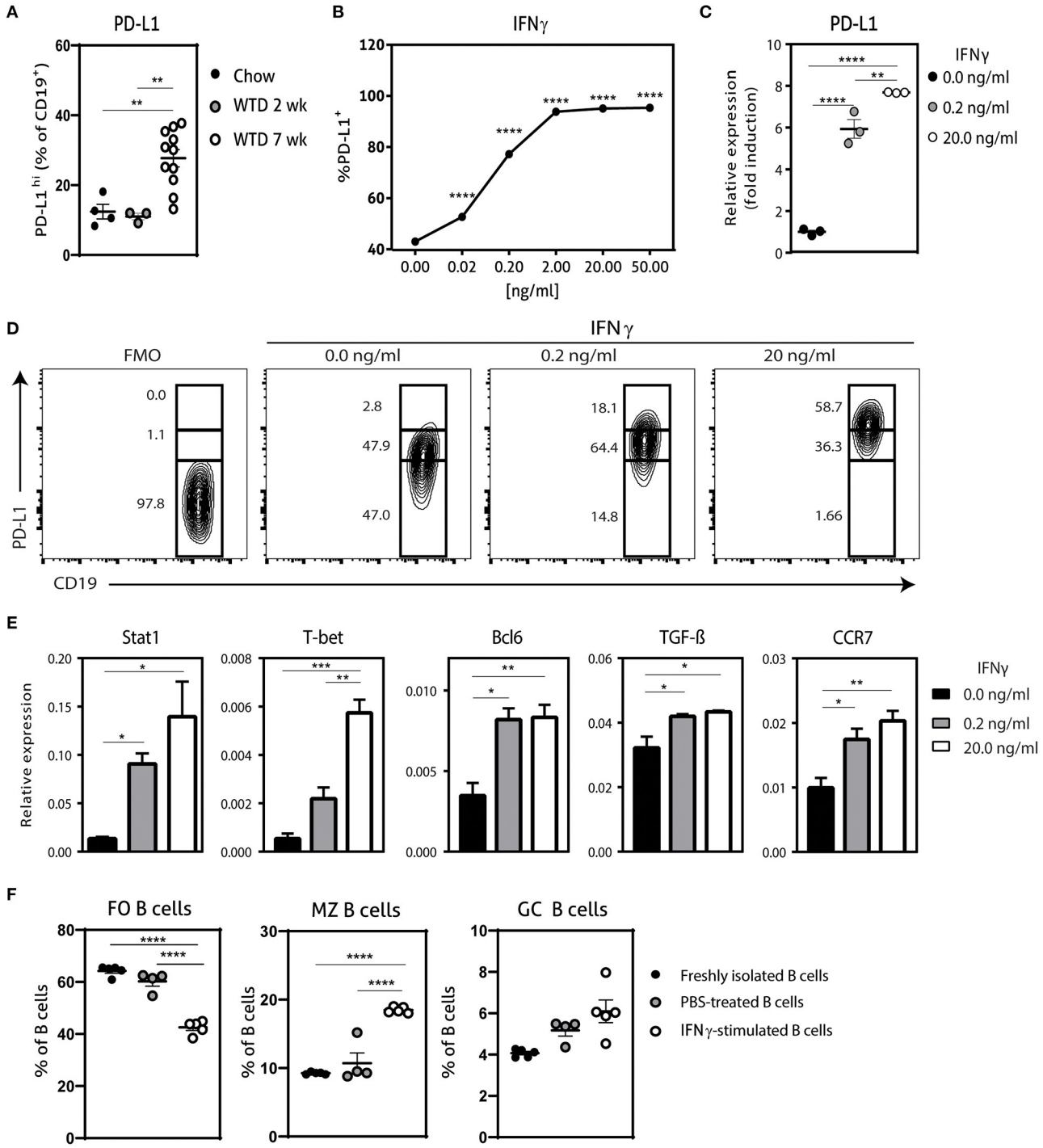

FIGURE 1 | Characterization of IFNγ-stimulated B cells. **(A)** PD-L1^{hi} expressing CD19^{+} B cells were determined in spleens of *apoE*$^{-/-}$ mice fed a regular Chow diet or Western type diet for 2 or 7 weeks using flow cytometry. **(B)** CD19^{+} B cells were isolated from C57BL/6 mice and stimulated for 24 h with different doses interferon-gamma (IFNγ) after which PD-L1 protein expression was measured with flow cytometry. **(C)** CD19^{+} B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 h, after which mRNA expression of PD-L1 was assessed using qPCR. **(D)** B cells as stimulated in (C) were analyzed for PD-L1^{lo}, PD-L1^{int} and PD-L1^{hi} expression with flow cytometry. **(E)** mRNA expression was analyzed for depicted genes in B cells as stimulated in **(C)**. **(F)** FO B cells (CD23^{+}) MZ B cells (CD23^{-} CD21^{+}) and GC B cells (GL-7^{+} CD95+) were determined in CD19^{+} B cells stimulated with 20.0 ng/ml IFNγ for 24 h. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.00001$). $n = 3$–11/group.

Interestingly, IFNγ stimulation resulted in a strong significant increase in TGF-β expression (**Figure 1E**). We also investigated the chemokine receptor profile of these B cells and found a dose-dependent increase in CCR7 expression (**Figure 1E**), while there was no effect on CXCR5 or Ebi-2 (**Supplementary Figure 1D**). This change in chemokine receptor expression is typical of B cells that migrate toward the T-B cell border in lymphoid tissues in response to the CCL21 gradient (22). Hence, these data indicate that IFNγ-stimulated B cells express high levels of PD-L1 and TGF-β and a chemokine profile that homes B cells to the T-B cell border. In line with these findings, we observed that IFNγ stimulation of B cells not only induced coinhibitory PD-L1 expression on all B cells (**Figure 1C**) but also increased the percentage of GC B cells and MZ B cells (**Figure 1F**). Together with a decrease in FO B cells, we thus show that IFNγ stimulation generates a B cell population with an enhanced anti-inflammatory phenotype.

IFNγ-Stimulated B Cells Inhibit T_{FH} Cells *in vitro* and *in vivo*

Next, we explored the functional effects of IFNγ-stimulated B cells on T_{FH} cell development using a $CD4^+$ T cell and B cell coculture. We stimulated wild-type $CD4^+$ T cells for 72 h with anti-CD3 in the presence of unstimulated or IFNγ-stimulated $apoE^{-/-}$ B cells. Although we did not observe any difference in proliferative capacity of $CD4^+$ T cells (**Figure 2A**), we found a remarkable decrease of T_{FH} cells when $CD4^+$ T cells were cocultured with IFNγ-stimulated B cells compared to unstimulated B cells (**Figure 2B**). These findings illustrate that IFNγ-B cells are able to curb T_{FH} cell development *in vitro*. We subsequently tested IFNγ-stimulated B cells in an *in vivo* setting by adoptively transferring either freshly isolated B cells or B cells stimulated with 20.0 ng/ml of IFNγ for 24 h into $apoE^{-/-}$ mice. To induce initial T_{FH} cell accumulation, mice were fed a Western-type diet for 2 weeks before they received the adoptive transfers (**Figure 2C**) (13). We observed a remarkable reduction in effector $CD4^+$ T cells in mice treated with IFNγ-B cells compared to mice that received PBS or B cells (**Figure 2D**). Contrary, the number of naïve $CD4^+$ T cells was increased in IFNγ-B cells treated mice compared to mice receiving PBS (**Figure 2D**). Most importantly, treatment of mice with IFNγ-B cells resulted in a strong reduction in T_{FH} cells compared to mice that were administered with PBS or B cells (**Figure 2E**). These data demonstrate that IFNγ-B cells are also able to inhibit T_{FH} cells *in vivo*.

Adoptive Transfer of IFNγ-B Cells During Atherosclerosis Development Affects the T_{FH}–GC B Cell Axis

Given the *in vitro* and *in vivo* regulatory effects of IFNγ-B cells on T_{FH} cells, we further investigated whether these B cells would be able to restrict T_{FH} cell numbers during atherosclerosis development. We fed $apoE^{-/-}$ mice a Western-type diet for 2 weeks after which we placed a perivascular collar and started treatment with either PBS, B cells or IFNγ-B cells (**Figure 3A**). After a total of 3 injections and 7 weeks of Western-type diet, we harvested the organs and analyzed the immune cells associated with the T_{FH}–GC B cell axis. IFNγ-B cell-treated mice showed a trend toward reduced FO B cells (**Figure 3B**) and a significant increase in MZ B cells compared to mice receiving PBS (**Figure 3C**). Moreover, mice that received IFNγ-B cells showed a significant increase in the number of GC B cells compared to mice that were administered PBS (**Figures 3D,E**). By using the CD45 congenic marker system, we showed that adoptively transferred IFNγ-B cells indeed reached the germinal center (**Figure 3F**) and locally increased the number of PD-$L1^{hi}$ B cells (**Figure 3G**). As shown in **Figures 3D,E**, freshly isolated B cells also increased the number of GC B cells, but these were not derived from the adoptive transfer (**Figure 3F**) and were PD-$L1^-$ (**Figure 3H**). At sacrifice, we did not observe a difference in T_{FH} cells in IFNγ-B cell-treated mice, while adoptive transfer of B cells resulted in an increase in T_{FH} cells compared to PBS-treated mice (**Figure 3I**).

Next, we assessed the number of plasmablasts and plasma cells and found a significant reduction in plasmablasts when mice received IFNγ-B cells compared to mice receiving PBS (**Figure 4A**). Mice that received B cells showed a similar trend toward less plasmablasts and also a significant increase in plasma cells compared to PBS- and IFNγ-B cells-treated mice. Since BLIMP-1 is the driving transcription factor for plasma cell generation (23), we also measured BLIMP-1 expression which revealed a significant increase in BLIMP-1^+ cells in mice that received B cells compared to mice receiving PBS or IFNγ-B cells (**Figure 4B**). Since plasmablasts and plasma cells are responsible for the humoral immunity, we measured circulating antibodies. However, neither B cell treatments led to a significant difference in circulating antibodies (**Figure 4C**).

Adoptive Transfer of IFNγ-B Cells Promotes an Anti-inflammatory $CD4^+$ T Cell Response

The $CD4^+$ T cell response is also highly involved in the pathogenesis of atherosclerosis and PD-$L1^{hi}$ B cells have previously shown to restrict $CD4^+$ T cell differentiation (16). While we did not observe any differences in Th1 or Th2 $CD4^+$ T cells between mice treated with IFNγ-B cells, B cells or PBS in our study (**Supplementary Figure 2**), we observed a significant decrease in Th17 cells (**Figure 4D**) and a significant increase in atheroprotective regulatory T cells (**Figure 4E**; Tregs) in mice treated with IFNγ-B cells. We next measured cytokine levels of *ex vivo* anti-CD3 and anti-CD28 stimulated splenocytes for 72 h with a multiplex assay. In line with the increase in Tregs, we observed a significant increase in IL-10 production by splenocytes from IFNγ-B cells treated mice compared to splenocytes from mice treated with PBS or B cells (**Figure 4F**).

Adoptive Transfer of IFNγ-B Protects Against Lesion Formation

Given the immune-regulating effects of IFNγ-B cells, we subsequently assessed whether adoptive transfer of these IFNγ-B cells was able to reduce collar-induced atherosclerosis in $apoE^{-/-}$ mice. We quantified lesion development in the carotid arteries and found a significant reduction in total lesion

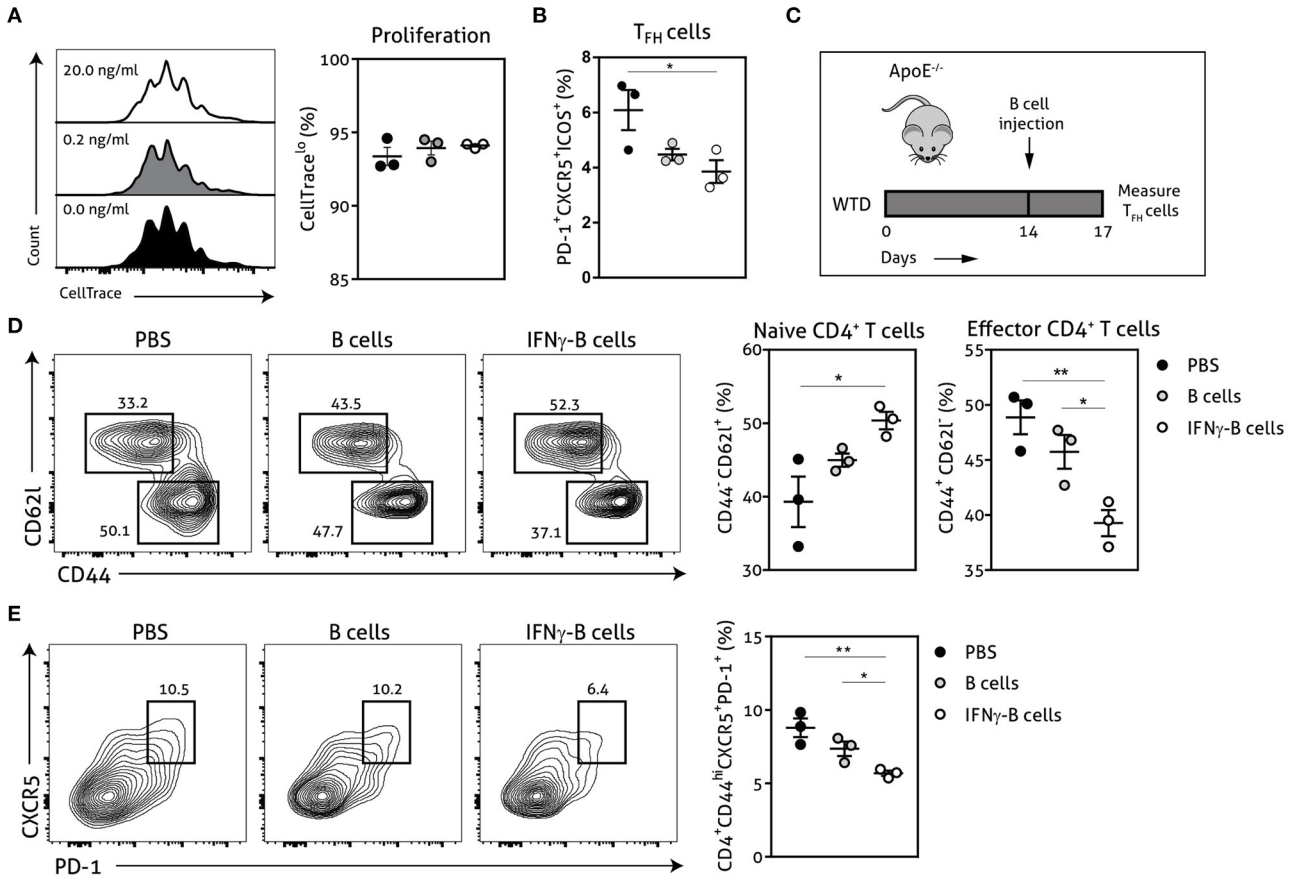

FIGURE 2 | IFNγ-B cells inhibit T follicular helper cells *in vitro* and *in vivo*. Isolated CD19$^+$ B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 h and co-cultured with isolated CD4$^+$ T cells. The coculture was stimulated with anti-CD3 (5 μg/ml) for 72 h after which **(A)** proliferation of CD4$^+$ T cells was analyzed with CellTrace Violet and **(B)** the number of follicular CD4$^+$ T helper (T_{FH}) cells were analyzed. **(C)** *apoE*$^{-/-}$ mice were fed a Western type diet for 2 weeks after which they either received PBS, freshly isolated CD19$^+$ B cells (B cells) or CD19$^+$ B cells stimulated with 20.0 ng/ml IFNγ for 24 h (IFNγ-B cells). After three days, mice were sacrificed and spleens were isolated for flow cytometry analysis for **(D)** naive (CD62l$^+$CD44$^-$) and effector (CD62l$^-$CD44^{hi}) CD4$^+$ T cells and **(E)** T follicular helper cells (CD4$^+$CD44^{hi}CXCR5$^+$PD-1$^+$). Representative flow charts of the CD4$^+$ T cell population are shown. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$). $n = 3$/group.

volume when mice were treated with IFNγ-B cells compared to PBS and a similar trend was found when compared to B cell-treated mice (**Figure 5A**). Media size did not differ between the treatment groups (**Figure 5B**). Furthermore, there were no differences in weight gain and serum cholesterol levels (**Supplementary Figures 3A,B**). We also assessed lesion phenotype at the site of the maximal lesion. This revealed that mice treated with IFNγ-B cells showed an early lesion phenotype with relatively more macrophages than collagen compared to mice that received B cells or PBS (**Figures 5C,D**), suggesting that adoptive transfer of IFNγ-B cells inhibited atherosclerotic lesion progression toward more advanced lesions. Evaluation of vascular smooth muscle cells (VSMCs) using α-smooth muscle actin did not reveal any differences (**Supplementary Figure 4A**) and we also did not observe altered necrotic core formation within the atherosclerotic plaque in IFNγ-B cell treated mice compared to the other groups (**Supplementary Figure 4B**). Finally, we did not observe significant differences in CD4$^+$ T cell infiltration between the treatment groups (**Supplementary Figure 4C**) and only very low numbers of CD4$^+$ T cells were found, suggesting that the reduction in lesion size is caused by systemic anti-atherogenic effects of PD-L1^{hi} B cells, rather than a local effect.

DISCUSSION

A proatherogenic role has been reported for the T_{FH}–GC B cell axis (12–14). Direct depletion of T_{FH} cells in *ldlr*$^{-/-}$ mice resulted in a reduction of atherosclerosis (14), and loss of control on the T_{FH}–GC B axis by depletion of MZ B cells (13) or CD8$^+$ regulatory T cells (12) aggravated atherosclerosis. We now show that adoptive transfer of PD-L1 expressing B cells inhibit T_{FH} cell responses both *in vitro* and *in vivo* and protect against atherosclerosis.

Using *ex vivo* stimulation of B cells with IFNγ we rapidly provided a large and almost pure population of PD-L1-expressing

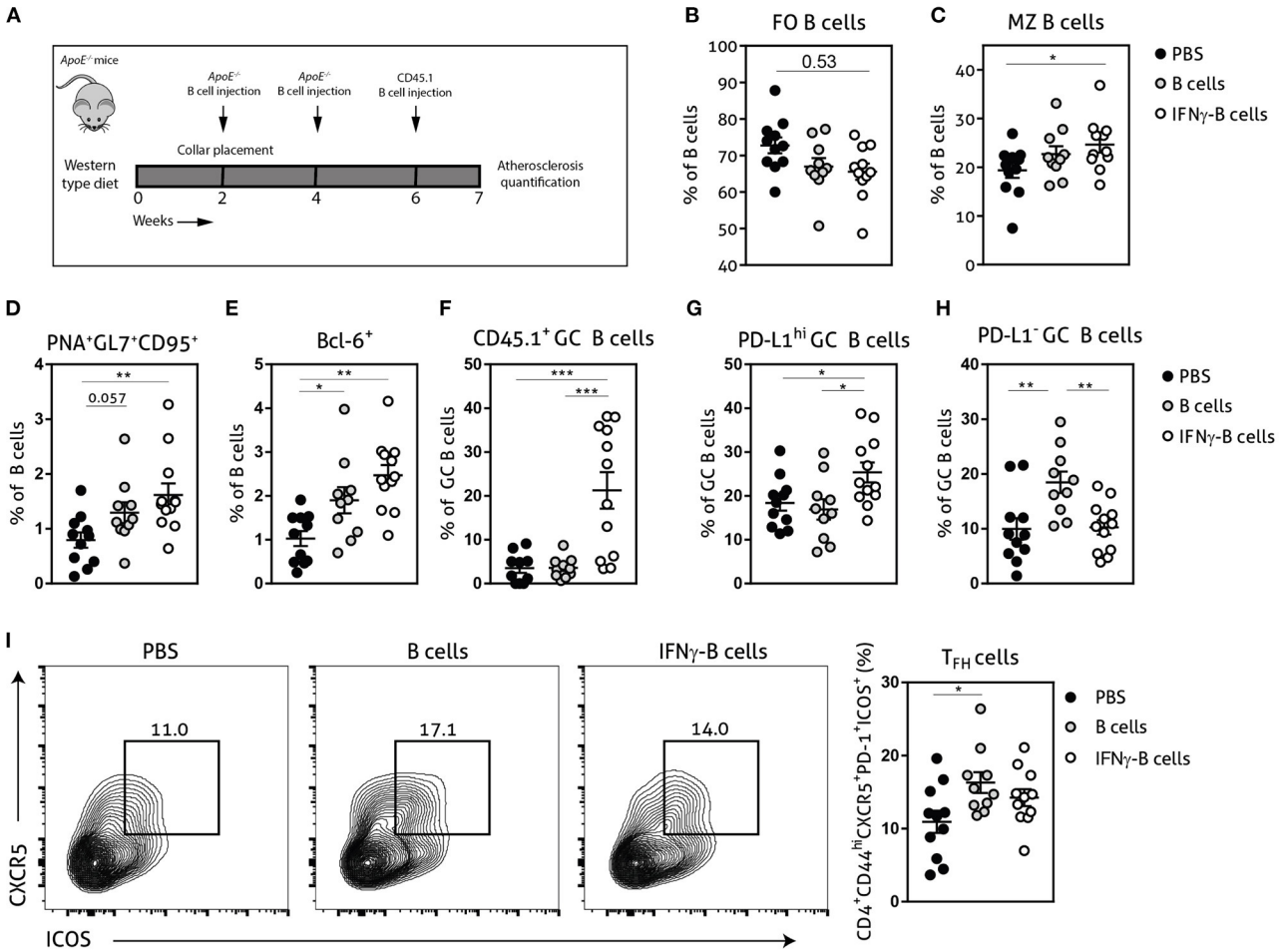

FIGURE 3 | Adoptive transfer of IFNγ-B cells during atherosclerosis development affects the T_{FH}–GC B cell axis. **(A)** *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 h (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry. Flow cytometry quantification of **(B)** follicular (FO) B cells, **(C)** marginal zone (MZ) B cells, **(D)** extracellular staining of germinal center B cells, **(E)** intracellular staining of germinal center B cells, **(F)** CD45.1$^+$ germinal center B cells, **(G)** PD-L1^{hi} germinal center B cells, **(H)** PD-L1$^-$ germinal center B cells and **(I)** flow charts and quantification of follicular helper T cells (CD44^{hi}PD-1$^+$CXCR5$^+$ICOS$^+$). Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). $n = 10$–12/group.

B cells. IFNγ is an extensively investigated cytokine with a broad range of immune actions. Interestingly, while we show here that IFNγ stimulation of B cells results in a regulatory phenotype that reduces the number of T_{FH} cells, earlier work identified a role for IFNγ in the generation of spontaneous germinal centers and B cell autoreactivity (20, 24). These previous data were, however, mainly acquired in the context of autoimmunity, where B cells receive a multitude of different signals and IFNγ signaling seems primarily to synergize with BCR-, CD40- and TLR-mediated stimuli to induce spontaneous germinal centers (24). Under these circumstances, IFNγ signaling in B cells results in increased IL-6 and IFNγ production which drives auto-immunity (20, 21). In contrast, our *ex vivo* stimulated B cells lacked additional stimuli and upregulated expression of PD-L1 and TGF-β, while we did not see any effects on IL-6 and IFNγ. We further showed that IFNγ-B cells express high levels of CCR7 with minimal changes in CXCR5 and Ebi-2. This expression profile is in line with a previous study that demonstrated that MZ B cells from *ldlr*$^{-/-}$ mice interact with pre-T_{FH} cells at the T-B cell border in response to a high-cholesterol diet (13). Moreover, we show that *ex vivo* IFNγ stimulation generates a PD-L1^{hi} B cell pool containing enhanced MZ and GC B cells, while pro-atherogenic FO B cells are decreased. During our atherosclerosis study, we indeed found that adoptively transferred IFNγ-B cells showed the characteristics of B cells that reside in or near the germinal center.

Similar to flow-sorted PD-L1^{hi} B cells (16), we next showed that IFNγ-B cells were able to inhibit T_{FH} cell numbers *in vitro* and in our short *in vivo* experiment. Notably, at the point of sacrifice in our atherosclerosis experiment we did not observe restriction of T_{FH} cells by IFNγ-B cells, but we did demonstrate

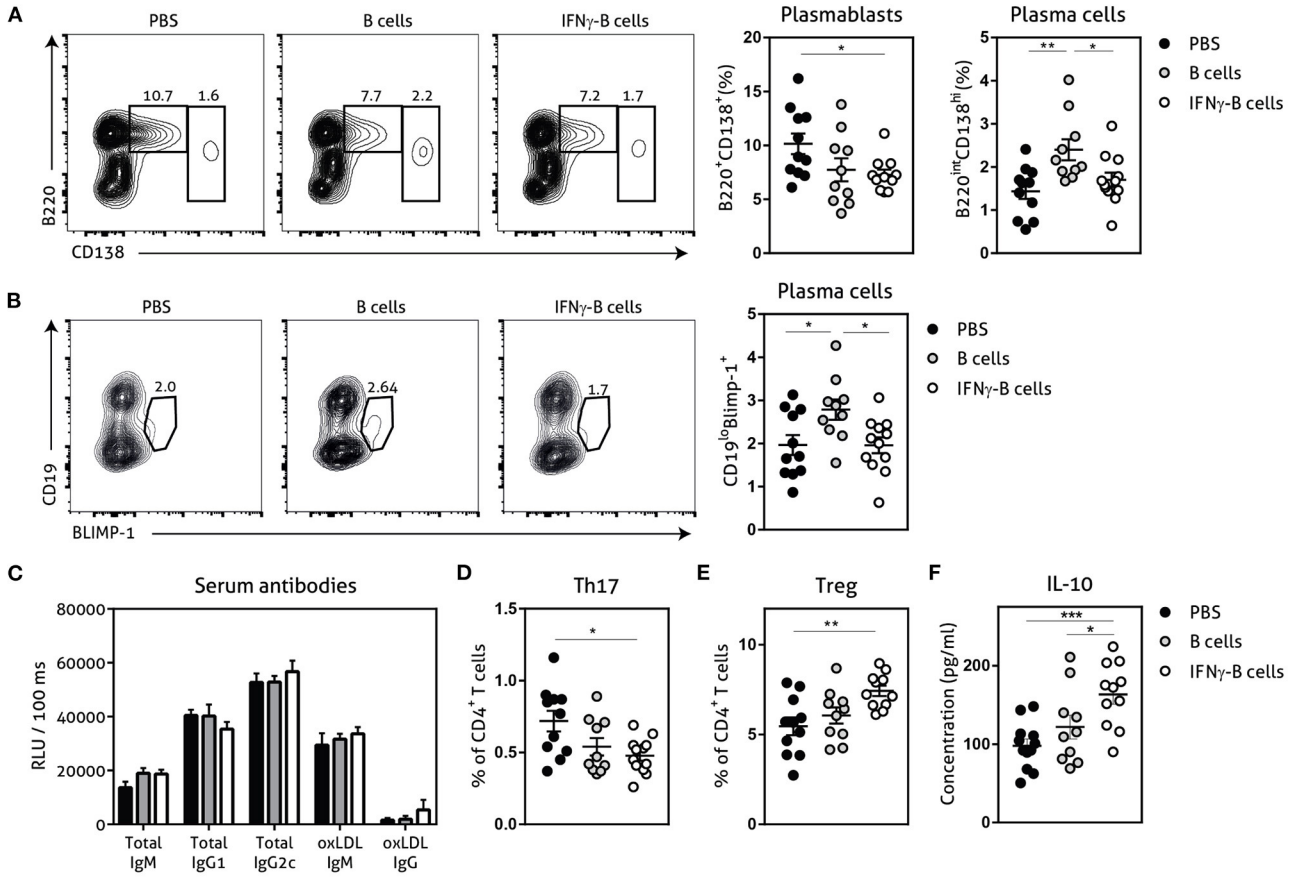

FIGURE 4 | Effects of adoptive transfer of IFNγ-B cells on the humoral immunity. *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 h (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry. **(A)** Flow charts and quantification of plasmablasts (B220^{+}CD138^{+}) and plasma cells (B220^{lo}CD138^{hi}). **(B)** Flow charts and quantification of CD19^{lo}BLIMP-1^{+} plasma cells. **(C)** Serum was analyzed for circulating antibodies by ELISA. Spleens were analyzed with flow cytometry for **(D)** Th17 cells (RORγt^{+}) or **(E)** Treg cells (FoxP3^{+}). **(F)** Splenocytes were isolated and stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for 72 h after which supernatant was collected and analyzed for cytokine expression with a multiplex analysis. Quantification of IL-10 concentration is shown. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$). $n = 10$–12/group, ***$p < 0.001$.

that IFNγ-B cells were able to promote anti-inflammatory CD4^{+} T cells. This corresponds with the effects found in experimental autoimmune encephalomyelitis using PD-L1^{hi} B cells, which restricted Th1 and Th17 differentiation (16). Along this line, we found that IFNγ-B cells resulted in decreased Th17 cells and a significant increase in atheroprotective Tregs and IL-10 production of CD4^{+} T cells. Interestingly, it has been reported that during atherosclerosis development there is a plasticity between Treg cells and T_{FH} cells and disturbances of this delicate balance greatly affected atherosclerosis development (14). We did not directly investigate this plasticity in our study, but our work shows that IFNγ-B cells are able to inhibit T_{FH} cells and increase Treg cells.

Furthermore, we demonstrated atheroprotective effects of IFNγ-B cells that express high levels of PD-L1.The observed reduction in atherosclerosis upon adoptive transfer of IFNγ-B cells was accompanied by changes in plaque morphology (relatively more macrophages, reduced collagen), indicating a more initial plaque phenotype. Reduced collagen content did not coincide with alterations in overall VSMCs content. Although we cannot specifically determine collagen production rate by SMCs locally in the plaque, the lack of difference in SMC content suggests that the difference in collagen content that we observed in the plaque is not explained by the amount of SMCs. Although we did not investigate this in our study, the reduced collagen content in IFNγ-B cell treated mice may also be attributed to an increase in collagen degradation through the production of matrix metalloproteinases by the macrophages in the plaque, which were relatively increased upon IFNγ-B cell transfer. The atheroprotective effect of PD-L1^{hi} B cells is in line with a general protective role of the PD-L1/PD-1 axis in atherosclerosis. Mice deficient in both PD-L1 and PD-L2 show increased atherosclerosis (25). Similarly, PD-1 knockout mice or mice treated with a PD-1 blocking antibody developed exacerbated atherosclerosis (26, 27), whereas stimulation of PD-1 signaling reduces atherosclerosis (28). Our data is further supported by

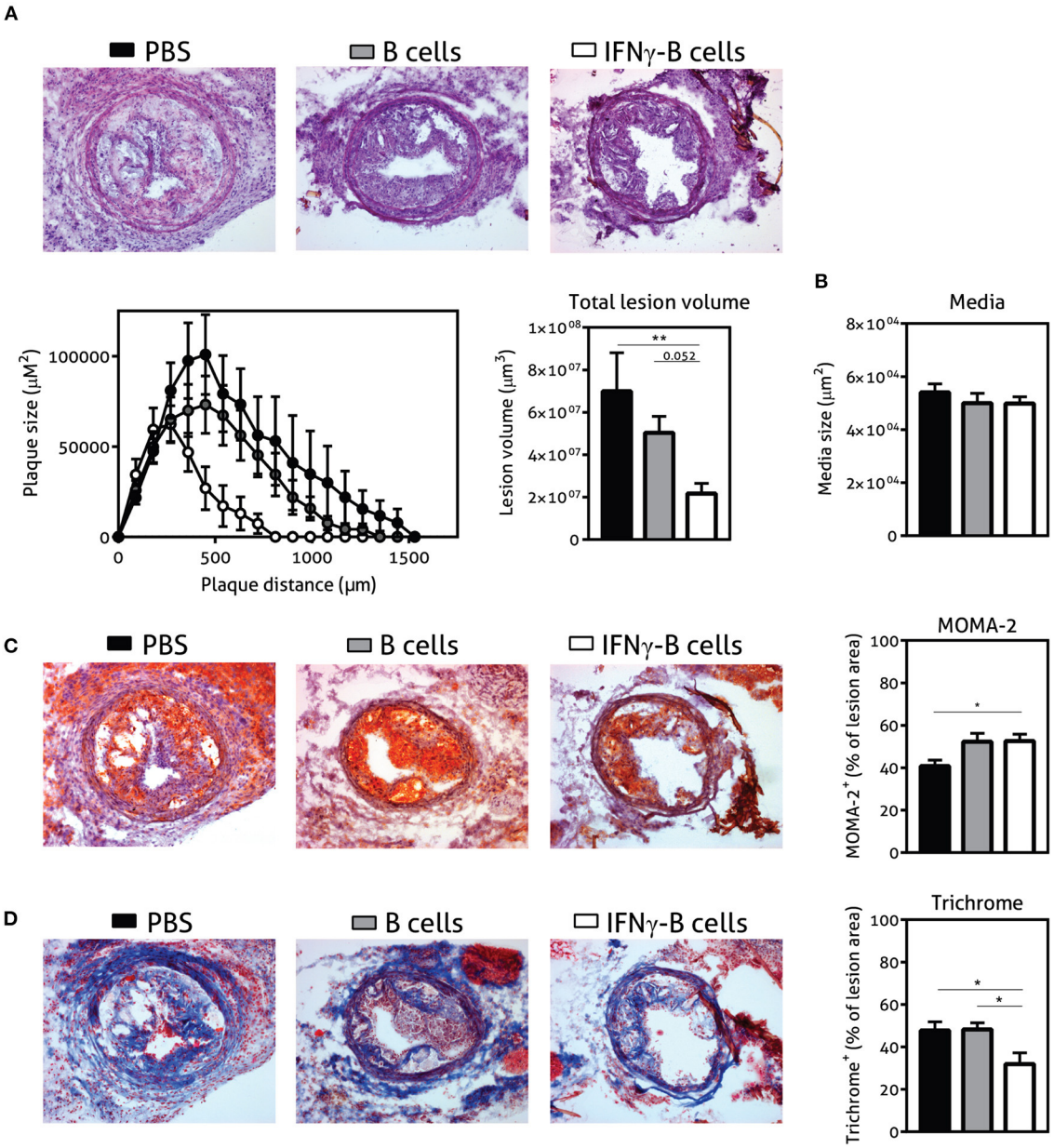

FIGURE 5 | Adoptive transfer of IFNγ-B protects against atherosclerosis. *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 h (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and **(A)** total lesion volume and **(B)** media size was determined in the right carotid artery with a hematoxylin and eosin staining. Lesion phenotype was determined in sections containing the largest lesions. **(C)** Macrophage content was analyzed using a MOMA-2 staining. **(D)** Collagen content was quantified with a Trichrome staining. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$). $n = 8$–12/group.

previous studies that demonstrated the proatherogenic role of T_{FH} cells (12–14).

However, due to the pleiotropic nature of IFNγ, other factors besides PD-L1 could also have contributed to our findings. Indeed, the observed increase in TGF-β expression could have contributed to both the T_{FH} inhibition and Treg induction, since TGF-β signaling is known to prevent T_{FH} cell accumulation and can promote Tregs (29). In addition, there have been a large number of studies with adoptive transfer of B cells expressing TGF-β that reported immune tolerance in autoimmune mouse models (30–33). The majority of these studies reported Treg induction, which is in line with our findings of increased Tregs and IL-10 production after adoptive transfer of IFNγ-B cells. PD-L1 is also known to be essential for the induction of Treg

cells (34), the Treg accumulation could thus be a combined effect of increased TGF-β and PD-L1 expression by IFNγ-B cells. Since the atheroprotective effects of Treg cells is well characterized (10, 35), the observed Treg induction undoubtedly contributed to the reduced atherosclerosis found with adoptive transfer of IFNγ-B cells. Moreover, we show that *ex vivo* IFNγ stimulation generates a pool of PD-L1^{hi} B cells which contains reduced FO B cells. FO B cells can contribute to atherosclerosis progression (15), and despite the increased co-inhibitory PD-L1 expression following IFNγ exposure, we cannot exclude that a decrease in FO B cells in the adoptively transferred IFNγ-stimulated B cells also contributed to the observed anti-atherogenic effect.

In conclusion, this study uncovers a new role for *ex vivo* stimulation of B cells with IFNγ for the induction of atheroprotective B cells. IFNγ-B cells show the genetic makeup of GC B cells with increased expression of PD-L1 and TGF-β and effectively inhibit T_{FH} cells *in vitro* and *in vivo* and ameliorate atherosclerosis development in *apoE*$^{-/-}$ mice. These results further emphasize the proatherogenic role of the T_{FH}–GC B axis and provide a novel way to regulate this axis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Leiden University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

HD, JM, GV, JK, and AF contributed to the conception and design of the study. HD, JM, JA, FS, BS, MKi, MKr, IB, GV, and AF carried out the experiments and acquired the data. HD and JM performed the data analysis. HD, JM, and AF wrote the manuscript. CB, JK, and AF provided critical feedback to the manuscript. JK and AF supervised the project. All authors read and approved the submitted version.

FUNDING

This work was supported by the European Union's Seventh Framework [Grant Number 603131], by contributions from Academic and SME/industrial partners, supported by the Dutch Heart Foundation [Grant Numbers 2016T008 and 2018T051 to AF] and by the European Research Area Network (ERA-CVD B-eatATHERO consortium) supported by the Dutch Heart Foundation [2019T107 to AF]. IB is an Established Investigator of the Dutch Heart Foundation [2019T067].

ACKNOWLEDGMENTS

We thank Maria Ozsvar Kozma for her technical assistance with the antibody measurements.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.781436/full#supplementary-material

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frontiers in Cardiovascular Medicine

REVIEW
published: 08 February 2022
doi: 10.3389/fcvm.2022.826103

The Applications of Single-Cell RNA Sequencing in Atherosclerotic Disease

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Atherosclerosis still is the primary cause of death worldwide. Our characterization of the atherosclerotic lesion is mainly rooted in definitions based on pathological descriptions. We often speak in absolutes regarding plaque phenotypes: vulnerable vs. stable plaques or plaque rupture vs. plaque erosion. By focusing on these concepts, we may have oversimplified the atherosclerotic disease and its mechanisms. The widely used definitions of pathology-based plaque phenotypes can be fine-tuned with observations made with various -omics techniques. Recent advancements in single-cell transcriptomics provide the opportunity to characterize the cellular composition of the atherosclerotic plaque. This additional layer of information facilitates the in-depth characterization of the atherosclerotic plaque. In this review, we discuss the impact that single-cell transcriptomics may exert on our current understanding of atherosclerosis.

Keywords: scRNA-sequencing, transcriptomics, -omics, single-cell, atherosclerosis

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Edited by:
Holger Winkels, Universitätsklinikum Köln, Germany

Reviewed by:
Judith Sluimer, Maastricht University, Netherlands
Jesse W. Williams, University of Minnesota, United States

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *30 November 2021*
Accepted: *03 January 2022*
Published: *08 February 2022*

Citation:
Slenders L, Tessels DE, van der Laan SW, Pasterkamp G and Mokry M (2022) The Applications of Single-Cell RNA Sequencing in Atherosclerotic Disease. Front. Cardiovasc. Med. 9:826103. doi: 10.3389/fcvm.2022.826103

INTRODUCTION

The classical concept of a "vulnerable plaque" originates from the 1980s and depicts plaque rupture in patients who died from coronary syndromes as the major pathological cause of acute myocardial infarction (1). The concept describes a lipid-rich, atheromatous plaque with a thin fibrous cap and local infiltration of inflammatory cells that cause proteolytic activity and degradation of the stabilizing extracellular matrix (ECM). Genome-wide association studies (GWAS) and lesion-based transcriptomic studies point to a diverse landscape of mechanisms leading to atherosclerotic lesion initiation and progression. Vascular obstructive lesions are characterized by significant variability in pathology-based characteristics. Thrombotic occlusions may arise from traditionally described ruptured vulnerable plaques, but also unruptured eroded plaques and microcalcifications can be complicated by destabilisation (2).

Bulk micro-array and sequencing of atherosclerotic plaques obtained from human patients (3–6) or animals (7) highlight networks and pathways that could accelerate lesion progression. The heterogeneous nature of the tissue complicates the interpretation and deconvolution of the transcriptomic signal. Therefore, it is difficult to pinpoint which cells are responsible for this signal, and the effect of rare but crucial cells is lost in the noise. Fine-tuning the representation of the cellular composition and concomitant gene expression profiles is facilitated by single-cell RNA sequencing (scRNA-seq). The research field is experiencing a rapid increase in scRNA-seq efforts in the domain of atherosclerosis (7–15). Numerous scRNA-seq datasets are already available for

the broad scientific community for reanalysis or *via* online tools like *PlaqView* (16). For both mice and humans, the present cell (sub-)populations have been meticulously described. These studies of atherosclerotic tissue may hold promise in the translation of mouse-to-human findings and provide clues to phenotypic changes in human cell populations that have so far only been described in animal models and cell cultures. In this review, we provide an overview of the findings made with scRNA-seq in atherosclerotic tissue. In addition, we discuss the possible answers that scRNA-seq will potentially provide to long-standing questions that have remained unanswered to date (**Figure 1**).

Going Solo

Exploring the cellular diversity of plaques is hindered by tissue heterogeneity. The broad spectrum of cells present in plaque tissue has been unraveled *via* histology, highlighting the classical image of the atherosclerotic plaque with the thin cap comprised of smooth muscle cells (SMCs) and infiltrating immune cells (1). Progress in various –omics techniques has elucidated protein content (17, 18) and transcriptomic content of plaque cells *via* DNA microarrays (3, 4, 6) and bulk RNA-seq (5, 19), providing more insight into the various processes. However, whole tissue (bulk) transcriptomics provides the sum of the transcriptome for all the cells in the tissue and provides no direct information about cell composition. Consequently, the most prevalent cell types dominate the outcomes and subsequent interpretations of bulk transcriptomics efforts, hiding the biology, and heterogeneity of individual cells in their numbers. Each cell type has its unique contribution to plaque manifestation, which is affected by both intrinsic and environmental factors -such as risk factors and medication. Therefore, it is not surprising that the current trend is leaning toward methods that allow for more optimal resolution at the cellular level, evident from the rise in papers using single-cell methods. Flow cytometry and mass cytometry can provide single-cell resolution. However, they are biased as they are dependent on prior knowledge about the phenotype and markers of the cells present in the sample. Predetermined antibodies have to be added to the cell suspension, which can subsequently be analyzed. These methods require in-depth knowledge of the available cell types and sparsely allow for discovering unexpected new or rare cell populations.

In contrast to flow cytometry, scRNA-seq does not require any prior knowledge of cell composition. Rapid developments in the–omics field have benefitted the progression from bulk RNA-seq to single-cell (sc)RNA-seq. The first scRNA-seq effort in 2009 (20) has opened the gates to a rapid increase in applying this technique to various fields, with no exception to atherosclerotic research (8–11, 21, 22). The main advantages are that it can be applied unbiasedly, highlighting new and known cell populations from heterogenic tissue (23). At the same time, they simultaneously uncover the (often subtle) transcriptomic differences created by their unique environment. The rising popularity of scRNA-seq has resulted in efforts to describe the transcriptome of heterogenic tissues and drove the development of bioinformatics tools to analyse the data that follows—ranging from tools to pre-process, analyse and visualize data (24–26), to in-depth specialized follow-up experiments such as identifying rare cell types (27), cell trajectory analysis [evaluated by Saelens et al. (28)], RNA velocity (29) and cell-cell communication [reviewed by Armingol et al. (30)], amongst others. In the following text, we will focus on the discoveries made through scRNA-seq in atherosclerotic research and their implications for plaque characterization. We will discuss the current knowledge on cellular composition of lesions, lineage tracing experiments in mice, cell-cell communication, the importance of sex-stratified research, and examine the technical considerations of scRNA-seq research. For the future perspectives we examine spatial scRNA-seq, the impact of scRNA-seq on pathological specification of atherosclerosis, cell plasticity, clonal expansion, integration of genetic and transcriptomic research, and finally the translation from mice to men.

CURRENT KNOWLEDGE ABOUT CELLULAR COMPOSITION: INSIGHTS FROM SCRNA-SEQ

Cellular Composition in Human and Mouse Plaques

The most significant impact that scRNA-seq has on our understanding of the atherosclerotic plaque is the meticulous dissection of cell populations that are present (8–14)—including multiple types of SMCs, endothelial cells (ECs), and immune cell sub-sets. The cell composition and cell state affect the plaque stability and may lead to different clinical manifestations. The bulk RNA-seq analysis of 654 human lesions uncovered five main plaque types that correlate with symptoms at admission (19). Deconvolution of this bulk data revealed that these plaque types have different underlying cell compositions. Indicating that underlying clinical symptoms can potentially be detected in individual cell populations using scRNA-seq. Here we will briefly discuss findings on the major cell populations of lesions reported in mice and humans. More comprehensive descriptions of the cellular landscape of atherosclerotic lesions are discussed elsewhere (31–33).

The vascular SMCs are located in the vessel wall. They provide structural support and regulate blood flow and blood pressure. Although vascular SMCs are omnipresent in the vasculature, in atherosclerosis, they exhibit distinct phenotypes. In human plaques, we (10) found *two* major subclasses of SMCs, one with contractile and the other showcasing synthetic characteristics. The synthetic phenotype showed low expression of typical SMC markers and upregulation of ECM genes, suggesting that these cells are cap-derived. In a recent review, authors challenge the black and white division between contractile and synthetic SMCs in the vasculature (34). Pan et al. (11) report SMCs (corresponding to a contractile phenotype), fibrochondrocytes, and an intermediate cell population named the intermediate cell state. They also report the presence of fibroblasts in carotid arteries. In aortic arteries and mice, Wirka et al. (8) report fibroblasts and phenotypically modulated SMCs. The value of scRNA-seq in unraveling the fate and origin of transdifferentiating SMCs is underlined by the association of *TCF21* expression with the phenotypic switch of SMC into

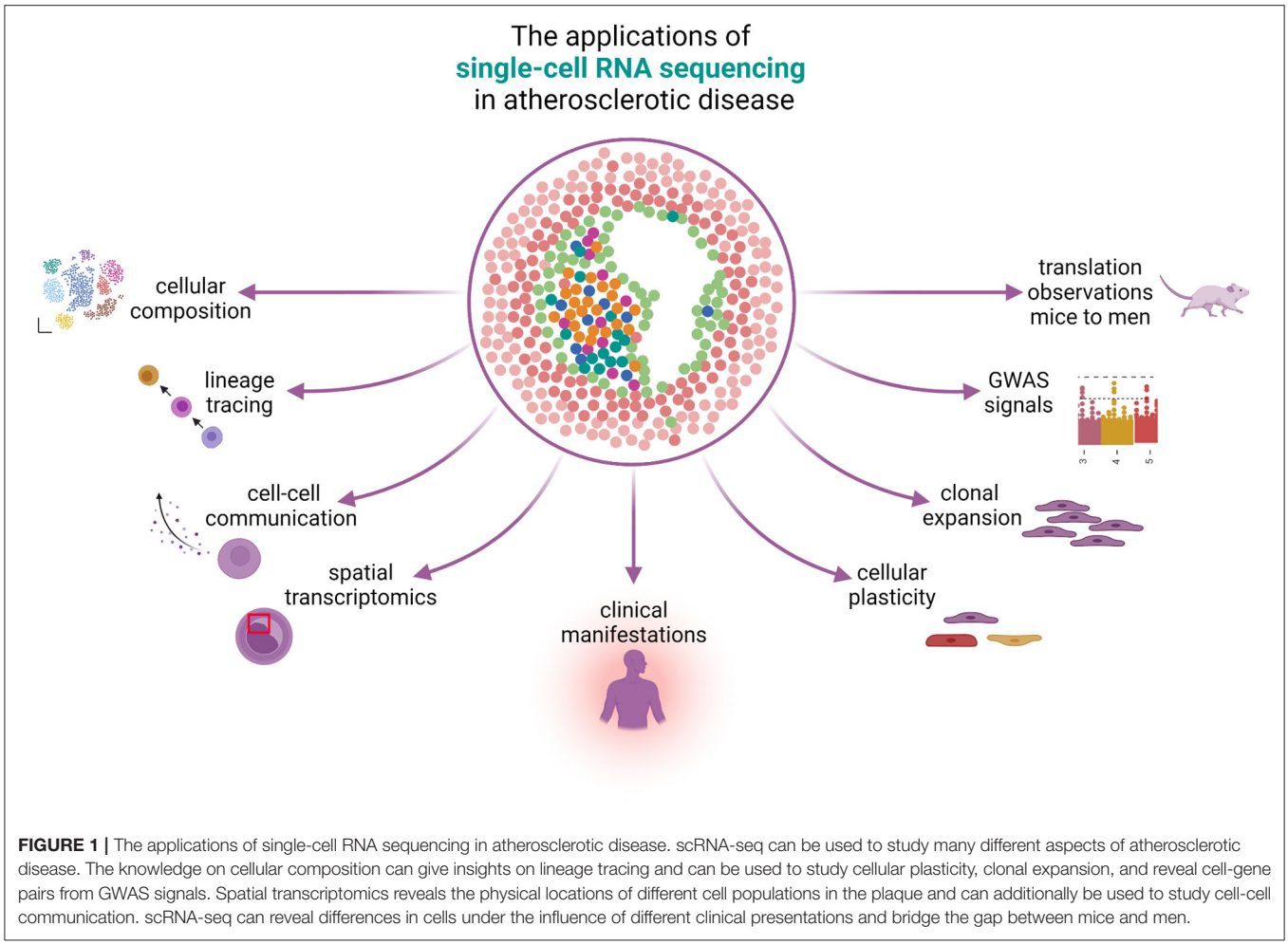

FIGURE 1 | The applications of single-cell RNA sequencing in atherosclerotic disease. scRNA-seq can be used to study many different aspects of atherosclerotic disease. The knowledge on cellular composition can give insights on lineage tracing and can be used to study cellular plasticity, clonal expansion, and reveal cell-gene pairs from GWAS signals. Spatial transcriptomics reveals the physical locations of different cell populations in the plaque and can additionally be used to study cell-cell communication. scRNA-seq can reveal differences in cells under the influence of different clinical presentations and bridge the gap between mice and men.

fibrocytes. With the subsequent strengthening of the fibrous cap and stabilization of the lesion (8).

A thin layer of ECs coats the inner layer of the artery, forming a barrier between the lumen and the artery. Endothelial dysfunction aggravates vascular diseases such as atherosclerosis (35). The role of these cells in disease progression ranges from athero-protective to inflammatory. Our group (10) and others (11) report two main distinct EC populations. Hidden within are also cells undergoing active angiogenesis or those that display the signs of epithelial to mesenchymal transition (10).

The infiltration of inflammatory cells into the intima is a hallmark of disease progression. Macrophages (Mϕs) can be observed during all phases of atherosclerotic lesion formation. Monocytes are rapidly recruited into the lesion and can differentiate into Mϕs or dendritic cells. In a recent meta-analysis (31), authors discriminate between five sub-populations of Mϕs in murine atherosclerosis: resident-like macrophages, foamy Trem2 macrophages, inflammatory macrophages, IFNIC macrophages, and cavity macrophages. Of note, not all Mϕs found in lesions are of monocyte origin (36, 37). In human lesions, Fernandez et al. (9) reported classically activated M1 macrophages. Further transcriptional analysis identified four different Mϕ populations with distinct functions. They reported activated Mϕs, and a population expressing inflammatory genes, and an MMP inhibitor, which could impair ECM degradation and subsequently a more stable plaque. The third population was pro-inflammatory, and the final population showed the transcriptional signature of foam cells with anti-inflammatory signaling. We (10), found three Mϕ populations. Two of which were pro-inflammatory and expressed *IL-1β* or *TNF*. The third population was foam cell-like and exhibited a fibrosis promoting phenotype. This population also showed alpha-actin expression, which potentially indicates a shift to SMC or from SMC to Mϕs. Cochain et al. (13) reported three Mϕ populations in mice. The first population represented a resident-like Mϕ, which is also present in healthy aortic tissue. The other two populations were atherosclerosis specific. The presence of these Mϕ populations was confirmed in human tissue (10).

T cells exhibit significant heterogeneity within the plaque. Fernandez et al. (9) reported that T cells in the plaque demonstrate cytotoxicity, activation, and exhaustion, whereas T cells in the blood circulation show cytokine inhibition, active RNA synthesis, and metabolic reprogramming. They also reported that T cells from symptomatic patients differ from

symptomatic patients. However, the number of patients used is small ($n = 3$). Similarly, we (10) found a diverse landscape of $CD4^+$ and $CD8^+$ T cells populations, the main difference being their activation state. The subclass phenotypes varied from cytotoxic to more quiescent. scRNA-seq of plaques has established the existence of unforeseen T cell clusters with mixed Th1 and Treg cell transcriptional programs such as ApoB reactive T cells (38). In mice, single-cell RNA-sequencing of plaque immune cells revealed differential expression patterns of Tregs in progressing vs. regressing plaques (39).

The spectrum of cell populations uncovered with scRNA-seq is complex and, without a doubt, still incomplete. The community will significantly benefit from a unified nomenclature of cell populations since transcriptome-based names of cell populations vary across multiple studies (40). The aggregation of this knowledge would simplify further research into understanding the molecular mechanisms in atherosclerosis.

Lineage Tracing

The cells in human atherosclerotic plaques have long been considered fully differentiated and non-plastic. However, *in vitro* and mice experiments have revealed the potential of vascular cell types to undergo phenotypic switching (a process also called dedifferentiation or phenotypic modulation) (36, 41, 42) and revealed the enormous differentiation potential of vascular cells. Endothelial cells can become mesenchymal cells by a process called EndoMT, and SMCs can undergo phenotypic modulation and differentiate into Mϕ-like, fibroblast-like, osteoblast-like, or chondrocyte-like cells. EndoMT can occur under the influence of disturbed blood flow (43, 44) and strongly affect the number of SMCs that stabilize a fibrous cap (45). However, the role and existence of EndoMT in disease is not without controversy (46). When the mechanisms are clear, the current definition of an atherosclerotic plaque eventually can be fine-tuned or altered. Determining the transcriptomic landscape of single cells in human atherosclerotic plaques with scRNA-seq may identify cells in a transitional state, and the trajectory and fate of phenotypic cell switching can be depicted.

One of the particular advantages of using mouse model systems is the ability to trace cells over time, providing a unique viewpoint on atherosclerotic lesion progression (47, 48). This is not directly possible in humans but can be inferred using the appropriate methods. For example, previous attempts at human lineage tracing involved the tracing of histone modifications on histological slides (49). Human atherosclerotic research is hampered by a cross-sectional design, but the (temporary) preservation of transcriptomic features may point to cells in transition. In addition, bioinformatics tools such as the inference of cell trajectories, also known as "pseudotime" may facilitate the capturing of cells in transition. Pseudotime is a computational technique used to determine the pattern of a dynamic process experienced by cells and then arrange the cells based on their progression through the process. Differences in gene expression between cells result from dynamic processes such as simple cell cycle progression or cell differentiation. In pseudotime such differences are depicted by placing cells along a continuous path that represents the evolution of the process rather than dividing cells into discrete clusters. To find evidence of EndoMT or phenotypic switching in heterogeneous tissue is not unambiguous, but knowledge from lineage tracing experiments in mice can be used to impute this (**Figure 2**). The assumption then is, that these processes are continuously active and cells from transient stages are always present. However, mice lesions are harvested in an earlier stage of atherosclerosis, whilst human plaques have progressed much further and are end-stage, often symptomatic, lesions. Naturally occurring markers fluctuate during cell transitions and cell fates are harder to trace without a reporter gene. For this reason, it is possible that the intermediate stages are not captured in either case and it is difficult to determine if they were present at all. Lineage tracing is a powerful tool to investigate putative cell transitions in time. A common practice is to label individual cells at an early or induced time point to trace the lineage of labeled daughter cells at a later time point. Lineage tracing is particularly insightful when the process at hand is less unambiguous to impute, such as phenotypic switching. The limitations of this analysis are reviewed elsewhere (50).

The fate of bone marrow derived cells can be followed in mice *via* bone marrow transplant. This method is often combined with lineage tracing techniques where the labeled bone marrow is grafted into wild-type or sex-mismatched mice. These studies show that bone marrow derived cells mainly give rise to inflammatory cells in the plaques and do not transdifferentiate into SMCs or ECs (51). However, supportive scRNA-seq evidence is lacking. A lineage tracing study following the fate of myeloid cells revealed that specialized aortic intima resident macrophages reside in the intima of the aortic arch (52). These resident macrophages give rise to early foam cells of the lesion but were not sustained during atherosclerotic progression. FoxP3 lineage tracing mice experiments showed that Treg cells switch to pro-atherogenic T follicular helper cells during atherosclerosis (53, 54). In order to determine which cells in plaque have SMC origin and how these cells contribute to disease, Shankman et al. (47) used an SMC $YFP^{+/+}Apoe^{-/-}$ mouse model and labeled medial SMCs with YFP labels after tamoxifen injection. This revealed that 36% of $LGALS3^+$ cells were of SMC origin and rather than of myeloid origin. Furthermore, they state that around 80% of cells with SMC origin are phenotypically modulated. A study from the same group with $SMC^{-\mathrm{DualLineage}}$ tracing mouse models showed that the percentage of $LGALS3^+$ cells was lower at around 25% (45). scRNA-seq data from these mouse models made it possible to compare cells across species. This revealed that all cell populations observed in the mice could be identified in human data, indicating that the transcriptional signatures can be used to identify cells that cannot be lineage traced in humans. The tamoxifen-induced $ROSA26^{ZsGreen1/+}$; $Myh11\text{-}CreER^{T2}$ mouse model of Pan et al. (11) revealed that SMCs in the lesion give rise to multiple cell-populations or cell states, including fibrochondrocytes and Mϕ-like cells. In a recent meta-analysis of scRNA-seq and scATAC-seq lineage tracing experiments, Conklin et al. (40) conclude that lipid loading of SMCs *in vivo* does not correlate to the changes observed *in vitro*. Highlighting

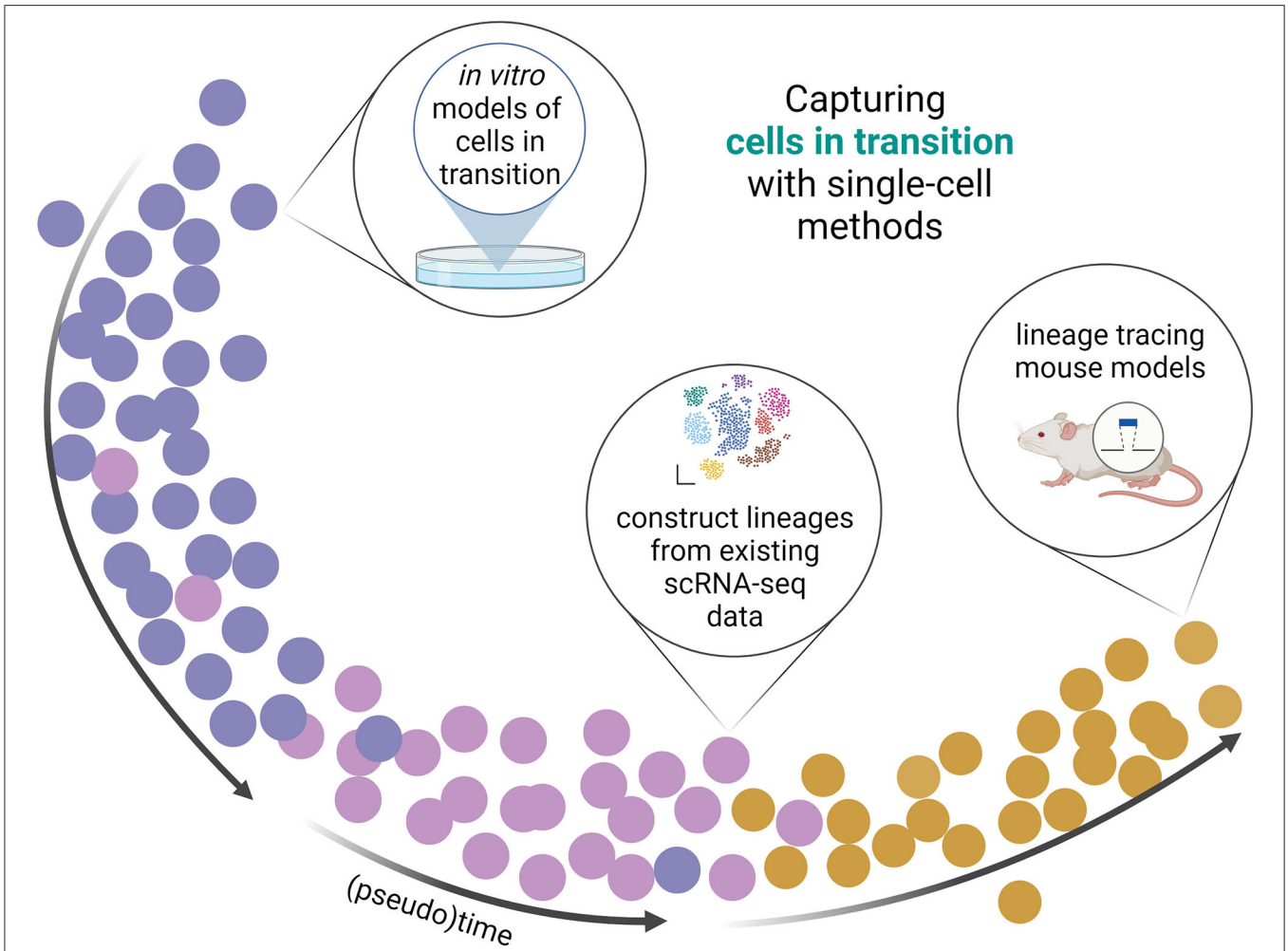

FIGURE 2 | Capturing cells in transition with single-cell methods. Single-cell methods fascilitate the detection of cells in transition. scRNA and/or scATAC facilitate (re)constructing or confirmation of lineages of cells transitioning between phenotypes or cell states. With bioinformatical tools, data from *in vitro, in silico*, and *in vivo* experiments can be used to place cells on an artificial timeline (pseudotime) from which the cell transitions can be studied.

the added value of lineage-tracing experiments compared to *in vitro*.

Cell-Cell Communication

Cell-cell communication (CCC) and cell-cell interactions are interactions between cells regulated by biochemical signaling. Molecules excreted by cells can be used to establish CCC or are part of the structural integrity of tissue, such as the extracellular matrix. scRNA-seq offers the opportunity to study the communicative links between cells in the lesions. Although a proxy for actual protein-protein interactions, the detailed RNA profile of cells can be leveraged to infer CCC from a transcriptomic viewpoint. The bioinformatical tools to impute CCC from this data have been rapidly maturing [reviewed by Almet et al. (55)], varying in their sensitivity and approach.

Previously, we examined the potential receptor-ligand interactions in carotid lesions. Overall, the majority of the interactions were between myeloid cells, SMCs, and ECs (10). The low number of potential interactions between immune cells by the lack of T Cell receptor-related genes can be possibly accounted to their absence in the receptor-ligand interaction database. The occurrence of false-negative or false-positive communication is not just a result of incomplete databases. For a large part, cells can communicate over a limited spatial distance, limiting the number of interactions that can physically take place within the plaque. Interactions by secreted molecules such as cytokines can, indeed, facilitate communications over larger distances. However, interactions where both ligand and receptor are present on the surface of the cells, such as the interaction between CD36 and THBS1, require physical proximity. This spatial information is lost with regular scRNA-seq. A novel sequencing technique makes use of doublets occurring in scRNA-seq data leveraging the idea that cells that are stuck together are likely to be physically interacting. By deconvoluting the single counterpart of doublet cells, the relationship between the physically interacting cells can be studied (56).

Single-Cell Sequencing and Understanding of Sex Differences in Atherosclerotic Disease

Next to the well-known risk factors for atherosclerotic disease [e.g., diabetes, smoking, hypertension, hypercholesterolemia, ethnicity (57) etc.] (58) also sex (59, 60) strongly associates with different clinical presentations and underlying atherosclerotic pathology. Understanding the underlying molecular and cellular mechanisms remains a significant challenge.

In murine *BXH ApoE*$^{-/-}$ model, the lesion size was increased in females (61). In humans, the sex-stratified gene regulatory networks derived from bulk RNA-sequencing data from atherosclerotic aortic root tissue showed large sex specific differences (62). The genes involved in female-specific networks have higher expression in SMCs, ECs, and Mϕs and are involved in epithelial to mesenchymal transition, KRAS signaling, and estrogen response, respectively. The next step will be to assess the cell types that are responsible for this transcriptomic diversity between sexes. This highlights the importance of sex stratification in studying risk factors.

Sex-specific atherosclerotic murine (12–14) or multiple samples of both sexes human scRNA-seq data (10) is available. However, for these analyses, a sufficient number of samples is required to reach statistical power and based on lessons from histopathological biobank studies, hundreds of individual samples might be required. Intuitively, this explains the limited amount of studies addressing this topic. It is not unlikely that the scRNA-seq field will face a *déjà vu* when researchers start merging data sets, just like the ongoing GWAS efforts to reach sufficient power for stratified analyses. If sample numbers increase, there are no doubt that the exploration of cell-based gene transcriptomes in humans may resolve many unanswered questions regarding the underlying mechanisms that explain the complex diversity and the mechanisms of how risk factors like sex modify the disease progression.

scRNA-seq Observations Associate With Hemodynamics

Blood flow is of major influence on the formation of atherosclerosis. Lesions develop primarily in vessel regions where blood flow is disturbed by branching or curvature of the artery. Disturbed flow affects the behavior of arterial cells, but how it affects their respective transcriptome was poorly investigated. In a study on flow effect, Andueza et al. (43) performed scRNA-seq and the single-cell assay for transposase accessible chromatin sequencing (scATAC-seq) in a partial carotid ligation mouse model. Trajectory analysis showed that ECs are capable of dramatic phenotypical changes. The already heterogeneous EC population was found to be transitioning to a mesenchymal, hematopoietic stem cell, endothelial stem/progenitor cell, and unexpected immune cell-like phenotypes (43).

Similarly, Li et al. (44) performed scRNA-seq on mice lesions after partial carotid ligation. Compared to the laminar flow condition, they found cell populations specific for both models. A disturbed flow EC population was found to be more enriched in processes such as epithelial to mesenchymal transition and TGF-β signaling. Disturbed flow-specific SMC and Mϕs were also reported (44). Current studies rarely take into account the influence of flow on the outcome of scRNA-seq experiments. However, with the notion that most studied plaque locations are subjected to disturbed flow (carotid artery and aortic arch), it is reasonable to assume that the disturbed flow could influence expression patterns of EC populations found in these lesions. In the future, spatial transcriptomics can help to resolve these questions.

Technical Considerations and Limitations

Source of Lesions

Atherosclerotic plaques develop in coronary, carotid, cerebral, aortic, iliac, and femoral arteries. The most commonly studied human plaques are located in the coronary and carotid arteries (32, 63). When it comes to defining the plaque on a cellular level, location matters (64). Although the arterial systems share characteristics in plaque morphology, there is a difference is the underlying substrate of the ischemic event. In coronary arteries, a local thrombosis caused by either plaque rupture or erosion leads to ischemia of downstream tissue. In the cerebral circulation, an embolization may take place from a plaque upstream of the cerebrovascular occlusion. In the carotid artery, intraplaque bleedings are observed more frequently, and in a significant number of cases, a fibrous lesion underlies the thrombotic event (65, 66). Human coronary plaque samples availability relies on the use of donor's hearts from recipients during transplantation. On the other hand, carotid plaques are dissected from living patients that can still be asymptomatic at the time of surgery. In murine research, aortic tissue is the most prevalent source (32, 63). Although these differences make comparing the coronary and carotid plaque and interspecies comparisons difficult (12), scRNA-seq can potentially unravel differential processes underlying a clinically relevant event. The common hurdle is tissue preservation prior to sequencing. In order to get the full potential from the lesion, sample handling has to be quick to keep it as fresh as possible, in contrast to histological samples where there is less time constraint for processing.

During carotid endarterectomy, the intima with the plaque is removed from the vessel while the tunica media stays majorly intact. Plaques derived from the aorta are likely clippings of diseased samples taken after heart transplants. This is ultimately reflected in the scRNA-seq data, where aortic plaques often show more significant SMCs numbers and phenotypic variety (8). Cochain et al. (13) observed that macrophages are the most dominant immune cell type in atherosclerotic plaques by scRNA-seq. But researchers using CD45^{+} positive selection with FACS will not report CD45^{-} populations such as SMCs (9). Different digestion protocols can heavily skew cell selection and survival before sequencing, even if there is no prior selection. This can dramatically affect cells with poor survival rates *ex-vivo,* such as foam cells and neutrophils. This way, cell populations can become over and under-represented in scRNA-seq (40, 67). Therefore, caution is needed when interpreting results, and reports of cell ratios should always be carefully considered. Additionally, because of the need for digestion of solid tissues

prior to scRNA-seq, the spatial location of cells is lost during the process.

Technical Limitations

A major limitation of scRNA-seq is the biases in transcript coverage and low capture efficiency, which additionally vary between protocols (33, 68, 69). Incomplete reverse transcription during second-strand synthesis and amplification of the samples can lead to fewer transcripts with the complete 5′ and/or 3′ ends. Gene length influences efficacy, and shorter genes generally have lower counts in single-cell sequencing compared to bulk RNA sequencing (70). As a consequence, genes with a low expression may not be detected due to dropouts and potentially create false negatives. A related obstacle is the use of poly(T) primer, resulting in only RNAs with a poly-A-tail being sequenced (68). Sequencing non-polyadenylated RNAs would be beneficial since these RNAs often serve regulatory functions.

The cost of scRNA-seq is another limiting factor in comparison to traditional bulk sequencing. Even though since 2017, the costs of single-cell sequencing have dropped significantly, single-cell sequencing is still 10 to 200 times more expensive per sample than bulk sequencing (52). Details on different methodologies are reviewed elsewhere (52).

A natural consequence of the growing popularity of scRNA-seq is the increasing need for specialized experimentalists, developers, and bioinformaticians as scRNA-seq gains popularity. However, finding the right tools to implement scRNA-seq into our research is not without caveats for experienced researchers. For example, the scRNA-tools database tracks 1,124 specialized tools for scRNA-seq analysis across 30 categories at the time of writing (71).

FUTURE PERSPECTIVES

Spatial Location

One of the current limitations of single-cell transcriptomics is that spatial information of cells is lost, but therein also lies an opportunity for the future. Not only can spatial context provide insight into the mechanisms of atherosclerotic disease, but it also greatly influences our interpretation of cell-cell communication. Multiple ways can be used to visualize the spatial gene expression in plaque tissue. Spatial barcoding, *in situ* hybridization, and *in situ* sequencing can all visualize spatial gene expression. However, none of these techniques can cover the full spectrum of the transcriptome like RNA-seq can provide [reviewed by Longo et al. (72)]. Laser-capture microdissection coupled with RNA-seq and downstream analysis can give a detailed view of specific regions of interest like plaque cap or pinpoint to cells with specific phenotypes, under the condition that these cells can be located. This method has been applied successfully in the past on mouse tissue (73). However, the technical challenges to keep plaque tissue in a condition where it is possible to preserve, locate, isolate, and sequence cells of interest are limiting. The growing interest in these techniques is already reflected in implementing these techniques in the major sequencing platforms (74) and data analysis tools (71, 75). However, the current state of spatial technology sequencing does not fully allow for sequencing at individual cell resolution. Future advancements in spatial sequencing can possibly make it a powerful tool to shine new light on spatio-temporal gene expression in atherosclerotic disease.

Underlying Mechanisms Explaining the Diversity in Pathological Substrates of Clinically Relevant Plaques

One of the most prominent dogmas in atherosclerotic research is the concept of plaque rupture vs. plaque erosion. A concept well-described through histology and clinical presentation. The atherosclerotic plaque may rupture, which leads to red thrombosis due to blood coagulation components getting access to the core of the plaque. If the thrombus arises in coronary arteries or embolises the cerebral arteries, it results in a myocardial infarction or stroke. Plaques that rupture often have a large lipid core with a thin and weak fibrous cap and are called vulnerable plaques or thin-capped fibroatheromas (TCFAs). Besides plaque rupture, plaque erosion may arise (76, 77). These plaques are rich in ECM, have a thick cap and have little lipid deposits with superimposed (white) thrombi. The characteristics described in the TCFA model serve to date as the surrogate endpoints for a vulnerable plaque in animal models. Among other processes, this has led to valuable insights into the role of LDL oxidation, plaque hemorrhage, accumulation, SMC migration, proliferation and apoptosis, senescence, and the role of the variation of local inflammatory cells in the diseased vessel wall. The mechanisms leading to plaque erosion are less well-understood and are one of the major upcoming challenges of the vascular biology community. Animals models hardly develop a thrombotic response, and the lesion underlying the thrombus is thus far categorized as "stable." For both processes, the mechanisms on a single cell level have not been revealed. The variety of sub-populations identified could be partly explained by these phenomena. Future research may reveal what cells contribute to a pro-thrombotic micro-environment and provide clues for underlying mechanisms.

Cell Plasticity

In lesions, cells can switch to a new phenotypical identity. These switches include EndoMT (78) and transition from SMC to a Mϕ-like phenotype or foam cells (36). scRNA-seq allows studying cellular plasticity in atherosclerosis in more detail and higher resolution (79). Tools such as the earlier-mentioned pseudotime can aid in studying these processes in mouse model systems and humans.

Dobnikar et al. (80) looked into the SMCs in healthy vessels and atherosclerotic plaque tissue from mice. A remarkable finding was a distinct SMC cluster that expressed *Sca1*—implying that these cells are transitioning from SMC to a Mϕ-like cell. Indications for the transitional capacity of vascular cells was also demonstrated in mice (45). Three SMC clusters expressed both endothelial cell and SMC marker genes, which suggests a transition between phenotypes. In addition, we found that a small subset of human SMCs was $KLF4^{+}$ (10), which points to cells that differentiate into a cell with a more synthetic or Mϕ-like phenotype. The value of scRNA-seq to unravel the fate

of and origin of transdifferentiating SMC was underlined by a study by Wirka et al. (8), who found that *TCF21* expression was associated with the phenotypic switch of SMC into fibrocytes, with the subsequent strengthening of the fibrous cap and stabilization of the lesion (8). Another mouse study detected an unexpected cluster of proliferating monocytes with a stem cell-like signature, suggesting that monocytes may persist in a proliferating self-renewal state in inflamed tissue and that not all monocytes differentiate immediately into Mϕs after entering the vascular tissue (15). A recent meta-analysis (40) addressed the SMC heterogeneity between four different murine scRNA-seq datasets. Researchers conclude that SMC are capable of sourcing multiple different trajectories within lesions, with many of these transitioning through a de-differentiated SMC state defined by Pan et al. (11) as stem-cell, endothelial cell, monocyte (SEM) cells. The field would benefit from a coalescent review of plastic cells found in lesions to understand their source and impact on plaque development.

Attack of the Clones

Clonal expansion theory is derived from cancer research. It is a process where one parent cell multiplies as a consequence of acquired mutations. The notion of clonality in atherosclerosis is not a novel theory (81), but there are doubts on the true monoclonality and its consequence on atherosclerotic cells. The clonal environment is likely already present around the plaque, which contributes to the expansion of the lesion (82). Mice with a *Jak2*^{VF} mutation showed increased inflammatory and proliferative myeloid populations in their lesions, as confirmed with scRNA-seq. In addition, these plaques show more pronounced necrotic cores (83). It is unclear whether these plaques exhibit clonal regions. Clonal expansion is mainly described for leukocytes, but in plaque, the concept applies similarly to SMCs. Mice fed with a high-fat diet showed an increase of dedifferentiated SMCs expressing the stem cell marker *Sca1*, forming hubs of clonally expanded cells that tended to form around the necrotic core. Researchers also report that clonally expanding SMCs aggravate atherosclerosis by activating the complement system (84). scRNA-seq confirmed that these cells exhibit a different phenotype from regular SMCs. Other findings in mice describe sheets of clonally expanded SMCs derived from the media rather than patches (85). The origin of these clonal progenitor cells giving rise to these clonal patches is still unclear, and the occurrence of clonal expansion is seemingly random. Whether or not this clonal expansion of SMCs is beneficial for plaque stability is still up for debate.

The use of lineage tracing techniques has been majorly beneficial for the research on clonality in plaque tissue. A privilege that cannot be extended to human research. The alternatives are tracing naturally occurring markers in know cell differentiation pathways or detecting somatic mutations (**Figure 3**). A novel approach integrates scRNA-seq and scATAC-seq of mitochondrial RNA for both clonal and lineage tracing (86). This method can be applied to already existing single-cell datasets of lesion tissue and can confirm these long-standing observations of clonal expansion in plaque.

Integrating scRNA-seq in Post-GWAS Analyses

The reported number of common genetic variants associated with coronary artery disease (87) ischemic stroke (88), and intermediate traits of atherosclerosis, such as carotid intima-media thickness (89), is increasing as the meta-analysis efforts of genome-wide association studies (GWAS) grow more prominent and larger (90). However, the functions of many genes that are considered to be causally related to cardiovascular diseases are unknown for roughly 50% of the loci discovered (90). The expression of these potentially causal genes or other downstream interacting genes can originate from different organs of which the (atherosclerotic) vasculature is one. Much research currently aims to prioritize the "right" genes underlying the GWAS loci through integration with molecular quantitative trait locus (molQTL) analyses in various tissues. In contrast, these efforts prioritize genes but they do not provide information on the cellular context (91). As we (92) and others (21) recently showed, the integration of GWAS summary statistics with plaque-derived scRNA-seq and/or snATAC-seq data aids in the prioritization of genes and cell populations relevant to disease, which in turn can be used as a guideline for *in vitro* and *in vivo* mechanistic research.

This could further be augmented by single-cell molQTL (sc-molQTL) analyses. Indeed, heritable genetic effects that modulate expression at a single-cell resolution may affect specific cellular processes in disease (93, 94). Studies focused on discovering genetic effects on proximal genes, *cis*-acting single-cell expression QTLs (*cis*-sc-eQTLs), across populations will face two challenges. First, most analytical tools are not designed to consider the sparsity (the zero-inflated expression) in single-cell data. New methods are developed, but this field has not matured (95). Second, expression and genetic variation is also population-specific, and many datasets are now mainly derived from European ancestral populations. An inclusive approach to population diversity will provide a fine-grained map of genetic variation affecting cellular processes at a single cell level in disease tissue.

From Mice to Men

The extrapolation of results from atherosclerotic mouse models to human advanced stages of atherosclerosis is still an ongoing matter of debate. Indeed, the translation from mouse to human regarding the mechanisms of initiation and progression of atherosclerosis has been shown to be complex. Small-scale comparison between specific murine and human cell-subsets have been attempted (8, 10, 11, 52), and to a smaller extent, larger scale comparison efforts have been made (16, 40). However, animals models hardly develop a thrombotic response, and the lesion underlying the thrombus is thus far categorized as "stable." Nonetheless, the determinants of end-stage atherosclerosis in humans have been used as benchmarks in atherosclerotic mice. However, verification in human plaques was mainly performed by demonstrating gene or protein expression differences between symptomatic and asymptomatic plaques (9). This single gene/protein cross-sectional approach was difficult to translate

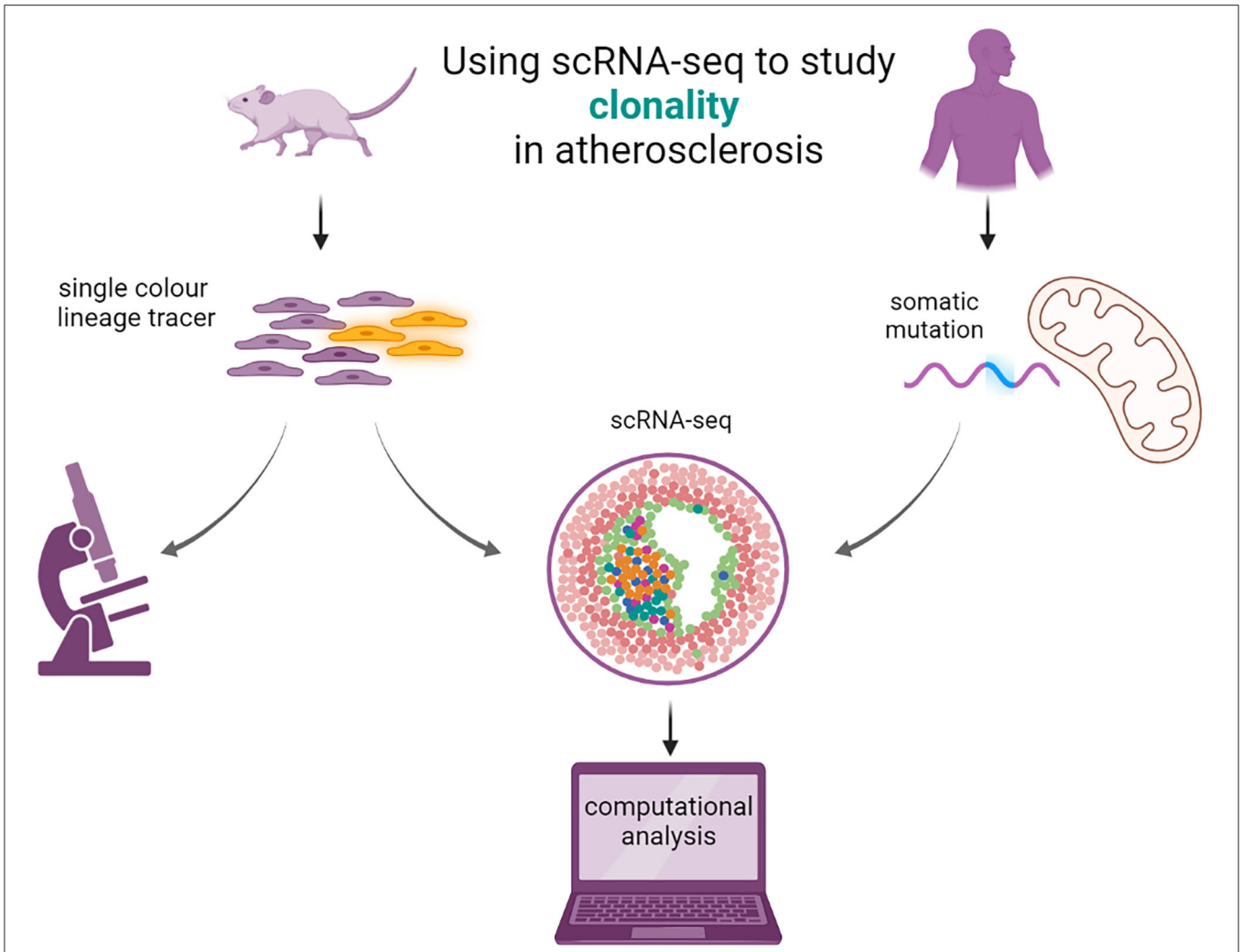

FIGURE 3 | Using scRNA-seq to study clonal cell expansion in atherosclerosis. Single color lineage tracers are added to reporter genes to study the clonality in mice (left). The mice will develop atherosclerosis and patches of clonal cells are visible from microscopy images. scRNA seq can be performed, and computational analysis can elucidate the transcriptomic properties of the cells expressing the lineage tracer. In humans (right), clonality can be imputed from studying the somatic mutations in mitochondria with scRNA- or scATAC-seq from existing data with computational analysis.

into a specific role in either plaque stabilization or destabilization. While still observational, scRNA-seq could indicate in which cell a gene is expressed, given the transcriptomic context in which the gene is located. This location is not limited to cell-population-specific expression but extends to their position in networks and pathways. This could provide the opportunity to better translate and verify the value of the mouse models to human plaques. Further comparative research will reveal the extent to which the gene expressions found occurring from a genetically identical mouse strain can be translated to the genetically heterogeneous human population and whether expression patterns are generic or individual-specific. A recent study compared mice and human vascular SMCs and reported that the many similarities favor the continued use of mice models to extrapolate the fate of the human counterpart cells (40). However, in a systematic review, we reported that genes involved in human genetic variants for coronary artery disease, large artery ischemic stroke, or atherosclerotic plaque characteristics seldom show associations in mice (96). The vast body of work describing the cellular composition of mice compared to human findings offers the opportunity to investigate population similarities and could contribute to the translation from murine to human atherosclerosis.

CONCLUDING REMARKS

The value of scRNA-seq in elucidating the mechanisms of arteriosclerosis is beyond doubt. The current literature already indicates a much greater diversity of inflammatory and resident cell types than previously believed. The plasticity of vascular cells has been demonstrated thanks to scRNA-seq, and the first steps to spatial gene expression in plaque are taking place. However,

the field would benefit from a consensus in cell-population nomenclature to match findings from different groups and facilitate inter-species comparisons. The rapid development of scRNA-seq will drive down the prices and therefore, more samples can be analyzed, which is currently the biggest limitation. A new limitation will arise, which is inevitably tied to the ever-growing popularity of big data: the expert (man) power to analyse and biologically interpret the data.

AUTHOR CONTRIBUTIONS

LS, DT, and GP prepared the original draft. LS drafted the manuscript and designed figures. SL and MM contributed to manuscript extension and revision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from: Fondation Leducq (PlaqOmics 18CVD02) to GP. SL was funded through grants from the Netherlands Cardiovascular Research Initiative of the Netherlands Heart Foundation (CVON 2011/B019 and CVON 2017-20: Generating the best evidence-based pharmaceutical targets for atherosclerosis [GENIUS I&II]). We are thankful for the support of the ERA-CVD program druggable-MI-targets (grant number: 01KL1802) and the EU H2020 TO_AITION (grant number: 848146).

ACKNOWLEDGMENTS

Figures were created with BioRender.com.

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frontiers in Cardiovascular Medicine

REVIEW
published: 17 February 2022
doi: 10.3389/fcvm.2022.826630

The Role of Tumor Necrosis Factor Associated Factors (TRAFs) in Vascular Inflammation and Atherosclerosis

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TNF receptor associated factors (TRAFs) represent a family of cytoplasmic signaling adaptor proteins that regulate, bundle, and transduce inflammatory signals downstream of TNF- (TNF-Rs), interleukin (IL)-1-, Toll-like- (TLRs), and IL-17 receptors. TRAFs play a pivotal role in regulating cell survival and immune cell function and are fundamental regulators of acute and chronic inflammation. Lately, the inhibition of inflammation by anti-cytokine therapy has emerged as novel treatment strategy in patients with atherosclerosis. Likewise, growing evidence from preclinical experiments proposes TRAFs as potent modulators of inflammation in atherosclerosis and vascular inflammation. Yet, TRAFs show a highly complex interplay between different TRAF-family members with partially opposing and overlapping functions that are determined by the level of cellular expression, concomitant signaling events, and the context of the disease. Therefore, inhibition of specific TRAFs may be beneficial in one condition and harmful in others. Here, we carefully discuss the cellular expression and signaling events of TRAFs and evaluate their role in vascular inflammation and atherosclerosis. We also highlight metabolic effects of TRAFs and discuss the development of TRAF-based therapeutics in the future.

Keywords: TRAF, atherosclerosis, immunity, inflammation, TNF, signaling

OPEN ACCESS

Edited by:
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Reviewed by:
Alexander Akhmedov,
University of Zurich, Switzerland
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *01 December 2021*
Accepted: *27 January 2022*
Published: *17 February 2022*

Citation:
Gissler MC, Stachon P, Wolf D and Marchini T (2022) The Role of Tumor Necrosis Factor Associated Factors (TRAFs) in Vascular Inflammation and Atherosclerosis. Front. Cardiovasc. Med. 9:826630. doi: 10.3389/fcvm.2022.826630

ATHEROSCLEROSIS IS A CHRONIC INFLAMMATORY DISEASE OF ARTERIES

Atherosclerosis is a disease of medium- to large-sized arteries that leads to the build-up of vessel occluding atherosclerotic plaques. Endothelial erosion and plaque rupture represent frequent and fatal complications of atherosclerosis that may lead to the formation of arterial thrombi, vessel occlusion, and subsequent tissue ischemia (1, 2). The prevalence of diseases caused by atherosclerosis, including coronary heart disease, stroke, and peripheral arterial occlusive disease, continues to rise and doubled from 271 million in 1990 to 523 million in 2019 (3, 4). The accumulation of low-density lipoprotein (LDL) cholesterol in the subintimal space of arteries triggered by cardiovascular risk factors, such as hypertension and diabetes mellitus, is the major culprit of atherosclerotic disease. Beyond the passive accumulation of LDL in the atherosclerotic plaque and its uptake by tissue-resident macrophages that fuels plaque growth, cumulating

evidence of numerous preclinical and clinical studies have stablished that atherosclerosis is a chronic inflammatory and immune-driven disease of the arterial wall (5). This response involves stromal cells, such as endothelial cells (EC) and smooth muscle cells (SMC), and cells of the innate and adaptive immune system. Recent evidence suggests that a part of the immune response in atherosclerosis is caused by autoimmunity against LDL and Apolipoprotein B (6, 7). Immune cells accumulate in the subintimal space of atherosclerotic arteries and interact with stromal cells by direct cellular contact and cytokines (8, 9). It is now established that inflammation is a local and systemic process that promotes atherosclerosis at all stages, from initial endothelial dysfunction to thrombotic complications of acute plaque rupture (10). Therapeutic inhibition of inflammation has the potential to prevent atherosclerosis progression and improve cardiovascular outcomes, as recently demonstrated by a neutralizing antibody against the pro-inflammatory master cytokine interleukin (IL)-1β by canakinumab (11), the pro-inflammatory cytokine IL-6 (12), and by colchicine that has broad anti-inflammatory properties (13, 14). Neutralization of other pro-inflammatory cytokines, such as of TNF-α and IL-17, revealed inconsistent effects on cardiovascular end-points (15) or worsened cardiovascular risk factors as exemplified by the increased risk for hypertension during anti-TNF therapy of rheumatoid arthritis (16). In addition, potential side-effects of live-long immune-modulating therapy, such as infection, have not yet been systematically evaluated albeit first safety signals from canakinumab and colchicine trials were promising (17, 18). It is therefore instrumental to define alternative therapeutic targets that are confined to atherosclerosis-relevant cell types and broadly regulate inflammatory signaling cascades without the risk of severe immunosuppression. Mostly preclinical evidence has suggested that TRAFs may represent such inflammatory targets.

PRO-INFLAMMATORY AND CELLULAR NETWORKS IN ATHEROSCLEROTIC DISEASE

It is now well-established that inflammatory signaling cascades are potent modulators of atherosclerosis (19). Besides their clinical use as biomarkers (20), several cytokines have been shown to orchestrate the inflammatory response in the atherosclerotic plaque, such as TNF-α, IL-1β IL-6, and IL-12 (21). Cytokines are soluble factors that are locally produced in atherosclerotic plaques and in remote organs, mostly, yet not exclusively, by immune cells (22). They circulate through the blood stream and modulate immune mechanisms in a plethora of stromal and immune cells in the plaque and cardiometabolic key organs. Distinct outcomes of cytokine signaling pathways are determined by their specific binding to a range of receptors with cell type-specific expression, and partially synergistic and antagonistic functions. Cytokines are involved in all stages of atherosclerotic disease, from initial endothelial dysfunction to pro-thrombotic events (21). Consistently, it has been established that a therapeutic modulation of some pro-inflammatory master cytokines and their receptors may efficiently interfere with atherosclerosis development in preclinical mouse models. CD40L, TNF-α, and IL-1β have been extensively evaluated in experimental atherosclerosis (23–25) and the development of underlying cardiometabolic risk factors (26, 27). One relevant limitation of targeting single receptor-ligand pairs, however, is their fundamental role in host-defense, regeneration, and other physiological processes, such as haemostasis. For instance, mice genetically deficient for CD40L or wild type mice treated with a blocking anti-CD40L antibody are protected from atherosclerosis (28). However, clinical treatment with a neutralizing anti-CD40L antibody in patients with Systemic Lupus Erythematosus has failed due to a higher rate of thrombotic complications (29). Likewise, inhibition of TNF-α reduces experimental atherosclerosis (30), but long term inhibition of TNF-α in patients increases the risk for opportunistic infections and non-melanoma skin cancers (31). It has therefore been proposed that targeting inflammatory signaling cascades downstream of several pro-inflammatory receptors may overcome some of these limitations.

TRAFS ARE INTRACELLULAR ADAPTER PROTEINS THAT REGULATE INFLAMMATORY SIGNALING

TNF receptor associated factors (TRAFs) are a family of cytoplasmic molecules that transduce, regulate, and bundle receptor-mediated signaling by distinct classes of cell-surface receptors that can activate or inhibit downstream signaling pathways mostly by canonical and non-canonical Nuclear Factor kappa B (NF-κB) signaling (32). TRAFs associate with TNF receptors (TNFRs), interleukin 1 receptor (IL-1R), Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and receptors for IL-2, IL-17, IFN, and TGF-β (33–37) (**Table 1**). The biological role of TRAFs is to regulate cell survival, immunity, and inflammation. To this date, seven different TRAFs, TRAF1 to 7, have been described (32, 38). Because of their ability to modulate downstream signaling of the above mentioned pro-inflammatory receptors, and their broad expression patterns among immune and stromal cell types, TRAFs have gained increasing attention as central inflammatory hubs that may become accessible to a therapeutic modulation of inflammatory disease (39, 40).

Members of the TRAF family are structurally homologous (**Figure 1**). All TRAFs except for TRAF7 share a common domain of 180 amino acids at the C-terminal end, the "TRAF domain," which is required for oligomerization of TRAF proteins and for the interaction with upstream receptors and downstream effector proteins (41). The TRAF domain consists of two subdomains: a variable coiled-coil TRAF-N domain and a highly conserved TRAF-C domain formed by seven to eight anti-parallel β-strands, known as the Meprin or the TRAF-C homology (MATH) domain (42). The TRAF-C subdomain allows TRAF molecules to form homo or heterodimers, and to recognize cytoplasmic tails of associated receptors. At the N-terminal end, a flexible α-helical structure mediates downstream signaling

TABLE 1 | TRAFs-associated receptor and ligand pairs.

TRAF1		TRAF2		TRAF3		TRAF5		TRAF6	
Receptor	**Ligand**	**Receptor**	**Ligand**	**Receptor**	**Ligand**	**Receptor**	**Ligand**	**Receptor**	**Ligand**
TNFR1	TNF, LTα	TNFR1	TNF, LTα	TNFR2	TNF, LTα	TNFR1	TNF, LTα	TNFR2	TNF, LTα
TNFR2	TNF, LTα	TNFR2	TNF, LTα	4-1BB	4-1BB ligand	TNFR2	TNF, LTα	CD40	CD40 ligand
4-1BB	4-1BB ligand	4-1BB	4-1BB ligand	AITR	AITR ligand	CD27	CD27 ligand	p75	NGF, BDNF, neurotrophins
AITR	AITR ligand	AITR	AITR ligand	BCMA	APRIL, THANK	CD40	CD40 ligand	RANK	RANKL
BCMA	APRIL, THANK	BCMA	APRIL, THANK	CD27	CD27 ligand	HVEM	LIGHT, LTα	TACI	APRIL, THANK
HVEM	LIGHT, LTα	CD27	CD27 ligand	CD40	CD40 ligand	LTβR	LTβ	TLR2	PAMPs
OX40	OX40 ligand	GITR	GITR ligand	HVEM	LIGHT, LTα	OX40	OX40 ligand	TLR3	dsRNA
p75	NGF, BDNF, neurotrophins	CD40	CD40 ligand	OX40	OX40 ligand	p75	NGF, BDNF, neurotrophins	TLR4	LPS
RANK	RANKL	HVEM	LIGHT, LTα	LTβR	LTβ	RANK	RANKL	TLR7	ssRNA
CD40	CD40 ligand	OX40	OX40 ligand	p75	NGF, BDNF, neurotrophins	TACI	APRIL, THANK	TLR9	CpG DNA
XEDAR	EDA	p75	NGF, BDNF, neurotrophins	RANK	RANKL	NOD1	PAMPs/DAMPs	NOD1	PAMPs/DAMPs
TLR3	dsRNA	RANK	RANKL	TLR3	dsRNA	NOD2	PAMPs/DAMPs, viral ssRNA	NOD2	PAMPs/DAMPs, viral ssRNA
TLR4	LPS	TACI	APRIL, THANK	TLR4	LPS	RIG-I	PAMPs/DAMPs, viral RNA	RIG-I	PAMPs/DAMPs, viral RNA
		CD30	CD30 ligand	TLR7	ssRNA	IL-17R	IL-17	IFNλR1,	IFNλ
		NOD1	PAMPs/DAMPs	TLR9	CpG DNA	IL-6R	IL-6	TβRI, TβRII	TGF-β
		NOD2	PAMPs/DAMPs, viral ssRNA	NOD1	PAMPs/DAMPs	Troy		IL-1R	IL-1β
		RIG-I	PAMPs/DAMPs, viral RNA	RIG-I	PAMPs/DAMPs, viral RNA			IL-2R	IL-2
		IFNAR1	IFN	IL-6R	IL-6			IL-17R	IL-17
		IL-6R	IL-6	IL-17R	IL-17			Troy	
		IL-17R	IL-17						
		Troy							

TRAFs-associated receptors are shown in the left column while ligands for TRAFs-associated receptors are shown in the right column.

and the recruitment of effector enzymes by Zinc- and RING-finger motifs (43). The N-terminal "Really Interesting New Gene" (RING) domain represents the second structurally homologous elements shared by most TRAF members, except for TRAF1. It allows TRAFs to act as an E3 ubiquitin ligase in addition to their scaffolding function (44). Following receptor activation, TRAFs either bind directly to the cytoplasmic tail of the receptor via the TRAF-C domain or indirectly via other adapter proteins such as TNFR type 1 Associated Death Domain (TRADD), Myeloid Differentiation primary response gene 88 (MyD88), or IL-1R Associated Kinase (IRAK). These intermediate signaling adaptors regulate the activation of different kinases including Mitogen Activated Protein Kinase (MAPK) such as JNK, p38, ERK-1/2 (45), and IκB Kinase (IKK). As a result, TRAFs can activate or suppress transcription factor NF-κB and regulate pro-inflammatory cytokine-, chemokine-, and adhesion molecule expression (46, 47). In addition, TRAFs can activate Apoptosis Protein 1 (AP-1), a transcription factor that promotes cellular response to stress (48). Interaction with cellular Inhibitors of Apoptosis Proteins (cIAP1/2) during NOD-like receptor signaling (49) regulates cell survival. Notably, TRAF molecules can interact in homo- or heterooligomers of TRAF family members to increase their avidity and form receptor signaling complexes (40). Importantly, different TRAFs interact with each other to amplify or inhibit distinct inflammatory signaling pathways and may compensate the function of other TRAFs. Given that each TRAF is differentially regulated, interacts with distinct receptors, and activates a variety of downstream signaling pathways, TRAFs represent individual branching points in inflammatory signaling cascades (**Figure 2**). Different inflammatory stimuli can converge in one TRAF; on the other hand, one inflammatory signal can be divided into different downstream signals through multiple TRAFs (43). This can be exemplified by IL-17R signaling: Upon binding of IL-17, heteromeric IL-17RA and IL-17RC recruit TRAF2, TRAF5, and TRAF6 via the adaptor protein Act1. While TRAF2 and TRAF5 induce activation of MAPKs, TRAF6 mediates activation of NF-κB1, IkBζ, C/EBPβ, and C/EBPδ. TRAF3 and TRAF4, on the other hand, inhibit IL-17 signaling by interacting with IL-17RA and IL-17RC or Act1, respectively (40).

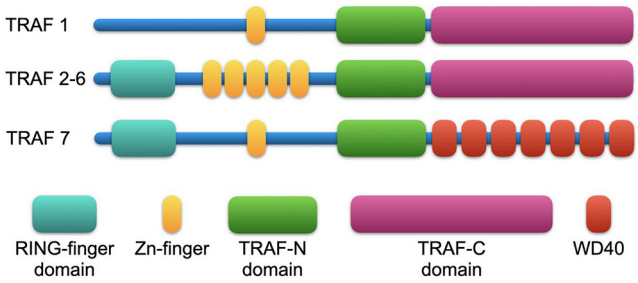

FIGURE 1 | Structural features of TRAF proteins. All TRAF family members (except for TRAF7) share a common domain of 180 amino acids at the C-terminal end, the "TRAF domain," which consists of two subdomains: a variable coiled-coil TRAF-N domain and a highly conserved TRAF-C domain, known as the Meprin or the TRAF-C homology (MATH) domain, that is required for oligomerization of TRAF proteins and for the interaction with upstream receptors and downstream effector proteins. At the N-terminal end, a Really Interesting New Gene (RING) domain hosts TRAFs E3 ubiquitin ligase function (except for TRAF1). TRAFs also recruit effector proteins by a variable number of Zn-finger domains: TRAF1 and TRAF7 have one Zn-finger domain; TRAF2, 3, 5, and 6 have five Zn-finger domains; and TRAF4 has seven Zn-finger domains. In TRAF7, the TRAF-C domain is replaced by WD40-repeats.

EXPRESSION OF TRAFS IN ATHEROSCLEROSIS-RELEVANT CELL TYPES

Immune, endothelial, and smooth muscle cells are the most abundant cell types that build the microarchitecture of atherosclerotic plaques (50). TRAFs have distinct expression profiles across these cell types and other stromal cell types: In the human transcriptome, expression of TRAF1 and TRAF5 is mostly confined to immune cells with the strongest expression in monocytes and lymphocytes, respectively (**Figure 3A**). Other TRAFs, such as TRAF2, TRAF6, and TRAF7, are ubiquitously expressed in stromal and immune cells. TRAF3 is mostly expressed in glial cells, neuronal cells, T and B cells; TRAF4 in epithelial and trophoblast cells (51). Expression of mRNAs coding for TRAF4, TRAF5, and TRAF7 can be detected in ECs, SMC, and adipocytes (**Figure 3B**). There is also a considerable variation among different immune cell types: TRAF1, TRAF4, and TRAF5 are the most expressed TRAFs in human T cells, while B cells express high levels of TRAF3, TRAF4, and TRAF5 but not of TRAF1. In human monocytes, TRAF1 is strongly expressed and shows the highest relative expression of all cell types available in the human protein atlas. Human dendritic cells (DCs) contain most TRAF4 transcripts compared to all tested immune cell types, while NK cells are generally only weak TRAF-expressors. Human expression patterns are distinct to mouse transcriptomic atlases, such as the Immunological Genome Project (ImmGen) (52), and suggest differential functional repertoires in mice and humans (**Figure 3C**). While these findings inferred from TRAF mRNA expression suggest clear patterns of cell type specificities and function, it is important to note that gene expression may not ultimately predict protein expression, post-translational modification, or function. This is highlighted by numerous reports demonstrating functional protein expression of TRAFs in B cells (53), T cells (54), DCs (55), neutrophils (56), macrophages (57), platelets (58), adipocytes (59), SMC (57), and EC (60), even if mRNAs are expressed at only low levels in resting cells. In addition, expression of TRAFs in macrophages, SMC, and EC (57) increases during inflammation. Whether tissue expression of TRAFs is modulated at atherosclerotic predilection sites, such as arterial branching points, or parts of the vasculature that are protected from atherosclerosis in humans, such as the internal thoracic artery (ITA), remains to be investigated (61). In the following sections, we will discuss expression, signaling networks (**Figure 2**), and functional roles of TRAFs in vascular inflammation and associated pathologies (**Figure 4**).

TRAF1

Expression of TRAF1 (46 kDa) is mainly regulated by NF-κB activation (62). While most resting cells lack TRAF1 protein expression, TRAF1 is strongly upregulated in activated immune cells, mostly in mononuclear cells and lymphocytes, as well as in EC and SMC from atherosclerotic lesions (57, 63, 64). In contrast to other TRAFs, TRAF1 lacks a RING-finger domain and expresses only one Zinc-finger domain (32). Intracellular signaling proteins such as TRAF-Interacting Protein (TRIP), Receptor-Interacting Protein (RIP), caspase 8, members of the cellular inhibitors of apoptosis (cIAP) family, as well as TRAF2, interact with TRAF1 (41, 65–68). TRAF1 and TRAF2 were the first TRAFs to be characterized, notably by their interaction with TNF receptor 2 (TNFR2) (69, 70).

TRAF1 contributes to signaling events by TNF-receptor superfamily members such as TNFR1, TNFR2, or CD40, and inhibits TLR-induced TRIFF signaling (71). Members of the TNFR superfamily stimulate cell survival by the activation of canonical NF-κB signaling and pro-inflammatory MAPK pathways (40). TRAF1 supports canonical NF-κB signaling events from TNFR1, TNFR2, CD40, 4-1BB, and LMP-1 by forming heterotrimers with TRAF2 and possibly through stabilizing TRAF2 by inhibiting its degradation by proteasomes (72–74). The complex of TRAF1 and TRAF2 is part of the E3 ubiquitin protein ligase and positively signals TNF-α/TNFR-2-dependent NF-κB and MAPK8/JNK activation and pro-inflammatory gene expression, which is considered a pro-survival event (75, 76). TRAF1, together with TRAF2 and cIAPs, supresses apoptosis (77), which is also supported by the observation that TRAF1 is instrumental for antigen-specific $CD8^+$ T cell responses during HIV and influenza virus infection, partially by promoting T cell survival and memory (62, 78). The absence of TRAF1, thus, impairs NF-κB signaling and favors the accumulation of pro-apoptotic signals in the cell, while transgenic over-expression of TRAF1 supports cell survival (54, 79). In hepatic ischemia/reperfusion injury, TRAF1-deficiency is protective through an inhibition of NF-κB mediated inflammation (80). The proposed pro-inflammatory role of TRAF1 has also been substantiated by reports showing attenuated lung inflammation after lipopolysaccharide (LPS) challenge in TRAF1-deficient mice (81). Taken together, these results argue for a pro-inflammatory and anti-apoptotic function of TRAF1. Other findings, however, have yielded contrasting

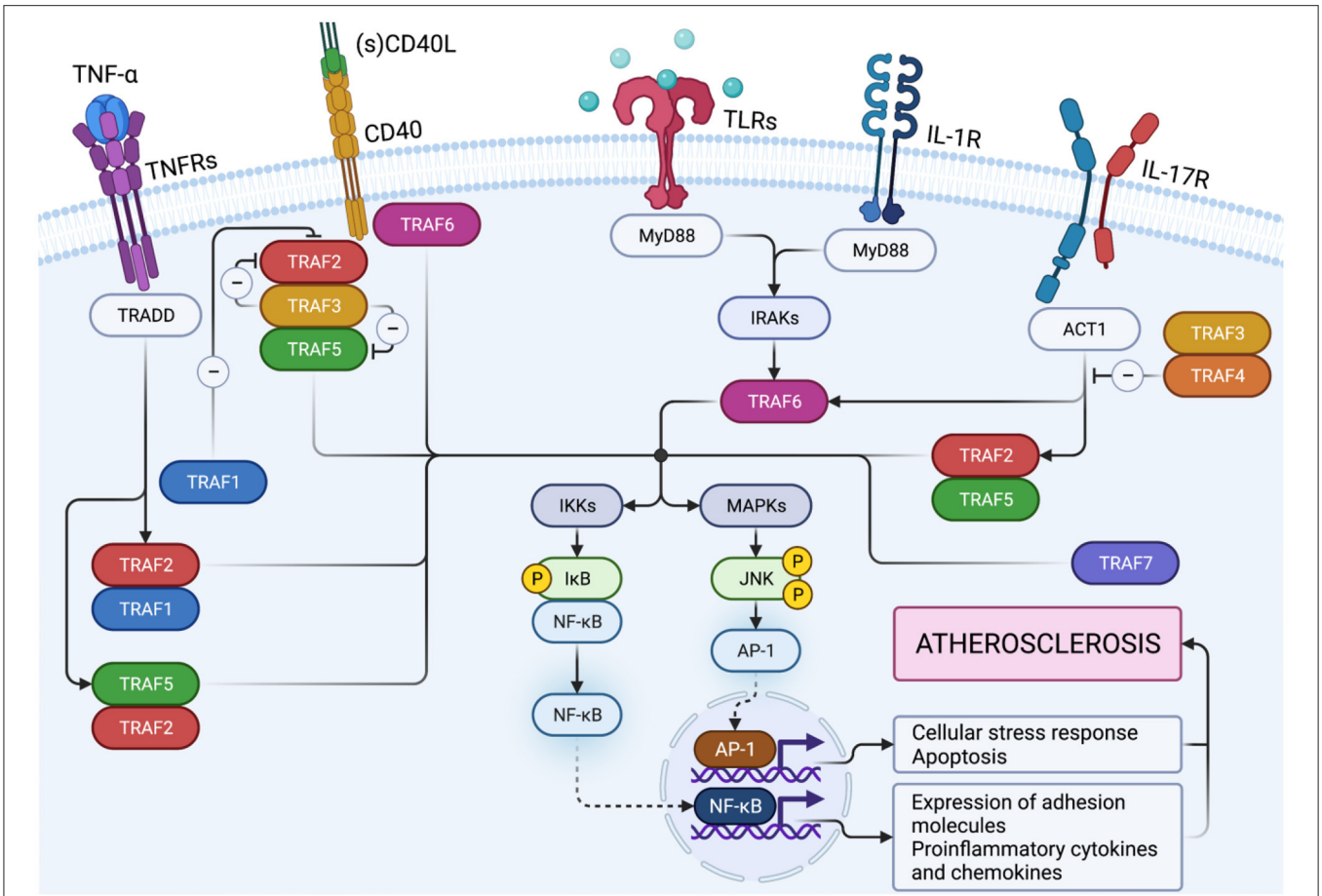

FIGURE 2 | TNF-receptor associated factors (TRAFs) link proximal receptor ligation and distal signaling pathways. Upon TNF-binding, TNFR1 activates inflammatory signaling pathways via TRAF1, TRAF2, and TRAF5. CD40 ligation induces canonical and non-canonical NF-κB activation by TRAF2/5 and canonical NF-κB activation by TRAF6. TRAF3 inhibits NF-κB activation mediated by TRAF2/5 but does not interfere with transcriptional activity of TRAF6-mediated NF-κB. TRAF1 inactivates TRAF2 by direct binding and is considered an inhibitory TRAF. Activation of TLRs and IL-1R can promote MyD88 dependent TRAF6 activity. Following binding of IL-17, heteromeric IL-17RA and IL-17RC recruit TRAF2, TRAF5, and TRAF6 via the adaptor protein Act1 to induce activation of downstream signaling pathways. TRAF3 and TRAF4 on the other hand, inhibit IL-17 signaling by interaction with IL-17R or Act1, respectively. Created with BioRender.com.

results that question a merely pro-inflammatory role of TRAF1. First, TRAF1-deficiency unexpectedly induced a hyper-proliferative phenotype in T cells after TNF/TNFR2-dependent stimulation in one report (82), suggesting TRAF1 is a negative regulator of TNFR-signaling. A possible explanation is that TRAF1 can restrain non-canonical NF-κB signaling (83) and T cell proliferation (84). Besides its positive regulation of NF-κB -signaling, TRAF1 may also limit NF-κB activation by several less frequent mechanisms (85, 86). In addition, TRAF1 can dampen TLR/NLR-dependent activation of NF-κB by sequestering the linear ubiquitin assembly complex (LUBAC). This process seems to be independent of TRAF2 and TNFRs (87). Notably, TRAF1-deficient mice are more susceptible to an LPS-induced septic shock (87). A single nucleotide polymorphism (SNP) at the C5-locus of TRAF1 is associated with rheumatoid arthritis in humans (88, 89). Patients with a homozygous TRAF1/C5 rs3761847 GG locus show increased mortality in sepsis and malignancies (90), establishing a clinical association with hyper-inflammation. In addition to its anti-inflammatory properties in TLR signaling, TRAF1 has been reported to disrupt the interaction between TRAF2 and CD40, leading to an attenuation of NF-κB activation (76). *In vivo*, TRAF1-deficient mice showed an increased responsiveness to TNF-induced skin necrosis. In this study, the authors accounted TRAF1 as a negative regulator of TRAF2-depending NF-κB activation (82). Down the same line, intratracheal TNF-α stimulation in TRAF1$^{-/-}$ mice exacerbates TNFR1-dependent liver injury (91). In conclusion, TRAF1 seems to exert differential and partially opposing roles in inflammation and apoptosis, likely caused by cell type and receptor-specificity and the precise signaling context.

Role of TRAF1 in Vascular Inflammation

TRAF1 is overexpressed in murine atherosclerotic lesions and in neointima formation after arterial injury (57, 92). Atherosclerosis-prone LDLR-deficient mice with a genetic deficiency of TRAF1 develop significantly smaller atherosclerotic lesions after 8 or 18 weeks of high cholesterol diet, suggesting a pro-atherogenic role of TRAF1 (60). This decrease of *de*

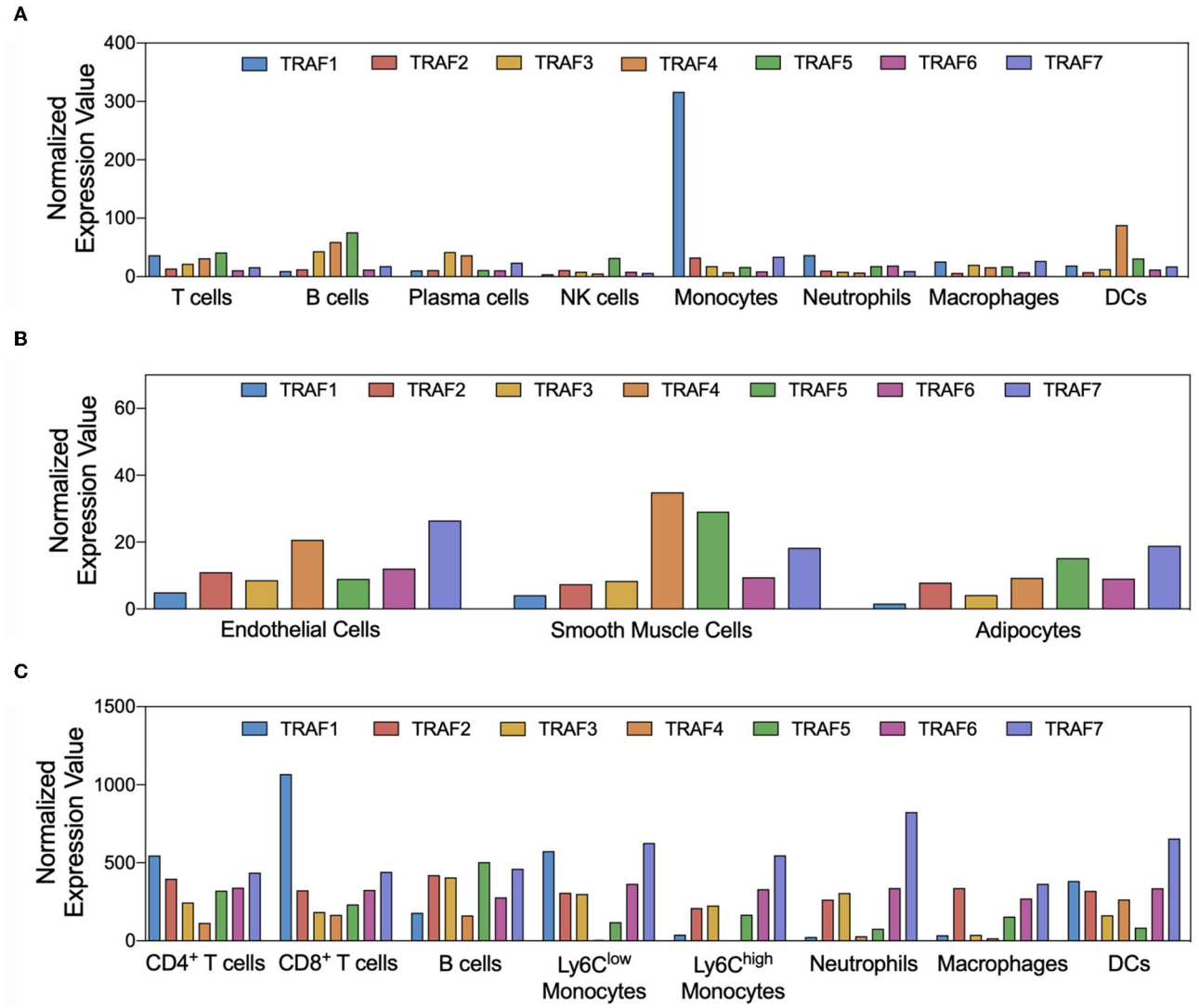

FIGURE 3 | Cell-type specific expression of TRAFs. Gene expression patterns of TRAF1-7 in atherosclerosis-relevant cell types are shown. Normalized expression values for human immune **(A)** and stromal **(B)** cell types were extracted from the Human Protein Atlas project (51), not further adjusted, and plotted as given. Normalized expression of TRAF mRNA in mouse cell types was extracted from the ImmGen database **(C)**.

novo atherosclerosis in the absence of TRAF1 is accompanied by a lower content of macrophages and lipids in TRAF1-deficient plaques, an effect likely caused by reduced VCAM-1 and ICAM-1 expression on EC and reduced β1-integrin expression on macrophages. This observation confirms previous studies that found that TRAF1 is required for the expression of ICAM-1 and chemokines in the lung (81). Bone marrow transplantation studies further indicated a requirement for TRAF1 on bone marrow-derived immune cells and stromal cells in maintaining adhesion factor expression and leukocyte recruitment to inflammatory sites (60). Notably, general immune responses seem not to be affected by a lack of TRAF1 (60), as opposed to previous findings that indicated a hyper-proliferative phenotype in T cells with TRAF1-deficiency (82). In humans, expression of TRAF1 is increased in fibrous, atheromatous, and aneurysmal atherosclerotic lesions of carotid arteries (57). TRAF1 blood mRNA expression is elevated in patients with acute coronary syndrome (60). Furthermore, the rs2416804 allele in the TRAF1 gene was associated with carotid intima-media thickness, a marker for subclinical atherosclerosis that predicts subsequent clinical cardiovascular events (93). Likewise, TRAF1 promoted inflammatory pathways through an activation of the ASK1-mediated JNK/p38 pathway in a model of myocardial ischemia/reperfusion (94). Recent results, however, have questioned a mere pro-inflammatory role of TRAF1 in cardiovascular pathologies: In diet-induced obesity (DIO), TRAF1-deficient mice were protected from diet-induced weight gain and associated metabolic derangements by an increased breakdown of lipids in adipocytes and UCP1-enabled thermogenesis (59). Notably, this effect seems to be caused

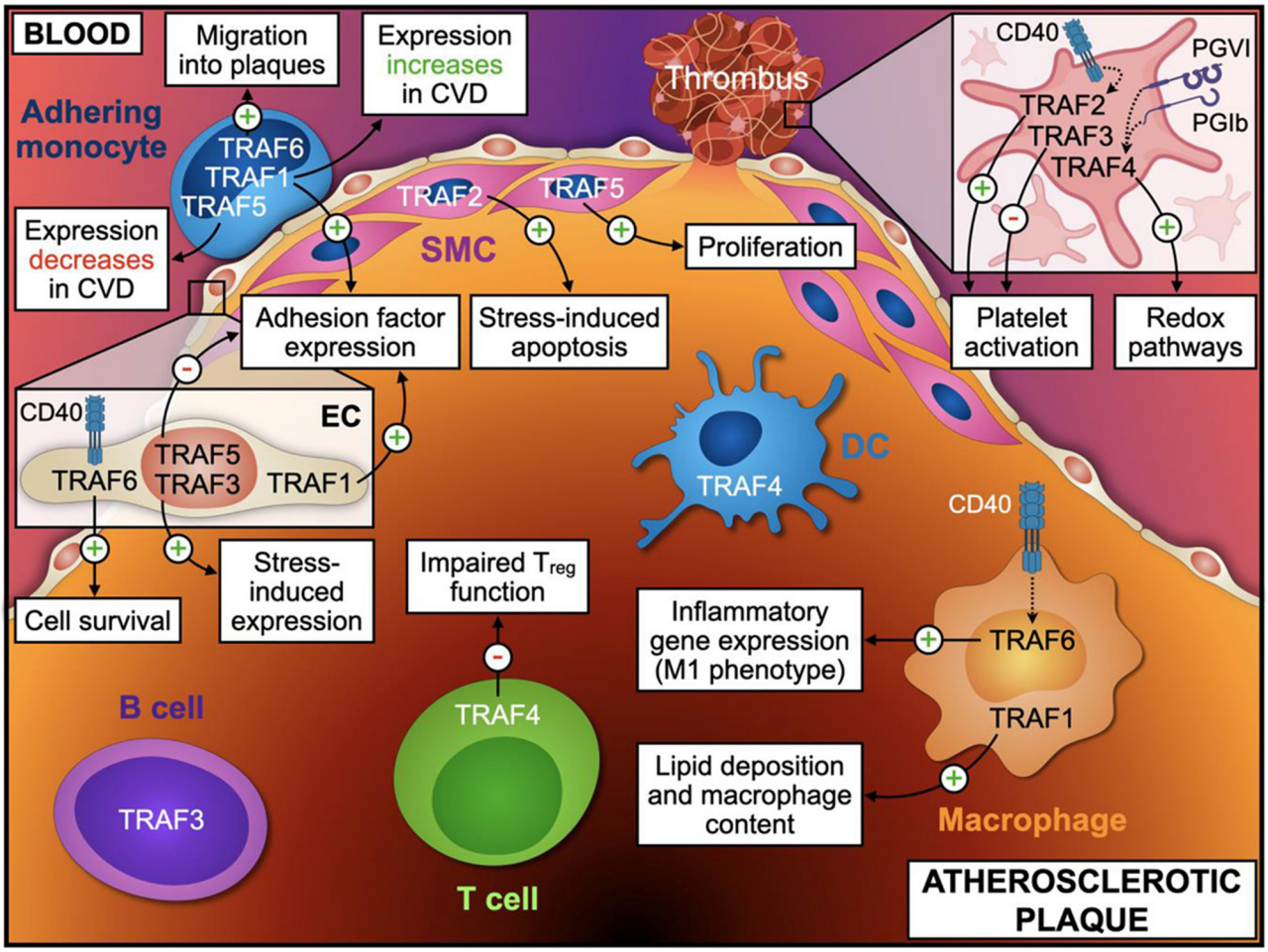

FIGURE 4 | TRAF-effector functions in atherosclerosis**.** Distinct cellular phenotypes and functions regulated by TRAF-associated signaling modulate vascular inflammation and atherosclerosis. The atherosclerotic plaque is separated from the blood by a thin layer of endothelial cells (EC) and smooth muscle cells (SMC). Monocytes adhere to the vascular endothelium and migrate into the plaque, where they differentiate into macrophages. Additionally, T cells, B cells, and Dendritic cells (DC) accumulate within the vessel wall and take part in regulation of local inflammatory processes. Upon rupture of the fibrotic cap, platelet activation precipitates thrombus formation and subsequent clinical complications. Only TRAFs with a proven effect or validated expression in the respective cell types are shown. Relevant TRAF-dependent processes in EC and platelets are indicated in the inlays. This figure was generated with schematics from BioRender.com.

by hyper-inflammation of adipocytes and adipose tissue, and subsequent induction of catabolic pathways. As atherosclerosis is driven by obesity and hyperlipidemia, improved metabolism in TRAF1 deficiency may contribute to its protective properties in atherosclerotic CVD, even in the light of enhanced inflammation.

TRAF2

TRAF2, a 56 kDa protein, is ubiquitously expressed (69). TRAF2 is involved in TNFR1-, TNFR2-, Receptor Activator of NF-κB (RANK)-, OX40-, and CD40-signaling, and regulates inflammatory responses mediated by NLRs, RIG-I, and other cytokine receptors such as for IL-6R and IL-17R (44). TRAF2 binds to its receptors through the TRAF-N and TRAF-C subdomains (41). The latter also mediate binding to TRAF1 and to itself to form homodimers. The TRAF2 RING-finger domain acts as an ubiquitin ligase in the canonical pathway of NF-κB activation, which activates IKKβ and releases NF-κB (95, 96). In addition, TRAF2 mediates JNK and p38 signaling (44). These processes trigger inflammatory gene expression and promote cell survival (44). Prolonged stimulation of TNFR2 leads to the degradation of TRAF2, which also negatively regulates non-canonical NF-κB signaling (97). Notably, TRAF1 stabilizes TRAF2 and prevents its degradation (62). TRAF2 also contributes to NF-κB activation after CD40 and OX40 ligation, two pro-atherogenic receptors (98–100). Although a deficiency of TRAF2 abolishes TNF-α signaling (101), TRAF2-deficiency results in chronic inflammation characterized by infiltration of activated T effector and T memory cells. This is caused by a dysregulation of the NF-κB pathway (101). TRAF2-deficiency is lethal in mice due to hyper-inflammation caused by TNF-α (98).

Role of TRAF2 in Vascular Inflammation

Several studies have demonstrated a crucial role of balanced TRAF2 levels in regulating T cell homeostasis, with either increased or decreased TRAF2 levels leading to inflammatory

disorders (44). TRAF2 expression is elevated in murine atherosclerotic lesions and neointima after arterial injury (57, 92). Human atherosclerotic lesions express higher levels of TRAF2 compared to healthy carotid arteries and stable atherosclerotic lesions (53). Because TRAF2-defcient cells are highly susceptible to TNF-α induced-death (98), TRAF2-deficient mice show an atrophy of the thymus and the spleen and die prematurely within 14 days after birth (101, 102). Only one study has tested the impact of TRAF2 in experimental atherosclerosis with $TRAF^{+/-}$ mice in bone marrow transplantations using TRAF2-deficient hematopoietic cells from fetal livers to generate viable mice with a deficiency of TRAF2 in hematopoietic cells (103). In these mice, atherosclerotic lesion size was similar to respective controls, suggesting that TRAF2 expressed in immune cells does not affect atherosclerosis (103). Furthermore, mice with a defective TRAF2-CD40 binding site did not show alterations in atherogenesis or in neointima formation after arterial injury (104, 105). The increased expression of TRAF2 in mouse and human atherosclerotic lesions could potentially be explained by the finding that porcine vascular SMC become apoptotic under mechanical stress by a TRAF2-dependent mechanism that activates the pro-apoptotic transcription factors JNK and p38 (106). Importantly, TRAF2 could contribute to plaque destabilization though the induction of SMC apoptosis. However, available data suggest that TNF-dependent anti-inflammatory properties of TRAF2 outweigh pro-inflammatory effects of TRAF2 in leukocytes and other cell types. In a murine ischemia/reperfusion model, cardiac-restricted overexpression of TRAF2 resulted in NF-κB activation and protection from ischemia-induced cardiomyocyte death (107). In murine DIO, hepatocyte-specific deletion of TRAF2 did not affect body weight or hepatic inflammation but attenuates the hyperglycaemic response to glucagon and protects against hyperglycaemia and hyperinsulinemia (108).

TRAF3

TRAF3 is a 65 kD protein expressed in various cell types, including EC, SMC, fibroblasts, and immune cells (44). Among immune cells, TRAF3 expression is the highest in B cells, plasma cells, macrophages, and T cells. At the N-terminal end, one RING-, five Zinc-fingers, and a leucine zipper domain mediate downstream signaling. TRAF3 inhibits CD27, CD30, CD40, OX40, and Latent Infection Membrane Protein (LMP)-1 induced canonical and non-canonical NF-κB activation mediated by TRAF2/5 (109–114), but does not interfere with transcriptional activity of TRAF6-mediated canonical NF-κB (115). TRAF3 induces the anti-inflammatory cytokine IL-10 after activation of IL-1/TLR-receptors (116). Consistently, mice with myeloid specific TRAF3-deficiency show decreased IL-10 and enhanced IL-6 and IL-12 cytokine levels following an *in vivo* challenge by LPS. In this model, TRAF3-deficiency results in spontaneous multi-organ inflammation (117). Collectively, these findings suggest that TRAF3 is a negative regulator of the above-mentioned pathways (118). Furthermore, TRAF3 plays an important role in the homeostasis of immune cells as murine TRAF3-defcient B cells, T cells, and DCs show a prolonged survival and constitutive activation (119). Mutations in the TRAF3 gene are associated with multiple myeloma and Waldenstrom's macroglobulinemia in humans (53, 120–122). TRAF3-deficient mice are characterized by impaired leukocyte development and die prematurely, likely caused by a permanent activation of non-canonical NF-κB signaling (123).

Role of TRAF3 in Vascular Inflammation

Previous studies pointed out anti-inflammatory characteristics of TRAF3 in vascular biology. Laminar blood flow, an atheroprotective stimulus, induces TRAF3 expression and inhibits CD40-induced endothelial activation and cytokine production (124). In EC from human atherosclerotic plaques, TRAF3 expression is upregulated in areas with high shear stress and downregulated in areas of turbulent blood flow (125). Overexpression of TRAF3 in EC inhibits endothelial expression of proinflammatory cytokines and tissue factor, blocks DNA-binding activity of the transcription factor AP-1, and prevents CD40-induced endothelial activation (125), a known driver of atherogenesis (126). Silencing TRAF3 expression by small interference RNA (siRNA) in EC increases CD40L-induced cytokine production (53). In addition, TRAF3 expression is upregulated in mice after arterial injury, as well as in murine and human atherosclerotic lesions (57, 92). These data suggest an overall anti-inflammatory role of TRAF3 in vascular inflammation. The fact that TRAF3 expression is elevated in human atherosclerotic lesions (53) may be explained by a compensatory regulation. Interestingly, epigenetic regulation of TRAF3 is associated with vascular recurrence in patients with ischemic stroke (127). Due to the limited viability of $TRAF3^{-/-}$ mice, an *in vivo* atherosclerosis study is missing (128). In cardiac ischemia/reperfusion injury, a knock-down of TRAF3 attenuates infarct size and inflammation by inhibition of NF-κB and xanthine oxidase (XO) signaling pathways and restraining JNK activation (129). In mouse models of high fat diet-induced and genetic obesity, hepatocyte-specific TRAF3 deficiency decreases insulin resistance, hepatic steatosis, and expression of pro-inflammatory cytokines in the liver, while development of obesity *per se* is not altered. These data imply that hepatic TRAF3 has anti-inflammatory properties in the setting of metabolic syndrome (130).

TRAF4

Unlike other members of the TRAF family, TRAF4 is mostly involved in morphogenic and developmental processes (131). A loss of TRAF4 results in upper respiratory tract deformities and is required for myelin homeostasis in the central nervous system (132). TRAF4 is expressed ubiquitously, but mainly found in thymus, spleen, and lymph nodes. Among leukocytes, B cells, T cells, and DCs express highest TRAF4 levels. Furthermore, TRAF4 is highly expressed in human breast cancer (133, 134). Apart from its stimulating role in DCs migration, TRAF4 does not affect immune cell development (135). In immune cells, TRAF4 increases NF-κB signaling after ligation of the T regulatory (T_{reg}) cell expressed "Glucorticoid Induced TNF-receptor" (GITR), which results in an impairment of T_{reg} function (136). Both, GITR (137) and T_{reg} cells (5), have established atheroprotective roles. The role of TRAF4 in

TNFR signaling remains unclear, despite a weak association to Lymphotoxin beta Receptor (LTβR) and the nerve growth factor p75 (138). In inflammatory diseases, TRAF4 has been described to exert both, adverse and protective functions: After IL-25/IL-25R ligation, TRAF4 facilitates the Act1/25R interaction and degrades the IL-25R inhibitory molecule DAZAP2, which increases airway inflammation (139). In T_H17-mediated autoimmune encephalomyelitis, TRAF4 restricts IL-17 induced production of GM-CSF, IL-6, CCL2, and CXCL1 (140). There is only spare information about the role of TRAF4 in atherosclerosis due to the limited viability of TRAF4$^{-/-}$ mice (141). Unstable atherosclerotic human plaques that are prone to rupture and to give rise to thrombotic complications show increased expression of TRAF4 (142). Evidence that TRAF4 is directly involved in vascular inflammation and atherosclerosis is missing.

TRAF5

The 64 kD protein TRAF5 interacts with multiple inflammatory and atherogenic surface receptors including CD27, CD30, CD40, LMP-1, LTβR, RANK, and OX40 (143–145). It is expressed in lymphoid tissues such as spleen and thymus, but also in epidermis, lungs (146), muscle, and adipose tissue (147). The expression of TRAF5 is highest in immune cells. Structurally, the C-terminal TRAF-domain is responsible for the associations to its receptor and other TRAFs. TRAF5 is highly similar to TRAF2 and TRAF3, and can form heterodimers with TRAF3 to facilitate their recruitment to inflammatory receptors such as CD40 (148, 149). TRAF5 has one RING- and five Zinc-finger domains at the N-terminal end for downstream signaling and activation of JNK and NF-κB (150). Overexpression of TRAF5 in fibroblasts enhances LTβR-dependent NF-κB and JNK signaling, indicating a pro-inflammatory role (143). In patients, a SNP within the TRAF5 gene is associated with rheumatoid arthritis (151). TRAF5 supports T cell immunity against *L. monocytogenes* (152) and enhances LMP-1 induced B cell responses (153). While these findings argue for an overall pro-inflammatory role, other reports have established a regulatory and anti-inflammatory function of TRAF5: TRAF5-deficient ECs show an enhanced activation of JNK after stimulation with TNF-α (103). While TRAF2 binding to OX40 induces NF-κB activation, the interaction of TRAF5 with OX40 seems to have an inhibitory and regulatory role. Following ligation of OX40, a known pro-atherogenic receptor (154), with an agonistic anti-OX40 antibody, TRAF5$^{-/-}$ CD4^{+} T cells upregulated T_H2-cytokines. The anti-inflammatory and regulatory properties of TRAF5 were further validated in a mouse model of asthma, where TRAF5-deficiency exacerbated lung inflammation by enhanced infiltration of eosinophils and an increased T_H2 response (155). If and how T_H2 immunity affects atherosclerosis is still under debate (5). In addition, TRAF5 limits autoimmune encephalitis in mice (156) and is protective in murine experimental colitis (157).

Role of TRAF5 in Vascular Inflammation

TRAF5 is overexpressed in human and murine atherosclerotic plaques, with a higher expression in fibrous atherosclerotic lesions from human carotid arteries compared to vulnerable lesions (57), suggesting a protective role of TRAF5. Consistently, TRAF5-deficient mice present with increased atherosclerosis, an effect likely caused by enhanced VCAM-1-dependent leukocyte migration and enhanced expression of MCP-1 and C-X-C motif ligand 1 (CXCL1), resulting in accelerated leukocyte recruitment into atherosclerotic lesions (103). In addition, the authors of this study found increased CD36-dependent foam cell formation and detected lower numbers of atheroprotective T_{reg} cells (103). While it has been proposed that TRAF5 can be partially compensated by TRAF2 (158), a deficiency of TRAF5 in bone-marrow derived cells did not affect atherosclerosis in LDLR$^{-/-}$ mice (103), rendering effects on lipid deposition and lower numbers of T_{reg} cells in plaques as indirect effects. An overall anti-atherogenic role of TRAF5 is supported by the fact that TRAF5 mRNA expression is decreased in patients with stable coronary heart disease compared to healthy individuals (103). In another study, the lack of the TRAF2, TRAF3, and TRAF5 binding site on CD40 in MHCII^{+} cells did not interfere with neointima formation after arterial injury and atherosclerosis (104, 105). However, in another report, the lack of TRAF5 protected from neointima formation and vascular SMC proliferation (159). In myocardial ischemia/reperfusion injury, TRAF5 protects against inflammation and associated cardiomyocyte damage via AKT signaling (160). Recently, we have shown that a genetic deficiency of TRAF5 in mice aggravates DIO and its metabolic derangements by a proinflammatory response in adipocytes, pointing toward an anti-inflammatory role of TRAF5 in cardiometabolic disease (147). Down the same line, other reports found that CD40-TRAF2/3/5 signaling in MHCII^{+} cells protects against adipose tissue inflammation and metabolic complications associated with obesity (161, 162). Thus, TRAF5 signaling seems to exert an overall anti-inflammatory effect. TRAF5 reduces atherosclerosis and improves cardiometabolic risk factors. Interestingly, TRAF5 is downregulated during aging (163). Restoring or boosting TRAF5 function may therefore represent a promising therapeutic approach in cardiovascular disease.

TRAF6

TRAF6, a 63 kD protein, is involved in multiple NF-κB-dependent processes and mediates signaling of the TNFR superfamily members, such as of CD40 and RANK, and members of the IL-1/TLR superfamily (146, 164). TRAF6 was first described as a signaling intermediate of IL-1R and CD40 (164). In addition, TRAF6 seems to be important for signal transduction of IL-17 and IL-25 receptors (165, 166). Overexpression of TRAF6 leads to p38- and JNK-activation by an interaction with TRAF2 (167). TRAF6-deficient mice show severe defects in organogenesis, lack secondary lymphoid organs, have thymic atrophy, and are characterized by decreased numbers of T_{reg} cells and exaggerated inflammatory cell accumulation in most organs (40, 168). CD40 receptor exhibits two distinguished cytoplasmic TRAF-binding sites: a proximal binding site for TRAF6 and a distal binding site for TRAF2, TRAF3, and TRAF5 (169). TRAF6 and TRAF2/3/5 cooperate in the differentiation of B cells (170), while CD40-TRAF6 signaling seemed to be relatively more important in inducing inflammation in a model

of murine autoimmune encephalitis (171). A lack of CD40-TRAF6 binding abrogated NF-κB, JNK, and p38 activation, and blunted inflammatory cytokine production after CD40 activation in monocytes and macrophages (172).

Role of TRAF6 in Vascular Inflammation

Among all TRAFs, TRAF6 has the highest relative expression in human arterial tissue (57), a finding consistent in murine atherosclerotic lesions (57). This modulation seems to be site-specific, since mRNA expression of TRAF6 remains unchanged in blood of patients with or without coronary heart disease (173). TRAF6 deficiency of hematopoietic cells does neither change the size of atherosclerotic plaques in mice nor alters plaque composition (173). This sparked the idea that distinct pro- and anti-inflammatory TRAF6-dependent signaling pathways with a net zero effect would exist. Interestingly, a selective lack of the CD40-TRAF6 binding site, as tested with a chimeric CD40 transgene carrying specific mutations within the TRAF6 binding site in $MHCII^{+}$ monocytes, macrophages, dendritic cells, and B cells (171), reduced neointima formation after carotid arterial injury, whereas the TRAF2/3/5 binding did not affect neointima formation in mice (92, 104). The CD40-TRAF6 axis also contributes to atherosclerosis by an activation of mononuclear cells, whereas CD40-TRAF2/3/5 interactions are crucial in CD40-driven immunity in other cell types, including B cells (174). Mice with a defective TRAF6 binding site on CD40 develop smaller atherosclerotic lesions compared to wildtype and CD40-TRAF2/3/5 deficient mice (105). Mice with a defective CD40-TRAF6 binding site also show reduced numbers of $Ly6G^{high}$ monocytes in peripheral blood, a reduced migration of monocytes to the arterial wall, and increased levels of anti-inflammatory M2-macrophages. This overall atheroprotective phenotype coincides with a higher content of collagen and SMC in atherosclerotic plaques, which resembles a more stable plaque phenotype in humans (105, 175). Beyond this MHCII-restricted effect, endothelial TRAF6-deficiency protects from atherosclerosis in female ApoE-deficient mice by inhibiting NF-κB-dependent proinflammatory gene expression and monocyte adhesion to EC (176). Likewise, murine ECs deficient for TRAF6 display a markedly reduced expression of VCAM-1, ICAM-1, E-selection, MCP-1, and MCP-3 levels after stimulation with oxLDL. Contrastingly, myeloid cell-specific TRAF6-deficiency caused exacerbated atherosclerosis with larger plaques containing more necrotic areas in both in male and female ApoE-deficient mice (176). ApoA-I, the core protein of high-density lipoprotein (HDL) cholesterol suppresses CD40 signaling in macrophages, by preventing TRAF6 translocation to lipid rafts through ABCA1-dependent regulation of free cholesterol efflux, which may present a novel mechanism of ApoA-I-mediated suppression of inflammation in macrophages (177). In vascular SMCs, TRAF6 induces SM22β ubiquitination, which maintains survival through increased G6PD activity and $NADP^{+}$ production (178). Thereby, TRAF6 participates in the regulation of glucose homeostasis during vascular repair after injury (178).

In summary, a growing body of evidence demonstrates a significant involvement of TRAF6-signaling in the pathogenesis of atherosclerotic CVD. Partially opposing results may well be explained by the distinct and cell-type specific expression of TRAF6-associated upstream receptors, i.e., mostly CD40-associated TRAF signaling has been demonstrated to exacerbate inflammation. In this context, the specific targeting of CD40-TRAF6 interactions remains highly desirable. It is noteworthy to mention that a selective blocker of the interaction between CD40 and TRAF6 ("TRAF-STOPs") that does not affect CD40-TRAF2/3/5 interactions and preserves CD40-mediated immunity reduces atherosclerosis, likely by impairing inflammatory leukocyte recruitment (39). In murine obesity, coinhibitory suppression of T cell activation by CD40 protects against obesity and adipose tissue inflammation, while CD40-deficient mice display an aggravated phenotype during DIO (27). While a genetic deficiency of CD40-TRAF2/3/5 signaling aggravates obesity and promotes metabolic dysfunction and hepatic steatosis, mice lacking the CD40-TRAF6 binding site are protected from obesity-associated complications, arguing for opposite roles of CD40-TRAF2/3/5 and CD40-TRAF6 signaling in obesity-associated metabolic dysregulation. Notably, pharmacologic inhibition of the CD40-TRAF6 pathway ameliorates obesity-related metabolic complications (161).

TRAF7

TRAF7, a 74 kDa protein, was originally described as a positive regulator of the stress-induced transcription factors AP-1 and CHOP (179). In contrast to other TRAFs, TRAF7 lacks the TRAF-domain and contains seven WD40-repeats at the C-terminal end followed by a RING-finger domain, potentially implying that it evolved separately (40). Expression of TRAF7 is found ubiquitously but relatively higher in the heart, spleen, kidney, liver, colon, skeletal muscle, and placenta (179, 180). Clinically, a TRAF7 mutation causes defects in the heart, intellectual disability, and facial defects (181). TRAF7-dependent p38 and JNK activation contributes to cellular apoptosis, while an overexpression of TRAF7 does not induce activation of NF-κB (38). siRNA targeting TRAF7 show inhibitory and agonistic effects on NF-κB activation depending on the cellular model. TLR2-dependent NF-κB activation is promoted by TRAF6 and TRAF7 (182). Because TLR2 is known to promote atherosclerosis (183), it may be speculated that TRAF7 inhibition impairs atherogenesis. However, no human or *in vivo* animal data addressing the role of TRAF7 in inflammatory or vascular disease have been published to date.

TRAFS IN PLATELET FUNCTION AND HOMEOSTASIS

Beyond their known role in homeostasis, platelets have been proposed to contribute to acute and chronic inflammatory pathologies, including atherosclerosis and atherothrombosis in the later stages of the disease (184, 185). Key mechanism of platelets that promote inflammation include the adhesion to the injured endothelium, leukocyte activation, and the formation of leukocyte-platelet aggregates (186). It has also been recognized

that platelets have an active secretome, allowing either rapid secretion of pre-formed soluble mediators or *de novo* synthesis upon platelet activation. Major platelet-derived factors are pro-inflammatory, such as RANTES, CXCL4, IL-1β, or CD40L (187). TRAF-associated receptors, such as CD40 and TLRs, have been detected on platelets (188, 189). Ligation of platelet CD40 by CD40L induced platelet activation by yet not clarified downstream signaling pathways (190) that may also involve TRAFs. Notably, expression of TRAFs in megakaryocytes—the bone marrow derived precursors of circulating platelets—was demonstrated by different studies (53, 56). Consistently, platelets express several TRAF members, including TRAF1, TRAF2, TRAF3, TRAF4, and TRAF6 (191). TRAF4 has been identified as a novel binding site for the platelet glycoproteins GPVI and GP1β (58). It has been proposed that engagement of both glycoproteins during platelet adhesion and binding of TRAF4 to their cytoplasmic tail provides a functional link to TRAF4-related downstream effector functions, such as activation of NADPH-related redox pathways (192). Whether TRAFs can be expressed at later stages of platelet differentiation is not clarified yet. It has been shown that soluble CD40L potentiates platelet activation and aggregation through a TRAF2/Rac1/p38 MAPK signaling pathway (193), while TRAF3 was demonstrated to play a negative role in regulating platelet activation (194). Mice with a genetic inhibition of CD40-TRAF6 signaling did not show altered platelet deposition and thrombus formation in an *in vitro* flow chamber assay, suggesting no functional role for TRAF6 in platelet function (195). These data unveil that some TRAFs directly affect platelet function and thereby provide direct signaling links between atherosclerosis and atherothrombosis. However, future studies are required to understand the participation of the TRAF family members in regulation of platelets primary functional repertoire.

CONCLUSION AND THERAPEUTIC PERSPECTIVE

TRAFs are potent regulators of several pro-inflammatory and pro-atherogenic signaling pathways (**Figure 4**), including these initiated by TNF-, IL-1-, IL-6-, IL-17-, and TLR-signaling. Its fundamental role in regulating these signaling cascades in either a pro- or anti-atherosclerotic fashion makes the TRAF family an attractive target for the treatment of atherosclerosis and its complications including myocardial infarction and ischemic stroke. Traditional anti-inflammatory therapies aim for the disruption of one inflammatory signaling cascade, either of soluble ligands or receptors by monoclonal antibodies or small molecule inhibitors, which is exemplified by monoclonal antibodies inhibiting IL-1β (11) or IL-6 (12). Moreover, broad anti-inflammatory therapy with low-dose colchicine has substantiated the role of anti-inflammatory treatments (18). However, these therapies are at the potential risk of side-effects, e.g., of an impairments of host-defense, compensatory up-regulation of other inflammatory pathways, and relapsed inflammation after discontinuation (196, 197). TRAF-directed therapies may be designed to specifically promote or inhibit downstream receptor signaling events. Such therapies may overcome some of the limitations of conventional anti-inflammatory therapies, but raise important considerations as well: First, TRAFs show a highly complex interplay with distinct downstream signaling cascades and associated upstream receptors with partially opposing and overlapping functions. TRAFs complex with other TRAFs and thereby inhibit or augment signaling capacities. Second, this complexity seems to be determined by the levels of cellular expression, concomitant signaling events, and the context of the disease. Third, numerous animal experiments highlight that unspecific neutralization of TRAFs, e.g., in genetic knock-outs, may cause lethal complications and drives unconventional, often unforeseeable signaling events. These conclusions argue for a more careful evaluation of TRAFs in the context of inflammatory disease. However, recent studies have shown that TRAFs can potently limit atherosclerotic and vascular disease. Their broad action on different cell types, stromal, and bone-marrow derived cells, may be of advantage in the multi-factorial and multi-cell type pathogenesis of atherosclerosis. At present, most promising targets for an anti-atherosclerotic therapy are TRAF1, TRAF5, and the CD40-TRAF6 axis as suggested by preclinical mouse models: TRAF1 acts as a pro-inflammatory, atherosclerosis-promoting factor, while TRAF5 serves as an atheroprotective factor; the inhibition of the CD40-TRAF6 axis attenuates atherosclerosis and neointima formation by limiting accumulation of inflammatory monocytes at sites of inflammation. These TRAFs have also demonstrated powerful effects on associated cardiometabolic conditions, such as hepatic steatosis, adipose tissue inflammation, and insulin resistance, which may in part explain atheroprotective effects. The development of small molecule inhibitors that specifically target receptor-TRAF interactions (TRAFs-STOP) demonstrates the feasibility of specific pharmacological compounds to modify TRAF function *in vivo*. However, other TRAFs and their functions remain potentially relevant in vascular biology: TRAF2 induces apoptosis of vascular SMC and promotes plaque destabilization, but lesion size is not affected by TRAF2-deficiency on hematopoietic cells. TRAF3 has some atheroprotective properties, but an *in vivo* mouse study is missing due to the limited viability of TRAF3-deficient mice. To date, there is only little evidence that TRAF4 and TRAF7 influence atherosclerosis, which calls for future investigations. Besides the development of novel, likely cell-type specific TRAF-modulators, it will also be necessary to interrogate the role of TRAFs in atherosclerosis-associated cell types and organs, such as in the bone marrow and platelets. The regulation of TRAFs in human cells, tissues, and disease models represents another important gap of knowledge. Addressing these challenges could result in the clinical development of TRAF-based inhibition of inflammation in future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), SFB1425, project #422681845. This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 853425). MCG was funded by the IMM-PACT-Programme for Clinician Scientists, Department of Medicine II, Medical Center - University of Freiburg and Faculty of Medicine, University of Freiburg, funded by the DFG - 413517907.

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frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 23 February 2022
doi: 10.3389/fcvm.2022.808732

Establishment of a New Abdominal Aortic Aneurysm Model in Rats by a Retroperitoneal Approach

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Background: Constructing an ideal model of abdominal aortic aneurysm (AAA) is of great significance to elucidate its complex pathogenesis. Therefore, we introduce a new and simple method to simulate human AAA and construct a rat AAA model through a retroperitoneal approach.

Methods: Forty healthy adult Sprague Dawley (SD) rats were randomly divided into a control group, elastase + calcium chloride group (PPE+$CaCl_2$), elastase group (PPE), and elastase + beta aminopropionitrile group (PPE+BAPN) according to a male-female ratio of 1:1, with 10 rats in each group. A retroperitoneal approach was used to free the infrarenal abdominal aorta in all four groups. In the PPE + $CaCl_2$ group, 0.1 ml of elastase (approximately 5 U) was perfused into the arterial cavity for 20 min, and 1.0 mol/L calcium chloride was infiltrated out of the arterial cavity for 10 min. In the PPE group, 0.1 mL of elastase (approximately 5U) was perfused into the arterial cavity for 20 min, and normal saline was infiltrated out of arterial cavity for 10 min; the PPE + BAPN group combined with 0.3% BAPN drinking water/day on the basis of PPE group; the control group was treated with saline instead of elastase and calcium chloride. Abdominal aortic specimens were collected after 4 weeks of feeding. The diagnostic criteria of AAA were 50% dilation of the abdominal aorta or rupture of the aneurysm at 4 weeks after the operation. Histopathology, immunohistochemistry, quantitative PCR (qPCR), western blotting assay, gelatine zymogram, and other methods were used.

Results: The operation time of the four groups was controlled at approximately 40 min, and the success rate of the operation was 100%. Survival rate: Control Group (100%) = PPE Group (100%) > PPE + $CaCl_2$ Group (90%) > PPE + BAPN Group (40%); Aneurysm formation rate: PPE + BAPN Group (100%) > PPE + $CaCl_2$ Group (80%) > PPE Group (60%) > Control Group (0%); Aneurysm rupture rate: PPE + BAPN group (60%) > PPE + $CaCl_2$ group (12.5%) > PPE group (0%);Inflammatory cells (macrophages, T cells, B cells, dendritic cells) infiltrated in different degrees in the PPE + $CaCl_2$, PPE and PPE + BAPN groups. Vascular thickness, elastic fiber content, collagen fiber content, and vascular smooth muscle cell content in the PPE + $CaCl_2$ group and PPE + BNPA group were significantly lower than those in Control group ($P < 0.05$). The content of elastic fibers and vascular smooth muscle cells in the PPE

OPEN ACCESS

Edited by:
Norbert Gerdes, Heinrich Heine University Düsseldorf, Germany

Reviewed by:
Marie Le Borgne, INSERM U1148 Laboratory for Vascular Translational Science, France
Markus Udo Wagenhäuser, Heinrich Heine University of Düsseldorf, Germany

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *03 November 2021*
Accepted: *17 January 2022*
Published: *23 February 2022*

Citation:
Zhu J-X, Tang Q-Q, Zhou C, Shi X-C, Yi S-Y and Yang Y (2022) Establishment of a New Abdominal Aortic Aneurysm Model in Rats by a Retroperitoneal Approach. Front. Cardiovasc. Med. 9:808732. doi: 10.3389/fcvm.2022.808732

group were significantly lower than that in Control group ($P < 0.05$). The expression and activity of matrix metalloproteinase 2 (MMP2) and MMP9 in the PPE + $CaCl_2$ group, PPE group, and PPE + BNPA group were significantly higher than those in the control group ($P < 0.05$).

Conclusions: A new, simple, and reproducible rat AAA model can be constructed by a retroperitoneal approach. The pathological features of the three models are effective simulation of human AAA inflammatory cell infiltration, protease activity enhancement, and extracellular matrix destruction. The PPE+ $CaCl_2$ model has the advantages of a high survival rate, high aneurysm formation rate, good stability, and reproducibility. It is an ideal animal model for studying the pathogenesis of AAA. The PPE + BAPN model can simulate the characteristics of spontaneous rupture of aneurysms. It is an ideal animal model to study the mechanism of AAA rupture.

Keywords: abdominal aortic aneurysm, animal model, retroperitoneal approach, elastase, calcium chloride, beta aminopropionitrile

BACKGROUND

Abdominal aortic aneurysm (AAA) is one of the most challenging cardiovascular diseases and usually has no symptoms but has the risk of spontaneous dilation and rupture. Once the aneurysm ruptures, the mortality rate is as high as 80% (1). Because of its potential lethality, AAA has always been a research hotspot. At present, the occurrence and development mechanism of AAA remains unclear. Therefore, it is of great significance to construct an ideal aneurysm model for studying the pathogenesis of AAA. A large number of studies have confirmed that the mixed chemical model based on high-pressure intra-arterial perfusion of elastase can better simulate the characteristics of inflammatory cell infiltration, endogenous protease activation, extracellular matrix degradation, smooth muscle cell apoptosis, and thrombosis, which is an ideal modeling method (2, 3). Unfortunately, this kind of modeling method is performed through an intraperitoneal approach, which destroys the integrity of the peritoneal cavity and loses extra body fluids and heat, which increases the chance of postoperative low-temperature reaction and abdominal infection (4). It is necessary to separate the peritoneum and retroperitoneum during the operation to prolong the operation time and increase the difficulty of the operation (5). The intestinal tract needs to be pulled during the operation, which is easily complicated by intestinal obstruction and increases postoperative complications (2). There are high mortality rates during the operation due to long-term and high-pressure perfusion and high disability rates due to lower limb ischemia after the operation (6). The difficulty of modeling, low success rate of operation, and high mortality during and after operation limit the wide application of this kind of model. However, the retroperitoneal approach can directly reach the abdominal aorta, avoiding the risk caused by the transabdominal approach. The retroperitoneal approach can be performed in the lateral position through a lumbar incision or prone position through a back incision. Therefore, in this study, a retroperitoneal approach was used to simplify the operation. At the same time, the models of elastase combined with calcium chloride, single elastase, and elastase combined with beta aminopropionitrile were compared, and it was expected to build a new rat AAA model with a high success rate, high aneurysm formation rate, low mortality rate, strong anthropomorphism, and replicability.

Abbreviations: AAA, abdominal aortic aneurysm; PPE+$CaCL_2$, elastase + calcium chloride; PPE, elastase; PPE+BAPN, elastase + beta aminopropionitrile; MMPs, matrix metalloproteinases.

METHODS

Rats and Ethics Statement

Forty healthy adult SD rats were purchased from the Laboratory Animal Center of North Sichuan Medical College. The experiments involving animals were approved by the Animal Care and Use Ethical Committee of General North Sichuan Medical College and complied with the Guide for the Care and Use of Laboratory Animals approved by the National Institutes of Health.

Experimental Grouping

Forty rats were randomly divided into the control group, elastase + calcium chloride group (PPE + $CaCl_2$), elastase group (PPE), and elastase + beta aminopropionitrile group (PPE + BAPN) according to a male-female ratio of 1:1, with 10 rats in each group. All the rats were fed for 4 weeks after the operation, and abdominal aorta specimens were collected by laparotomy 4 weeks after the operation.

Establishment of Animal Model

PPE Model

① The rats were fasted 12 h before the operation and anesthetized with pentobarbital sodium at 40–50 mg/kg. The right lateral position was taken after anesthesia. Conventional skin preparation, disinfection, and towel laying occurred. The line from the costal edge of the left posterior axillary line to the thigh root was taken as an incision, with a

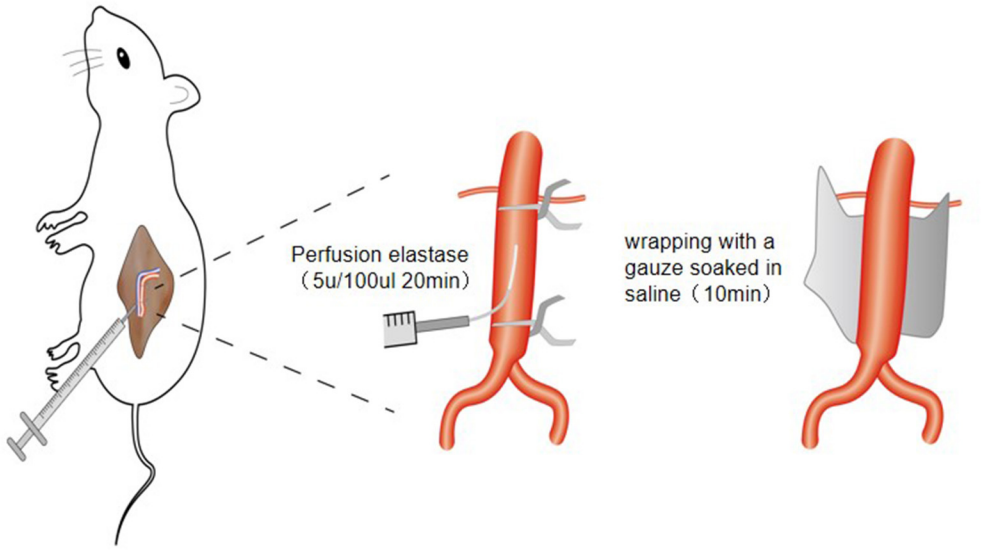

FIGURE 1 | Schematic diagram of establishing animal model of abdominal aortic aneurysm in rats through retroperitoneal approach.

length of approximately 3–3.5 cm. After cutting the skin, subcutaneous tissue, internal oblique muscles, external oblique muscles, and transverse abdominal fascia layer by layer, retroperitoneal adipose tissue was observed. The potential gap between the peritoneum and lumbar dorsal muscles could be found along the adipose tissue. Separating this gap with cotton swabs allowed us to reach the abdominal aorta directly. The abdominal aorta and inferior vena cava were separated carefully, and the 1 cm main abdominal aorta was dissociated below the left kidney. The diameter of the abdominal aorta under the kidney was measured with a Vernier caliper and recorded. The average value was measured by two experimenters.

② The abdominal aortic branch of the perfusion segment was fully freed and ligated with mousse thread. The surgical field of vision was fully exposed with a mastoid distractor. First, the proximal end of the abdominal aorta was blocked with a microvascular clamp, then the blood in the perfusion section was squeezed to the distal end with a microvascular clamp, and the distal end of the abdominal aorta was blocked with a microvascular clamp (the distance between the two vascular clamps should be greater than 0.5 cm). The blood vessels collapsed completely, indicating that the blood vessels were well sealed. Then, 0.2 mL of elastase (approximately 10 U) was extracted with a disposable insulin syringe (1 mL 30G). The syringe needle was bent slightly with vascular forceps to make it slightly "L" shaped, and then used to puncture the abdominal aorta. After successful puncture, 0.1 mL of elastase (approximately 5 U) was slowly injected to fully fill the abdominal aorta in the perfusion segment. The puncture needle was fixed and maintained for 20 min. During this period, if there was little extravasation of elastase, it was supplemented in time to keep the abdominal aorta in a full filling state at all times. After the operation, the medicine was drawn in the blood tube, the needle was pulled out, the puncture point was covered with a gelatine sponge, and the tube was pressurized with a cotton ball. The distal vascular clip was removed first, followed by the proximal vascular clip. After observation for several minutes, no active bleeding was found. A small gauze soaked in normal saline was applied to the surface of the abdominal aorta in the perfusion section, and the small gauze was removed after 10 min. The retroperitoneal space was cleaned, checked for bleeding, and the incision closed layer by layer. After the operation, the rats were raised in a single cage and fed routinely (**Figure 1**).

PPE + $CaCl_2$ Model

On the basis of the PPE model, the normal saline small gauze was replaced with a 1.0 mol/L sterile calcium chloride small gauze.

PPE + BAPN Model

Combined with 0.3% BAPN drinking water/day on the basis of the PPE model, the success rate, survival rate, aneurysm formation rate, and aneurysm rupture rate were calculated. Success rate of operation = number of rats surviving intraoperatively/total number of rats × 100%; Survival rate = number of surviving rats during and after operation/total number of rats × 100%; Aneurysm formation rate = number of rats meeting aneurysm standard/total number of rats surviving the operation × 100%; Aneurysm rupture rate = number of aneurysm ruptured rats/total number rats with aneurysms × 100%; Abdominal aortic dilatation rate = (abdominal aortic diameter 4 weeks after operation-abdominal aortic diameter before perfusion)/abdominal aortic diameter before perfusion × 100%, and abdominal aortic dilatation rate over 50% or aneurysm rupture 4 weeks after operation was taken as the standard for diagnosis of AAA (7).

TABLE 1 | Primer sequences for quantitative PCR (qPCR).

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-Actin	CAGGCATTGCTGACAGGATG	TGCTGATCCACATCTGCTGG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
MMP2	GACCCTGGAGCTTTGATGGC	GTGTAGGCGTGGGTCCAGTA
MMP9	GCTCGGATGGTTATCGCTGG	CCAGTTACAGTGACGTCGGC

Hematoxylin and Eosin, Masson, Verhoeff's Van Gieson (EVG) Staining, and Immunohistochemical Detection

Paraffin-embedded 5-μm-thick sections were stained with hematoxylin and eosin (HE) for general histology. Masson staining and Verhoeff's Van Gieson (EVG) staining were used to evaluate the integrity of collagen fibers and elastic fibers of the abdominal aorta, respectively. To detect the target protein expression, primary antibodies against CD3 (1:1000; Proteintech), CD20 (1:100; Invitrogen), CD68 (1:1000; Proteintech), OX-62 (1:500; BD), and smooth muscle actin (SMA) (1:3000; Proteintech) were used. Each section was randomly selected from five visual fields under high power to count cells or measure the area of positive areas, with PBS as the negative control.

Fluorescence Quantitative PCR (QPCR) Assay

The total RNA of the perfused abdominal aorta in each group was extracted according to the instructions of the TRIzol kit (Takara), and cDNA was synthesized by reverse transcription according to the instructions of the reverse transcription kit (Takara). The primer sequences are shown in **Table 1**. PCR conditions: the first step: predenaturation at 95°C 30 s for one cycle, PCR at 95°C 5 s → 60°C 30 s for 40 cycles, the second step: dissolution curve at 95°C 6 s → 65°C 5 s → 95°C 30 s for one cycle. Using β-Actin and GAPDH as reference genes, the relative expression level of target genes was calculated by extended ΔCt-method.

Western Blotting Assay

The aortic tissues were homogenized, and total protein was extracted. To detect the target protein expression, primary antibodies against matrix metalloproteinase 2 (MMP2) (1:1000 Proteintech), MMP9 (1:500 Proteintech), and GAPDH (1:20000 Proteintech) were used. After the images were collected by a Bio–Rad imaging system, the density of the gray value was analyzed by ImageJ, and the relative protein expression levels of MMP2 and MMP9 in the perfused abdominal aorta were calculated with GAPDH as the internal reference.

Gelatine Zymogram

The activities of MMP-2 and MMP-9 in rat abdominal aortae were detected by a MMP Zymography Assay Kit (Applygen). The kit instructions were followed.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21 statistical analysis software was used to analyze the data. The measurement data were expressed by M (P25, P75). The Mann–Whitney test was used to compare the measurement data between the two groups, and the Kruskal–Wallis H test was used to compare the measurement data between multiple groups. The x^2 test or Fisher's exact probability method was used for counting data. If P was < 0.05, the difference was statistically significant. ImageJ and Image-Pro Plus 6.0 software were used for image analysis and blood vessel parameter measurement.

RESULTS

Success Rate of Operation and Survival Rate of Animals

The operation time of the four groups was controlled at approximately 40 min, and the success rate of the operation was 100%. There were no deaths during or after the operation in the control group and PPE group, and the survival rate was 100%. In the PPE + $CaCl_2$ group, there were no deaths during the operation, and one rat died on the 7th day after the operation. The cause of death was aneurysm rupture and hemorrhage, and the survival rate was 90%. In the PPE + BAPN group, there were no deaths during the operation; one rat died on the 3rd, 10th, 21st, and 22nd days after the operation, and two rats died on the 26th day after the operation. The cause of death was rupture and hemorrhage of the aneurysm, and the survival rate was 40%.

Dilatation Degree of the Abdominal Aorta, Aneurysm Formation Rate, and Aneurysm Rupture Rate

There was no significant difference in the diameter of the abdominal aorta before perfusion among the control group, PPE + $CaCl_2$ group, PPE group, and PPE + BAPN group ($p > 0.05$). At 4 weeks after the operation, the diameter and dilatation rate of the perfusion abdominal aorta in the PPE group, PEE + $CaCl_2$ group, and PPE + BNPA group were significantly larger than those in the control group ($P < 0.05$), but there was no significant difference among the other groups ($P > 0.05$). No aneurysm was found in the control group, and the aneurysm formation rate was 0%. In the PPE group, there were 6 aneurysms (4 males and 2 females, aneurysm formation rate = 60%), and there was no aneurysm rupture, the aneurysm rupture rate was 0%. In the PPE + CaCl2 group, 8 aneurysms were formed (5 males and 3 females, aneurysm formation rate = 80%), and one of the aneurysms ruptured; the rupture rate of aneurysms was 12.5%. In the PPE + BAPN group, 10 aneurysms were formed (5 males and 5 females, aneurysm formation rate = 100%), and six of the aneurysms ruptured, the rupture rate of aneurysms was 60% (**Table 2**, **Figure 2**).

TABLE 2 | Diameter of abdominal aorta of rats in each group.

Group	Number of rats	Preoperative diameter (mm)	Diameter when taking specimens (mm)	Diameter expansion rate (%)	Aneurysm formation rate (%)	Aneurysm rupture rate (%)
Control group	10	1.20 (1.20, 1.30)	1.30 (1.28, 1.32)	8.01 (0.00, 8.33)	0 (0/10)	0 (0/0)
PPE+$CaCL_2$ group	10	1.30 (1.15, 1.35)	2.20 (1.85, 3.90)$^{\#\#}$	116.67 (51.75, 200.00)$^{\#\#}$	80 (8/10)$^{\#\#}$	12.5 (1/8)
PPE group	10	1.25 (1.10, 1.40)	2.10 (1.68,3.25)$^{\#\#}$	68.94 (37.94, 132.14)$^{\#\#}$	60 (6/10)$^{\#}$	0 (0/6)
PPE+BNPA group	10	1.25 (1.13,1.45)	4.20 (3.40, 5.00)$^{\#\#}$	198.97 (177.40, 326.06)$^{\#\#}$	100 (10/10)$^{\#\#}$	60 (6/10)

The measurement data were expressed by M (P25, P75); M, Median; P25, 1st quartile; P75, 3rd quartile; #: P < 0.05, ##: P < 0.01 compared with the control group.

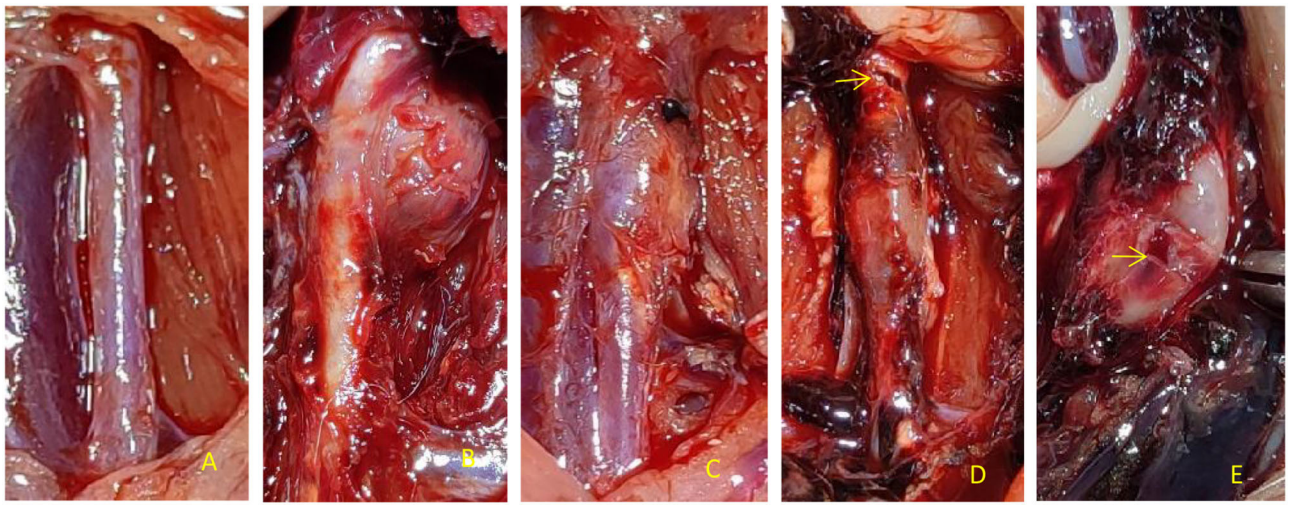

FIGURE 2 | Dilatation degree and aneurysm appearance of the abdominal aorta in rats. **(A)** There was no dilation of abdominal aorta in perfusion segment in Control group, **(B)** PPE + $CaCl_2$ group perfusion segment abdominal aortic saccular aneurysm local, **(C)** The fusiform aneurysm of abdominal aorta was perfused in PEE group, **(D)** In the PPE + BNPA group, the rupture of early aneurysm was in the anterior wall; **(E)** In the PPE + BNPA group, the rupture of late aneurysm was in the posterior wall.

Morphological and Histological Changes in the Abdominal Aorta in the Perfusion Segment

The abdominal aortic aneurysms in the PPE group and PPE + BAPN group were mostly fusiform. All AAAs in the PPE + $CaCl_2$ group were cystic. There were obvious adhesions around the abdominal aorta in the perfusion segment, and local calcifications could be seen. Microscopically, the vascular wall became thinner, the extracellular matrix of the media was degraded, some dissections and thrombosis were formed, the elastic fibers lost continuity, some were broken and missing, the smooth muscle cells decreased, and the inflammatory cells of the media and adventitia infiltrated. The control group did not have the above phenomenon. Compared with control group, the content of elastic fibers and smooth muscle cells in PPE + $CaCl_2$, PPE, and PPE + BAPN groups decreased significantly ($P < 0.05$), the thickness of blood vessels and the content of collagen fibers in PPE + $CaCl_2$ and PPE + BAPN groups decreased significantly ($P < 0.05$), but there was no significant difference in the thickness of blood vessels and the content of collagen fibers in PPE group ($P > 0.05$) (**Table 3**, **Figure 3**).

Immunohistochemical Analysis of Inflammatory Cells in the Abdominal Aortic Wall

There was no significant infiltration of inflammatory cells in the control group, but T cells, B cells, macrophages, and dendritic cells infiltrated to different degrees in the other three groups. The number of inflammatory cells in the PPE + $CaCl_2$ group was as follows: B cells > T cells > macrophages > dendritic cells. The number of inflammatory cells in the PPE group and PPE + BAPN group was as follows: T cells > B cells > macrophages > dendritic cells. The number of T cells, B cells, macrophages, and dendritic cells in the PPE + BNPA group and PPE + $CaCl_2$ group was significantly higher than those in PPE group ($P < 0.05$), but there was no significant difference in other groups ($P > 0.05$) (**Figure 4**).

Expression and Activity of MMP2 and MMP9 in the Abdominal Aorta Wall

Compared with the control group, the expression of MMP2 and MMP9 mRNA and protein in the PPE + $CaCl_2$ group, PPE group, and PPE + BAPN group increased significantly

TABLE 3 | Comparison of vascular thickness, elastic fiber content, collagen fiber content, and smooth muscle cell content in the abdominal aorta of rats in each group.

Group	Number of rats	Thickness of blood vessel (μm)	Elastic fiber content (%)	Collagen fiber content (%)	Smooth muscle cell content (%)
Control group	10	48.49 (40.27, 76.24)	32.21 (29.80, 41.80)	24.79 (22.33, 28.14)	35.37 (31.08, 37.94)
PPE+$CaCL_2$ group	10	17.39 (13.66, 23.73)##	15.44 (10.27, 19.15)##	18.92 (16.22, 22.80)#	21.48 (17.28, 23.95)##
PPE group	10	28.42 (22.21, 39.56)	21.57 (11.68, 26.02)##	25.08 (22.64, 27.29)	23.76 (19.60, 25.59)#
PPE+BNPA group	10	14.78 (13.07, 15.66)##	10.75 (9.14, 13.57)##	16.64 (11.89,20.58)#	17.28 (15.66, 20.30)##

The measurement data were expressed by M (P25, P75); M, Median; P25, 1st quartile; P75, 3rd quartile; #: $P < 0.05$, ##: $P < 0.01$ compared with the control group.

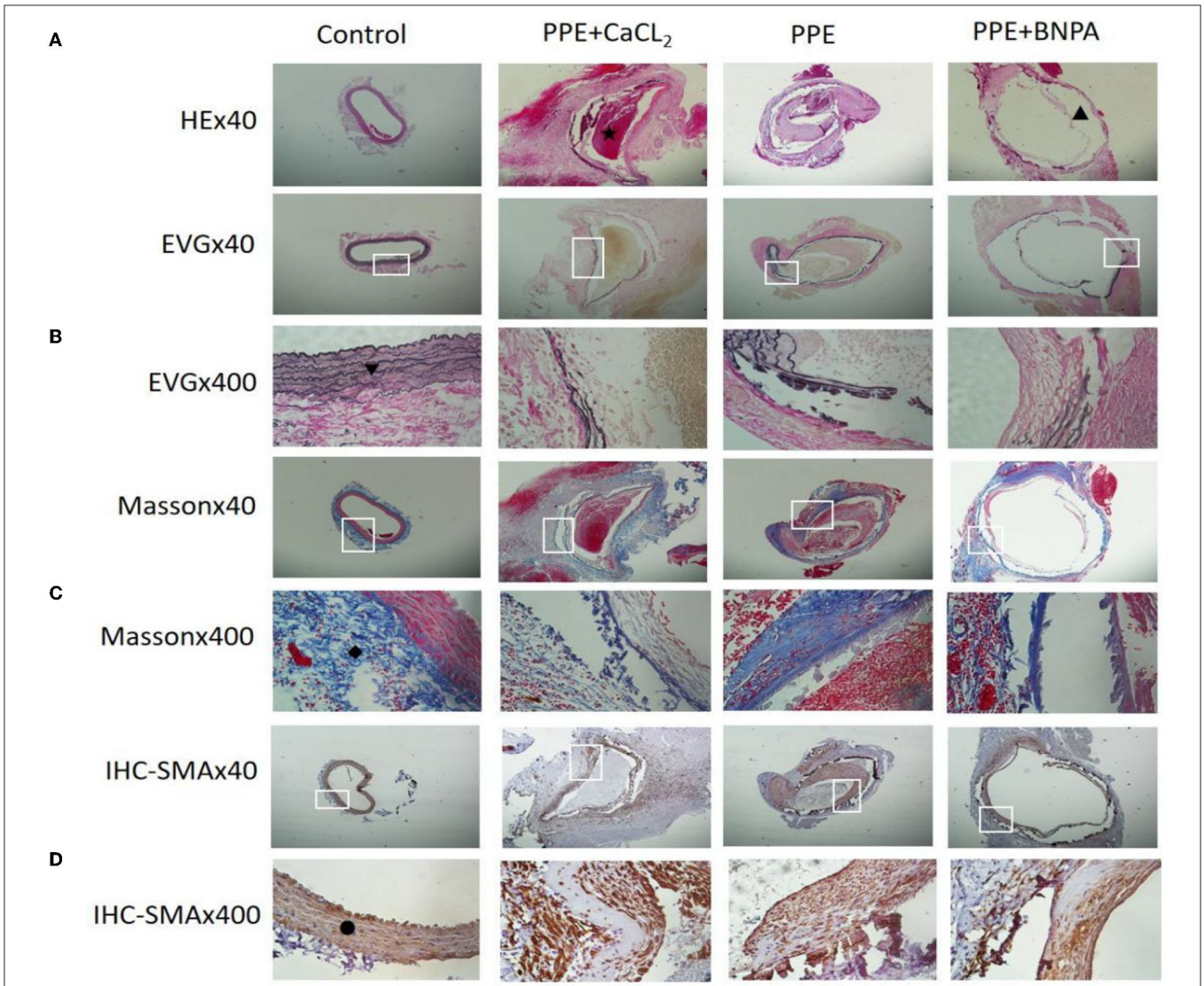

FIGURE 3 | Histopathological changes of abdominal aortas in rats. **(A)** Representative pictures of HE staining in each group; **(B)** Representative pictures of EVG staining in each group; **(C)** Representative pictures of Masson staining in each group; **(D)** Representative pictures of SMA-IHC staining in each group. ★: Indicates thrombosis; ▲: Indicates interlayer; ▼: Indicates elastic fibre; ♦: Indicates collagen fibre; ●: Indicates smooth muscle cells.

($P < 0.05$), and the activity of MMP2 and MMP9 increased significantly ($P < 0.05$). Compared with the PPE group, the expression of MMP2 mRNA and protein in the PPE + BAPN group increased significantly ($P < 0.05$), and the activity of MMP2 increased significantly ($P < 0.05$). There was no significant difference in MMP2 mRNA, protein expression, or MMP2 activity among the other groups ($P > 0.05$). There was no significant difference in MMP9 mRNA or protein expression

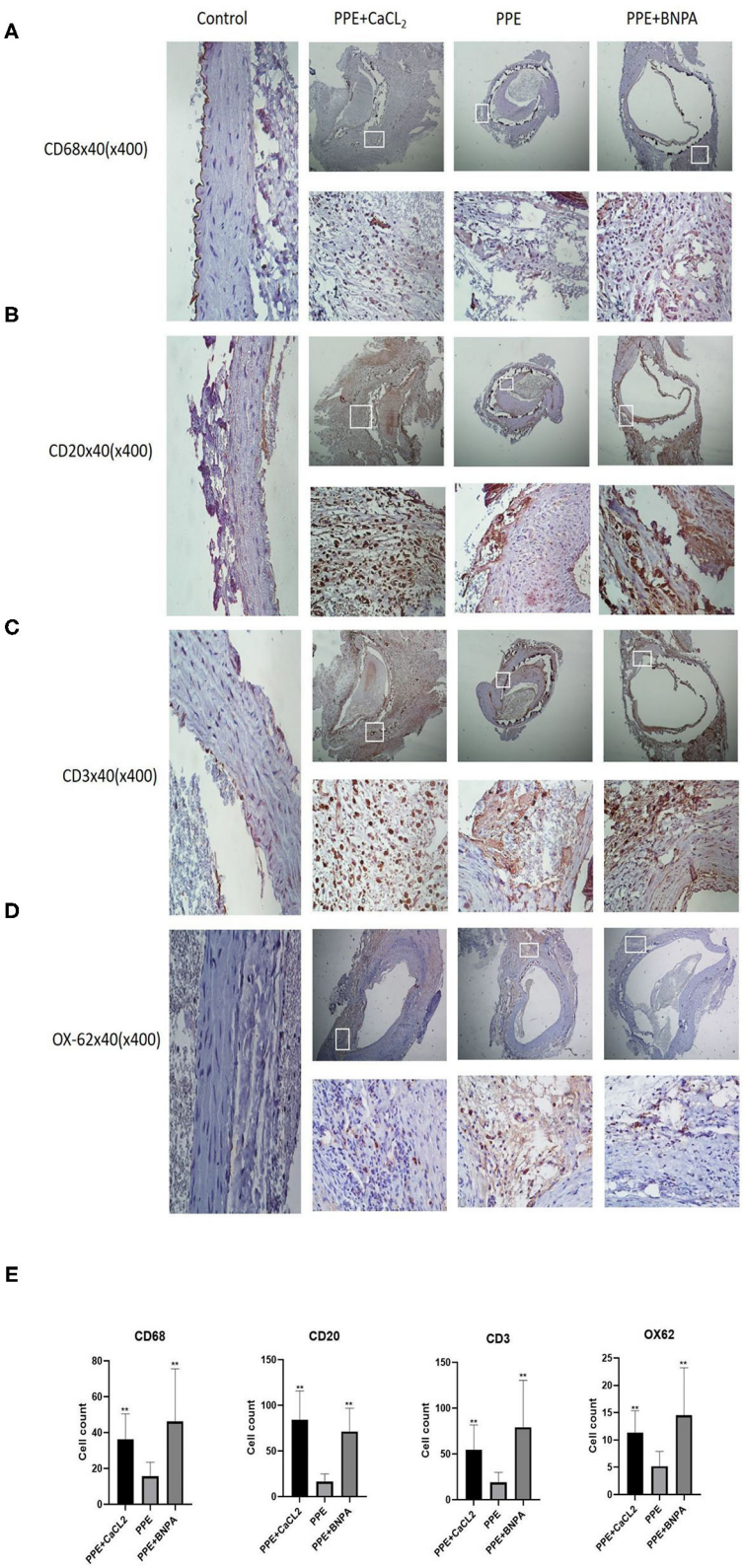

FIGURE 4 | Infiltration of inflammatory cells in the abdominal aorta wall of rats. **(A–D)** Representative pictures of immunohistochemical (IHC) staining of CD68, CD20, CD3, and OX-62 cells; **(E)** CD68, CD20, CD3, and OX-62 cell count analysis. *: $P < 0.05$, **: $P < 0.01$ compared with the PPE group; $n = 10$.

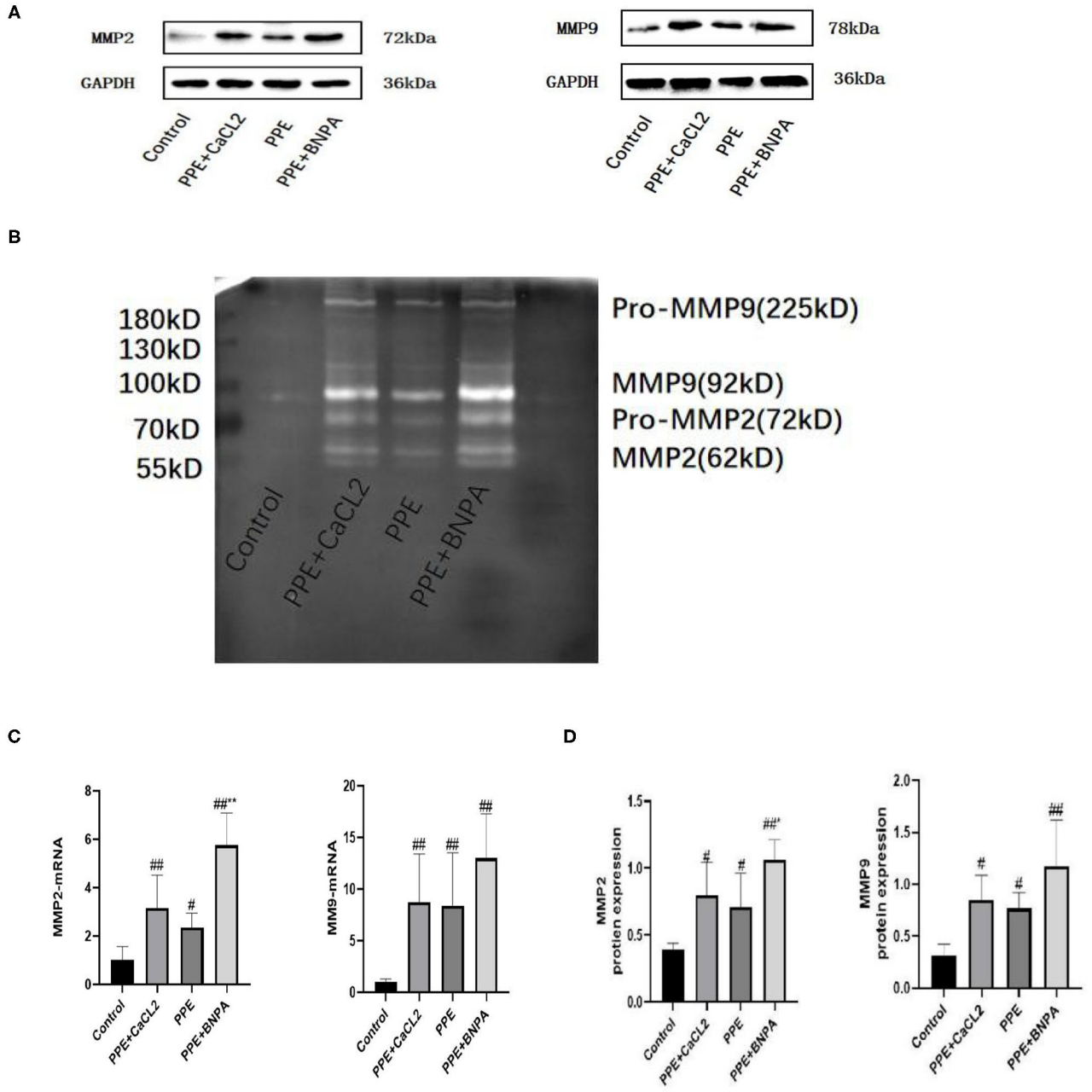

FIGURE 5 | Expression and activity of MMP2 and MMP9 in the abdominal aorta wall of rats. **(A)** Typical Western blot analysis of MMP9 and MMP2 protein expression in each group; **(B)** Typical zymogram analysis of MMP9 and MMP2 protein activities in each group; **(C)** Quantitative analysis of relative expression of MMP2 and MMP9mRNA, $n = 6$; **(D)** Quantitative analysis of relative expression of MMP2 and MMP9 protein, $n = 4$; *: $P < 0.05$, **: $P < 0.01$ compared with the PPE group; #: $P < 0.05$, ##: $P < 0.01$ compared with the control group.

or MMP9 activity among the PPE + $CaCl_2$ group, PPE group, and PPE + BAPN group ($P > 0.05$) (**Figure 5**).

DISCUSSIONS

At present, the pathogenesis of AAA is not clear. Constructing a simple and reliable animal model of AAA provides a theoretical and practical basis for studying the pathogenesis and treatment of AAA. The animal models of AAA can be divided into three categories: physical, chemical, and gene induction methods, among which the chemical induction (angiotensin II, elastase, calcium chloride, beta aminopropionitrile) models are mainly used to study the pathogenesis (8).

The pressurized perfusion of elastase established by Anidjar et al. (6) is one of the most commonly used chemical modeling

methods at present and simulates the degradation process of extracellular matrix elastase during the formation of AAA. Although a large number of reports prove the feasibility of this method, this method still has many shortcomings. This method adopts an abdominal approach. The operation is complicated, the integrity of the peritoneal cavity is destroyed, and the body fluids and heat are lost, which increases the risk of postoperative hypothermia reaction and abdominal infection. The intestinal tract should be pulled during the operation, which easily causes intestinal obstruction and injury to abdominal organs. The peritoneum and retroperitoneum should be separated to prolong the operation time and increase the risk of death. For a long time (30 min-2 h), high pressure (>100 mmHg) and high dose (12 U) of elastase perfusion led to an intraoperative and postoperative mortality rate of 40% (6, 9–11). In this study, the surgical path and some details were improved on the basis of the classical elastase model. ① The retroperitoneal approach is a common surgical method in urology (12). Because the abdominal aorta and kidney are adjacent to each other and belong to extraperitoneal organs, it is possible to construct a rat model of infrarenal AAA through a retroperitoneal approach. The advantage of the retroperitoneal approach is that it can reach the abdominal aorta directly. There was no interference from internal organs, and the operation was relatively simple. It can avoid the risk caused by the intraperitoneal approach and significantly improve the success rate of the operation. However, there are the following limitations: (a) The retroperitoneal space is an unnatural space and lacks body surface markers. Therefore, it is more difficult to find the surgical entrance than traditional abdominal surgery. (b) The retroperitoneal space is narrow, which limits visual field exposure and operating space, and is difficult to completely free and expose the entire segment of the abdominal aorta. ② It is not necessary to completely free and expose the whole infrarenal abdominal aorta and ensure that the perfusion abdominal aorta is larger than 0.5 cm. Regardless of which approach is adopted, the excessive free and exposed blood vessels will increase the side injury and lead to massive hemorrhage, or local tissue and spinal cord ischemia due to ligation of too many branches and lumbar arteries, which will increase the risk of death in rats. ③ Classical elastase perfusion needs to maintain a perfusion pressure of 100 mmHg or higher by hydraulic or micropump syringe, but it is difficult to accurately reach and maintain the same pressure. Excessive pressure can cause elastase to enter the circulation and lead to the death of rats (10, 13, 14). After perfusion, it is necessary to ligate the punctured iliac artery or femoral artery, which is prone to unilateral limb ischemia and necrosis, and the disability rate is high (2, 6). Although there are reports that the puncture site is changed to the abdominal aorta, after perfusion, the absorbable vascular sutures are replaced to repair the puncture site of the abdominal aorta and restore the original anatomical structure of the abdominal aorta (15). This method requires microsurgical technology, which is difficult to operate, and the operator needs a certain vascular surgical foundation, which limits its wide application. The pressurized perfusion was not used in this study because there is no external mechanical pressure in the formation of human AAA (2). It is only necessary to slowly inject elastase into the abdominal aorta of the perfusion section to keep it full. The selected puncture needle was an insulin needle (30G), which causes minimal damage to blood vessels. After perfusion, local compression can stop bleeding. The success rate of operation is significantly improved, and the model is more in line with the formation process of human AAA. This method is simple and easy to use, without the help of microsurgical techniques and expensive instruments such as micropumps and is easy to popularize. ④ The dosage of elastase was reduced (5 U), and an excessive dosage of elastase easily led to the death of rats (14). ⑤ The perfusion time was shortened (20 min). The classical elastase perfusion time is 2 h. Too long of a time to block the abdominal aorta easily forms thrombi, and a long-term concentration of blood above the blocking site easily leads to brain oedema and lower limb ischemia, with high mortality (16). Sinha et al. (17) shortened the perfusion time to 30 min and successfully established AAA, which significantly reduced the occurrence of brain oedema and mortality, but there remained a certain proportion of lower limb ischemia incidence. In this study, the perfusion time was shortened to 20 min, and no lower limb ischemia occurred.

This study confirmed that it is feasible to construct a rat elastase model through a retroperitoneal approach. However, the aneurysm formation rate is only 60%, which is related to the perfusion of elastase at only 0.1 mL (5 U). To further improve the aneurysm formation rate, we should consider moderately increasing the perfusion amount and concentration of elastase and prolonging the perfusion time, but these changes may lead to an increase in mortality. In this study, to avoid affecting the survival rate while further increasing the rate of aneurysm formation, two methods of adding calcium chloride and beta aminopropionitrile to elastase perfusion were investigated separately. Calcium chloride (18) induced the inflammatory reaction of the arterial wall, while beta aminopropionitrile (19) inhibited the cross-linking process of collagen and elastin to construct an AAA animal model. Using these two methods alone has the disadvantage of a low aneurysm formation rate. Therefore, in this study, two chemical induction methods were combined to construct an AAA animal model to improve the rate of aneurysm formation. In this study, in the elastase combined with calcium chloride group, the success rate of the operation was 100%, the survival rate was 90%, the total aneurysm formation rate was 80%, the male aneurysm formation rate was 100%, and the female aneurysm formation rate was 60%. Consistent with the epidemiological investigation of human AAA, the incidence rate of males was higher than that of females (20). The model has good stability and is suitable for studying the pathogenesis of AAA. In the elastase combined with beta aminopropionitrile group, although the formation rate of aneurysms was 100%, the rupture rate of aneurysms was as high as 60%. The model has poor stability and is suitable for the study of dynamic AAA fracture mechanisms. There is also an angiotensin II (21) model that can simulate the unique characteristic atherosclerosis phenomenon of human beings, but it needs to use gene knockout

mice, which has limited sources, strict requirements on animal feeding and reproduction conditions, and certain restrictions on experimental research. Therefore, this study did not use angiotensin II for discussion.

In addition, the present study compared the morphology and pathology of the three models. The aneurysms in the Elastase Group and Elastase Combined with beta aminopropionitrile Group were mainly spindle-shaped, which was consistent with most AAAs found in human pathology. The aneurysms in the elastase and calcium chloride groups were all saccular. The synergistic effect of elastase and calcium chloride stimulates the inflammatory reaction and elastolytic cascade reaction of the abdominal aorta wall. The aneurysms in the three models were characterized by disordered arterial wall structure, thinning of the media, serious degradation of elastic fibers, loss of continuity or even disappearance, reduction of nucleated smooth muscle cells in the media, and dissection with mural thrombosis in some of the aneurysms. Inflammatory cells (macrophages, dendritic cells, T cells, and B cells) infiltrated to different degrees in both media and adventitia, especially T and B cells. However, the classical elastase model is dominated by macrophage infiltration (22, 23). This difference occurs because the time nodes observed are different. Macrophages mediate the innate immune response, which occurs immediately in the early stage of disease. T and B cells mediate the adaptive immune response, which occurs relatively late but runs through the disease all the time. The time node observed in this study is the 4th week, which is the late model, while the time node observed in the classical elastase model is the 1st week and the 2nd week, which is the early model. The infiltration of inflammatory cells in the elastase combined with calcium chloride group and elastase combined with beta aminopropionitrile group was greater than that in the elastase group. Inflammatory cells are the main source of MMPs in the arterial wall, and MMP2 and MMP9 are important proteases that degrade the extracellular matrix of arterioles, which are the main causes of AAA (24). The results of this study also confirmed that the expression and activity of MMP2 and MMP9 were significantly increased in the three groups of aneurysms. It is worth mentioning that the expression and activity of MMP2 in the elastase combined with beta aminopropionitrile group (aneurysm rupture group) were significantly higher than those in the elastase group (no aneurysm rupture group), and the content of elastic and collagen fibers decreased most significantly in the three groups of models. The existing studies have shown that MMP2 degrades both elastic and collagen fibers, and the degradation of elastic fibers is associated with aneurysm dilation, while the degradation of collagen fibers is associated with aneurysm rupture (25). It can be concluded that MMP2 may play an important role in aneurysm rupture. This result is consistent with the research results of Lu et al. (26) and Sean et al. (27) believed that the increased activity of MMP9 was closely related to aneurysm rupture. The reasons for the different conclusions may be the different methods adopted by researchers, the different observation time nodes, and the great individual differences of the specimens taken.

CONCLUSIONS

Through the change of surgical approach and the improvement of intraoperative details, the mortality of experimental animals was obviously reduced, and the survival rate was improved. In this study, the elastase combined with calcium chloride model and the elastase combined with beta aminopropionitrile model were similar to human AAA in histomorphology, inflammatory cell infiltration, and vascular matrix destruction. Elastase combined with calcium chloride has the advantages of a high survival rate, high tumorigenic rate, good stability, and reproducibility. It is an ideal animal model for studying the pathogenesis of AAA. Elastase combined with a beta aminopropionitrile model can simulate the characteristics of spontaneous rupture of aneurysms. It is an ideal animal model to study the mechanism of AAA rupture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Ethical Committee of North Sichuan Medical college. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

J-XZ performed experiments, interpreted the data, and wrote the manuscript. YY conceived the research design, experimental plan, and manuscript revision. Q-QT participated in the experiment. X-CS, S-YY, and CZ participated in the data analysis. All the authors read and approved the final draft.

FUNDING

This work was supported by the Sichuan Province Science and Technology Planning Project (2016JY0172) and the Scientific Research Project of Sichuan Provincial Health and Family Planning Commission (16PJ121).

ACKNOWLEDGMENTS

The author thanks Master Feng Song for his help in exploring the establishment of animal models in the early stage.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.808732/full#supplementary-material

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frontiers | Frontiers in Cardiovascular Medicine

REVIEW
published: 07 April 2022
doi: 10.3389/fcvm.2022.823549

Platelet Subtypes in Inflammatory Settings

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In addition to their essential role in hemostasis and thrombosis, platelets also modulate inflammatory reactions and immune responses. This is achieved by specialized surface receptors as well as secretory products including inflammatory mediators and cytokines. Platelets can support and facilitate the recruitment of leukocytes into inflamed tissue. The various properties of platelet function make it less surprising that circulating platelets are different within one individual. Platelets have different physical properties leading to distinct subtypes of platelets based either on their function (procoagulant, aggregatory, secretory) or their age (reticulated/immature, non-reticulated/mature). To understand the significance of platelet phenotypic variation, qualitatively distinguishable platelet phenotypes should be studied in a variety of physiological and pathological circumstances. The advancement in proteomics instrumentation and tools (such as mass spectrometry-driven approaches) improved the ability to perform studies beyond that of foundational work. Despite the wealth of knowledge around molecular processes in platelets, knowledge gaps in understanding platelet phenotypes in health and disease exist. In this review, we report an overview of the role of platelet subpopulations in inflammation and a selection of tools for investigating the role of platelet subpopulations in inflammation.

Keywords: platelets, reticulated platelets, procoagulant platelets, vascular, immunology, inflammation

OPEN ACCESS

Edited by:
Florian Kahles, University Hospital RWTH Aachen, Germany

Reviewed by:
Alice Assinger, Medical University of Vienna, Austria
Matthew Dean Linden, University of Western Australia, Australia

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *27 November 2021*
Accepted: *09 March 2022*
Published: *07 April 2022*

Citation:
Hamad MA, Krauel K, Schanze N, Gauchel N, Stachon P, Nuehrenberg T, Zurek M and Duerschmied D (2022) Platelet Subtypes in Inflammatory Settings. Front. Cardiovasc. Med. 9:823549. doi: 10.3389/fcvm.2022.823549

INTRODUCTION

Platelets promptly initiate a set of responses at the endothelium upon encountering molecular or biophysical cues of aberrations in vascular flow, form, or function. Such responses include platelet adhesion to endothelium, shape change, secretion, and aggregation which is physiologically critical to limit vessel leakage and prevent bleeding (1, 2). There are roughly 300,000 platelets per μl of blood, with a cell volume of 7 fl and a mean surface area of 8 μm^2, which makes them display a larger total volume and surface area compared to all other leukocyte subtypes. Platelet involvement in inflammatory or immune processes *via* their proinflammatory mediators as well as surface receptors clearly shows that they have a role that exceeds being mere players in hemostasis and thrombosis. Thrombus formation can be divided into 3 distinct phases: adhesion,

activation, and aggregation of platelets (3). Upon activation, platelets release considerable quantities of secretory products and express a multitude of immune receptors on their membrane giving them the ability to support the recruitment of leukocytes into inflamed tissue and regulate their function. Platelets are able to form aggregates with neutrophils (Platelet-Neutrophil Complexes, PNCs), which leads to mutual activation of both cells resulting in cytokine release, exposition of certain adhesion molecules, and receptors on the cell surface which in turn facilitates extravasation of these cells into inflamed tissue (4). The PNC formation is mainly mediated by the platelet's P-selectin (CD62P) and its ligand P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils (4, 5). The importance of the P-selectin/PSGL-1 axis has been shown, as blocking platelet CD62P could abolish PNC formation in murine and human whole blood samples (6). Hence, platelets provide an ideal and crucial link to explain the inseparability of thrombotic and inflammatory events such as atherosclerosis or atherothrombosis. Circulating platelets differ one from another with respect to their (a) size (7, 8), (b) surface receptor expression (9–11), (c) glycosylation (12), (d) granule content (13, 14), (e) response to agonist stimulation (15–17), and (f) participation in thrombus formation (18), meaning that within the normal platelet pool there are some distinct subpopulations each performing a certain role in different settings. Indeed, in contrast to rapid shape change and other responses platelets can also undergo more extended transitions in phenotype that are increasingly associated with chronic disease (1, 19, 20). The phenotype in its generalized concept refers to the observable, distinguishable, or measurable type of phenomenon exhibited by a biological entity resulting from the interaction of its genotype and environment (21, 22). The notion to describe single-cell properties of platelets or platelet subpopulations that deviate from normal is gaining more attention to evaluate whether these phenotypes are indicative or causative agents of disease. Both *in vitro* as well as *in vivo* studies have begun to catalog heterogeneous subpopulations of platelets described as procoagulant, "angry," coated, secretory, exhausted, or sticky – in different pathological settings. Despite the wealth of knowledge around molecular processes in platelets, knowledge gaps in understanding platelet phenotypes in health and disease exist. Here, we present an overview of different platelet phenotypes and their behavior during an inflammatory response.

PLATELET SUBTYPES

Platelets have different physical properties leading to distinct subtypes of platelets based either on their function (procoagulant, aggregatory, secretory) (**Figure 1**) or their age (reticulated/immature, non-reticulated/mature). Indeed, it has been described that at wound sites there is a subpopulation that is the first to adhere to collagen and spread to form a monolayer known as "vanguard platelets" and a second population that adheres to and spread onto nearby collagen or over the vanguard platelets described as "follower platelets" (23). When "vanguard platelets" adhere to collagen, they rapidly begin to spread and lose the distinctive mound-shaped structure. Then, this process is usually followed by additional adhesion of vanguard platelets as well as other platelets (follower platelets) (23). The platelet-platelet interactions are crucial for follower platelets deposition thus, functional GPIIb/IIIa receptors are indispensable. From another perspective, another platelet subpopulation lacks endothelial nitric oxide synthase (eNOS), fails to produce nitric oxide, and has a down-regulated soluble guanylate cyclase signaling pathway. In turn, this subpopulation of platelets shows greater activation of αIIbβ3 and adhesion to collagen, resulting in larger aggregates than eNOS-positive platelets (24). In the concept of heterocellular control of coagulation, platelets can be distinguished in different tasks such as control of thrombin generation, support of fibrin formation, and regulation of fibrin clot retraction (25). Within the functional scope, there are two distinct phenotypes of platelets with distinct surface properties facilitating these coagulant functions. One is a phenotype that externalizes phosphatidylserine (PS) and binds tenase and prothrombinase complexes, leading to accelerated coagulation at the wound site and controlling thrombin and fibrin generation (25). A second phenotype is characterized by active integrin αIIbβ3, which tightens the clot into an impermeable cell mass by pulling fibrin over the platelet plug (25). The youngest platelet subtype released into the circulation appears to be more reactive and shows an increased tendency to recruit other platelets and immune cells to the site of injury. The newly formed platelets contain a residual amount of the megakaryocytic messenger RNA (mRNA) that gives them a greater array of functional pathways (26). As platelets age, the total protein content is degraded or lost without the possibility for replacement leaving old platelets with several biological alterations in function (26). Differences related to platelet age propose a young platelet subpopulation that are rapid hemostatic responders and an old platelet population with higher apoptosis and senescence. Some data also showed that lung megakaryocytes (Mks) have immune cell characteristics that differ from bone marrow (BM) Mks characterized by antigen-presenting-cell-like cell markers and functions (27). These site-specific cell characteristics may in part be driven by the tissue environment as lungs and BM are very different tissue environments. In the BM, Mks face a few pathogen challenges and the environment is relatively hypoxic, while in the lung there's high oxygen (O_2) as well as a microbiome. Such immune regulatory functions of Mks described here are likely to be forwarded to the platelet progeny. It is clear that there are intrinsic platelet factors (such as platelet size and structure, protein composition, genetic factors, and platelet age), and environmental factors (such as the local rheology, exposure to agonists, surrounding cells, and plasma) that account for the response heterogeneity. The evidence that supports the concept of functionally different subpopulations of platelets is well-reported and targeting platelet subpopulations might be an encouraging antithrombotic approach.

Procoagulant and Aggregatory Platelets

There are major differences between aggregatory and procoagulant platelets which leads to the question of how a platelet becomes procoagulant while another does not. For a platelet to become procoagulant, it is required to have a

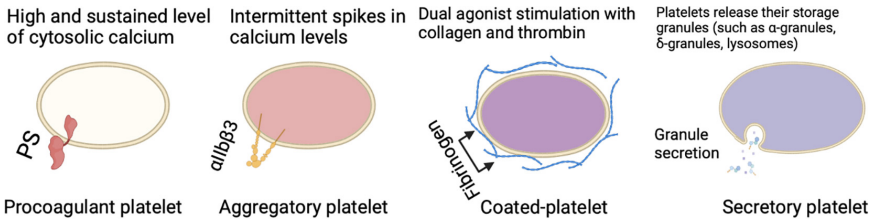

FIGURE 1 | Different circulating platelet subtypes each perform a certain role in different settings and participation in thrombus formation.

high and sustained calcium rise leading to PS externalization, coagulation factor binding, and calpain-mediated inactivation of $\alpha_{IIb}\beta_3$ integrin (23, 28–30). The fundamental calcium rise for the procoagulant response is led by calcium mobilization from intracellular stores, which is associated with the activation of calcium (Ca^{2+}) activated chloride channels, resulting in an initial salt entry, which is then followed by the influx of water (23, 25, 31, 32). The electrochemical drive for Ca^{2+} entry is enhanced as well as membrane hyperpolarization as a result of the chloride ion entry, and that's achieved through both store-operated and store-independent pathways (23, 29, 31–33). Jointly these responses guarantee a high and sustained level of cytosolic calcium required to drive the procoagulant response. It is important to emphasize that the irreversible membrane swelling or ballooning that results from the physical disruption of the membrane-cytoskeleton interaction and an increase in internal hydrostatic pressure provided by a coordinated fluid entry system is a key event during procoagulant platelet formation (23). All these changes lead to a distinct population of highly activated platelets characterized by surface-exposed PS, prolonged cytosolic Ca^{2+} rises, a rounded structure, and the ability to bind coagulation factors such as factor V (FV) and factor X (FX) (25). Meanwhile, a different pattern of calcium signaling is found in aggregate-forming platelets, which is rather characterized by intermittent spikes in calcium levels or oscillatory calcium responses (18, 23, 29, 34). Aggregatory platelets have active $\alpha_{IIb}\beta_3$ integrins on their surface which is a major difference to PS-exposing platelets enabling them to consolidate the plug by clot retraction (25). This might be seen as a mechanism for narrowing the gaps between platelets to allow contact-dependent signaling (35). Upon dual agonist stimulation of platelets with collagen and thrombin, a subpopulation of cells is observed known as coated-platelets (formerly known as COAT-platelets), which retains high levels of several procoagulant proteins on its surface resulting in an unparalleled ability to promote thrombin generation (36).

Secretory Platelets

Besides the procoagulant and aggregatory roles that platelets play, upon activation, platelets act as secretory cells. Platelets contain multiple storage granules (such as α-granules, δ-granules, and lysosomes) that release their content when activated by fusing the intracellular granules with the plasma membrane. Besides the intracellular vesicles, platelets are able to produce extracellular vesicles, these secretions in turn can influence many physiological and pathophysiological processes. The importance of platelet secretion granules and their content (such as growth factors, chemokines, cytokines, and microbicidal proteins) can be further elucidated by looking at platelets lacking α-granules (such as in gray platelet syndrome), δ-granules (such as in Hermansky-Pudlak syndrome), or both can result in bleeding, reduced inflammation, and impaired vascular remodeling and wound healing (37). The extracellular vesicles in turn which can be further classified to exosomes and microvesicles also seem to play a role in blood-related processes (38). In the context of inflammation, platelet-derived extracellular vesicles interact with leukocytes and their inflammatory role can be observed in rheumatoid arthritis stimulating cytokine production from synovial fibroblasts (37). Not only they are able to secrete multiple products, but platelets are also able to take up plasma-derived or cell-derived components such as RNA species from tumor cells (39). Taken together, all of these multiple mechanisms indicate that there is bidirectional communication of platelets and platelet-derived mediators with components of the inflammatory pathways, in a manner that platelets influence their environment, and their environment in return has an influence on them. This concept can be further supported by the observation of platelets interaction with leukocytes and the coagulation system during thromboinflammation. The so-called "exhausted platelets" which is a phenotype seen in patients with solid tumors, sepsis, or stroke, characterized by low platelet activation responses *in vitro,* is also another example of how the environment affects platelets (40).

Young and Senescent Platelets

Platelets are anucleate cells that circulate for approximately 7–10 days during which their protein composition change as they age leading to alterations in structure and function. Reticulated platelets (RP) (also known as immature platelets) represent the youngest platelets released into the circulation from Mks and are referred to as "reticulated," analogous to reticulocytes in erythropoiesis (41). These young platelets appear to have increased RNA content compared to mature platelets as well as more dense granules and higher levels of surface activation markers upon stimulation (**Figure 2**) (37). Hence, this platelet fraction is considered to show increased reactivity and is associated with impaired response to antiplatelet therapy (42–44). RP is about 2–3 times higher in the BM compared to peripheral blood where they are present for ≤24 h in humans and count for around 12% of the total platelet population in a steady-state (45–47). Platelet aging is linked to a decrease in cytoskeletal

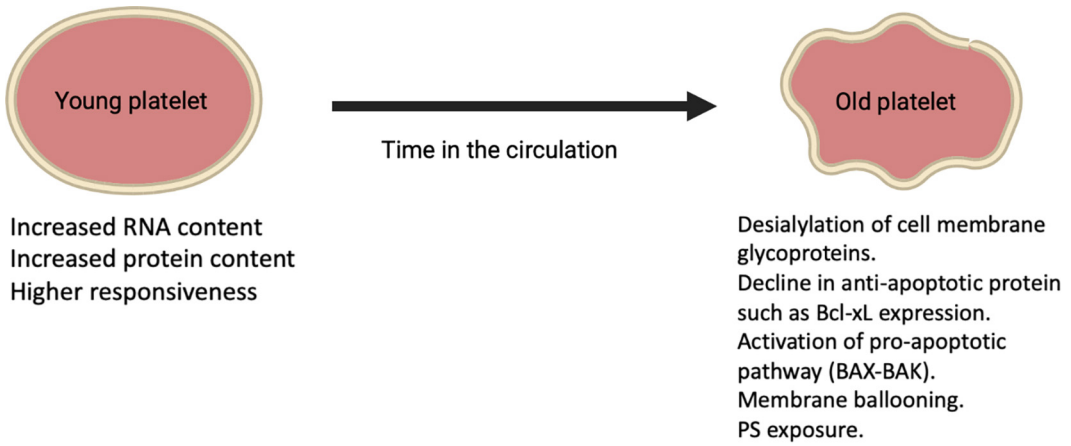

FIGURE 2 | Alterations on platelets during their lifetime in the circulation.

protein, lower mitochondria number, as well as lower calcium dynamics and granule secretion (26). A recent study showed that the total protein content was almost 50% lower in old platelets compared to young platelets (26). Besides, during conditions with increased platelet turnover, RP appear to be larger than mature platelets, for instance, in humans after chemotherapy (48). On the other hand, platelet size may not correlate with platelet age under steady-state platelet production and clearance further confirmed using the Abbott Sapphire analyzer showing a negative association between RP and mean platelet volume (MPV) (49). Despite the fact that platelets are anucleate, they still share some similarities in mechanisms that are used by nucleated cells for programmed cell death resulting in a steady state of platelet production and clearance in health. With aging in the circulation, platelets appear to show a gradual decline in Bcl-xL expression, which is an anti-apoptotic protein that in turn liberates the proapoptotic Bak/Bax proteins leading to Bak/Bax pathway activation and starting mitochondrial-dependent apoptosis and subsequent PS exposure (25). After PS exposure on their outer membrane surface, platelets are cleared *via* scavenging receptors on phagocytic cells in the liver and other organs. The apoptotic PS exposure differs mechanically from that of agonist-induced, as apoptotic PS exposure appears to rely on caspase activation (25). These clear distinctions between apoptotic and agonist-stimulated PS-exposing platelets have led to the suggestion that the latter are activated by a necrotic cell death pathway. The loss of the negatively charged sugar moiety sialic acid from the surface of senescent platelets is another way by which platelets are cleared from the circulation by the hepatic asialoglycoprotein receptor 1 (76).

PLATELET SUBPOPULATIONS IN CARDIOVASCULAR DISEASES

In the scope of cardiovascular diseases (CVD), different platelet subpopulations have different roles in the prognoses of the disease, and some of them are linked to a higher risk of major adverse cardiovascular events and death (48, 50). While the procoagulant activity of platelets is vital for hemostasis after vessel injury, it has been linked to stroke and coronary artery disease (51–53). Indeed, stable coronary artery disease (CAD) has been associated with a heightened procoagulant platelet response when compared to healthy controls, and this response is not even inhibited by aspirin alone (54). High levels of coated platelets were also associated with an increased risk for recurrent infarction in non-lacunar stroke (55). RP might have a significant role in myocardial ischemia/reperfusion (I/R) injury, caused by the interventional reopening of an occluded coronary vessel in the context of myocardial infarction (MI) especially as RP seem to exhibit resistance to common antiplatelet therapies at least to some extent (56). Beyond providing therapeutic targets, measuring these heterogeneous subpopulations of platelets with specific molecular properties may offer the means to define, predict and diagnose platelet-associated conditions – especially vasculopathy that is progressed by inflammatory, procoagulant, and other platelet responses.

PLATELET SUBPOPULATIONS IN INFECTIOUS DISEASES

In infections, the formation of an intravascular thrombus might be part of the process of pathogen containment which is also known as "immunothrombosis," and platelets are key players in promoting this process. Although platelets and their products suppress infection, during an infection platelet consumption and removal are increased often leading to thrombocytopenia. Platelets can be immunomodulatory cells during an infection regulating and/or participating in the inflammatory response with certain dysregulation in platelets subpopulation such as higher levels of young/reticulated platelets which can be reported as high immature platelet fraction (IPF) levels during infection. For instance, during dengue infection thrombocytopenia is a common complication and IPF can be used as an indicator to predict platelet recovery 24–48 h earlier (57). Another example is

the significant correlation between higher IPF and the diagnosis of sepsis as well as a predictor of severe thrombocytopenia and mortality (43). One more example is the COVID-19 caused by the severe acute respiratory syndrome corona virus-2 (SARS-CoV-2), which is associated with a high incidence of venous and arterial thromboembolic events and the pathophysiology seems to be multifactorial. During COVID-19 infection platelets express procoagulant phenotype, which shows enhanced PS externalization and increased apoptotic markers (58, 59). There's a strong need for markers to guide antithrombotic therapy in COVID-19 patients and to somehow monitor the platelets' dynamics. IPF provides indices of platelet turnover and reactivity in patients with COVID-19 respiratory disease which might serve as a prognostic marker for disease severity. Several studies have shown that patients with COVID-19 had higher levels of IPF and immature platelet count (IPC) than healthy controls and patients with cardiovascular risk factors (60–64). IPF has been a useful tool not only in detecting an infectious state, but also in differentiating a serious state from a minor one (65). Such findings suggest that platelets are refractory to the inflammatory process that is happening which makes them (platelet population as whole or subpopulation markers) a very good candidate to be used as a diagnostic/prognostic marker in certain conditions such as vascular diseases, cancer, infectious diseases (65), pregnancy complications, liver diseases (66–68). More recent findings on the immunomodulatory role of platelets during an infectious status are the platelet's role during the hand, foot, and mouth disease (HFMD) caused by enterovirus 71 (EV71). It has been shown that platelets have distinct roles in the pathogenesis of HFMD by regulating the pathogenic CD4 + T cell differentiation and function (69). When exploring the mechanism by which platelets regulate CD4 + T cell differentiation, gene expression of the T cell surface molecule CD40 was found to be decreased in the mild group of patients while it increases gradually in the severe group. PSGL-1 gene expression on the other hand, which binds to the platelet's P-selectin was also found to increase significantly in the severe group. Such findings suggest that platelets in severe patients with HFMD mainly regulate T cells through CD40L, GPIbα, and CD62P. Not only that, but plateletcrit and platelet count levels both were positively associated with the severity of HFMD (69). Alongside the change in the total platelet count, platelet subpopulations would also show certain trends of dysregulation during different disease states.

TOOLS TO INVESTIGATE PLATELET SUBSETS

Advances in research methodology and technology such as the application of flow cytometry to platelet studies have enhanced our ability to study platelet subpopulations. The platelets are stained with receptor-specific monoclonal antibodies conjugated to fluorescent probes and evaluated with fluorescence flow cytometry (FFC). FFC offers the possibility to evaluate platelets and their function in small blood volumes and very rapidly (∼10,000 platelets/min), and hence, FFC has been traditionally used in clinical and research settings (70, 71). In terms of platelet subpopulations, certain markers could be applied to distinguish them and evaluate their dynamics. For instance, young/reticulated can be evaluated and differentiated from the older platelets in the circulation depending on their RNA content using nucleic acid dyes. Thiazole orange or SYTO13 both have been reported as indicators of reticulated platelets (72, 73). Another method to evaluate reticulated platelets is using automated hematology analyzers such as the Sysmex analyzers reported as IPF% or #IPC (74). There are several pre- and post-analytical considerations when evaluating platelets using these techniques and we have reviewed all these considerations in greater detail in a previous review which readers might refer to (41). Annexin V could be used in FC to report the levels of procoagulant or apoptotic platelets due to its ability to bind to PS (75). Aggregatory platelets could also be evaluated using antibodies against the active αIIbβ3 integrins. While desialylated platelets can be detected by conjugating the ricinus communis agglutinin I (RCA-1) lectins with a fluorochrom, which specifically target exposed galactose residues following GP desialylation (76). FFC comes with a major limitation which is the limited number of parameters that can be simultaneously analyzed due to emission spectra overlap increasing the complexity of the compensation required for accurate analysis (77). The way to solve the inherited limitation of FFC is to overcome the spectral overlap and have the possibility to evaluate different markers simultaneously on individual platelets. One way to achieve that is by applying mass cytometry (MC), which is a next-generation flow cytometry platform and using probes that are conjugated to heavy metal isotopes instead of fluorescent dyes and time-of-flight as a detection technique (78, 79). Using MC there will be no need for compensation as this detection technique has minimal spectral or channel overlap resulting in an increase in the number of cellular parameters that can be analyzed simultaneously on individual cells. MC enables simultaneous phenotypic and functional analysis of multiple parameters applying panels of up to 45 different cellular parameters (80), and in theory up to 100 different parameters (81, 82). The possibility to evaluate multiple markers can be a great way to evaluate platelet heterogeneity within the platelet pool of healthy donors and patients. Platelets could be evaluated in whole blood or in the form of platelet-rich plasma (PRP), which allows the evaluation of high numbers of events enabling the investigation of previously unappreciated small platelet subgroups. One of the limitations with platelet subsets studies, in general, is the lack of standardized protocols that are easily reproducible. Recently, a structured method to stain and evaluate platelets from PRP using CyTOF was published which allows the acquisition of 300,000 to 500,000 events and recording the expression of up to 40 markers at once (78). MC data can be analyzed using Visual stochastic neighbor embedding (viSNE) to visualize high-dimensional single-cell data, for platelet-specific analysis some groups have developed freely available analysis pipelines such as CYANUS (83). As expected, such a detailed evaluation of the platelet pool revealed some differences between baseline and stimulated samples in healthy donors. For instance, studies have shown that the expression of CD42a and CD42b receptors goes down after TRAP stimulation (84). MC analysis of

platelets from Glanzmann Thrombasthenia (GT) patients show a significant reduction in CD41, CD61, and activated integrin αIIbβ3 surface expression (84).

THERAPEUTIC TARGETING OF PLATELET SUBPOPULATIONS

Dual antiplatelet therapy is recommended for secondary prevention of coronary artery disease, including a cyclo-oxygenase-1 inhibitor, and a platelet adenosine diphosphate ($P2Y_{12}$) receptor inhibitor (85). Aspirin is a dose-dependent cyclooxygenase (COX) inhibitor that inhibits COX-1, and at higher concentrations, it inhibits COX-2, through irreversible acetylation of a serine residue in the catalytic channel (86, 87). This inhibition will translate into permanent suppression of thromboxane A2 (TXA2) generation in platelets. On the other hand, inhibiting the $P2Y_{12}$ receptor on platelets prevents platelet activation by ADP. There are several $P2Y_{12}$ inhibitors such as clopidogrel which has an antiaggregatory effect and is beneficial in the treatment of MI. Subpopulations of platelets exist as a result of variability in surface molecules expression which might be attributed to differences related to platelet age or differences in exposure to local *in vivo* activating conditions. The activated procoagulant platelets come with unique challenges for drug therapy such as aspirin and $P2Y_{12}$ blockers that usually target the inhibition of platelet secretion, which in turn demonstrates a need for alternative targets. Now, with the advancement in mass spectrometry instrumentation, it is possible to perform quantitative studies beyond that of earlier work allowing the discoveries of the importance of every receptor or platelet state and possibly targeting them more beneficially. The existing literature suggests a need for a clinically effective antiplatelet-antiprocoagulant regimen to limit the procoagulant response of platelets. For instance, it has been observed that cyclic-adenosine-monophosphate (cAMP) elevation can sufficiently inhibit the initiation of COVID-19 antibody-mediated procoagulant platelet generation thus reducing subsequent thrombus formation (88). Indeed, inducing increased intracellular cAMP levels in platelets using clinically approved therapeutic agents such as iloprost was shown to prevent COVID-19 antibody-mediated coagulopathy. A different potential agent might be acetazolamide, which is a mild diuretic that is already in clinical use and has been shown as a potent antithrombotic (89). Acetazolamide is a carbonic anhydrase inhibitor that suppresses platelet procoagulant responses and thrombus formation by distinct mechanisms and is also capable of blocking water entry *via* the water channel aquaporin 1 (AQP1) (89, 90). Another attractive target for the development of new antithrombotic drugs would be the PAR1 system, which mediates human platelet activation at low thrombin concentration, unlike PAR4 which requires a higher concentration of thrombin for platelet activation and thus preserve a protective mechanism in situations such as trauma (91). Indeed, preclinical and early clinical work on PAR1 inhibition was promising in terms of safety profile and did not affect primary hemostasis. Vorapaxar (SCH530348), developed by Schering Plough is one of the anti-PAR1 molecules used in clinical trials (92). Another molecule is atopaxar (E5555), developed by Eisai pharmaceuticals. It is a small organic molecule, orally active, an inhibitor that binds at the tethered ligand binding site of PAR1 (93). An increase of bleeding events in the study group seems to be reported when compared to the placebo group. On the other hand, adding a third antiplatelet drug to the standard dual antiplatelet therapy is a higher risk of bleeding thus, these agents should be considered differently in future trials not only as an "add-on" therapy. Targeting primary platelet activation pathways is also one of the recent efforts to develop new classes of antiplatelet drugs. Targeting the immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor GPVI/FcRγ-chain complex would provide platelet inhibition due to the role of these receptors in the downregulation of platelet ITAM-receptor signaling (94). The results from targeting GPVI are encouraging with reduced aggregation and smaller arterial thrombi, with no major bleeding complications. It has been suggested in the literature that both PECAM-1 (which inhibit signaling downstream of the collagen receptor GPVI and other platelet activation pathways, such as those mediated by ADP and thrombin), and G6b-B (which inhibits platelet activation by the ITAM-bearing receptors GPVI and CLEC-2) are worthy of consideration as targets for new antiplatelet therapy. For greater details on targeting PECAM-1 and G6b-B as antithrombotic targets, readers may refer to (94).

CONCLUSION

The responsive transitions in form and function platelets undergo are essential to repair vascular endothelium and mediate hemostasis. Platelets are central players in immunosurveillance and vascular inflammation as they facilitate the recruitment of leukocytes into the inflamed tissue as well as enhancing leukocytes' contact with endothelium, which is achieved by the different adhesion molecules and soluble immune mediators. In response to a variety of physiological and pathological circumstances, qualitatively distinguishable platelet phenotypes are increasingly reported in the circulation with conceptually vague origins and significance. It is of great importance to have a meaningful and practical manner where platelets themselves can serve as important puzzle components and also provide physiologically relevant examples on cellular function and vascular wellbeing.

AUTHOR CONTRIBUTIONS

MAH and DD: conceptualization. MAH: methodology. All authors: writing—original draft preparation and writing—review and editing. KK and DD: supervision. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was part of SFB1425, funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation #422681845).

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frontiers | Frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
published: 11 April 2022
doi: 10.3389/fcvm.2022.826729

Circulating Autoantibodies Recognizing Immunodominant Epitopes From Human Apolipoprotein B Associate With Cardiometabolic Risk Factors, but Not With Atherosclerotic Disease

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Rationale: Atherosclerosis is a chronic inflammatory disease of large arteries that involves an autoimmune response with autoreactive T cells and auto-antibodies recognizing Apolipoprotein B (ApoB), the core protein of low-density lipoprotein (LDL). Here, we aimed to establish a clinical association between circulating human ApoB auto-antibodies with atherosclerosis and its clinical risk factors using a novel assay to detect auto-antibodies against a pool of highly immunogenic ApoB-peptides.

Methods and Results: To detect polyclonal IgM- and IgG-antibodies recognizing ApoB, we developed a chemiluminescent sandwich ELISA with 30 ApoB peptides selected by an *in silico* assay for a high binding affinity to MHC-II, which cover more than 80% of known MHC-II variants in a Caucasian population. This pre-selection of immunogenic self-peptides accounted for the high variability of human MHC-II, which is fundamental to allow T cell dependent generation of IgG antibodies. We quantified levels of ApoB-autoantibodies in a clinical cohort of 307 patients that underwent coronary angiography. Plasma anti-ApoB IgG and IgM concentrations showed no differences across healthy individuals (n = 67), patients with coronary artery disease (n = 179), and patients with an acute coronary syndrome (n = 61). However, plasma levels of anti-ApoB IgG, which are considered pro-inflammatory, were significantly increased in patients with obesity (p = 0.044) and arterial hypertension (p < 0.0001). In addition, patients diagnosed with the metabolic syndrome showed significantly elevated Anti-ApoB IgG (p = 0.002). Even when normalized for total plasma IgG, anti-ApoB IgG

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Edited by:
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *01 December 2021*
Accepted: *15 March 2022*
Published: *11 April 2022*

Citation:
Marchini T, Malchow S, Caceres L, El Rabih AAH, Hansen S, Mwinyella T, Spiga L, Piepenburg S, Horstmann H, Olawale T, Li X, Mitre LS, Gissler MC, Bugger H, Zirlik A, Heidt T, Hilgendorf I, Stachon P, von zur Muehlen C, Bode C and Wolf D (2022) Circulating Autoantibodies Recognizing Immunodominant Epitopes From Human Apolipoprotein B Associate With Cardiometabolic Risk Factors, but Not With Atherosclerotic Disease. Front. Cardiovasc. Med. 9:826729. doi: 10.3389/fcvm.2022.826729

remained highly upregulated in hypertensive patients ($p < 0.0001$). We observed no association with triglycerides, total cholesterol, VLDL, or LDL plasma levels. However, total and normalized anti-ApoB IgG levels negatively correlated with HDL. In contrast, total and normalized anti-ApoB IgM, that have been suggested as anti-inflammatory, were significantly lower in diabetic patients ($p = 0.012$) and in patients with the metabolic syndrome ($p = 0.005$).

Conclusion: Using a novel ELISA method to detect auto-antibodies against ApoB in humans, we show that anti-ApoB IgG associate with cardiovascular risk factors but not with the clinical appearance of atherosclerosis, suggesting that humoral immune responses against ApoB are shaped by cardiovascular risk factors but not disease status itself. This novel tool will be helpful to develop immune-based risk stratification for clinical atherosclerosis in the future.

Keywords: atherosclerosis, cardiovascular disease, ApoB, auto-antibodies, immunity

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide (1) and most frequently caused by atherosclerosis, a chronic inflammatory disease characterized by lipid accumulation in the subendothelial space of middle- to large-sized arteries that form vessel-obstructing atherosclerotic plaques (2). The spontaneous rupture of atherosclerotic plaques may ultimately cause an occlusive arterial thrombi that restricts blood flow and precipitates myocardial infarction (MI) and stroke (3). Atherosclerotic lesions develop in arteries with high shear stress, turbulent blood flow, and endothelial dysfunction (4). This process is promoted by traditional cardiovascular risk factors, such as smoking, hypertension, obesity, diabetes, and environmental stressors (5). In atherosclerotic arteries, low-density lipoprotein (LDL) cholesterol particles from the blood circulation are deposited in the subendothelial space and modified by oxidative processes (6). Oxidized LDL (oxLDL) is taken up by tissue-resident macrophages and initiates a pro-inflammatory response that drives disease progression (7). Levels of plasma cholesterol, LDL, and apolipoproteins including Apolipoprotein B (ApoB), the protein backbone of LDL, correlate with clinical atherosclerosis (8). LDL lowering strategies by pharmacological inhibition of the HMG-CoA reductase are recommended in the primary and secondary prevention of atherosclerotic disease (9). However, a relevant inflammatory risk remains even if LDL levels are within the current target ranges (10). Multiple discordance analysis has demonstrated that ApoB has a stronger association with cardiovascular risk than other plasma lipoproteins (11, 12).

Increasing clinical and experimental evidence has highlighted the contribution of an ongoing autoimmune response in atherosclerosis that involves T cells and B cell derived autoantibodies (13). Both $CD4^+$ and $CD8^+$ T cells accumulate in mouse and human atherosclerotic lesions (14). In $Apoe^{-/-}$ mice, a population of $CD4^+$ T cells recognizes ApoB *self*-peptides presented in an MHC-II dependent fashion (15, 16). Autoreactive T cells expand within the atherosclerotic plaque of mice and humans (17, 18). In addition to an autoreactive T cell response, autoantibodies recognizing LDL, oxLDL, and ApoB have been detected in the plasma of mice and humans with atherosclerosis (6, 19). Naturally occurring IgM antibodies directed against oxLDL epitopes are generally considered atheroprotective (20). The role of IgG in the pathogenesis of atherosclerosis is still under debate as IgGs recognizing oxLDL have been shown to aggravate (21) or protect (22) from atherosclerosis in mice. Likewise, studies in humans have suggested that anti-ApoB autoantibodies are atheroprotective (23–27) or pro-atherogenic (28). Although several studies have tested the association of IgG and IgM autoantibodies in a clinical setting [reviewed here (29)], most studies interrogated humoral responses to single self-peptides from ApoB, thus, not allowing to assess the net effect of a polyclonal antibody response against the complex antigen ApoB. In addition, previous studies have not accounted for the genetic diversity of MHC-II allele expression in humans that may affect the generation of autoantibodies against specific ApoB-peptides. Collectively, the association of anti-ApoB autoantibodies and cardiovascular risk factors remains only poorly defined. Here, we aim to circumvent these limitations by the quantification of autoantibodies recognizing a pool of ApoB derived-peptides with proven MHC-II affinity in a single-centre study with 307 participants.

MATERIALS AND METHODS

The study workflow is summarized in **Figure 1**. An Expanded Methods Section is available in the online-only Data Supplement.

Patient Cohort

Human plasma samples were obtained from the Tumor Necrosis Factor Receptor Associated Factors in Cardiovascular Risk Study (TRAFIC) at the University Heart Center Freiburg, University of Freiburg, Germany, that underwent coronary angiography (30). We analyzed plasma samples from 67 patients without relevant coronary atherosclerosis (healthy), 179 patients with

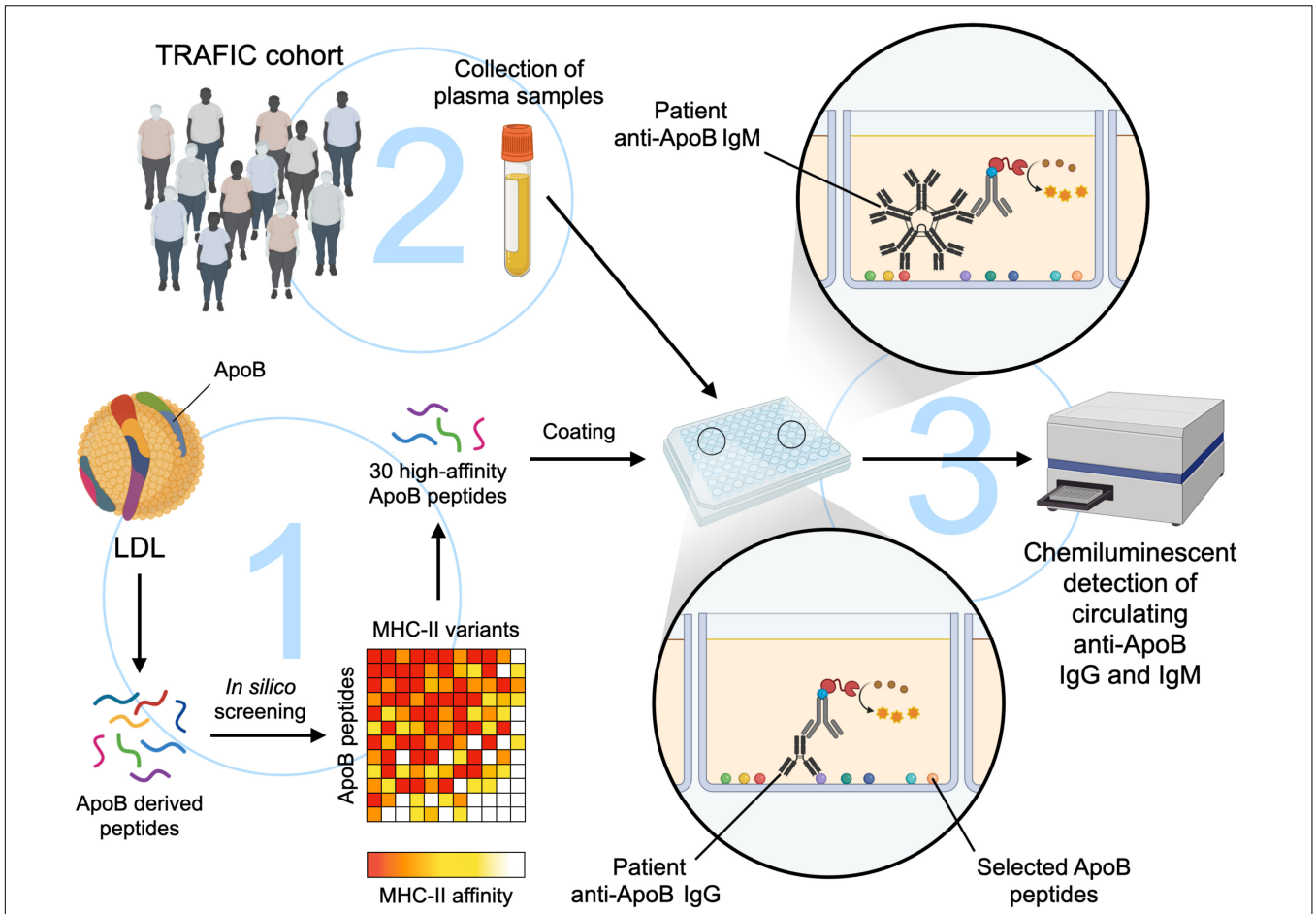

FIGURE 1 | Study workflow for the development of a novel ELISA to detect anti-ApoB IgG- and IgM- plasma levels. (1) ApoB-derived peptides with a length of 15 amino acids were screened *in silico* for their binding affinity to MHC-II variants (18). A pool of 30 peptides that showed a high affinity to MHC-II in direct *in vitro* affinity measurements were used to coat a microplate at 5 μg/mL. (2) A total of 307 plasma samples from the TRAFIC cohort were analyzed by an *in-house* chemiluminescent ELISA (3) to detect circulating anti-ApoB IgG and IgM auto-antibodies. The figure was generated with schematics from Biorender.com.

coronary artery disease (CAD), and 61 patients presenting with a coronary syndrome (ACS). CAD was defined as the presence of at least one coronary stenosis >50% in coronary angiography. ACS was defined as the presence of unstable angina, non-ST-segment elevation myocardial infarction (NSTEMI), or ST-segment elevation myocardial infarction (STEMI). Type 2 diabetes mellitus (T2DM) and hypertension (HTN) were either defined by pre-documented disease or HbA1c > 6.5% (T2DM) or blood pressure >140/90 mmHg (hypertension) at the time of presentation. Obesity was defined by a body mass index (BMI) >35. Metabolic Syndrome (MS) was defined by the presence of at least 3 metabolic conditions (obesity, arterial hypertension, hypercholesterolemia, T2DM, and hypertriglyceridemia) according to a modified definition of the IDF (31). Standard laboratory assays were performed by the local clinical laboratory at the University Hospital Freiburg, Germany. All procedures were carried out according to institutional guidelines (approval number 75/06 and 22-1046 by the local ethics committee, University Hospital of Freiburg, Germany) and the declaration of Helsinki.

Total Plasma IgG and IgM Levels

Total plasma IgG and IgM levels were quantified using a Flex Set Cytometric Bead Array (BD Biosciences, San José, CA, United States) according to the manufacturer's protocol. Briefly, human plasma samples were diluted in assay buffer at 1:200,000 for total IgG and 1:4,000 for total IgM and incubated for 1 h with the supplied beads in a V-Bottom 96 well plate at room temperature. Subsequently, samples were washed and incubated with a PE-detection reagent for 2 h at room temperature. Samples were acquired on a FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software v10 (Tree Star, Ashland, United States). Absolute concentrations (in μg/mL) were calculated using a concentration curve with purified IgG and IgM.

Plasma ApoB$_{100}$ Levels

Patient plasma ApoB$_{100}$ levels were measured using a Human ApoB Quantikine ELISA Kit (R&D systems, Minneapolis, MN, United States) according to the manufacturer's protocol. Samples

were denatured, diluted at 1:2,000, and incubated with the capture and detection antibodies for 1 h at room temperature. After washing, TMB development solution was added and incubated for 15 min. Sample optic density (OD) was determined at 450 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, San Jose, CA, United States).

Development of an ELISA to Detect Circulating Anti-ApoB IgG and IgM

30 $ApoB_{100}$ peptides with a high affinity to several human MHC-II variants were chosen by *in silico* screening and direct *in vitro* affinity measurements (18). Peptides were manufactured by Peptide Specialty Laboratories GmbH (Heidelberg, Germany), diluted in DMSO at 20 mg/mL, pooled, and frozen at −80°C until used. To detect plasma levels of anti-ApoB auto-antibodies by ELISA, the peptide pool was used to coat white U-bottom microplates (BrandTech Scientific, Essex, CT, United States) at 5 μg/mL. A standard curve was prepared by coating the plate with increasing concentrations of pure human IgG or IgM (Jackson ImmunoResearch, Cambridgeshire, United Kingdom). After overnight incubation at 4°C, plates were washed and blocked with 1% BSA. After 1 h at room temperature, plates were washed and 50 μL of diluted plasma samples (1:50) were added to the wells in duplicates. After 90 min at room temperature, plates were washed and incubated with a horseradish peroxidase (HRP) conjugated anti-human IgG or IgM antibody (1:20,000, Jackson ImmunoResearch). After 1 h at room temperature, plates were washed and incubated with the SuperSignal ELISA Femto Substrate (Thermo Fisher Scientific, Waltham, MA, United States). Luminescence was measured 1 min after addition of the substrate in an Infinite 200 PRO microplate reader (Tecan, Switzerland). Patient anti-ApoB IgG or IgM plasma levels were obtained using the corresponding standard curve adjusted to a 5-parameter logistic curve fit, resulting in a dynamic range of 1.16–66.67 ng/mL for the IgG assay and of 0.51–29.63 ng/mL for the IgM assay. Alternatively, background signal in DPBS coated wells (blank) was subtracted from raw RLU values (RLU-blank). As indicated, single ApoB-peptides were used instead of the pool of 30 peptides at the same total peptide concentration.

Statistics

Statistical analysis was performed using GraphPad Prism software v8.0. As data was not normally distributed, a Mann–Whitney test was used for the comparison of two groups and the Kruskal–Wallis test followed by Dunn's multiple comparisons for the comparison of more than two groups. Statistical significance was considered at $p < 0.05$. Data are presented as median or as otherwise indicated.

RESULTS

Clinical Characteristics of the Patient Cohort

The TRAFIC study population comprises a total of 307 patients that underwent coronary angiography at the University Heart Center, Freiburg. 58.3% of patients were diagnosed with CAD, 19.9% presented with an ACS. Expectedly, we detected an increased frequency of T2DM, smoking, arterial hypertension, and dyslipidaemia among patients with CAD/ACS compared to subjects absent of CAD. Patients with ACS had higher concentrations of circulating C-Reactive Protein (CRP). Body Mass Index (BMI), the prevalence of obesity, and levels of serum glucose, HbA1c, and triglycerides were not significantly changed between the groups. Clinical characteristics are presented in **Table 1**.

In a subgroup analysis, patients with T2DM showed increased BMI, serum glucose, and HbA1c (**Supplementary Table 1**). Among smokers, a higher frequency of males and dyslipidaemia was detected (**Supplementary Table 2**) as well as higher circulating leukocyte numbers. In patients with arterial hypertension, obesity, and MS, we detected increased BMI, serum glucose, HbA1c, as well as a higher prevalence of hypertriglyceridemia and dyslipidaemia (**Supplementary Tables 3–5**).

TABLE 1 | Clinical characteristics of the TRAFIC study population.

	no CAD (*n* = 67)	CAD (*n* = 179)	ACS (*n* = 61)
BMI (kg/m^2)	27.6 ± 4.5	27.6 ± 4.4	28.3 ± 4.3
Age (years)	62.5 ± 8.7	65.7 ± 8.3*	64.3 ± 8.9
CRP (mg/L)	3.2 ± 10.6	3.6 ± 8.6$^{\#\#\#}$	17.6 ± 40.7§§§
Creatinine (mg/dL)	0.9 ± 0.2	1.1 ± 0.7**	1.0 ± 0.3§§
Prior MI (%)	0.0 (0)	40.2 (72)*** $^{\#\#}$	20.0 (12)§§§
Sex (% male)	64.2 (43)	84.4 (151)**	77.1 (47)
Diabetes Mellitus Type 2 (%)	9.0 (6)	25.1 (45)**	27.9 (17)§
Smoking (%)	32.8 (22)	52.0 (93)*,$^{\#\#}$	72.1 (44)§§§
Serum Glucose (mg/dL)	111 ± 21	120 ± 39	121 ± 32
HbA1c (%)	5.9 ± 0.6	6.2 ± 0.8	6.4 ± 0.9
Leukocytes ($x10^6$/mL)	6.8 ± 1.8	7.0 ± 2.1$^{\#\#\#}$	8.8 ± 3.1§§§
Total Cholesterol (mg/dL)	199 ± 42	181 ± 42*	195 ± 51
Triglycerides (mg/dL)	127 ± 69	162 ± 99	166 ± 107
LDL (mg/dL)	116 ± 31	99 ± 34*	99 ± 43
VLDL (mg/dL)	30.0 ± 15.2	34.9 ± 17.7	39.7 ± 19.9§
HDL (mg/dL)	53.7 ± 16.1	45.3 ± 13.3*	47.7 ± 18.5
Arterial Hypertension (%)	55.2 (37)	74.3 (133)**	77.1 (47)§
Hypercholesterolemia (%)	19.4 (13)	51.4 (92)***	47.5 (29)§§§
Obesity (%)	22.4 (15)	25.7 (46)	27.9 (17)
Metabolic Syndrome (%)	32.8 (22)	52.5 (94)**	57.4 (35)§§

Categorical variables are expressed in percentages within the groups (total number depicted in brackets), continuous variables as mean ± SD. Statistical significance was tested using Kruskal–Wallis test followed by multiple comparisons for continuous variables, using Fisher's exact test for categorical variables. MI, myocardial infarction.

**Indicates significance between no CAD and CAD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).*

§indicates significance between no CAD and ACS ($^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$).

$^{\#}$indicates significance between CAD and ACS ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

Total Plasma IgG and IgM Levels Are Not Regulated in Patients With Clinical Atherosclerosis

To investigate differences in ApoB-specific autoantibodies, we first aimed to clarify total IgG and IgM levels. Importantly, we detected no significant differences in total IgG or IgM plasma levels among healthy (no CAD), CAD, and ACS patients (**Figure 2**). Quartile distribution of total IgG and IgM plasma levels according to patient diagnosis is provided in **Supplementary Table 6**. Our data indicate that total concentrations of immunoglobulins do not significantly differ between patients with and without clinically relevant coronary atherosclerosis.

Anti-ApoB IgG Plasma Levels Are Increased in Patients at High Cardiovascular Risk

We next tested the association of ApoB-specific antibodies and the clinical appearance of coronary atherosclerosis. Surprisingly, the concentration of specific IgGs binding to the ApoB peptide pool (anti-ApoB IgG) was not changed between patients without CAD (no CAD) and those with CAD and presenting with an ACS (**Figure 3A**). This was consistent even after normalization for total IgG levels in each participant (**Supplementary Figure 1A**). Because angiographically-defined coronary atherosclerosis may not be the major determinant of autoantibody generation, we next grouped the study participants according to the presence of cardiometabolic risk factors: While anti-ApoB IgG showed no significant regulation in patients with T2DM (**Figure 3B**) and smoking status (**Figure 3C**), plasma anti-ApoB IgG was significantly increased in patients with arterial hypertension [1.27 ± 0.17 μg/mL (no hypertension) vs. 3.42 ± 0.30 μg/mL (hypertension)], obesity [2.81 ± 0.29 μg/mL (lean) vs. 3.72 ± 0.53 μg/mL (obese)], and the Metabolic Syndrome [2.81 ± 0.29 μg/mL (no MS) vs. 3.72 ± 0.53 μg/mL (MS)], suggesting a positive association of these cardiometabolic risk factors and anti-ApoB IgG levels (**Figures 3D–F**). After normalizing for total IgG levels, anti-ApoB IgG levels remained highly upregulated in hypertensive patients (**Supplementary Figure 1D**). We detected no association of anti-ApoB IgG with plasma CRP levels (**Supplementary Figure 2**). These data indicate that anti-ApoB IgG antibody levels are specifically increased in patients at high cardiometabolic risk, particularly in those with arterial hypertension.

Because total anti-ApoB IgG levels reflect a mix of several monoclonal IgG-antibody clones that can bind to one of the 30 ApoB-peptides, we next interrogated whether the increase of total anti-ApoB IgGs was driven by a small number of predominant ApoB-peptides only. We therefore selected a subgroup of patients with the 10 highest individual anti-ApoB IgG levels with clinical obesity and hypertension ("high" CVD risk) and with the 10 lowest individual anti-ApoB IgG levels without clinical obesity and hypertension ("low" CVD risk). We observed relevant anti-ApoB IgG titers against all ApoB-peptides (**Supplementary Figure 3A**) with a low standard deviation (41.2% of mean values) and an overall 5.8-fold difference between the peptide with the lowest (peptide 19) and highest (peptide 24) average anti-ApoB IgG signals. While we detected varying patterns of high anti-ApoB IgG titers against a small or larger number of ApoB-peptides within one patient (**Supplementary Figure 3B**), anti-ApoB IgG titers against 19 of the 30 ApoB-peptides were higher or showed a strong tendency to be increased in patients with a high CVD risk compared with those with a low CVD risk (**Supplementary Figure 4**). These data indicate that although patients may preferably express antibodies against specific peptides, the observed increase of anti-ApoB IgG antibodies in obese and hypertensive patients is caused by a broad range of ApoB self-peptides.

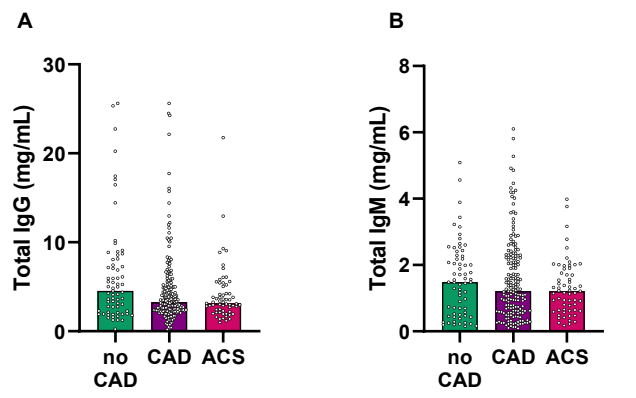

FIGURE 2 | Total IgG and IgM plasma levels do not associate with coronary atherosclerosis in coronary angiography. **(A)** Total IgG and **(B)** IgM levels were quantified in plasma samples using a Flex Set Cytometric Bead Array (BD Biosciences) and grouped according to the presence of coronary atherosclerosis in coronary angiography. CAD, coronary artery disease; ACS, acute coronary syndrome.

Anti-ApoB IgG Plasma Levels Are Associated With Lower HDL and Apolipoprotein B Levels

Since the secretion of autoantibodies is instructed by the presence of the cognate autoantigen, we speculated that levels of anti-ApoB IgG would associate with the plasma concentration of the ApoB-containing apolipoproteins LDL and very low-density lipoprotein (VLDL). Expectedly, we did not find an association to non-ApoB containing triglycerides (**Figure 4A**). However, we did not detect a relevant difference of anti-ApoB IgG levels to quartiles of cholesterol (**Figure 4B**), VLDL (**Figure 4C**), and LDL (**Figure 4D**). Interestingly, we detected an inverse correlation of anti-ApoB IgG concentrations and high-density lipoprotein (HDL, **Figure 4E**). HDL is known to negatively correlate to CVD but does not carry ApoB itself (32). Furthermore, we detected a negative association of anti-ApoB IgG levels with total ApoB concentrations in plasma (**Figure 4F**). These effects remained significant after normalizing to total IgG levels (**Supplementary Figure 5**) and support a clinically relevant association between ApoB-autoantibodies and apolipoprotein metabolism.

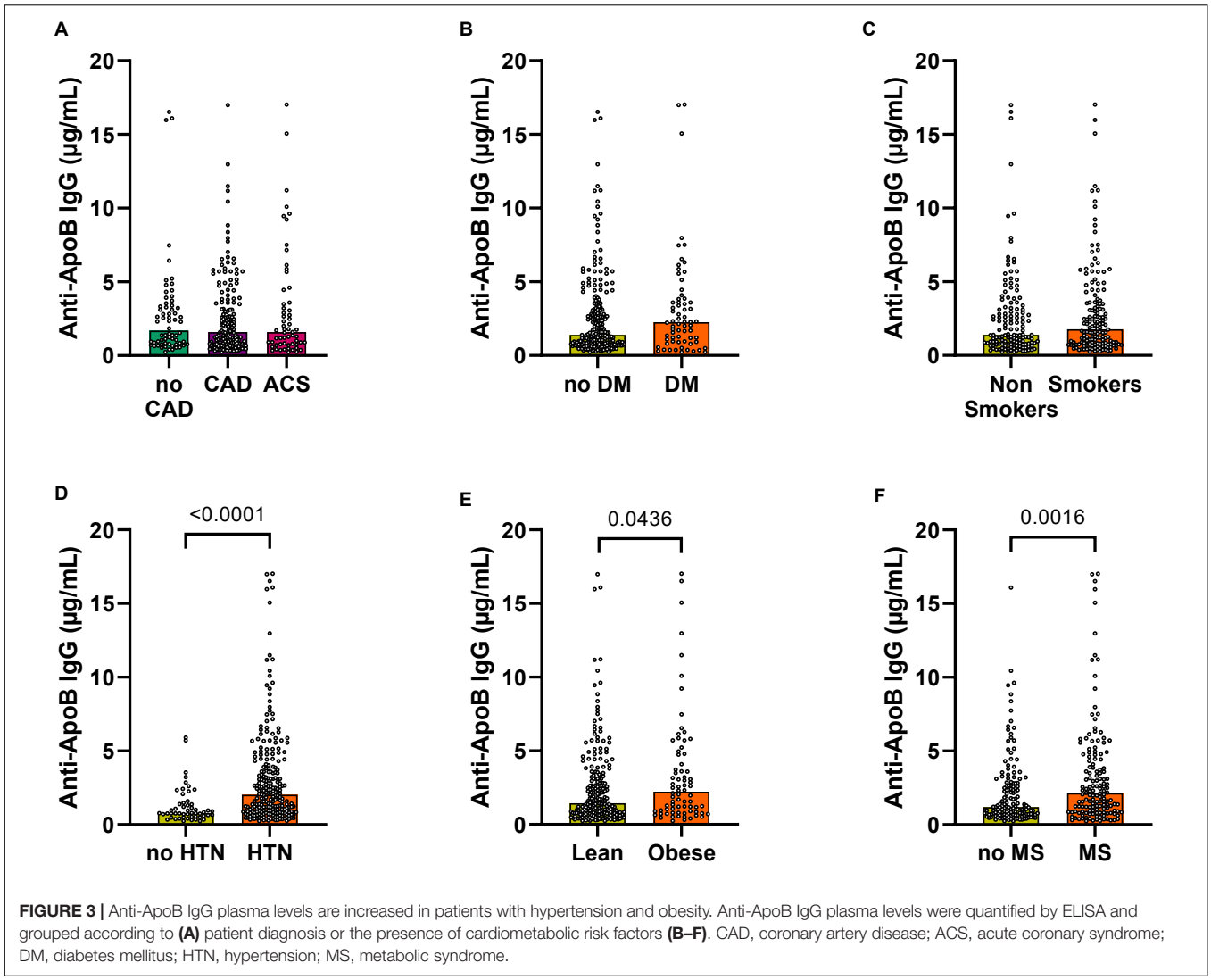

FIGURE 3 | Anti-ApoB IgG plasma levels are increased in patients with hypertension and obesity. Anti-ApoB IgG plasma levels were quantified by ELISA and grouped according to **(A)** patient diagnosis or the presence of cardiometabolic risk factors **(B–F)**. CAD, coronary artery disease; ACS, acute coronary syndrome; DM, diabetes mellitus; HTN, hypertension; MS, metabolic syndrome.

Anti-ApoB IgM Plasma Levels Are Decreased in Patients With a High Cardiovascular Risk

Anti-LDL IgM antibodies mostly recognize oxLDL epitopes with oxidized phosphocholine and malondialdehyde (MDA)—modified amino groups. Anti-LDL IgM antibodies have been detected in mice and humans (33) and seem to have atheroprotective effects (20). To clarify the relation of IgM autoantibodies recognizing unmodified ApoB-peptides, we quantified IgM antibodies in parallel to anti-ApoB IgG antibodies. Both, total IgM plasma levels (**Figure 2B**) and specific anti-ApoB IgM levels, showed no relevant differences among patients without CAD (no CAD), CAD, and these presenting with an ACS (**Figure 5A**). We obtained similar findings after normalizing for total IgM levels (**Supplementary Figure 6**). We detected that anti-ApoB IgM plasma levels were significantly decreased in patients with T2DM (**Figure 5B**) and the MS (**Figure 5F**) but were not modulated by smoking status (**Figure 5C**), arterial hypertension (HTN, **Figure 5D**), or obesity (**Figure 5E**). The negative association with T2DM and MS remained significant after controlling for total IgM levels (**Supplementary Figure 6**). In contrast to anti-ApoB IgG antibodies, anti-ApoB IgM plasma levels did not associate with the lipoprotein profile (**Supplementary Figures 7**, **8**) but where significantly higher in patients within the highest ApoB quartile normalized for total IgM (**Supplementary Figure 8F**). Collectively, these data suggest an opposing regulation of anti-ApoB IgM and IgG by cardiometabolic risk factors and ApoB in patients with CVD.

DISCUSSION

This study interrogated the relationship between IgM and IgG anti-ApoB autoantibodies in patients with coronary artery disease (CAD) and traditional cardiometabolic risk factors. We found – partially in contrast to existing evidence (23–25, 27) – that antibodies binding to ApoB-peptides do not primarily associate with clinically relevant coronary atherosclerotic disease

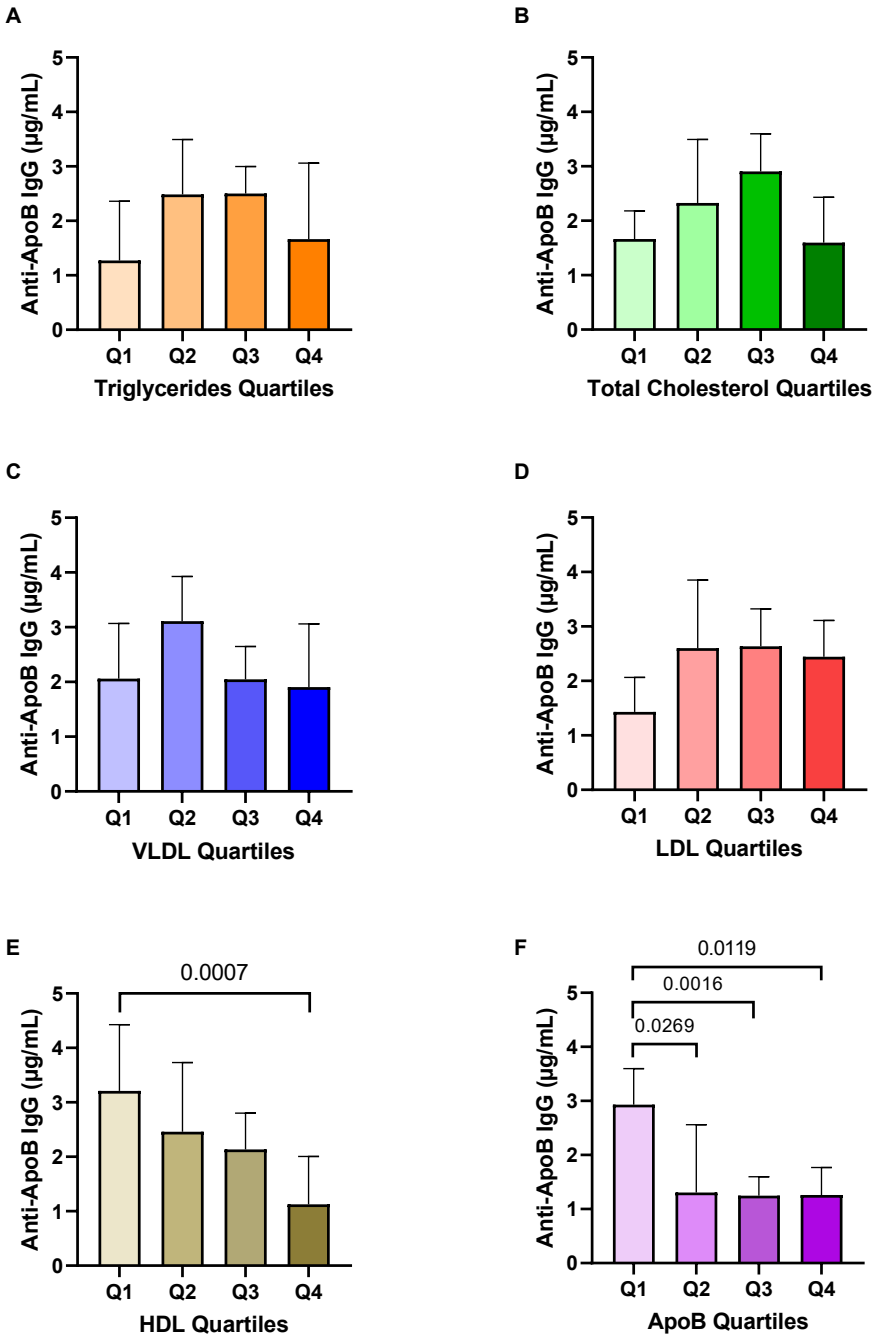

FIGURE 4 | Associations of anti-ApoB IgG plasma levels with triglycerides, cholesterol, and apolipoproteins. Anti-ApoB IgG plasma levels were quantified by ELISA and divided into quartiles (Q) of patient plasma levels of **(A)** triglycerides, **(B)** total cholesterol, **(C)** VLDL, **(D)** LDL, **(E)** HDL, and **(F)** ApoB. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Data are presented as median ± 95%CI.

but with cardiometabolic risk factors. We observed the strongest regulation in patients with arterial hypertension. Along with the concept that IgM immunity is atheroprotective, while IgG antibodies recognizing LDL, ApoB, or peptides from ApoB are pathogenic mediators (20), we found an opposing regulation of IgG and IgM. In addition, our study is the first to demonstrate a positive correlation between obesity and anti-ApoB IgG levels, while previous studies have proposed a negative association with metabolic risk factors (23, 25, 26). These findings emphasize the relation of metabolism and atherosclerosis-relevant autoimmunity, particularly in the light of the emerging field of immunometabolism (34) and novel anti-diabetic and anti-obesity therapies that improve the outcome of atherosclerotic disease (35).

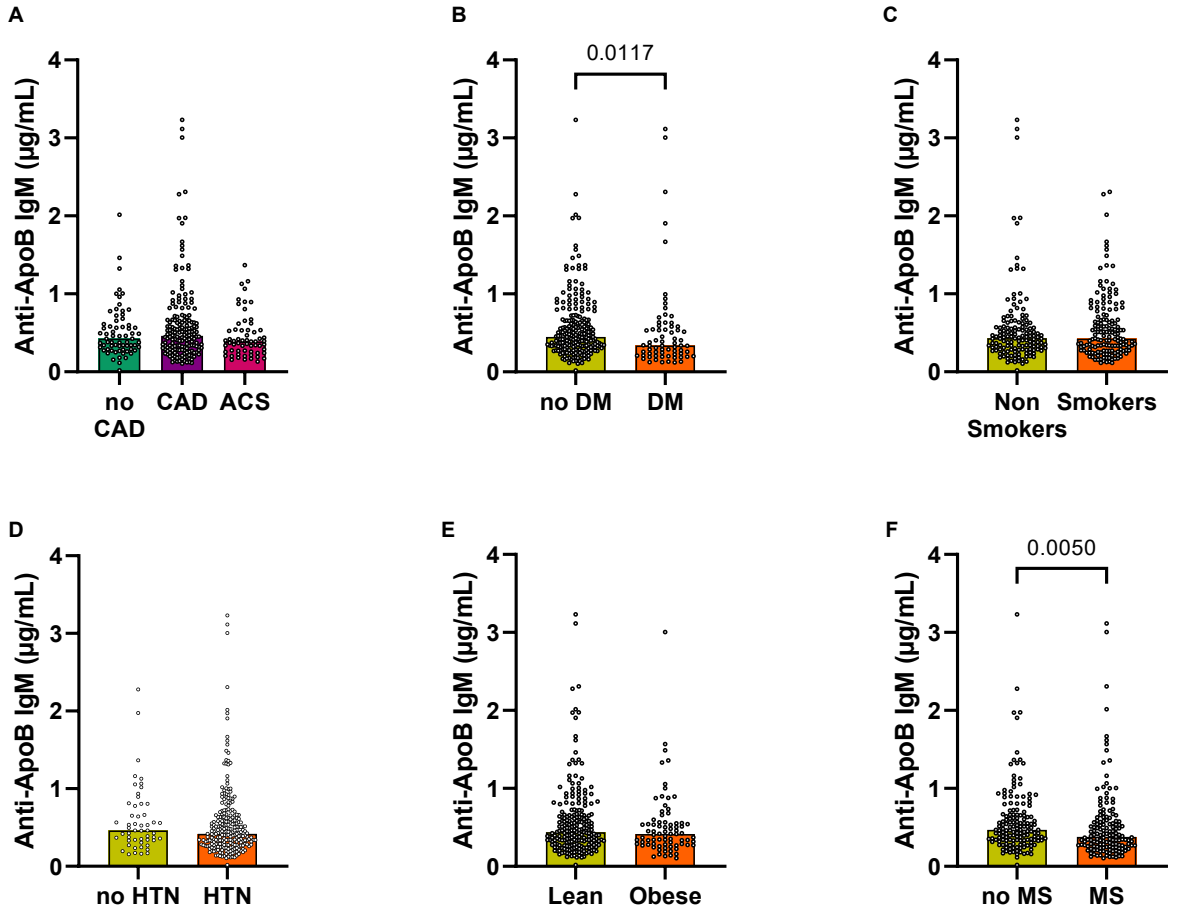

FIGURE 5 | Associations of anti-ApoB IgM plasma levels with cardiovascular risk factors. Anti-ApoB IgM plasma levels were quantified by ELISA and grouped according to **(A)** patient diagnosis or **(B–F)** cardiometabolic risk factors. CAD, coronary artery disease; ACS, acute coronary syndrome; DM, diabetes mellitus; HTN, hypertension; MS, metabolic syndrome.

Autoimmunity in atherosclerosis is a delicate balance of protective and pathogenic events that has been best established in T cellular immunity against ApoB (13). Growing evidence has demonstrated the existence of autoreactive $CD4^+$ T-helper cells in the blood of mice and humans (15, 18). These autoreactive T cells mostly express surface markers, cytokines, and transcriptomes reminiscent of immune-suppressive T-regulatory cells (16, 18). In mouse models, adoptive transfers of these cells protect from atherosclerosis. In the later stages, however, ApoB-specific T cells transform into their pathogenic counterparts that are closer to pathogenic T_H1-polarized cells than T_{regs} (18). In addition, a subpopulation of T cells in the atherosclerotic plaque and in lymph nodes differentiates into T follicular helper cells (T_{FH}) that aid B cells in maturation, IgG class switch, and their differentiation in plasma cells, which express anti-ApoB autoantibodies. A lack of T_{FH} protects from atherosclerosis (36). *Per se*, the generation of ApoB-autoantibodies represents an event of late-stage atherosclerosis, in which pathogenic T cell phenotypes and effector functions dominate protective limbs of autoimmunity (37). In line, it has been postulated that IgG-mediated LDL complex formation and opsonization may enhance lipid uptake into plaque phagocytes and promote atherosclerosis (21, 38), Conversely, vaccination with ApoB-peptides protects from atherosclerosis but it is unclear whether this is an effect of vaccination-induced T_{reg}-like $CD4^+$ T-helper cells or of a humoral response involving ApoB-autoantibodies (39). Notably, anti-ApoB antibodies could help clearing LDL particles from the circulation (40) or block its uptake by macrophages and prevent atherosclerosis (20, 22, 41–43). Reports on vaccination-induced mechanisms have been controversial and there is still a considerable lack of knowledge. Likewise, clinical findings remain hard to interpret. For instance, in most studies non-modified ApoB-IgGs inversely correlate with clinical disease (23, 24, 26–28), while antibodies to modified LDL positively correlates with disease in one study allowing a direct comparison (28). Associations with cardiometabolic risk factors in these studies remain highly heterogenous. The largest clinical association study with more than 3,500 participants highlighted a fine-tuned relationship to sex, age, ethnicity and partially opposing effects for ApoB and LDL-specific antibodies (28). Apart from these associative studies, one clinical trial tested the direct transfer of a monoclonal antibody recognizing

oxidized LDL in patients with CAD but failed to reduce plaque inflammation in FDG-PET imaging (44).

There are several important differences among the available studies that may explain such heterogeneity: First, it is important to note that the immune response against complex LDL particles and single ApoB-peptides may substantially differ. This is exemplified by the observation that germ-line encoded, naturally occurring IgM recognize modified lipids, which share structural homologies with evolutionary conserved foreign patterns, such as from bacteria or viruses (20). Therefore, lipid-specific (oxidations-specific) and T cell dependent, anti-peptide antibodies may have fundamentally different roles in host defense, inflammation, and autoimmunity. Second, the fact that antibodies recognize peptides that are located in the inner core of LDL particles or peptides that are exposed to the hydrated surface and therefore accessible to circulating antibodies may relevantly impact on their biological function. Vaccination studies have demonstrated antibody-responses to a wide range of peptides independent of their location within LDL (39). While antibodies may represent a biomarker of vaccination, they may not necessarily exhibit biological function, in particular when the respective peptide is not accessible to circulating antibodies because of its location inside LDL particles. During the intracellular processing of LDL-containing peptides by APCs, however, antibodies without a biological function may be generated as bystander-response and used as biomarker of the specific immune risk. Third, to quantify antibodies with a specificity against a single peptide in a mixed population is potentially under-estimating potential effects. Since the generation of these antibodies depends on T_{FH} that provide help to differentiating B cells it is more likely that a peptide with a high-affinity for MHC-II is presented by respective B cells to a T cell with a high-affinity TCR for this MHC-II:peptide interaction. Because several thousands of different MHC-II alleles are expressed in the human population, it is unlikely that a single peptide would exhibit high-affinities across all MHC-II variants. Notably, MHC-II typing has not been performed in the available ApoB antibody trials so far. In our study, we used a pool of 30 ApoB-peptides that have been carefully selected to represent sufficient binding affinities across a wide range of MHC-II alleles (18). We suggest that the multi-peptide design of our study is more representative and potentially biologically relevant than single-peptide IgG targets. We detected a clear predominance of antibodies against some peptides in individual patients. This pattern showed a considerable variation across patients, which supports the idea that individual MHC-II variants respond differently to certain ApoB-peptides. It is also important to note that even IgG-antibodies recognizing only one ApoB-peptide may consist of a polyclonal repertoire of different IgG-antibody clones with varying affinities toward the same peptide. Our findings therefore suggest that reported clinical associations may be under-estimated in available studies testing auto-antibodies against one peptide only. On the contrary, studies quantifying autoantibodies to the entire ApoB protein are at the risk of lower specificity. Interestingly, our study revealed that the peptide with highest individual ApoB-IgG signal (peptide 24) was not regulated between patients with a low and high risk for CVD. Therefore, a pool of selected peptides should be broad enough to account for naturally occurring MHC-II alleles and small enough to allow tracking of single peptide-specificities and to minimize unspecific peptide-specific IgGs that are not regulated in disease.

Our study raises some important questions: For instance, it is intriguing to ask whether hypertension remains independently associated with ApoB IgG levels and how arterial hypertension may drive IgG generation. Associations with arterial hypertension have (26, 28) and have not been demonstrated before (25). It will also be important to clarify the relation between autoantigens, humoral, and cellular responses. While we have previously shown the existence of a T cell population circulating in human blood that recognizes the same pool of ApoB peptides, it will be necessary to clarify the association of autoantibodies and antigen-specific T cells on a single peptide basis. Particularly, it would be interesting to interrogate whether T_{FH} with the same peptide specificity would exist and associate with antibodies recognizing the same peptide. Although the inverse correlation of ApoB concentrations and ApoB-autoantibodies seems to be counter-intuitive, it is plausible to speculate whether the chronic stimulation of humoral and cellular limbs of autoimmunity would result in immune exhaustion with a subsequent loss of ApoB-specific plasma cells and a decline of respective auto-antibodies over time. In this case, an inverse correlation of disease progression and antibody concentrations should not be interpreted as causal. Eventually, the understanding of the precise interplay of humoral and cellular autoimmune mechanisms will allow the development of novel humoral and cellular risk stratification tools to identify CVD patients at high immune risk.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee, University Hospital of Freiburg, Germany (Approval numbers 75/06 and 22-1046). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed made a substantial, direct, and intellectual contribution to this work, and approved it for publication.

FUNDING

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Program (grant agreement no 853425). TM, LM, IH, TH, CM, and DW are members of the SFB1425 (project #422681845), funded by the Deutsche

Forschungsgemeinschaft (DFG, German Research Foundation). LC was funded by a fellowship from the German Academic Exchange Service (DAAD) and from the University of Buenos Aires.

ACKNOWLEDGMENTS

We would like to thank Christoph Borner (Institute of Molecular Medicine and Cell Research, University of Freiburg, Germany) for his support and access to research facilities and equipment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.826729/full#supplementary-material

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frontiers | Frontiers in Cardiovascular Medicine

MINI REVIEW
published: 15 April 2022
doi: 10.3389/fcvm.2022.864602

The Spectrum of B Cell Functions in Atherosclerotic Cardiovascular Disease

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B cells are a core element of the pathophysiology of atherosclerotic cardiovascular disease (ASCVD). Multiple experimental and epidemiological studies have revealed both protective and deleterious functions of B cells in atherosclerotic plaque formation. The spearhead property of B cells that influences the development of atherosclerosis is their unique ability to produce and secrete high amounts of antigen-specific antibodies that can act at distant sites. Exposure to an atherogenic milieu impacts B cell homeostasis, cell differentiation and antibody production. However, it is not clear whether B cell responses in atherosclerosis are instructed by atherosclerosis-specific antigens (ASA). Dissecting the full spectrum of the B cell properties in atherosclerosis will pave the way for designing innovative therapies against the devastating consequences of ASCVD.

Keywords: B cells, atherosclerosis, antibodies, lipids, inflammation, cardiovascular disease

OPEN ACCESS

Edited by:
Norbert Gerdes, Heinrich Heine University Düsseldorf, Germany

Reviewed by:
Jürgen Bernhagen, Ludwig Maximilian University of Munich, Germany
Kosuke Kataoka, Tokushima University, Japan

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *28 January 2022*
Accepted: *02 March 2022*
Published: *15 April 2022*

Citation:
Smeets D, Gisterå A, Malin SG and Tsiantoulas D (2022) The Spectrum of B Cell Functions in Atherosclerotic Cardiovascular Disease. Front. Cardiovasc. Med. 9:864602. doi: 10.3389/fcvm.2022.864602

INTRODUCTION

Heart attacks and strokes are the leading causes of mortality and morbidity worldwide (1–3). The main underlying pathology of these clinical manifestations is ASCVD, which leads to the formation of plaques in large and medium-sized arteries. Rupture or erosion of atherosclerotic plaques triggers thrombus formation thereby causing myocardial infarction (MI) or stroke (4, 5). Atherosclerosis is a lipid-driven chronic inflammatory disease characterized by progressive retention of cholesterol-carrying low-density lipoprotein (LDL) particles in the subendothelial space of arteries (6, 7) followed by a chronic maladaptive immune response (4, 8–12) and remodeling of the artery wall (13), fueled by genetic (14) and lifestyle risk factors (13). Local enzymes act on retained lipoproteins, which leads to LDL aggregation and oxidation (OxLDL) characterized by the formation of lipid peroxidation-derived products called oxidation-specific epitopes (OSE) (15, 16). The accumulating modified LDL particles stimulate endothelial cells to produce adhesion molecules and chemokines (17), which attract circulating leukocytes such as T lymphocytes (18) and monocytes (19) to the vessel wall.

The controlled double-blind clinical trials CANTOS (20), COLCOT (21), and LoDoCo2 (22) have demonstrated the therapeutic value of immunomodulation in secondary prevention of ASCVD. While these studies have shown that inflammation is crucially involved in human ASCVD, they also revealed the need for the development of precise immunotherapies that would limit side effects, such as the risk for fatal infections (23).

B Cells Are Key Pieces of the CVD "Immune-Mosaic"

Patients with autoimmune rheumatic diseases, who display dysregulated responses of adaptive immunity (B and T lymphocytes), are at high risk for premature ischemic heart disease due to accelerated development of atherosclerosis that cannot be fully explained by the traditional Framingham risk factors such as cholesterol levels, smoking and systolic blood pressure (24). Furthermore, mice lacking adaptive immunity display reduced atherosclerosis (25). These findings have highlighted the crucial role of adaptive immunity in modulating atherosclerosis. Several studies have revealed a broad spectrum of T cell [reviewed elsewhere; (18)] and B cell properties that affect atherosclerosis (26–28).

B cells have the unique ability to generate immunoglobulins that can be displayed on the cell surface in the form of the B cell receptor (BCR) or secreted as antibodies. In mouse B lymphopoiesis, B-cell-biased lymphoid progenitors (BLPs) differentiate via the pre-pro-B cell stage to committed pro-B cells, with commitment regulated by the transcription factor *Pax5* (29). The successful display of a recombined heavy chain together with surrogate light chains on the cell surface provides proliferative signals to large pre-B cells and this cell division is followed by rearrangement of the light chain genes at the small pre-B cell stage, hence completing V(D)J recombination and resulting in an immature B cell that displays IgM on the cell surface. Upon completion of recombination events, the B cells can leave the bone marrow to further mature in secondary lymphoid organs. Although the marrow of long bones is often considered the predominant site of B lymphopoiesis, other locations are noticeable for B cell development, including the fetal liver, the calvaria of the skull (30), and also the mouse intestinal lamina propria (31).

Mature B cells consist of two main subsets, the conventional B-2 cells, and the less frequent B-1 cell subset (26). B-1 and B-2 cells display differences in their activation requirements, anatomical localization, and surface markers. B-1 cells are subdivided into B-1a and B-1b cells. B-1a cells are long-lived and self-renewing innate-like B cells that are derived from the fetal liver hematopoiesis, and are enriched within the peritoneal and pleural cavities, although a substantial population also can be found in the spleen (32). Notably, $CD20^+CD27^+CD43^+CD70^-$ B cells were proposed to be the equivalent of mouse B-1 cells in humans (33). However, this remains unsettled considering the similarities of $CD20^+CD27^+CD43^+CD70^-$ B cells with preplasmablasts (34, 35). On the other hand, B-2 cells display many similarities between mice and humans concerning their localization and function (36). B-2 cells include the follicular (FO) B cells and the marginal zone (MZ) B cells. Both subsets are generated through the maturation of splenic immature B cells, which have successfully escaped the bone marrow selection, via pertinent BCR signaling (37). In contrast to MZ B cells, FO B cells display circulating properties, which allow them to home to distant sites (37).

Early evidence supporting a role for B cells in human atherosclerosis is derived from studies more than 40 years ago that demonstrated the presence of immunoglobulins in atherosclerotic arteries (38, 39). Based on histological analyses, B cells are commonly detected in adventitia surrounding atherosclerotic regions with the ability to recirculate to draining lymph nodes (40), while they are an infrequent cell type in atherosclerotic plaques (41). However, although a technical contamination of circulating B cells cannot be excluded, a mass-cytometry analysis of human carotid atherosclerotic plaques revealed a substantial portion of plaque B cells (42). Besides being present in atherosclerotic arteries, a systems biology investigation of whole blood gene expression data from Framingham Heart Study participants and genome-wide association studies coupled to the construction of co-expression networks, identified coronary heart disease-specific causative modules enriched in genes regulating B-cell activation (43), thereby providing indications for a functional role of B cells in human atherosclerosis. In line with this, numbers of activated $CD19^+CD86^+$ B cells or IgM^+ unswitched memory B cells display a positive and negative association, respectively, with increased risk for stroke in humans (44, 45), suggesting that B cell activation may be involved in the progression of atherosclerosis.

The first experimental evidence that B cells impact atherosclerosis was provided by Caligiuri et al., who showed that splenectomy-induced acceleration of atherosclerosis in Apolipoprotein E deficient ($Apoe^{-/-}$) mice could be rescued upon transfer of splenic B cells that were isolated either from wild type or $Apoe^{-/-}$ donors (46). Next, Major et al. reported that lethally irradiated LDL receptor-deficient ($Ldlr^{-/-}$) mice that were injected with bone marrow from B cell-deficient (μMT) donor mice developed increased atherosclerosis compared to controls (47). However, in a recent study Tay et al., reported that $Apoe^{-/-}$ μMT mice developed decreased atherosclerosis compared to control $Apoe^{-/-}$mice (48). $Apoe^{-/-}$ mice accumulated predominately VLDL remnants in their circulation whereas in $Ldlr^{-/-}$ mice the main accumulating lipoprotein in plasma is the LDL (49). Apart from the obvious reasons, such as different experimental settings, the differences in lipoprotein profile and metabolism may be, at least in part, responsible for the differential effect in atherosclerosis upon B cell deficiency between $Ldlr^{-/-}$ and $Apoe^{-/-}$mice.

B cell subsets exhibit distinct effects in atherosclerosis, which further emphasizes the sophisticated involvement of B cells in this disease (**Figure 1**). B-1 cells confer protection in atherosclerosis (50, 51). On the other hand, treatment of $Apoe^{-/-}$ or $Ldlr^{-/-}$ mice with a B cell depleting anti-CD20 antibody, which preferentially depletes B-2 cells, reduced atherosclerosis, and prevented the MI-induced acceleration of atherosclerosis (52–54). In addition, genetic deletion of the B cell transcription factor *Pax5* in CD23-expressing cells (primarily mature B2 cells) (55), or treatment with an agonistic antibody specific for B- and T-lymphocyte attenuator (56) that reduced mature B-2 cells, also resulted in decreased atherosclerosis. Disruption of the B cell-activating factor receptor (BAFFR) pathway, which is essential for B-2 (but not B-1) cell survival (57), also conferred an atheroprotective effect (58–61). However, selective ablation of MZ B cells increases atherosclerosis (62), which indicates that therapeutic strategies targeting the entire B-2 cell compartment

may not be optimal, and thus, dissecting the functions of B cell responses is essential for the designing of precise therapies in atherosclerosis.

Antibody-Mediated Functions of B Cells in Atherosclerosis

The main property of B cells that plays a crucial role in atherosclerosis is antibody production. B-1 cells secrete high amounts of natural IgM antibodies, which are produced in absence of a foreign microbial threat (63). On the other hand, FO B cells can enter the germinal center (GC) reaction, which is notable for producing high-affinity antibodies through the process of somatic hypermutation, although this process is not exclusive to the GC. GCs can be found in the secondary lymphoid organs of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (27, 55, 62, 64). Upon exit from the GC reaction, B cells can differentiate into short- and long-lived memory B cells as well as short- and long-lived antibody-producing cells (65).

Immunoglobulin M, a Trustworthy Groundkeeper

There is a consensus that IgM exhibits atheroprotective properties. For instance, transfer of B-1a cells into splenectomized mice, which exhibit a severe reduction in peritoneal B-1a cells and circulating IgM antibodies, reversed splenectomy-accelerated atherosclerosis (50). However, the protective effect of B-1a cell transfer in this setting was absent when B-1a cells deficient in secreted IgM (sIgM) were injected (50). Consistent with this, mice lacking sIgM develop aggravated atherosclerosis (66–68). Moreover, B cell-specific CXCR4 (C-X-C chemokine receptor type 4) deficiency, which resulted in reduced IgM levels in plasma, led to increased atherosclerosis in female mice (69). Thus, dissecting the molecular pathways that regulate the production of atheroprotective IgM antibodies may reveal new therapeutic strategies for atherosclerosis. Along this line, apoptotic cell injection (64), infusion of liposomes decorated with phosphatidylserine moieties (70), and genetically induced inhibition of antibody class-switching (71) led to increased total IgM levels in plasma and reduced atherosclerosis. In addition, the transfer of B-1b cells into lymphocyte-deficient atherosclerotic mice also led to increased plasma IgM and reduced plaque size (51). Thus, strategies directly promoting the expansion of B-1a or B-1b cells could be of interest. For instance, reduced atherosclerosis along with increased B-1a cell numbers and circulating IgM levels were reported in atherosclerosis-prone mice that were deficient in sialic acid-binding immunoglobulin-like lectin G (72) or had been treated with an antibody against the phosphatidylserine receptor T-cell immunoglobulin and mucin domain-1 (73). However, therapeutic strategies for the expansion of B-1a cells have to be considered with caution as they may be accompanied by an increase of the proatherogenic B-1a cell-derived subset, the innate response activator (IRA) B cells (74), which via producing granulocyte-macrophage colony-stimulating factor instruct a dendritic cell-mediated promotion of proatherogenic Th1 immunity (75).

The identification of atherosclerosis-specific antigens (ASA) will allow the designing of precise therapeutic strategies in atherosclerosis. Clinical studies have shown an inverse correlation of OSE-specific IgM against malondialdehyde and phosphorylcholine (PC), which are present on oxidized LDL (16, 76) and apoptotic cellular debris (77, 78), with atherosclerotic burden and cardiovascular outcomes (28, 79–82). The implication of OSE-specific IgM antibodies in atherosclerosis was originally shown by using the E06 IgM antibody that binds oxidized phospholipids (OxPLs) and has an identical CDR3 region to the germline-encoded B-1 cell-derived T15 clone (83). Immunization with heat-killed pneumococcal extracts led to a strong increase of the PC-specific T15/E06 IgM clonotype and decreased lesion formation (84). Furthermore, passive infusion of T15/E06 IgM antibodies reduced vein graft atherosclerosis in atherosclerotic *Apoe*$^{-/-}$ mice thereby providing direct evidence that the E06 IgM confers an atheroprotective effect (85). In a seminal study by Prof. Witztum's lab, it has been shown that transgenic overexpression of the single-chain variable fragment of E06 strongly decreased atherosclerosis in *Ldlr*$^{-/-}$ mice (86). These data suggest that E06 acts as a blocking antibody limiting the proinflammatory effect of OxPLs in atherosclerosis *in vivo*. This is supported by the capacity of E06 to block OxLDL uptake by macrophages (87) and proinflammatory cytokine production by OxPL-stimulated macrophages (88) *in vitro*. While the expansion of OSE-specific IgM could be considered therapeutically in atherosclerosis, it is important to identify its right "therapeutic window." This is essential as endogenous OSE-specific IgMs are present at high levels in both mice and humans (89) and increase over time in hypercholesterolemia (90). In fact, infusion of purified T15/E06 preparations (91) or the OxPL-neutralizing 10C12 IgM clone (68) had no effect in advanced atherosclerosis. Furthermore, it appears likely that long exposure to an atherogenic milieu might induce the expansion of IgM with shared antigen specificities. For instance, genetic deficiency of the *V_HS107.1.42* locus, which is essential for the successful production of T15 antibodies, did not affect experimental atherosclerosis in mice with marked dyslipidemia (92). IgMs also recognize other self-antigens and thereby regulate the maturation of B-2 cells (93–96) and circulating levels of other immunoglobulins (67, 97, 98). Notably, mice lacking sIgM, display high levels of IgE antibodies, which are responsible for the accelerated atherosclerosis in this setting (67). Taken together, the properties of IgM in atherosclerosis demonstrate the important role of (neo)-self-antigens in this disease.

Immunoglobulin E, a Powerful Assailant

The most well-documented properties of IgE antibodies are their role in triggering an allergic reaction and fighting microbial infections (99, 100). These properties of IgE are mediated via binding to high-affinity IgE receptor FcεRI, which is mainly present on mast cells, basophils, and eosinophils (101). Mice lacking the FcεRI receptor display reduced atherosclerotic plaque size, thereby suggesting that IgE antibodies play a role in atherosclerosis (102). The proatherogenic role of IgE antibodies was directly shown using a neutralizing anti-IgE antibody specific for free IgE that, as mentioned above, completely reversed the accelerated atherosclerosis in atherosclerotic sIgM deficient mice, which display high plasma IgE antibodies (67). In agreement with this, mice deficient in IgE antibodies developed

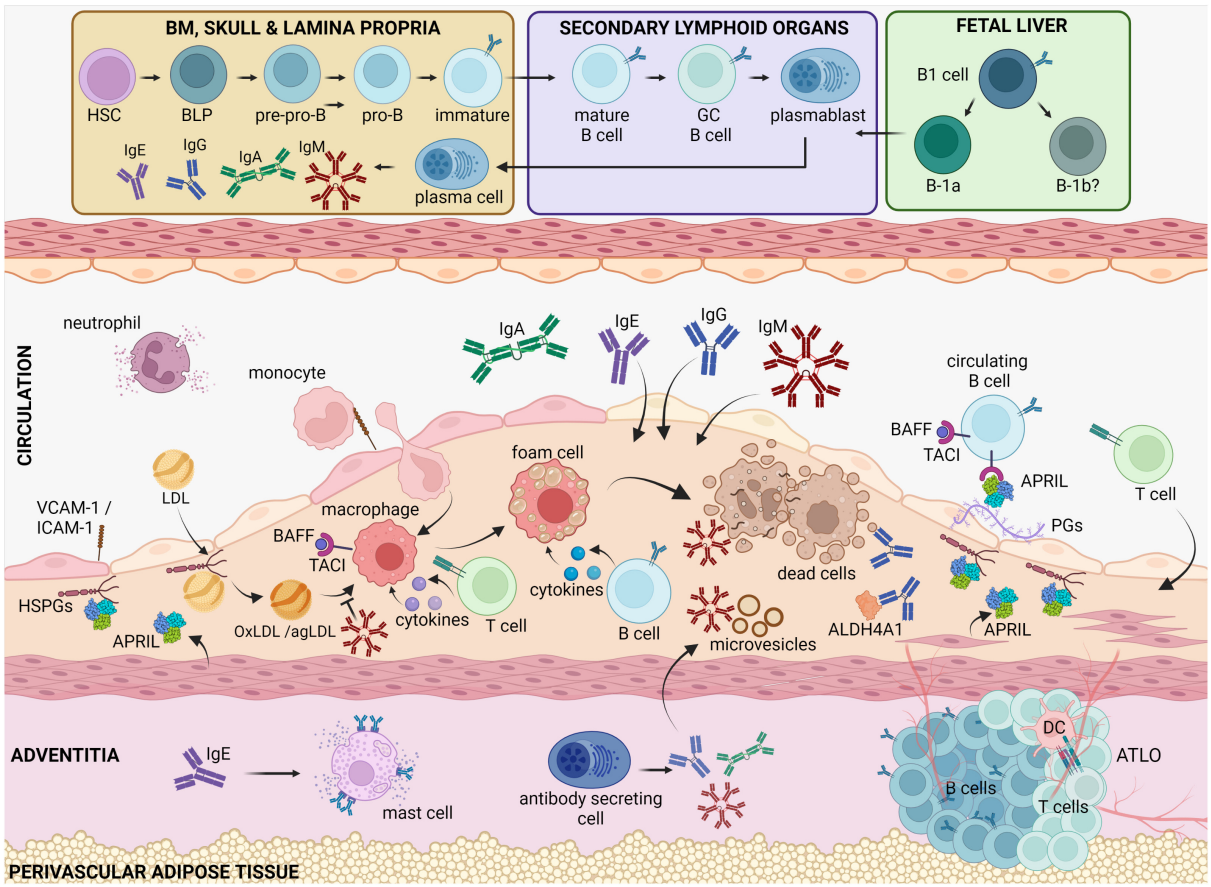

FIGURE 1 | B cell functions in atherosclerotic disease. Circulating cholesterol-containing LDL particles are progressively retained in the subendothelial space of arteries. Oxidized or aggregated LDL particles are taken up by arterial macrophages, which turn to foam cells because of uncontrolled lipid uptake and thereby undergo apoptosis or necrosis. Antibody-producing cells that reside in the bone marrow, the spleen and in the adventitia, produce high amounts of antibodies that are deposited in the plaque. IgM is known to bind and block the proinflammatory effects of oxidized LDL, apoptotic cells and microvesicles. IgE antibodies by binding to FcεRI receptors activate powerful proinflammatory responses by mast cells and macrophages. IgG antibodies can also bind OxLDL as well as self-proteins, such as ALDH4A1, and modulate macrophage activation. BAFF and APRIL, which both bind the TACI receptor in B cells, dampen the proinflammatory responses by macrophages, and limit the LDL retention in the intima, respectively, thereby revealing an indirect property of B cells to regulate plaque inflammation. BLPs, B-cell-biased lymphoid progenitors; PGs, proteoglycans, TACI, Transmembrane activator and CAML interactor; BAFF, B cell activating factor; APRIL, A Proliferation Inducing Ligand, HSPGs, heparan sulfate proteoglycans; BM, bone marrow; ATLO, artery tertiary lymphoid organ; OxLDL, oxidized LDL; agLDL, aggregated LDL.

decreased atherosclerosis (103). Furthermore, a systemic IgE-mediated mast cell activation in atherosclerotic mice lacking B cells resulted in increased lesion size (104). Mechanistically, IgEs promote mast cell and neutrophil activation, and the production of proinflammatory cytokines by macrophages and smooth muscle cells (67, 102, 103), which could be responsible for their effect in atherosclerosis *in vivo*. The detrimental role of IgE antibodies in atherosclerosis is also supported by several epidemiological studies (105, 106) that also implicate the mammalian oligosaccharide galactose-α-1,3-galactose as a candidate antigen (107). Future studies are required to identify the spectrum of proatherogenic IgE-specific antigens.

Immunoglobulin G, a Vault for Atherosclerosis-Specific Antigens?

IgG antibodies are produced in different subclasses: IgG1, IgG2, IgG3, and IgG4 in humans and IgG1, IgG2a/c, IgG2b, and IgG3 in mice (108). Tay et al., provided the first direct evidence on the role of IgG antibodies in atherosclerosis, by showing that mice lacking most endogenous immunoglobulins developed increased plaque size upon injection of purified total IgG from atherosclerotic mice compared to IgG from non-atherosclerotic donors (109). This study also suggests that exposure to an atherosclerotic milieu alters the antigen specificities of the IgG repertoire by inducing the expansion or even the *de novo* generation of B cell clonotypes that are likely to include specificities against ASA. In line with this, a protein array analysis revealed an altered repertoire of IgG1 protein targets in the serum of *Apoe*$^{-/-}$ vs. C57BL/6 mice fed an atherogenic diet (71). It is not clear whether the altered IgG repertoire is triggered upon chronic exposure to atherogenic pressure or already emerges at the initiation of the disease. In this regard, abrupt loss of APOE, which results in acute onset of dyslipidemia, triggered a rapid increase in IgG antibodies levels enriched in specificities against common

autoantigens (55). These data suggest that the GC reaction is involved in atherosclerosis. In agreement with this, elimination of GC B cells achieved upon deletion of the key B cell transcription factor *Pax5* in AID-expressing B cells, reduced atherosclerosis (55, 110). Furthermore, B cell-specific overexpression of the FcγRIIB receptor limited GC B cell responses and reduced atherosclerosis in male mice (111). Moreover, deletion of *Prdm1* encoding the key transcription factor BLIMP1, in all B cells (110) or selectively in mature B cells (109), caused impaired plasma cell differentiation and a dramatic reduction in all immunoglobulin isotypes (particularly in IgG) and led to reduced atherosclerotic plaque size (109, 110). While these data show that the IgG antibodies confer an overall proatherogenic effect, B cell-specific deletion of the transcription factor x-box binding protein-1, which similarly to BLIMP-1 deficiency resulted in reduced levels of all immunoglobulins, increased early atherosclerosis (112). Thus, it is conceivable that the IgG repertoire also includes atheroprotective clones. Indeed, Lorenzo et al. have recently shown that GC-derived antibodies from hypercholesterolemic mice against mitochondrial dehydrogenase ALDH4A1 protect from atherosclerosis (27), thereby demonstrating that the repertoire of the antigen specificities of GC B cells includes also protective responses in atherosclerosis. This conclusion is also supported by Centa et al. showing that IgG antibodies could promote plaque stability (110). It appears promising that the identification of ASA recognized by IgG would reveal new mechanistic layers for the role of B cell responses in atherosclerosis.

Antibody-Independent Functions of B Cells in Atherosclerosis

B cells are an important source of cytokines (113). Transfer of $CD21^{hi}CD23^{hi}CD24^{hi}$ IL-10 secreting B cells isolated from renal lymph nodes into syngeneic mice increased plaque size in a perivascular collar injury model of the carotid artery (114). In contrast, B cell-specific IL-10 deficiency did not affect atherosclerosis in the aortic root (115). These data suggest that B cells may exhibit distinct effects in different atherosclerosis-prone sites. B-2 cell functions that affect atherosclerosis, such as antigen presentation via MHCII complexes (48, 109), CD40 (48, 109), and GITRL (116) signaling, involve interaction with T cells. In addition, MZ B cells mediate their protective effect in atherosclerosis via suppressing the proatherogenic responses of T follicular helper cells (62). However, lymphocyte-deficient mice that were injected with splenic B-2 cells developed increased atherosclerosis (53), which shows that B-2 cells can affect plaque formation in absence of T cells, for example via the production of TNF (117). Furthermore, BAFFR deficiency or blockage (that leads to dramatically reduced B cell numbers) limits atherosclerosis (58–60), whereas soluble BAFF neutralization aggravates atherosclerotic plaque size (118). Both B cell deficiency (48) as well BAFFR blockage (60) result in increased soluble BAFF levels that could be responsible for the atheroprotective effect in these settings. Similarly, A Proliferation Inducing Ligand (APRIL), which is recognized by B cells through shared receptors with BAFF, confers atheroprotection via binding to heparan-sulfate proteoglycans (HSPGs) in the artery wall (119). Therefore, it is likely that B cell depletion in the vessel wall (120, 121) would increase the availability of APRIL for binding to HSPGs.

Summary and Future Perspectives

B cells have the capacity to sense and respond to atherosclerosis. The revolutionary development of high-throughput technologies and methods for gene editing will allow the dissection of the mechanisms by which B cells impact atherosclerotic plaque formation in an unprecedented depth. A key point in this effort would be to identify if and how the B cell response in atherosclerosis is driven by ASA. This will set the ground for the development of precise therapies that will target selectively culprit or atheroprotective B cell clones.

AUTHOR CONTRIBUTIONS

DS, AG, SM, and DT researched the data, wrote, and reviewed the manuscript. DS designed the figure (created with BioRender.com) with input from the other authors. DT supervised the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

DT was supported by the European Research Area Network on Cardiovascular Diseases (I4647), the Austrian Science Fund (I4963 and P35233) and the Austrian Heart Foundation. SM was funded by the Swedish Research Council (2020-01593), the Swedish Heart-Lung Foundation (20210520 and 20210532) and the Leducq Foundation Networks of Excellence Program grant B cells in Cardiovascular Disease. AG was funded by the Swedish Research Council (2020-01789), the Swedish Heart-Lung Foundation (20200333 and 20210469), Loo and Hans Osterman Foundation for Medical Research, Foundation for Geriatric Diseases at Karolinska Institutet, and Stiftelsen för Gamla Tjänarinnor.

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Conflict of Interest: DT was a named inventor on a patent application (EP20217536.0) to exploit APRIL for diagnostic and therapeutic purposes in cardiovascular diseases that has been filed by the Medical University of Vienna (Austria) and CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences (Austria).

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frontiers | Frontiers in Cardiovascular Medicine

REVIEW
published: 25 April 2022
doi: 10.3389/fcvm.2022.865367

Macrophages in Atheromatous Plaque Developmental Stages

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Atherosclerosis is the main pathomechanism leading to cardiovascular diseases such as myocardial infarction or stroke. There is consensus that atherosclerosis is not only a metabolic disorder but rather a chronic inflammatory disease influenced by various immune cells of the innate and adaptive immune system. Macrophages constitute the largest population of inflammatory cells in atherosclerotic lesions. They play a critical role in all stages of atherogenesis. The heterogenous macrophage population can be subdivided on the basis of their origins into resident, yolk sac and fetal liver monocyte-derived macrophages and postnatal monocyte-derived, recruited macrophages. Recent transcriptomic analyses revealed that the major macrophage populations in atherosclerosis include resident, inflammatory and foamy macrophages, representing a more functional classification. The aim of this review is to provide an overview of the trafficking, fate, and functional aspects of the different macrophage populations in the "life cycle" of an atheromatous plaque. Understanding the chronic inflammatory state in atherosclerotic lesions is an important basis for developing new therapeutic approaches to abolish lesion growth and promote plaque regression in addition to general cholesterol lowering.

Keywords: macrophage, monocyte, regression, trafficking, fate, atherosclerosis

OPEN ACCESS

Edited by:
Partha Dutta,
University of Pittsburgh, United States

Reviewed by:
Esther Lutgens,
Academic Medical Center, Netherlands
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *29 January 2022*
Accepted: *31 March 2022*
Published: *25 April 2022*

Citation:
von Ehr A, Bode C and Hilgendorf I (2022) Macrophages in Atheromatous Plaque Developmental Stages. Front. Cardiovasc. Med. 9:865367. doi: 10.3389/fcvm.2022.865367

INTRODUCTION

Atherosclerotic cardiovascular diseases such as myocardial infarction, stroke or peripheral artery disease remain the major cause of morbidity and mortality worldwide to date (1, 2). Despite scientific and therapeutic advance in recent years, there is still an endemic increase in cardiovascular diseases, especially in developing countries (1). Atherosclerotic lesions may occur in any arterial vessel, preferably at regions of non-laminar, low-shear flow (3), and progress slowly causing chronic symptoms such as stable angina or intermittent claudication. The rupture of a plaque or superficial erosions may lead to acute atherothrombotic vascular occlusions with life-threatening consequences such as myocardial infarction or stroke. The standard therapeutic pillars of atherosclerosis primary and secondary prevention therefore encompass antithrombotic and cholesterol lowering treatment options. Yet, despite ever lower target levels for low density lipoprotein cholesterol, residual cardiovascular risk remains prevalent. Vascular inflammation is considered to contribute to the persistent risk of recurrent atherothrombotic events. Macrophages are critically involved in the formation and progress of atherosclerotic lesions. Although some argue that without cholesterol there is no atherogenesis, experimental studies with C-C chemokine

receptor 2 (CCR2) deficient mice indicate that lack of lesion infiltrating monocytes, which give rise to plaque macrophages, protects against atherosclerosis even in the presence of hypercholesterolemia (4, 5). Monocytes are also attracted to atherosclerotic lesions *via* chemokine receptors CCR5 and CX3CR1 (4, 6, 7). Overall restriction of leukocyte infiltration by disruption of CD40 and CD40L interaction and other adhesion molecules attenuates atherosclerosis formation (8–10). According to the current pathomechanistic concept, endothelial dysfunction and activation alongside accumulation of oxidatively modified lipoproteins in the subintimal space of arterial vessels facilitate inflammatory cell recruited to the nascent atheromatous plaque. Beyond new cell recruitment, macrophages and transdifferentiated vascular smooth muscle cells proliferate locally, form foam, cells and propagate disease progression (11–13). In this work we review the roles of arterial macrophage subtypes during the course of atherogenesis highlighting novel insights from fate mapping studies.

MACROPHAGE ORIGINS IN THE VASCULATURE

For decades macrophages, first described in the late nineteenth century, were believed to arise exclusively from monocytes infiltrating tissues and giving rise to tissue macrophages. While monocyte differentiation into macrophages appears to be particularly relevant in the context of tissue inflammation, recent fate mapping, proteomic, and transcriptomic single cell profiling studies in mice portray a far more divers story on macrophage origins and heterogeneity. Broadly speaking, both embryonic, yolk sac (YS)- and fetal liver monocyte-derived resident macrophages, and postnatal monocyte-derived recruited macrophages contribute to varying degrees to the pool of macrophages in different tissues (14–18). Macrophages are the first innate immune cells to seed the tissues between day E9 and E12 of embryonic development in mice, including the arterial vascular system (19–22). These prenatally derived, resident macrophages are long-lived and their population self-sustains by local proliferation predominantly in the adventitia in adulthood (**Figure 1**) (21). Other macrophage populations in arteries arise after birth from infiltrating monocytes that originate from bone marrow (BM) hematopoiesis and populate both the intima and adventitia (21, 23). Monocyte-derived macrophages become even more prevalent during atheromatous plaque development, phagocytizing oxidized lipoproteins and apoptotic cells inside the plaque, and thereby critically regulating local inflammation. These leukocyte-derived macrophages in the plaque are complemented by macrophage-like cells that originate from infiltrating vascular smooth muscle cells (SMC) through transdifferentiation, adopting some macrophage signature markers and forming foam cells (**Figure 1**) (24). Much of our current knowledge of macrophage ontogeny is based on fate mapping studies in animal models such as mice or zebrafish. Because similar studies in early human embryos are limited, embryonic development and characterization of the various macrophage subtypes in humans remain poorly understood. Phenotypic analyses showed that macrophages in the human YS at week 9 of gestation appear as spherical as murine macrophages and acquire a typical phagocytic phenotype with multiple dendrites during maturation. Furthermore they express similar macrophage markers (CD68, CX3CR1) as their counterparts in mice (20). Recent single-cell RNA sequencing from early human embryos obtained after abortion at different time points of development revealed two waves of YS-derived embryonic tissue resident macrophages. The first wave of primitive yolk sac-derived macrophages were detected in the yolk sac at Carnegie stage 11 (CS11), whereas yolk sac derived myeloid progenitor cells give rise to a second monocyte-derived macrophage population after CS17 (25). These findings resemble data acquired in murine studies, suggesting a relevant concordance in macrophage development between mice and human. In the following we discuss how macrophages of diverse origins contribute to vascular health and disease.

MACROPHAGES IN HEALTHY ARTERIES

The arterial vessel wall is composed of three layers: the innermost intima containing the endothelium as a barrier to the blood stream, the media predominantly consisting of vascular smooth muscle cells (VSMCs) and the outer and mostly fibrous adventitia. Under physiological conditions, the vast majority of arterial wall macrophages reside in the adventitia. The intima, the site where atheromatous plaques develop, harbors less than 10% of the overall arterial macrophage population under healthy conditions (19, 31). In mice the YS gives rise to erythro-myeloid progenitors (EMP) which colonize the cardiovascular system and develop into tissue resident macrophages (**Figure 1**) (21, 32). Around birth, almost all macrophages in the arterial wall derive from YS EMP and fetal liver monocytes (21). Postnatally, bone marrow-derived blood monocytes invade the arterial wall and contribute to the macrophage population both in the adventitia and intima (21, 23). Interestingly, the dual origin of adventitial macrophages is maintained by local proliferation and cell recruitment at a ratio of about 70% prenatally derived and 30% monocyte-derived macrophages which slowly declines with age (19). Recent scRNA-sequencing analyses of murine aortic cell suspensions identified different subsets among $CD45^+$, $Csf1r^+$, $MerTK^+$, and $CD64^+$ macrophages in the healthy aorta. Resident macrophages in the adventitia typically express high levels of Lymphatic vessel hyaluronan receptor-1 (Lyve-1) with some subsets presenting with an interferon-inducible signature (Isg15+, Stat1+) or specialized in antigen presentation. The role of resident adventitial macrophages for maintaining vascular health has yet to be fully elucidated, but scRNA-Seq-analyses point toward homeostatic functions based on high expression levels of Lyve-1, Growth arrest-specific protein 6 (Gas6) and Stabilin-1 (Stab1). Csf1r blockade in mice leads to aortic macrophage depletion and results in increased arterial stiffness and fibrosis, effects reversed upon repopulation with arterial macrophages (33). Adventitial macrophage-derived collagenolytic MMP9 release depends on Lyve-1-hyaluronan

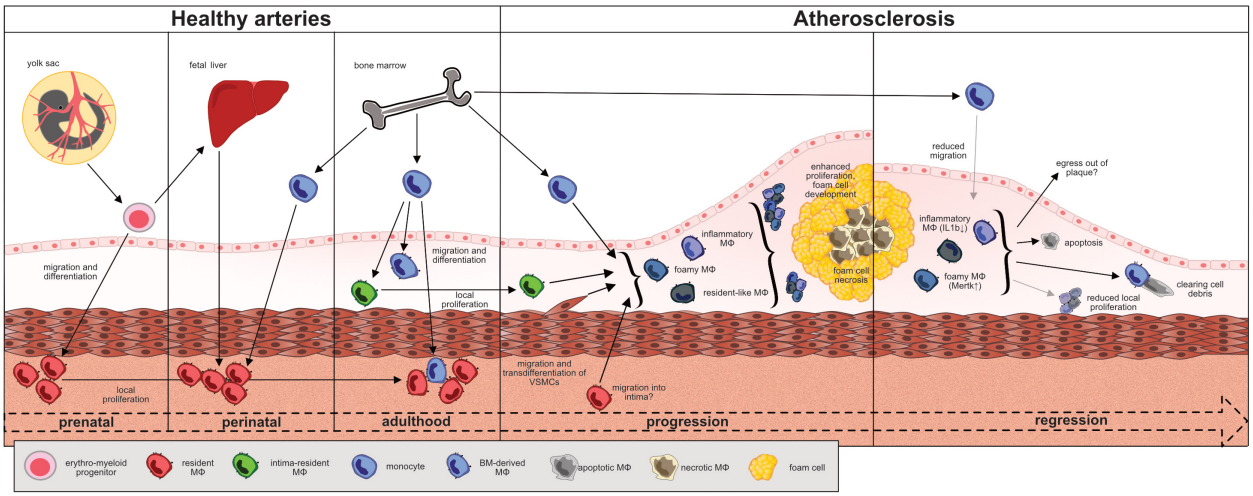

FIGURE 1 | Fate, trafficking and functional changes of macrophages during the "life cycle" of an atheromatous (murine) plaque. EMP-derived macrophages seed the tissues between day E9 and E12 of embryonic development. Macrophages reside primarily in the adventitia and sustain through local proliferation. Monocyte-derived macrophages from the fetal liver and, around birth, from the bone marrow seed the vessel wall and differentiate in the adventitia. The recently introduced intima resident macrophages (Mac^{AIR}) originate from the bone marrow and maintain through local proliferation. They are the first macrophages to get in contact with lipoproteins in the intima and are outnumbered and replaced by newly recruited monocyte-derived macrophages during atherogenesis. At advanced disease stages, local macrophage proliferation dominates cell turnover. Vascular smooth muscle cells may transdifferentiate and adapt a macrophage-like phenotype and contribute to the atheromatous foam cell population. The role of adventitial macrophages in this context remains to be elucidated. Major atheromatous plaque macrophage subpopulations, as defined by their transcriptomes, include resident (*Ccl8, Lyve-1, FOLR2,* CXCL4*, F13a1, Wfdc17*), inflammatory (*Cxcl2, Ccl2, Ccl3, Ccl4, Il-1b, TNF*), and TREM-2^+ foamy (*Lgals3, Cd9, Ctsd*) macrophages. The degree of plasticity in between subpopulations and their respective roles in disease progression are under investigation. In murine models of atherosclerotic regression, inflammatory macrophages showed less Nlrp3 and IL1β expression while $Trem2^{hi}$ foamy macrophages expressed more Mertk and less Mmp2 in line with a rather atheroprotective function.

TABLE 1 | scRNA-seq from murine and human arteries identified markers that classify macrophage subsets into inflammatory, resident-like, and foamy macrophages.

	Murine marker	Human marker	Proposed function
Inflammatory macrophages	Tnf, Ccl2, Ccl3, Ccl4, Cxcl2 (26, 27)	TNF, IL1b, CASP1, CASP4, CCL3, CCL4 (28, 29)	Pro-inflammatory
Resident-like macrophages	Lyve1, $Cx3cr1^{hi}$, Cd206, Gas6, Ccl8, CXCL4, Wfdc17 (26, 27)	TXNIP, Cd14, YWHAH (29)	Regulatory, endocytosis
Foamy macrophages	Trem2, Cd9, Lgals3, Fabp5, Ctsd, Abcg1 (26, 30)	ABCA1, ABCG1, MMP9, OLR1 (28, 30)	Both pro- and anti-inflammatory, cholesterol metabolism

The proposed functions of different subsets are yet to be confirmed experimentally.

interactions with smooth muscle cells, and Lyve-1 expressing macrophages can promote angiogenesis in mice and human (33, 34). Macrophage-expressed Gas6 and Stab1 act as scavenger receptors mediating the clearance of apoptotic cells (35, 36). A small subset of less than 10% of murine aortic macrophages features a distinct profile enriched for inflammatory markers such as interleukin-1β (IL-1b) and MMP12. It develops from monocytes, largely resides in the intima and was therefore termed "aorta intima resident macrophages" (Mac^{AIR}) (23). Mac^{AIR} are predominantly found in arterial branches, locations of turbulent flow, and more susceptible to atherosclerosis development (23). Monocytes, classified as $Ly6C^{high}$ $CCR_2{}^+$ and $Ly6C^{low}$ $CCR_2{}^-$ subsets in mice, play a subordinate role for aortic macrophage maintenance during homeostasis. Merely 20% of the arterial macrophage pool is constantly replenished by invading blood monocytes, predominantly by the $Ly6C^{high}$ subset (19, 31). Cycling $Ly6C^{low}$ monocytes constantly surveil the endothelium but rarely cross the endothelial barrier (37–39). Due to the pro-inflammatory properties of monocyte-derived macrophages it is conceivable that BM-derived macrophages represent the vascular first line of defense against invading pathogens whereas EMP-derived macrophages exert primarily homeostatic functions, as described above. In keeping with this concept, the population of resident adventitial macrophages outlasts acute inflammatory challenges (19, 21). A single injection of LPS or continuous infusion of Angiotensin II (Ang II) cause acute vascular inflammation in mice and a transitory surge in monocyte-derived macrophages in the arterial wall. However, within days to weeks the original, resident macrophage composition of predominantly embryonically-derived macrophages is being restored through enhanced local proliferation (19, 21). Functionally, EMP-derived macrophages in the adventitia express genes associated with

tissue repair during Ang II infusion whereas monocyte-derived macrophages activated inflammatory processes (19).

These findings are in line with previous murine studies from the heart. Macrophages populate the heart already during embryonic development, self-sustain through *in situ* proliferation and orchestrate cardiac tissue homeostasis (40). In an acute cardiac injury, the resident cardiac macrophages of prenatal origin support healing after myocardial infarction, whereas macrophages that descend from recruited monocytes aggravate adverse cardiac remodeling (41). In general, macrophage ontogeny defines distinct cellular programs in- and outside the vessel (18, 42).

Hu et al. recently analyzed the transcriptomic profile of macrophages in different healthy human cardiac arteries after heart transplantation (29). Four macrophage subsets were identified (inflammatory macrophage 1, inflammatory macrophage 2, resident macrophage, and dividing macrophage). Inflammatory macrophages mainly expressed typical M1 markers, such as TNF, IL1B, CCL3, and CXCL8 whereas M2 markers were scarcely expressed in any of the macrophage subsets (29). The authors concluded that even under physiological conditions a majority of macrophages in human cardiac arteries feature an inflammatory profile with activation of NFKB pathways and inflammasome regulation (29, 43).

MACROPHAGES AND THE PROGRESSION OF ATHEROSCLEROSIS

Atherosclerosis is a major pathomechanism underlying cardiovascular disease, including coronary and ischemic heart disease, peripheral artery disease, and stroke. Oxidized low density lipoproteins accumulate in the arterial intima sparking a chronic, lipid-driven inflammatory response, enhanced by shear stress, and hypoxia (44–48). In response, monocytes infiltrate the intima, differentiate into atheromatous plaque macrophages, proliferate locally, and phagocytize lipoproteins leading to foam cell formation (27, 49, 50). Hypercholesterolemia, inflammation, sedentary life style, and mental stress stimulate medullary and extramedullary hematopoiesis resulting in higher numbers of circulating pro-inflammatory monocytes (51–56). Ly6C^{low} monocytes appear to be better equipped for lipid uptake in the circulation which enhances their patrolling behavior and aids in atheroprotection (37, 57), while Ly6C^{high} monocytes are more prone to infiltrate the arterial vessel wall (11). The recruitment of monocytes from the bloodstream to the intima is mediated by chemokine receptors (CCRs), including CCR2, CCR5, and CX3CR1 in mice and human (7, 58, 59). Endothelial, smooth muscle, and immune cells as well as platelets secret chemokine ligands of the CC family (CCLs) that interact with the respective chemokine receptor on the monocyte surface (58, 60). Ly6C^{high} monocytes express low levels of CX3CR1 and high levels of CCR2 (61), but secretion of CCL2 by endothelial cells can enhance monocyte CX3CR1 expression and facilitate their adhesion *via* integrins (62–64). After crossing the endothelial barrier, Ly6C^{high} monocytes, beyond differentiating into tissue macrophages, may proliferate *in situ* or function as antigen-presenting cells themselves when migrating to lymph nodes (65, 66). Murine Ly6C^{high} monocytes resemble human CD14^{++}/CD16^{-} monocyte population, whereas Ly6C^{low} monocytes correspond to the CD14^{+}/CD16^{++} subset (60).

Recently described intima resident Mac^{AIR} become foam cells even before blood monocytes are recruited to the nascent plaque. Transgenic mice lacking Mac^{AIR} show less lipid deposition in the first stages of atherosclerosis suggesting a pioneering role at the onset of atherogenesis (31). As atherosclerosis progresses, Mac^{AIR} are marginalized by atheromatous plaque macrophages derived from newly recruited monocytes and transdifferentiating vascular smooth muscle cells (VSMC) (11, 31). In advanced plaques of Apoe$^{-/-}$ mice, all macrophages of monocyte origin renew within 4 weeks, but largely independent of newly recruited monocytes (13, 50). Instead, more than 85% of atheromatous plaque macrophages renew through local proliferation (13). Non-leukocytes expressing prototypical macrophage markers and forming foam cells may originate from VSMCs. Under physiological conditions, they express typical SMC markers such as α-smooth muscle actin (α-SMA). During atherogenesis in human carotid arteries VSMCs exhibit a loss in contractility, a higher proliferation rate and a reduced expression of α-SMA (67). VSMCs constitute at least 30% of all plaque cells (68) and their contribution may be even underestimated as they switch their phenotype. Lineage-tracing approaches in mice indicate that VSMC are able to adopt characteristics of osteoblast, fibroblasts, and macrophages (69). VSMC play a major role in cell-cell-interactions attracting various immune cells to the plaque by the secretion of cytokines such as IL-6, CCL2, and upregulation of ICAM-1 and fostering chronic inflammation of the arterial vessel wall (70). Equipped with scavenger receptors for lipoprotein uptake, VSMC account for the majority of foam cells in the plaque, although only a fraction of these cells co-expresses macrophage markers which classify them as macrophage-like cells (71). VSMC-specific deletion of the transcription factor Krüppel-like factor 4 (KLF4) limits transdifferentiation of VSMCs into macrophage-like cells and slows plaque progression (72). It is estimated that about 40% of cells expressing macrophage marker CD68 in human plaque derive from VSMC, but the majority of atheromatous plaque macrophages arises from monocytes and *via* local proliferation (73).

The traditional view was Ly6C^{high} monocytes differentiate into macrophages with M1 type features during the progression of atherosclerosis, releasing pro-inflammatory cytokines (TNF-α, IL-12, IL-6) and reactive oxygen species, while differentiation into alternatively activated M2 type macrophages facilitates plaque regression (74). Recent single cell analyses of atheromatous plaque immune cells using mass cytometry and RNA sequencing refuted the dichotomous M1/M2 classification and instead identified macrophage subsets with mixed phenotypes specialized in inflammation, lipid handling, and homeostasis (26, 28, 30). For example, in mice the expression of transcription factor Interferon regulatory factor 5 (IRF 5), mediating classical M1 type macrophage polarization *in vitro,* is not restricted to the inflammatory macrophage subpopulation in the atherosclerotic aorta (30). Loss of Irf5 in myeloid cells limits

lipid and macrophage accumulation in the plaque, decreases IL-12 and increases TGFβ, MerTK, and CD206 expression, promoting a stable plaque phenotype (30, 75, 76). Major macrophage populations in murine atherosclerotic aortas, as defined by their transcriptomes, include resident (*Ccl8, Lyve-1, FOLR2* CXCL4,*F13a1, Wfdc17*), inflammatory (*Cxcl2, Ccl2, Ccl3, Ccl4, Il-1b, TNF*), and TREM-2^{+} foamy (*Lgals3, Cd9, Ctsd*) macrophages (**Table 1**), and smaller populations such as interferon-inducible (*Ccl12, Isg15, Irf7, Ifit1, Ifit3*) and cavity macrophages (*Fn1, Clec4b1, Sept11, Ear2*) (26, 27). The latter shares transcriptional similarities with macrophages from the peritoneum and may actually infiltrate from pleural or pericardial cavities. Their role and impact in atherosclerosis remains elusive to date. Transcriptionally, proliferating atheromatous plaque macrophages represent a distinct subpopulation without preferential overlap with one of the aforementioned major macrophage subsets in mice and men (77, 78). These data do not indicate whether one macrophage subset is more prone to proliferate than others or what may trigger local proliferation, which dominates cell turnover in advanced atherosclerotic lesions. However, we have identified scavenger receptors Msr1 and CD36, involved in the uptake of modified lipoproteins, experimentally as mediators of local macrophage proliferation in atherosclerotic aortas using a mixed bone-marrow chimeric approach in mice (12). In line, *in situ* macrophage proliferation in human carotid artery plaques correlated with serum LDL-cholesterol levels and plaque lipid contents (12). In addition, macrophage colony-stimulating factor or uptake of apoptotic cells stimulate proliferation of plaque macrophages and the latter induces an inflammation-resolving phenotype (79–81). CD47 binding to inhibitory signal regulatory protein α (SIRPα) on macrophages induces a "don't eat me" signal. SIRPα deletion on macrophages enhances their efferocytic capacity and suppresses atherogenesis in mice similar to effects observed with the administration of CD47-blocking antibodies (82, 83).

Although foam cells are considered a hallmark of atherosclerotic plaques, and high plaque lipid loads represent a feature of plaque vulnerability, TREM-2 + foam cells feature a rather anti-inflammatory profile and enhanced lipid metabolism in contrast to inflammatory macrophage populations (84). TREM-2 is a transmembrane receptor that interacts with apolipoprotein E and its expression is enhanced by alternative macrophage activation *via* the IL-4 pathway and abolished by LPS and IFN-γ as pro-inflammatory cytokines (85, 86). Experimental models have shown that cholesterol mediated foam cell formation deviates the cellular cholesterol-biosynthetic pathway resulting in the preferential production of desmosterol, which suppresses inflammatory responses in macrophages (87). Conversely, overexpression of the enzyme converting desmosterol into cholesterol in an atherosclerosis mouse model stimulated inflammasome activation in plaque macrophages while even enhancing intracellular lipid accumulation, thus decoupling foam cell formation from inhibition of inflammation (88). Other means of foam cell formation, e.g., oxidized LDL uptake *via* CD36, have also demonstrated to trigger rather than suppress inflammation (89). Activation of the olfactory receptor 2 on vascular macrophages by octanal, a lipid aldehyde formed during oxidative stress and lipid accumulation in atherosclerotic plaques, stimulates the inflammasome and exacerbates atherosclerosis in mice (90). Finally, a more refined algorithm analyzing human atherosclerotic tissue single cell transcriptomic datasets was able to discriminate two distinct programs in plaque macrophages—homeostatic foaming and inflammatory pathogenic foaming. Gene expression profiles associated with inflammatory foaming correlate with cardiovascular events in the Multi-Ethic Study of Atherosclerosis, indicating that foam cell formation and inflammation are not mutually exclusive after all (91). The degree of cell plasticity which allows macrophages to adopt different phenotypes within atherosclerotic plaques remains an active field of investigation. Likewise, it is unclear to what extend the prominent population of resident-like Lyve-1 + macrophages, identified by unsupervised scRNA-Seq of atherosclerotic tissues, derives from adventitial macrophage precursors of largely prenatal origin or from recruited monocytes. A large fraction of resident-like macrophages expresses CLEC4A2, a C-type lectin receptor. Depletion of CLEC4A2-expressing macrophages, located mainly in the adventitia, increased atherosclerotic lesion formation in mice, pointing toward an atheroprotective role by preserving lipid handling and suppressing inflammatory responses (92).

In advanced atherosclerotic lesions, male $ApoE^{-/-}$ mice have greater numbers of macrophages than their female littermates. However, at week 22 of a proatherogenic diet, the number of macrophages in female $ApoE^{-/-}$ mice is similar to or higher than in males. Elizabeth Moss et al. recently showed that female Ldlr-deficient mice in an AAV-PCSK9 mouse model develop larger atherosclerotic plaques but are protected from vascular inflammation. Compared with males, they have 62% fewer myeloid cells in the aortic arch (93). The finding of lower plaque inflammation in female mice is consistent with a recent study that found lower expression of matrix metalloproteinase-12 (MMP12) by lesional macrophages in aortic root plaques from female mice compared with males (94). The lower plaque inflammation in female mice may indicate a more stable plaque phenotype and explain the protection from cardiovascular ischemia observed in premenopausal women (95). Various studies of human carotid endarterectomy specimen showed that atherosclerotic plaques from men contain more macrophages than women of the same age, regardless of disease severity or symptomatology (96–98). Although plaque macrophage numbers may differ between males and females, sex-dependent differences in transcriptional profiles require further studies and may be confounded by the extent of disease. Of note, in the heart, little to no difference between male and female human macrophages were detected apart from genes on X and Y chromosomes (99).

MACROPHAGES AND THE REGRESSION OF ATHEROSCLEROSIS

While we have not harbored the full potential of primary prevention, yet, many patients present to the doctor already with established atherosclerotic cardiovascular disease. Therefore,

promoting regression of atherosclerotic lesions is of utmost clinical importance. In contrast, mechanisms that govern plaque regression are less well studied than those driving plaque progression. Clinical trials using intravascular imaging tools have shown that plaque volumes decline minimally but plaque composition can change significantly upon reduction of LDL-cholesterol levels below 70 mg/dL (1.8 mmol/L), with fibrotic cap thickness increasing and lipid and macrophage contents decreasing (100, 101). Reversal of elevated cholesterol levels in atherosclerotic mouse models was used to study plaque regression, experimentally, by switching from high-cholesterol to cholesterol-free diets, transplanting atherosclerotic aortas into non-hypercholesterolemic mice or interfering with hyperlipidemia associated genes on a transcriptional level (102–104). In humans, however, significant and lasting reductions in LDL cholesterol levels depend on drug treatment which target cholesterol biosynthesis (e.g., statins, bempedoic acid), absorption (ezetimibe) or hepatic LDL particle clearance (PCSK9 inhibition) (105). The ApoE3L.CETP transgenic mouse model features a humanized lipid profile and responds to oral statin treatment with plasma cholesterol lowering unlike the more common atherosclerosis mouse models with ApoE and Ldlr-deficiency. In ApoE3L.CETP mice with established atherosclerosis, oral atorvastatin treatment induced plaque regression with marked decline in macrophage content. Although monocyte infiltration was reduced, as previously reported (23, 106), the reduction in atheromatous plaque macrophage numbers mostly depended on the suppression of local proliferation and constant cell death (12). Macrophage egress from sites of inflammation (107), an alternative mechanism to explain the decline in macrophage numbers during plaque regression, was observed in some (74, 108, 109), but not other studies (12, 23, 106, 110). Aside from numeric changes, transcriptional profiles of murine macrophages in regressing plaques were compared to those in progressing plaques. Interestingly, proportions of resident-like, inflammatory and foamy macrophages, as defined by bioinformatic cluster and marker gene analysis, were relatively similar in progressing and regressing plaques. Macrophage genes associated with plaque progression encoded for MHCII molecules and Malat1, while Cathepsins and Cxcr4 were overexpressed during regression (65). Inflammatory macrophages expressed less Nlrp3 and IL1b while foamy macrophages expressed more Mertk and less Mmp2 (111). Smaller subpopulations with interferon- and IL-4-signatures were more prevalent in progressing plaques, indicative of more diversified cell polarization states during plaque progression and revising a previous oversimplified concept of M1–M2 macrophage conversion during plaque regression (65, 74). Still, administration of M2-polarizing IL-13 can induce plaque regression or slow progression in mice with decreased plaque macrophage numbers and improved cellular lipid handling independent of systemic cholesterol levels (110). More physiologically, regulatory T cells accumulate in regressing plaques and support macrophage egress and apoptosis while suppressing macrophage proliferation. Tregs enhanced the efferocytic and inflammation-resolving capacity of plaque macrophages, and their depletion prevented plaque regression in mice even when serum cholesterol levels were normalized (111). Under hyperlipidemic conditions, however, plaques continue to progress as protective Tregs covert into pathogenic T_H1/T_H17 cells (112). These data encourage the design and study of atherosclerosis vaccines to support atherosclerosis regression (112, 113).

THERAPEUTIC APPROACHES TARGETING MACROPHAGES

As described above, macrophages play a crucial role in all phases of the atheromatous plaque's "life cycle," and thus represent potential therapeutic targets. In atherosclerotic mice, insufficient cholesterol efflux from cells was linked to enhanced hematopoietic activity and an increase in hematopoietic stem and progenitor cells (HSPC) and their progenies (52, 114). Systemic monocytosis leads to increased recruitment of macrophages to the vessel wall and the number of macrophages in the plaque correlates with plaque vulnerability: In general, the more macrophages in the plaque, the more susceptible it is to rupture (12, 115). Systemic therapy with anti-IL1b antibodies decreases monocyte and neutrophil blood counts in experimental models and patients post myocardial infarction, and suppresses endothelial cell activation (54, 116). Of note, in the CANTOS trial treatment with the anti-IL1b-antibody canakinumab significantly reduced rates of recurrent cardiovascular disease, independent of alterations in lipid levels (117).

Upstream of IL1b release by macrophages, the inflammasome, a macromolecular structure composed of multiple components responsive to intra- or extracellular danger signals (118), activates caspase1, which cleaves the pro-form of IL-1b. In particular, cholesterol crystals, hypoxia, and non-laminar flow can lead to inflammasome and caspase1 activation in the plaque; usually several of these stimuli are required for sustained activation (119–122). Expression of Nlrp3 inflammasome components in atherosclerotic plaques of patients correlates with cardiovascular disease severity (123), and NLRP3 expression in blood monocytes predicts adverse cardiac events (124). Several small molecular inhibitor trials targeting the NLPR3 inflammasome are currently underway. Current guidelines recommend considering low dose colchicine treatment for secondary prevention in patients at very high cardiovascular risk following a series of positive clinical trials (125–127). Although the exact mechanisms underlying colchicine's atheroprotective effects are not well understood, inhibition of the NLRP3 inflammasome appears to be a plausible mode of action.

We propose that inhibition of local macrophage proliferation in atherosclerotic lesions may support plaque regression. This may be achieved by reducing cholesterol-rich lipoprotein levels which we recently identified as triggers of macrophage proliferation (12) or, potentially, by promoting plaque cholesterol efflux with recombinant Apolipoprotein A infusions, an approach being tested in the AEGIS II cardiovascular outcome

trial. Alternatively, nanoparticles can be used to deploy antiproliferative or gene expression modifying agents in atherosclerotic lesions to target plaque macrophages directly (128, 129) and to prevent or even revert disease progression.

CONCLUDING REMARKS

Macrophages are prominent cells in the pathogenesis of atherosclerosis. Thanks to their versatile nature, they carry both inflammation propagating and resolving properties. Decreasing triggers of plaque progression, e.g., dyslipidemia, while fostering reparative cell responses through immunomodulation and targeted nanoparticle approaches may help to promote plaque regression in the future (112, 113, 130, 131).

AUTHOR CONTRIBUTIONS

AE and IH contributed equally to writing the manuscript. CB discussed and commented the research. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the German Research Foundation to IH (HI1573/2 and Collaborative Research Center SFB1425 grant 422681845) and to AE (grant 413517907, IMM-PACT Program for Clinician Scientists, Department of Medicine II, Medical Center—University of Freiburg and Faculty of Medicine, University of Freiburg).

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frontiers | Frontiers in Cardiovascular Medicine

REVIEW
published: 25 April 2022
doi: 10.3389/fcvm.2022.868466

Cellular Heterogeneity of the Heart

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Recent advances in technology such as the introduction of high throughput multidimensional tools like single cell sequencing help to characterize the cellular composition of the human heart. The diversity of cell types that has been uncovered by such approaches is by far greater than ever expected before. Accurate identification of the cellular variety and dynamics will not only facilitate a much deeper understanding of cardiac physiology but also provide important insights into mechanisms underlying its pathological transformation. Distinct cellular patterns of cardiac cell clusters may allow differentiation between a healthy heart and a sick heart while potentially predicting future disease at much earlier stages than currently possible. These advances have already extensively improved and will ultimately revolutionize our knowledge of the mechanisms underlying cardiovascular disease as such. In this review, we will provide an overview of the cells present in the human and rodent heart as well as genes that may be used for their identification.

Keywords: single cell RNA sequencing, heart, cell type, heterogeneity, genes

OPEN ACCESS

Edited by:
Norbert Gerdes,
Heinrich Heine University Düsseldorf, Germany

Reviewed by:
Petra Kleinbongard,
Essen University Hospital, Germany
Gustavo Campos Ramos,
University Hospital Würzburg, Germany

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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *02 February 2022*
Accepted: *23 March 2022*
Published: *25 April 2022*

Citation:
Anto Michel N, Ljubojevic-Holzer S, Bugger H and Zirlik A (2022) Cellular Heterogeneity of the Heart. Front. Cardiovasc. Med. 9:868466. doi: 10.3389/fcvm.2022.868466

INTRODUCTION

Cardiovascular disease (CVD) and its sequelae represent a major health and socioeconomic burden accounting for vast and continuously increasing morbidity, and roughly a third of all deaths in the world (1). There are many different types of CVD including coronary heart disease, stroke, peripheral arterial disease, myocardial disease, and aortic disease. Coronary heart disease can lead to angina, heart attacks, or heart failure. Although the exact cause of CVD is unknown, a solid body of experimental and clinical data identified inflammation as a common final pathomechanism. This is particularly well documented for atherosclerosis and its direct clinical consequences (2), however, increasing evidence suggests that traditional and non-traditional risk factors trigger this inflammatory process and thus ultimately drive initiation and progression of CVD (3, 4). These risk factors include smoking, hypertension, hypercholesterolemia, chronic kidney disease, and diabetes, but also systemic inflammation stemming from chronic inflammatory conditions (e.g., rheumatoid arthritis), infectious diseases, or obesity-derived visceral adipose tissue inflammation. However, how this pathogenic link between (multiple) risk factors, inflammation, and adverse cardiac phenotypes operates, and which cellular phenotypes or clusters mediate its action in the heart is largely unknown.

Identification of cellular heterogeneity and their intercommunication can play a vital role in differentiating a healthy from a diseased heart and it may predict future outcomes with superior precision and at much earlier stages than currently possible. Conventional ways used to identify specific cells include fluorescence-activated cell sorting (FACS). FACS is a powerful tool that allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. Nonetheless, FACS affords some limitations in sample

preparation, fluorescent parameters, antibodies selection and most importantly, paucity of markers that can be assayed at the same time. Therefore, FACS is not suited for an unbiased identification and further subclassification of unknown or poorly subcategorized cells and the definition of their potential roles in physiology and disease pathology.

In the last 10 years, analyses of cellular heterogeneity with single cell resolution have made an astounding progress. Developments in high parametric multiplex cell analysis such as cytometry by time of flight (CyToF) and single cell RNA sequencing (scRNAseq) have enabled us to gain a high-power view on novel individual cellular phenotypes as well as on distinct cellular expression patterns integrating the transcripts of thousands of genes (5). While such high-parametric data sets are still scarce in cardiovascular disease, scRNAseq techniques have been successfully employed to detail the cellular composition of whole organs, to identify new cell types, and/or characterize cellular expression patterns associating with disease, disease severity, outcome, or therapeutic response in other areas (6–10). Therefore, they have proven to be an excellent tool to advance our understanding of the cell types and populations involved in disease pathogenesis in various fields. Such knowledge is urgently needed to better understand cardiac physiology and its derangement through disease and to ultimately improve the treatment options and outcomes for cardiac patients.

In the following sections, we summarize current knowledge on the cellular composition of the heart, its relevance for physiology and pathologic transformation, as well as characteristic changes in pathological conditions reported. We also provide a comprehensive overview of the developmental stage-specific changes in cellular heterogeneity and most promising cellular markers that can increase robustness and reproducibility of single cell transcriptomic analyses in different experimental animal models and human biomaterials (**Figure 1** and **Supplementary Tables 1–3**). Given that methodological differences between scRNAseq and FACS complicate data interpretation and would require a large discussion, we predominantly focus on publications reporting data generated by scRNAseq.

CELLULAR COMPOSITION OF THE HEART

Cardiomyocytes – The Core of Cardiac Contraction

Cardiomyocytes are the engine of the heart where energy is converted to mechanical work by myosin ATPases consuming high energy phosphates, thereby driving the cross-bridge cycle of the cardiomyocyte. While the use of stereological and morphometric methods estimated that cardiomyocytes would cover 75% of the total cardiac cell volume in rats, studies based on flow cytometry and immunohistochemistry revealed that around 30% of total cardiac cells are cardiomyocytes, with significant species differences in the cellular composition between human, mouse and rat hearts (11–13). Confident identification of cardiomyocytes can be achieved by measuring the expression proteins involved in the contractile machinery such as myosin light (*MYL2-4, MYL7,* and *MYL9*) and heavy (*MYH6, MYH7,* and *MYH7B*) chains, myosin binding proteins (*MYBPC3*), troponins (*TNNT2, TNNTI1, TNNTI3,* and *TNNTC1*), and proteins involved in calcium-mediated processes [ryanodine receptor 2 (*RYR2*), phospholamban (*PLN*), Sodium ion/calcium exchanger (NCX)] (14–16). Integrity of cardiomyocytes is reflected by expression of the protocadherin 7 (PCDH7) gene which encodes a strong calcium-dependent adhesive molecule (17), and of SET (Suppressor of variegation, Enhancer of Zeste, Trithorax) and MYND (Myeloid-Nervy-DEAF1) domain containing 2 (*SMYD2*) corresponding to a lysine methyltransferase that promotes sarcomere formation and stabilization (18). It seems that the latter proteins are only significantly expressed in cardiomyocytes of the adult heart.

Among regularly contracting cardiomyocytes, atrial and ventricular cardiomyocytes participating in the cardiac conduction system can be distinguished which transduce electrical stimuli to drive cardiac contraction. These cardiomyocytes have been traditionally identified based on hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (*HCN4*) (19) and contactin-2 (*CNTN2*) expression (20). Additional markers include gap junction protein alpha-1 (*GJA1*), gap junction protein alpha-5 (*GJA5*), secreted protein acidic and cysteine rich (SPARC)-related modular calcium-binding protein 2 (*SMOC2*), ISL LIM homeobox 1 (*ISL1*) or T-box transcription factor 3 (*TBX3*), although the expression of these markers shows significant heterogeneity depending on their anatomical location within the conduction system (nodes, atrioventricular bundle, Purkinje fiber network) (21–23). Using scRNAseq technology, an even higher grade of heterogeneity within the conduction system has been revealed. Specific subpopulations of cardiomyocytes with particular expression of myozenin 2 (*MYOZ2*) in adult murine and developing human heart (24) have been identified, and the expression of insulin-like growth factor-binding protein 5 (*IGFBP5*), copine 5 (*CPNE5*), and neurotrimin (25) is enriched in the entire conduction system whereas *SMOC2* expression is exclusively observed in cells of the sinus node (26).

To distinguish the fetal and adult heart, *TNNTI1*, *TNNTI3*, and *TNNTC1* are accepted cellular markers in fetuses, whereas different troponin and myosin genes are commonly used to identify adult cardiomyocytes (*TTN, MYBPC3, TNNT2, MYH7,* and *MYL2*) (14–16). Of note, while the known switch toward predominant expression of *MYH6* in postnatal hearts (compared to predominant expression of *MYH7* in fetal hearts) is observed in rodents, humans mainly express *MYH7* throughout life without a significant isoform switch. Furthermore, α-skeletal actin (*ACTA1*) is mainly expressed in fetal and neonatal human hearts, whereas expression of *ACTC1* predominates in the adult heart (27, 28). Fetal hearts also show enhanced expression of compliant titin (*TTN*) isoforms (N2BA1/N2BA2), which are eventually replaced by adult isoforms in postnatal development (29).

A differentiation between fetal and adult cardiomyocytes can also be achieved by evaluating markers of cell proliferation, given the high and low proliferative capacity of fetal and

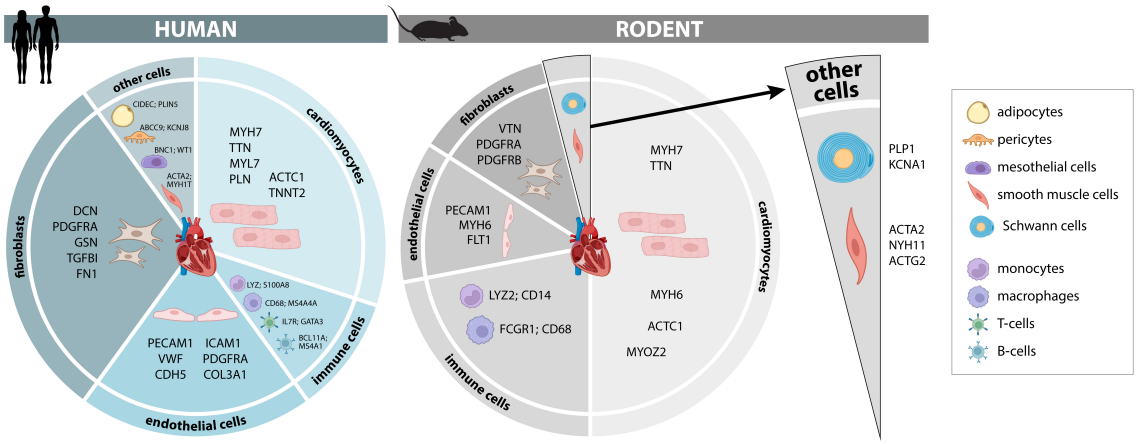

FIGURE 1 | Cellular composition and markers of the human and murine heart. Cellular composition and most consistently identified markers that may be used for identification and separation of major cellular lineages in adult human and murine hearts. A more comprehensive list of additional marker genes, including fetal human hearts, is provided in **Supplementary Tables 1–3**.

adult cardiomyocytes, respectively. Convenient markers to appreciate cardiomyocyte proliferation may include expression levels of DNA topoisomerase 2 alpha (*TOP2A*) and marker of proliferation Ki-67 (*MKI67*) (14, 30, 31). It remains to be noted that only few cardiomyocytes in the adult heart (0.4% of all cardiac cells) display markers of proliferation at all as revealed by an integrative analysis using a mouse cell type atlas in combination with single-nuclei RNA-seq (32).

Furthermore, a differentiation between fetal and postnatal cardiomyocytes is also possible by measuring markers or metabolites of energy metabolism (33). While fetal hearts mainly utilize carbohydrates for ATP regeneration and have low mitochondrial oxidative capacity, cardiomyocytes switch toward predominant fatty acid oxidation and undergo marked mitochondrial biogenesis once fatty acid and oxygen availability increase in the early postnatal period (34). The fetal metabolic program is characterized by expression of the liver isoform of carnitine palmitoyltransferase 1 (*CPT1*), whereas human postnatal cardiomyocytes express muscle *CPT1*, high levels of peroxisome proliferator-activated receptor alpha (*PPARα*; induced by fatty acid availability) and *PPAR* gamma coactivator 1-alpha (*PGC-1α*; driving physiological mitochondrial biogenesis). In addition, the content of glycogen (measurable by histology are electron microscopy) may be higher than 30% in fetal but only around 2% in adult cardiomyocytes (33).

Atrial and ventricular myocardium differs in developmental, structural, hemodynamic, and physiological properties, which is reflected by a distinct expression profile (35). Based on these differences, Hairy/enhancer-of-split related family bHLH transcription factor with YRPW motif 2 (*HEY2*) and *MYH7* may qualify as markers predominantly expressed in ventricular cardiomyocytes, whereas expression of natriuretic peptide A (*NPPA*) and *MYL4* is more evident in atrial cardiomyocytes (16). Furthermore, the atrial fibrillation susceptibility gene, paired like homeodomain 2 (*PITX2*), is only observed in left atrial cardiomyocytes (36). Interestingly, *HCN4* which encodes the ion channel responsible for spontaneous depolarization and which has also been associated with atrial fibrillation, is present in approximately 4% of right atrial, in less than 0.5% of left atrial and only ∼1% of ventricular cardiomyocytes (16).

In non-diseased myocardium, a significant heterogeneity of cardiomyocytes with gradients of specific gene expression has been observed, and additional changes in gene expression are superimposed by cardiac pathologies, complicating the interpretation of transcriptional profiling studies on the single cell level. Application of single-cell technology has revealed a more extensive heterogenic gene expression (e.g., *NPPA*; brain natriuretic peptide, *BNP*; MYH7) in failing hearts than previously identified using bulk-RNA sequencing (37). In another study, the combination of single-cell analysis and RNA *in situ* hybridization of human dilated cardiomyopathy samples uncovered transcriptional heterogeneity, allowed to distinguish distinct gene modules responsible for cardiomyocyte hypertrophy or failure, and elucidated coordinated molecular and morphological dynamics of cardiomyocytes that may promote heart failure development (38). Furthermore, studies in rodents with pressure overload-induced cardiac hypertrophy showed that *MYH7* genes were greatly expressed with smaller cardiomyocytes as opposed to larger cardiomyocytes, and that *MYH7* was markedly expressed in middle layers of the myocardium (38). These data support the concept of adaptive heterogeneity of cardiomyocytes, where cardiomyocytes that consume less energy undergo atrophy and express major histocompatibility complex (MHC) proteins to limit myofibrillar ATPase activity, whereas other cardiomyocytes with increased oxidative capacity may express MHC proteins to guarantee a high rate of myofibrillar ATP consumption Using these novel technologies to provide a spatial map of cells-of-interest within the heart or to identify cardiomyocytes of interest may have intriguing clinical value, e.g., by facilitating diagnostics of myocardial tissue or biopsy specimen by pathologists.

Cardiomyocytes are the main cellular population responsible for cardiac contraction, and their unique molecular composition allows them to accomplish their highly specialized roles. However, overall cardiac function needs to be investigated and understood in the context of cardiac tissue, in which other cell types operate together with cardiomyocytes to orchestrate periodic cardiac contractions that adapt to the physiological demands of the living system.

Fibroblasts – Frequent and Functional

Cardiac fibroblasts are involved in the synthesis and remodeling of extracellular matrix, communicate with cells of the immune system, participate in cardiac conductivity and rhythmicity, and take part in myocardial healing responses, e.g., following myocardial infarction or during chronic disease states (39–41). In absence of disease, fibroblasts are nearly equivalent to cardiomyocytes in cell number, accounting for approximately 25–32% of all cells in the heart (16, 42). In adult human heart tissue, classical fibroblast markers include decorin (*DCN*), gelsolin (*GSN*), transgelin (*TAGLN*), regulator of G protein signaling 5 (*RGS5*), Smooth muscle aortic alpha-actin 2 (*ACTA2*), Thy-1 cell surface antigen (*THY1.1*), platelet derived growth factor receptor alpha (*PDGFRA*), S100 calcium binding protein A4 (*S100A4*), discoidin domain receptor tyrosine kinase 2 (*DDR2*), lymphocyte antigen 6 complex, locus A (*LY6A*) also known as Sca-1, vimentin (*VIM*), and collagen type I alpha 1 chain (*COL1A1*) (41). However, once fibroblasts become activated or the heart suffers injury (e.g., myocardial infarction), fibroblasts may also strongly express other markers such as periostin (*POSTN*), alpha smooth muscle actin (α*SMA*) or mesenchyme homeobox 1 (*MEOX1*), among other genetic signatures. In this respect, a differentiation of three major types of fibroblasts in the heart by distinct expression profiles (mature fibroblasts, activated fibroblasts, myofibroblasts) has been provided in another excellent review (41). Fibroblasts of the fetal human heart express transcription factor 21 (*TCF21*), smooth muscle cells snail family transcriptional repressor 2 (*SNAI2*), *COL1A1*, collagen type I alpha 2 chain (*COL1A2*), *DCN*, delta like non-canonical Notch ligand 1 (*DLK1*), and lumican (*LUM*). Similar to cardiomyocytes, fibroblasts also exhibit differential expression between atria and ventricles, and between the left and right heart. Good examples are cartilage intermediate layer protein 2 (*CILP*) and integrin beta-like 1 (*ITGBL1*) which are upregulated in the left ventricle while downregulated in the right ventricle (41). Human and rodent genes involved in pathological remodeling of the heart or considered as profibrotic markers are cytoskeleton associated protein 4 (*CKAP4*), NADPH oxidase 4 (*NOX4*), insulin like growth factor 1 (*IGF1*), A disintegrin and metalloproteinase (ADAM) with thrombospondin type 1 motif 4 (*ADAMTS4*), vascular cell adhesion molecule (*VCAM*), and AXL receptor tyrosine kinase (*AXL*) (16, 24, 43, 44). For example, in the event of myocardial ischemia reperfusion, an increase in the expression of *CKAP4* was identified in activated fibroblasts (24), a protein considered also responsible for development of atrial fibrosis in the heart (45). Interestingly, myocardial biopsies of patients suffering from ischemic heart disease also show increased expression of *CKAP4* in fibroblasts, accompanied by activation of other genes such as *POSTN*, WNT1-inducible signaling pathway protein-1 (*WISP1*), and tenascin C (*TNC*) (24) as well AE binding protein 1 (*AEBP1*) a novel transcription factor identified in human cardiac fibrosis (46).

In mice, fibroblasts have been intensely studied. In young mice, fibroblasts constitute 15–19% of the healthy murine heart, whereas in adult mice, this percentage increases to more than 20% (47). Characteristic markers used to identify fibroblasts in murine hearts include *COL1A1*, *GSN*, *DCN*, WNT inhibitory factor 1 (*WIF1*), dickkopf WNT signaling pathway inhibitor 3 (*DKK3*), metallothionein 2 (*MT2*), TIMP metallopeptidase inhibitor 1 (*TIMP1*), *PDGFRA*, and *TCF21* (48). In the context of disease, expression of the fibrosis-associated extracellular matrix genes, *POSTN* and fibrillin 1 (*FBN1*), are increased in a mouse model of pediatric mitochondrial cardiomyopathy, although expression of these genes has been reported in other cell types as well (47). Upon ischemia reperfusion, fibroblasts show high expression of *POSTN*, *WISP1*, and *TNC*, associated with fibroblast activation (24). Another study in mice demonstrated that gene expression of fibroblasts was skewed toward Ki-67, *COL1A2,* collagen type III alpha 1 chain (*COL3A1*), collagen type V alpha 1 chain (*COL5A1*), *SPARC*, secreted frizzled related protein 2 (*SFRP2*), and *DKK3* following myocardial infarction (49). However, in pigs the infarct zone presented upregulation of ACTA2, COL1A1, TIMP2, POSTN, TAGLN, MMP2, and FN1 genes specifically in the infarct zone (50).

Aging has a significant impact on the fibroblast expression profile and function. Comparing transcriptomes of 12 week-old and 18 month-old mouse hearts using single-nucleus RNA Seq revealed that aging predominantly affected fibroblast gene expression, and a total of 12 age-dependent fibroblast subclusters were identified (51). Gene ontology analysis of differentially regulated genes elucidated that aging predominantly affected expression of genes related to inflammatory/immune responses, extracellular matrix organization, angiogenesis, and osteogenesis. In particular, expression of serine protease inhibitors (SERPIN) family E member 1 and 2 (*SERPINE1* and *2*) was increased in certain fibroblast clusters, promoting antiangiogenic effects upon their secretion. Furthermore, some fibroblast subclusters identified in this study showed higher expression of genes involved in osteoblast differentiation such as *DDR2,* runt related transcription factor 2 (*RUNX2*), glycoprotein M6B (*GPM6B*), JunB proto-oncogene, AP-1 transcription factor subunit (*JUNB*), and CCAAT enhancer binding protein beta (*CEBPB*), showing a transition toward an osteogenic fate. This osteogenic transition seems to be particularly evident within epicardial layers of the aged heart (51).

In a study focused specifically on the role of active fibroblasts in myocardial infarction in mice, a subpopulation called cells reparative cardiac fibroblasts (CFRs), was identified by the expression of POSTN and collagen triple helix repeat containing 1 (*CTHRC1*). CFR activity appears to be essential in scar healing following MI (52). Of note, the CFR signature was also found in MI model in swine, and at least in part in human myocardial biopsy specimen taken from the ischemic zone of ischemic cardiomyopathy or from dilated cardiomyopathy, indicating this

signature may be conserved across species and highlighting the translational significance of these findings.

Interestingly, specifically targeting fibroblast may reduce fibrosis in mice suffering myocardial injury (53, 54). Aghajanian et al. set out to identify proteins specifically expressed by activated cardiac fibroblasts in a MI mouse model. They found that fibroblast activation protein alpha (FAP) is one of the main responsible for fibrosis and that FAP was also previously observed in human and rat hearts after MI (55). Of note, targeting FAP by nanoparticle-mediated generation of chimeric antigen receptor (CAR) T cells, *in vivo* resulted in a reduction in cardiac fibrosis suggesting that this approach may hold promise for treatment of fibrosis in cardiac disease states (56, 57).

Approximately three decades ago, fibroblasts were suggested to be considered as immune cells because of their ability to produce cytokines when stimulated with IL1 or TNFα. They are also capable of producing prostaglandin E2 (PGE2), giving them the ability to regulate immune responses. Furthermore, fibroblasts produce several growth factors as for example platelet-derived growth factor, transforming growth factor-β and insulin-like growth factors that regulate the reparative response, possibly involving autocrine regulatory loops (58). Finally immune functions of fibroblast may include interaction with myeloid cells, lymphocyte mobilization as well as induction of pro-inflammatory attributes, as observed in inflammatory disorders and cancer (59). Although fibroblasts may take part in immune responses in these diseases, it remains largely unexplored whether and what immune cell functions they may exert in the heart.

Endothelial Cells – An Underrecognized Force

In the heart, endothelial cells cover the inner lumen of cardiac chambers, blood, and lymphatic vessels. Using immunohistochemistry, it has been estimated that endothelial cells cover more than 60% of non-myocyte cardiac cells in the adult mouse heart (60). Endothelial cells (ECs) fulfill a number of different tasks, including control of blood flow by modulating the degree of vascular relaxation and constriction, regulation of extravasation of solutes, fluid, macromolecules and hormones, participation in leukocyte trafficking and hemostasis, and contributions to thermoregulation and angiogenesis (61, 62). Based on the multitude of different functions within a variety of distinct tissues, endothelial cells show a high degree of heterogeneity, are equipped with specific properties, and exert different morphological features, all regulated by differences in the cellular gene expression programs (63, 64).

Despite the cellular heterogeneity of endothelial cells, markers could be identified to distinguish this cell type from other cardiac cells. Endothelial cells of the adult human heart can be identified by platelet and endothelial cell adhesion molecule 1 (*PECAM1*), cadherin 5 (*CDH5*), and von Willebrand factor (*VWF*), and subdivision into arterial endothelial cells is possible by prospero homeobox 1 (*PROX1*), FMS related tyrosine kinase 4 (*FLT4*), podoplanin (*PDPN*), B one M arrow tyrosine kinase gene in chromosome X non-receptor tyrosine kinase (*BMX*), and natriuretic peptide receptor 3 (*NPR3*) (16). In mice, *NPR3* is selectively expressed in adult endocardium (65). In the fetal human heart, endothelial cells can be identified using Sry-type box transcription factor (*SOX*) 7, 17, and 18, *PECAM*1, and *CDH5*. It is important to mention that these cells can undergo endothelial-mesenchymal transition and will then express genes that have been mostly used to identify fibroblasts (*COL3A1*; *COL1A2*; fibronectin 1, *FN1*; and biglycan, *BGN*). Thus, a deeper characterization of these cells is highly recommended. Other genes that are useful for the identification of endothelial cells are apolipoprotein E (*APOE*), intercellular adhesion molecule 2 (*ICAM2*), tyrosine kinase with immunoglobulin like and EGF-like domains 2 (*TIE2*), endoglin (*ENG*), and nitric oxide synthase 1 and 2 (*NOS1* and *NOS2*) (15, 66). Further classification between venous and arterial endothelial cells is feasible with the following genes: EPH receptor B4 (*EPHB4*), neuropilin 1 and 2 (*NRP1* and *NRP2*), nuclear receptor subfamily 2 group F member 2 (*NR2F2*), ephrin B1 (*EFNB*), delta like canonical Notch ligand 4 (*DLL4*) and *HEY1/2* (61). Lymphatic endothelial cells are found in a low percentage in the heart, and these are expressing *PROX1*, lymphatic vessel endothelial hyaluronan receptor 1 (*LYVEL*), *FLT4*, and *PDPN* (60). Interestingly, it was observed that endothelial cells are also able to switch on cardiomyocyte lineage genes such as *MYL2*, myoglobin (*MB*), *MYL3*, *TNNT2*, *TNNI3*, and ACTC1 following myocardial infarction, indicating the utility of transcriptional profiling and cell marker analysis in detecting cell type shifts, thus facilitating understanding of pathology, e.g., a cell type shift from endothelial cell phenotype to cardiomyocyte phenotype following myocardial infarction (67).

Some genes related to the role of endothelial cells in vascular tension, permeability and vessel formation are differentially expressed in young and adult mice. In healthy, 10-day-old mice, genes such as cytochrome c oxidase subunit 6A2 (*COX6A2*), cardiac myosin binding protein C (*MYBPC3*), myosin heavy chain associated RNA transcript (*MHRT*), *NPR3*, *TIE1,* and *TIE2* are clearly expressed (47). However, endothelial cells in adult mouse hearts express *CDH5*, *PECAM1*, fatty acid binding protein 4 (*FABP4*), *VWF,* and VCAM1 (48, 68–70). It is important to mention that although these markers are very specific for endothelial cells, other genes *GJA1*; ATPase sarcoplasmic/endoplasmic reticulum calcium transporting 2, *ATP2A2*; *TTN*; *RYR2*; *MYH6*) have also been reported that are related to cardiomyocyte function (71). Once cardiac tissue suffers a damage due to myocardial infarction, e.g., 3 days following coronary artery ligation, genes involved in leukocyte migration [e.g., chemokine (C–C motif) ligand 9 (*CCL9*), C–X–C motif chemokine ligand 2 (*CXCL2*)] are upregulated in endothelial cells. Intriguingly, different genes related to collagen production (*COLl3A1*), ribosome assembly and protein translation [ribosomal protein L9 (*RPL9*) and S12 (*RPS12*)], and cell proliferation [tumor protein, translationally controlled 1 (*TPT1*)], are enriched in endothelial cells 7 days following myocardial infarction (72). In heart failure, the most relevant genes upregulated in endothelial cells are mainly related to cell adhesion, angiogenesis, and cell migration (major histocompatibility complex, class I, B, *HLA-B*; EGF like domain multiple 7, *EGFL7*; receptor activity modifying protein 1 and 2, *RAMP1, RAMP2*; plasmalemma vesicle associated

protein, *PLVAP*; inhibitor of DNA binding 1, *ID1*; and formin like, *FMNL3*), inflammatory response (*CX3CL1*; cluster of differentiation 74, *CD74*), as well as development and maturation (*SOX17, SOX18*) (73).

Immune Cells – Regulators of Health and Disease in the Heart

The more frequent use of single-cell immune profiling in combination with advanced visualization technologies has profoundly deepened our understanding of the immune system of the heart, revealing the presence of a diverse landscape of innate and adaptive immune cells. Virtually all known types of immune cells have been described within the heart of both human and rodents including monocytes/macrophages, T-cells, B-cells, natural killer (NK) cells, and mast cells. Their precise roles often are yet to be fully defined (74). A recent study showed monocytes constituted 4.3% of all cells within fetal human hearts. They were identified by expression of basic leucine zipper ATF-like transcription factor 3 (*BATF3*), lysozyme (*LYZ*), *S100A8* and *S100A6*. Macrophages accounted for approximately 4.7% of all cells within the fetal human heart. Proliferating macrophages expressed the markers membrane spanning 4-domains A4A (*MS4A4A*), selenoprotein P (*SEPP1*), and *CD68*, while non-proliferating macrophages expressed additionally *MKI67*, *LYZ*, and *S100A6*. Around 5% of all cells were T cells, predominantly expressing GATA binding protein 3 (*GATA3*), lymphotoxin beta (*LTB*), and interleukin 7 receptor (*IL7R*), 2.4% of all cells were NK cells and expressed eomesodermin (*EOMES*), natural killer cell granule protein 7 (*NKG7*), granulysin (GNLY), granzyme A (*GZMA*), granzyme B (*GZMB*), and perforin 1 (*PRF1*). B cells represented 3.2% of all cells with signatures showing expression of B cell CLL/lymphoma 11A (*BCL11A*), membrane spanning 4-domains A1 (MS4A1), and immunoglobulin lambda like polypeptide 5 (*IGLL5*). Mast cells (1.3% all cells) could be separated by expression of tryptase beta 2 (*TPSB2*) and *GATA2* and dendritic cells by that of $CD1C^{+}$, respectively (15).

Macrophages can be commonly classified by their phenotype and function into M1 and M2 polarized macrophages. Classically activated macrophages (M1 polarization) express interleukin 1β (*IL1B*), *CCL2*, *CCL9,* CXCL3, and usually govern pro-inflammatory functions (16, 75). In contrast, M2 polarized, non-classical macrophages are more likely to express *APOE*, galectin-3 (*LGALS3*) and the transmembrane glycoprotein NMB (*GPNMB*) and largely contribute to resolution of inflammation and repair (75).

As in fetal human hearts, myeloid cells represent the most prominent cellular fraction in adult human hearts. Commonly they are classified according to their C-C-chemokine receptor type 2 (*CCR2*) expression status into locally proliferating, self-renewing tissue resident (TR) $CCR2^{-}$ macrophages, originally populating the heart from the yolk sac in early stages of embryonic development, and $CCR2^{+}$ tissue resident macrophages (TRMs) stemming from the monocyte blood pool. While $CCR2^{-}$ TRMs are abundant in the healthy state and instrumental for repair following damage, e.g. after myocardial infarction (MI), $CCR2^{+}$ TRMs are rare in healthy states but quickly recruited upon injury and frequently mediating disease (76). The latter are not only recruited from blood and bone marrow but also from other tissues functioning as a reservoir to ensure timely recruitment during onset of inflammation (77, 78). Combining genetic fate mapping with scRNAseq, Dick et al. identified three clusters of TRMs: T cell immunoglobulin and mucin domain containing 4 positive, *LYVE1* positive, major histocompatibility complex class II low and *CCR2* negative ($TIMD4^{+}LYVE1^{+}MHC\text{-}II^{lo}CCR2^{-}$) relying almost exclusively on self-renewing by proliferation, $TIMD4^{-}LYVE1^{+}MHC^{-}II^{hi}CCR2^{-}$ that are partially renewed from blood monocytes and finally $TIMD4^{-}LYVE1^{-}MHCII^{hi}CCR2^{+}$ that recruit themselves from exterior monocytes only. Interestingly, the $TIMD4^{+}CCR2^{-}$ group limited adverse remodeling in a mouse model of MI (79). TRMs have not only been implicated with myocardial infarction but with various other cardiac pathologies including myocarditis. Here, macrophages expressing mast cell immunoglobulin like receptor 1 (*MILR1*), *CXCL9*, lymphocyte antigen 6 complex, locus I (*LY6I*), *NOS2*, arginase 1 (*ARG1*), argininosuccinate synthase 1 (*ASS1*) appeared to entertain the inflammatory process (66). In addition, cardiac hypertrophy mimicked by the transverse aortic constriction has been linked to proinflammatory TRMs expressing Oncostatin M (80, 81). Important note has been found a correlation of human expression genes and mouse, $CCR2^{+}$ macrophage abundance is associated with left ventricle (LV) remodeling and systolic function in heart failure patients (77).

Macrophage gene signature characteristically differs between male and female human hearts. Male cardiac macrophages upregulate genes involved in responding to foreign antigens, antigen processing, and presentation via MHC class II molecules such as interferon regulatory factor 8 (*IRF8*), a gene linked to chronic inflammation (82). In contrast, female-upregulated genes in cardiac macrophages are involved in the response to stress and the electron transport chain, e.g., the TSC22 domain family member 3 (*TSC22D3*, also known as Gilz), the most upregulated gene in female macrophages and the most sexually dimorphic macrophage gene between both sexes (68), a transcription factor implicated in anti-inflammatory functions and a downstream driver of the potent anti-inflammatory effects of glucocorticoids (83–85).

Constructing a cell atlas of the human heart from scRNAseq data, Tucker et al. identified two main immunologic cell clusters: A) a cluster representing TRMs expressing the scavenger receptors *CD163* and collectin subfamily member 12 (*COLEC12*), the mannose receptor C-type 1 (*MRC1*), the E3 ubiquitin ligase membrane associated ring-CH-type finger 1 (*MARCH1*), and the natural resistance-associated macrophage protein 1 (*NRAMP1*) which could be further separated into two macrophage clusters, both M2-like, expressing recombination signal binding protein for immunoglobulin kappa J region (*RBPJ*) and coagulation factor XIII A chain (*F13A1*) on the one hand and the transmembrane collagen *COL23A1* on the other (16). B) an immune cell cluster showing a T cell phenotype expressing the T cell surface antigen *CD2*, the early T cell antigen *CD69*, and the T-cell receptor-associated transmembrane adaptor 1 (*TRAT1*),

the T cell immune adaptor src kinase associated phosphoprotein 1 (*SKAP1*), and the thymocyte selection marker *CD53* (16).

Immune cells of the adaptive immune system are the second largest cell fraction within the human heart and have been implicated with various cardiovascular diseases including myocardial infarction, myocarditis, and heart failure summarized elsewhere in a recent review by Steffens et al. (74). For example, T regulatory cells (Tregs) showing elevated expression of forkhead box P3 (*FOXP3)*, *CD25*, cytotoxic T-lymphocyte associated protein 4 (*CTLA4)*, and killer cell lectin like receptor G1 (*KLRG1)* were identified during MI in mouse hearts (86). However, interferon gamma (*IFNG*), tumor necrosis factor (*TNF*), IL3 and IL17 genes, related to classically polarized Th cells were not differentially expressed after MI in mice (87). In the same model, B cells presented with upregulation of activation markers such as *CD69*, *CCR7*, CXC-chemokine receptor type 5 (*CXCR5*), and transforming growth factor beta 1 (*TGFB1*) (88).

Other immune cell types found in the adult human heart include granulocytes expressing *CCR1*, colony stimulating factor 3 receptor (*CSF3R*), and *S100A9*, B-cells expressing *MS4A1* (14) and dendritic cells that – despite their very low number in cardiac tissue – may be identified by expression of *CD209a* (16). The knowledge about their significance for cardiac diseases is still very limited.

Other Cell Types – Rare and Underrecognized

Recently, it has become evident that the cellular diversity of the heart stretches beyond cardiomyocytes, endothelial cells, fibroblast, and immune cells. Some of the more untraditional cells found in the heart are:

Adipocytes

Epicardial adipose tissue covers up to 80% of a human heart while it is essentially absent in rodents (89). Therefore, adipocytes account for up to 20% of the total mass (90). Since epicardial adipose tissue is supplied with blood thought the coronary circulation and it has a common embryonic origin with the heart, it has been suggested that it might be important for cardiac physiology (89). Epicardial adipose tissue can be segregated into adipocytes, preadipocytes and the so-called stroma vascular fraction comprising various cell types such as vascular cells and fibroblasts. Although the exact role of adipocytes remains unknown, it is possible that they might serve as a local energy store or to protect cardiomyocytes from lipotoxicity and hypothermia (91). Marker genes used for identification of adipocytes are those regulating the size and lipid droplet stability, including cell death-inducing DNA fragmentation factor, alpha subunit like effector c (*CIDEC*) and perilipin 5 (*PLIN5*). Furthermore, cardiac adipocytes are notably enriched of adiponectin, C1Q and collagen domain containing (*ADIPOQ*), which plays a role in the regulation of fatty acid transport and intracellular calcium homeostasis, as well as in thyrotropin releasing hormone degrading enzyme (*TRHDE*) expression, a gene responsible for inactivation of the thyrotropin release hormone.

Surprisingly, these cells also overexpress *IGF1* and T cell-activated increased late expression (*TACTILE* also known as *CD96*), markers involved in cell growth and proliferation in different cell types (16). While the mentioned studies are supportive of adipocytes playing a role in the development of CVD, descriptive and mechanistic studies are scarce in this topic.

Pericytes/Smooth Muscle Cells

The main difference between pericytes and smooth muscle cells is that pericytes reside within micro vessels, whereas smooth muscle cells contribute to the vascular wall of larger vessels. In human hearts, pericytes are characterized by genetic expression of platelet-derived growth factor receptor beta (*PDFRB*), ATP binding cassette subfamily C member 9 (*ABCC9*), and potassium voltage-gated channel subfamily J member 8 (*KCNJ8*), and it is possible to subdivide them by the expression of some adhesion molecules such as neural cell adhesion molecule 2 (*NCAM2*) and *CD38*, or with a gene related in microvascular morphogenesis, chondroitin sulfate proteoglycan 4 (*CSPG4*). Expression of *MYH11*, known as smooth muscle actin, generates a debate on the specificity of this gene (14). A recent study provided additional evidence on reliable genetic tools that can be used to identify, label, and target cardiac pericytes in mice, thereby facilitating further investigation of the role of this understudied cell type in heart disease (92).

Proteins related with contractile function such as *MYH11*, *ACTA2*, *TAGLN*, *RGS5*, vitronectin (*VTN*), *KCNJ8,* and myocardin (*MYOCD*) are used to identify smooth muscle cells (48, 68, 72). Until now, no changes in smooth muscle cells have been reported in the heart during any CVD, but one study performed in smooth muscle cells of ascending aortic wall in patients with myocardial infarction showed that at least 21 genes were upregulated in comparison with the control group (non-myocardial infarction patients). Those genes were related to three different functions such as the regulation of smooth muscle cell contraction by ATPase Na^+/K^+ transporting subunit alpha 2 (*ATP1A2*), superoxide dismutase 1 (*SOD1*), and *MYOCD*, heart development by histone deacetylase 9 (*HDAC9*), polycystin 2, transient receptor potential cation channel (*PKD2*), hexamethylene bisacetamide inducible 1 (*HEXIM1*), *FOXP1*, and integrin subunit beta 1 (*ITGB1*), and not less important actin cytoskeleton organization by spectrin alpha, erythrocytic 1 (*SPTA1*), platelet activating factor acetylhydrolase 1b regulatory subunit 1 (*PAFAH1B1*), erythrocyte membrane protein band 4.1 like 2 (*EPB41L2*), and profilin 1 (*PFN1*) (93). This shows that these cells may play an important role in the development of CVD, but so far, they have not been identified.

Mesothelial Cells

The mesothelial layer covering the heart has a crucial role in cardiac development and repair after injury. The most recent insights into cellular composition and diversity of the epicardium have lately been summarized comprehensively (94). This distinct small population of mesothelial cells expresses Wilms tumor 1 (*WT1*), basonuclin 1 (*BNC1*), basonuclin 2 (*BNC2*), and odd-skipped related transcription factor 1 (*OSR1*) under normal conditions, while neuropeptide Y (*NPY*) has been

described to be responsible for cardiac remodeling, angiogenesis and vasoconstriction (14, 16). This cell subpopulation also expresses unspecific genes such as slow muscle troponin T1, slow skeletal type (*TNNT1*) or genes involved in immune response (complement C1r, *C1R*; complement factor I, *CFI*; complement C3, C3; and serpin family G member 1, *SERPING1*) (16).

Glia Cells/Schwann Cells

This cell type covers all surfaces of neuronal cells, and it has been shown to regulate tissue remodeling in a paracrine fashion (95). Peripheral glial cells can be separated into two main types; satellite glial cells covering neuron cell bodies located in ganglia, and Schwann cells which wrap nerve fibers. Since cardiac glial cells are dispersed throughout the heart, they were particularly difficult to analyze until the development of the scRNAseq methodology. However, scRNAseq studies have not yet examined sufficient cardiac glial cells to either detect subpopulations or investigate their change during heart development and disease. Although these cells are confirmed to be present in the heart (16), approaches to isolate these cells are limited. Generally used markers include *CSPG4* [also called Nerve/glial antigen 2 (*NG2*)], *PDGFRB*, and melanoma cell adhesion molecule (*MCAM*), which are non-specific; *CSPG4* and *MCAM* are expressed as well in mural cells, and *PDGFRB* expression is found in mural cells and fibroblasts. It is thought that *CD59a* might be a good marker (68), but this marker is also highly expressed in endothelial cells.

Progenitor/Progenerative Cells

Using radiocarbon (^{14}C) birth dating and design-based stereology Bergmann et al. provided compelling evidence for the strikingly low regeneration capacity of human cardiomyocytes, with less than 1% renewing yearly in an adult human heart (13). Nevertheless, identification and understanding of progenitor/progenerative cells is highly relevant, as unlocking regenerative potential of contractile cells could provide means to rescue an injured heart (96). Over the last decade, two potential sources of cardiomyocyte renewal were extensively studied—pre-existing cardiomyocytes undergoing dedifferentiation and duplication, and stem or progenitor cells that contribute to *de novo* generation of cardiomyocytes—with recent work (97, 98) and subsequent consensus (99) favoring the former. By combining genetic fate-mapping with stable isotope labeling and multi-isotope imaging mass spectrometry, it was demonstrated that cardiomyocyte turnover in adult heart is primarily driven by the division of pre-existing cardiomyocytes during normal aging and after myocardial injury (98). Furthermore, a population of cardiomyocytes with a high pro-regenerative profile was identified in infant patients with dilated cardiomyopathy but was absent in children >6 years of age (100). In addition, an integrative cluster analysis of adult murine hearts obtained from multiple data sets discovered a minor population of cardiomyocytes characterized by proliferation markers that could not be identified by analyzing the datasets individually (101), further supporting the idea that the renewal of the cardiomyocyte pool is driven by cytokines of resident cardiomyocytes rather than differentiation of progenitor cells.

On the other hand, cardiac progenitor cells are made up of different cell types characterized by the expression of proto-oncogene receptor tyrosine kinase (*KIT*), *LY6A*, ATP binding cassette subfamily G member 2 (*ABCG2*), *ISL1*, and *TBX18* (25, 100–103). However, recent genetic lineage tracing studies revealing that only a small fraction of endogenous cells expressing *LY6A* or *KIT* contribute to the adult cardiomyocyte population challenge the view that newly formed cardiomyocytes are predominantly derived from cardiac progenitor cells (69, 104, 105). Even with these data, it has not yet been possible to define whether the cardiomyocyte renewal, if any, is originated by the generation of new cardiomyocytes from a rare division of existing cardiomyocytes or from putative cardiac stem cells after cardiac injury (96, 106).

NKX2 Homeobox (*NKX2*) and *ISL1* expression has led to the identification of previously unknown progenitor subpopulations during the early phase of cardiac fate decision-making (107). In addition, a population of cardiomyocytes with a high pro-regenerative profile was identified in infant patients with dilated cardiomyopathy but was absent in children >6 years of age (108). It is broadly accepted that adult heart also has an, albeit very limited, regenerative potential. Its origin, however, is still matter of an ongoing debate. An integrative cluster analysis of adult murine hearts obtained from multiple data sets discovered a minor population of cardiomyocytes characterized by proliferation markers that could not be identified by analyzing the datasets individually (97).

It is now widely accepted that the heart has an, albeit very restricted, regenerative potential. However, further work in needed to identify and characterize populations of proliferative cardiomyocytes and mechanisms of endogenous renewal that could be exploited for repairing the injured myocardium.

DISCUSSION

In recent years, scRNAseq has made a quantum leap from large-scale cell population studies to single cell analysis. Despite its short history, scRNAseq has already begun to drive new discoveries in different disciplines that would not have been possible with traditional methods such as for example FACS analysis. International collaborative efforts of multiple laboratories aim to define the cellular heterogeneity in all organ systems. The Human Cell Atlas (109) and Human BioMolecular Atlas Program[1] are of particular importance for human physiology and pathophysiology, while the Tabula Muris project (32) allows for deconvolution of murine single cell subtype transcriptome. In addition, Asp et al. combined scRNAseq data of human embryonic cardiac cells, RNA-seq data of spatial transcriptomics, and *in situ* sequencing data to map cell-type distribution and spatial organization in the human embryonic heart and generate a 3D gene profile atlas of the developing human heart (110). Using a similar approach to study the development of the chicken heart from the early to late four-chambered heart stage, Mantri et al. identified diverse

[1]https://commonfund.nih.gov/hubmap

cellular lineages in developing hearts, their spatial organization, and their interactions during development (111). Although the atlases generated from scRNAseq technology are becoming more and more complete, one of the crucial remaining tasks that will facilitate their effective integration into future clinical trials, is standardization of the markers used for the different cells, as well as experimental and analytical pipelines.

Despite challenges associated to tissue availability and cellular isolation (112), in the cardiovascular field alone, numerous groups have used scRNAseq technology to identify new cell populations and key molecular players driving numerous physiological and pathophysiological processes in the heart. Using scRNAseq of isolated cardiomyocytes from heart failure patients with ventricular arrhythmia, Yamaguchi et al. recently identified a subpopulation of cardiomyocytes which readily expresses dopamine D1 receptor (113). Following the lead from untargeted transcriptomic analyses, they further generated cardiomyocyte-specific D1 receptor knockout and overexpressing mice and proved that cardiac D1R receptor upregulation is both necessary and sufficient for inducing life-threatening ventricular arrhythmia.

scRNAseq of cells from a commonly used heart failure model—transverse aortic constriction (114) mouse model—facilitated novel discoveries with important clinical implications. Transcriptome analysis of >11,000 single cells revealed that activation of proinflammatory macrophages is the key event in the transition from normal to reduced ejection fraction (115). Furthermore, macrophage activation and subtype switching, a key event at middle-stage of cardiac hypertrophy, was effectively attenuated by Dapagliflozin, a sodium glucose cotransporter 2 inhibitor known for its beneficial effects in heart failure patients, as well as two additional anti-inflammatory agents, inhibitor of galectin-3 (TD139) and Arglabin, which are rarely used in setting of cardiac diseases. Importantly, the authors could confirm similar molecular and cellular patterns in human samples of hypertrophic cardiomyopathy and heart failure. Nomura et al. manually isolated single cardiomyocytes from wild-type and *p53* cardiomyocyte-specific deficient mice in the presence or absence of TAC (38). They subsequently analyzed transcriptomes of 473 cardiomyocytes and found that continuous pressure overload leads to a cardiomyocyte divergence into adaptive and failing phenotypes, and that p53 signaling is specifically responsible for alterations typical for late cardiac remodeling. Again, accompanying human single-cardiomyocyte analysis validated the conservation of the pathogenic transcriptional signatures in heart failure patients. Satoh et al. applied three single-cell analysis methods, namely, sc-qPCR, scRNAseq, and single-molecule fluorescence *in situ* hybridization (smFISH) to study transcriptome profile in isolated cardiomyocytes and cross sections from TAC murine hearts at an early hypertrophy stage (2 weeks post-TAC) and at a late heart failure stage (8 weeks post-TAC) (116). In alignment with the idea of cardiomyocytes progressing into different phenotypes over the course of the remodeling, expression levels of *MYH7*, a representative fetal gene, greatly varied in hypertrophic cardiomyocytes and was more consistently found in failing cardiomyocytes. *MYH7*-expressing cardiomyocytes were significantly more abundant in the middle layer, compared with the inner or outer layers of hypertrophic hearts, while such spatial differences were not observed in failing hearts. Interestingly, expression of *MYH7* was negatively correlated with cellular size and abundance of mitochondria-related gene transcripts.

In a rat model of heart failure with preserved ejection fraction, scRNAseq transcriptome analyses of the sinoatrial node revealed significant alterations in both the "membrane clock" (ion channels) and the "calcium clock" (spontaneous calcium release events) which—when probed in functional experiments—further validated RNA-seq data (117).

Dong et al. performed meta-analyses of large-scale, publicly available bulk and single-cell RNA sequencing datasets to identify vascular smooth muscle cell (VSMC)-enriched long non-coding RNAs. The role of novel VSMC-expressed long non-coding RNA, cardiac mesoderm enhancer-associated non-coding RNA (*CARMN*), was then investigated in VSMC-specific *CARMN* knockout mice that underwent carotid artery injury. *In vivo*, *CARMN* deletion in VSMC exacerbated, while its overexpression markedly attenuated injury-induced neointima formation in two independent animal models, underscoring its potential clinical implication as a therapeutic target for intimal hyperplasia (80).

Although there are only a few reports on spatial transcriptomics in the heart, two areas of cardiac research are particularly dependent on detailed understanding of the spatial transcriptome patterns. First, cardiac development is a spatially complex process and comprehensive understanding of regional changes in gene expression during heart maturation is of great interest. Second, spatial information is crucial in myocardial infarction, given that localized occlusion of a coronary artery differentially affects the site directly adjacent to the infarct site, whereas the remote areas are only indirectly affected. Accordingly, care must be taken when developing treatment strategies. In conditions where only a portion of cells shows alterations in signaling pathways, we must learn more about the specific cell type, their localization in the heart, as well as the temporal resolution of their reprogramming in order to introduce the treatment, when and where this trigger is detrimental, and to reduce off-target effects.

Two important factors complicate interpretation of data on cellular heterogeneity of the heart. First, there are continuous fluctuations in abundance of diverse cellular lineages, their spatial organization and molecular composition, as well as their interactions during heart development. Second, well-documented inter-species differences must be considered when extrapolating data from experimental animal models to human cardiac physiology and pathophysiology. Therefore, the high-level standardization of markers for different cell types, their developmental stage and their host species is crucial for the comprehensive understanding of cellular heterogeneity in cardiac health and disease.

Overall, our knowledge on the cellular composition and its dynamic changes in cardiac health and disease is steadily increasing through the advent of powerful technologies such as scRNAseq. scRNAseq technology alone and particularly in combination with spatio-temporal genetic and/or proteomic data has a potential to transform our knowledge on disease mechanisms, more precisely predict patients at risk of developing adverse cardiac outcomes and reveal mechanisms underlying

distinct personalized therapeutic responses. It, therefore, holds a promise to become an integral and central part of future clinical trials (118).

AUTHOR CONTRIBUTIONS

NA and AZ conceptualized the manuscript. NA, SL-H, HB, and AZ contributed to the research for writing the manuscript. NA and SL-H designed the figure and tables. All authors contributed to the discussion, writing, and review of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.868466/full#supplementary-material

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frontiers | Frontiers in Cardiovascular Medicine

REVIEW
published: 29 June 2022
doi: 10.3389/fcvm.2022.907348

Modulation of mTOR Signaling in Cardiovascular Disease to Target Acute and Chronic Inflammation

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Inflammation is a key component in the pathogenesis of cardiovascular diseases causing a significant burden of morbidity and mortality worldwide. Recent research shows that mammalian target of rapamycin (mTOR) signaling plays an important role in the general and inflammation-driven mechanisms that underpin cardiovascular disease. mTOR kinase acts prominently in signaling pathways that govern essential cellular activities including growth, proliferation, motility, energy consumption, and survival. Since the development of drugs targeting mTOR, there is proven efficacy in terms of survival benefit in cancer and allograft rejection. This review presents current information and concepts of mTOR activity in myocardial infarction and atherosclerosis, two important instances of cardiovascular illness involving acute and chronic inflammation. In experimental models, inhibition of mTOR signaling reduces myocardial infarct size, enhances functional remodeling, and lowers the overall burden of atheroma. Aside from the well-known effects of mTOR inhibition, which are suppression of growth and general metabolic activity, mTOR also impacts on specific leukocyte subpopulations and inflammatory processes. Inflammatory cell abundance is decreased due to lower migratory capacity, decreased production of chemoattractants and cytokines, and attenuated proliferation. In contrast to the generally suppressed growth signals, anti-inflammatory cell types such as regulatory T cells and reparative macrophages are enriched and activated, promoting resolution of inflammation and tissue regeneration. Nonetheless, given its involvement in the control of major cellular pathways and the maintenance of a functional immune response, modification of this system necessitates a balanced and time-limited approach. Overall, this review will focus on the advancements, prospects, and limits of regulating mTOR signaling in cardiovascular disease.

Keywords: myocardial infarction, atherosclerosis, inflammation, metabolism, rapamycin, mTOR, anti-inflammatory treatment, cardiovascular disease

OPEN ACCESS

Edited by:
Emiel Van Der Vorst, Institute for Molecular Cardiovascular Research (IMCAR), Germany

Reviewed by:
Nhat Tu Le, Houston Methodist Research Institute, United States
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Specialty section:
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

Received: *29 March 2022*
Accepted: *30 May 2022*
Published: *29 June 2022*

Citation:
Kaldirim M, Lang A, Pfeiler S, Fiegenbaum P, Kelm M, Bönner F and Gerdes N (2022) Modulation of mTOR Signaling in Cardiovascular Disease to Target Acute and Chronic Inflammation. Front. Cardiovasc. Med. 9:907348. doi: 10.3389/fcvm.2022.907348

INTRODUCTION

Ischemic heart disease is still the leading cause of death worldwide (1). At present, risk reduction through lifestyle modifications, weight management, blood pressure control, and lipid-lowering treatment achieves just a part of the prevention goal. Furthermore, in acute myocardial infarction (AMI), rapid blood flow restoration and post-infarct treatment supported by dual antiplatelet

medication, ß-blockers, and angiotensin-converting enzyme (ACE) inhibitors lead to better outcomes (2).

However, in high-income nations, impaired cardiac function following myocardial infarction is the predominant cause of heart failure development, leading to chronic impairment and a decline in quality of life due to dyspnea and decreased physical ability (3). Years of healthy life lost due to disability (YLDs) increased by 106% between 1990 and 2019 (4). As a consequence, additional preventative and therapeutic strategies are required to enhance heart function following ischemic events.

The involvement of the immune system is well recognized as a key component of cardiovascular diseases (CVDs) such as atherosclerosis, a chronic inflammatory disease, but also of myocardial infarction (5). As a result, the regulation of inflammatory processes in vascular and myocardial illness has sparked significant attention as a treatment option for CVD.

Meanwhile, mTOR inhibitors have made significant progress in their utilization to modulate the immune response in a variety of disease disorders (6). The therapeutic applicability of these inhibitors is expanding, as indicated by the rising number of studies. Thus, it is critical to assess the impact of mTOR on acute and chronic types of inflammation in the setting of CVD.

In this review, the role of mTOR in acute and chronic CVD situations will be discussed. The precise function of mTOR in these pathologies will be emphasized using myocardial infarction (MI) and atherosclerosis as two notable instances of CVD defined by pathophysiological mechanisms comprising acute and chronic inflammation, respectively. The involvement of immune cells and their subtypes will be discussed in light of mTOR inhibition as a therapeutic option for reducing the excessive immune response in CVD.

mTOR, A MASTER REGULATOR OF METABOLISM

The mTOR signaling system is an important regulator of metabolism, cell survival, and cytoskeletal architecture (**Figure 1**). When the route is activated, it promotes anabolic activities like protein and lipid synthesis while inhibiting catabolic processes such as lysosome biogenesis and autophagy. The mTOR-complex itself exists as mTORc1 and mTORc2 in two distinct protein compositions. In addition to mTOR, the proteins mammalian lethal with SEC13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (DEPTOR), TELO2 Interacting Protein 1 (Tti1), and telomere length regulation protein TEL2 homolog (Tel2) are found in both complexes (7). Regulatory-associated protein of mTOR (RAPTOR) and proline-rich Akt substrate of 40 kDa (PRAS40) are specific proteins for mTORc1 generation, whereas Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), protein observed with Rictor-1 (Protor1), and mammalian ortholog of Stress-activated map kinase-interacting protein 1 (mSIN1) are specific proteins for mTORc2 formation (8, 9). Growth factors and insulin/Insulin-like growth factor 1 (IGF-1) operate as upstream regulators by binding to their receptor tyrosine kinase (RTK) and activating phosphoinositide 3-kinase (PI3K) and, ultimately, protein kinase B (AKT) (9, 10). The Ras/Erk/p90 ribosomal S6 kinase 1 (RSK1) signaling axis is used by alternative routes to send their signal from the RTK (7).

mTOR directly and indirectly senses nutrient levels and the cell's energy source. The lysosome is the cellular compartment where most nutrients are assembled and where the components of the mTOR-complex come together, resulting in mTOR pathway activation. By forming the complex at the lysosomal membrane with the activating protein Ras homolog enriched in brain (RHEB), amino acids induce Rag-Proteins (Ras-like small GTPase) to form heterodimers and activate mTORc1 (7, 10). Phosphatidic acid (PA) is a lipid second messenger derived from phosphatidylcholine that activates mTOR by removing the inhibitory protein DEPTOR from mTOR-complexes (10). Inactivation of mTORc1 by binding to hexokinase II can signal a shortage of glucose (11). Low energy levels in the form of low ATP abundance limit mTOR function by phosphorylating AMP-activated protein kinase (AMPK) thus activating the upstream inhibitor tuberous sclerosis complex 2 (TSC2) (12). Hypoxia interacts with mTOR via regulated in development and DNA damage responses 1 (REDD1), which also activates the upstream inhibitor TSC2 (12).

The downstream consequences of mTOR vary depending on whether mTORc1 or mTORc2 is activated. Indeed, activating mTORc1 enhances lipid and protein synthesis while inhibiting autophagy and lysosome biogenesis (7). In particular, it phosphorylates the ribosomal protein S6 Kinase-beta 1 (S6K1) which is involved in protein synthesis by promoting ribosome biogenesis (10) and inhibits AMPK, an important activator of autophagy through phosphorylation (13). This is why mTOR inhibitors are frequently used in studies of autophagy (14). mTORc1 suppresses the translation repressor 4EBP1 [eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1], which can enhance anabolic pathways (10). mTORc2 primarily affects cell survival, metabolism, and cytoskeletal architecture via regulating cAMP-dependent kinases, cGMP-dependent kinases, protein kinase C, protein kinase B, and SGK1 (serum/glucocorticoid-regulated-kinase1) (10). Feedback mechanisms between mTORc1 and mTORc2 allow for reciprocal fine-tuning. mTORc2 activation of AKT results in mTORc1 upregulation, whereas mTORc1 activation inhibits mTORc2 activity via S6K1 phosphorylation. Furthermore, mTORc1 regulates its own activity by feedback regulation, which involves the suppression of IRS1 (insulin receptor substrate 1), an upstream regulator of mTORc1 function (7). mTORc2, on the other hand, can activate itself via a positive feedback loop involving AKT and stress-activated map kinase SIN1 (SAPK-interacting 1) (10).

IMMUNE RESPONSE IN MYOCARDIAL INFARCTION

When the coronary blood flow is disrupted, most frequently due to coronary artery occlusion by a thrombus, the affected tissue lacks oxygen and nutrient supply and eventually enters apoptotic or necrotic programs. In this setting of sudden necrosis of

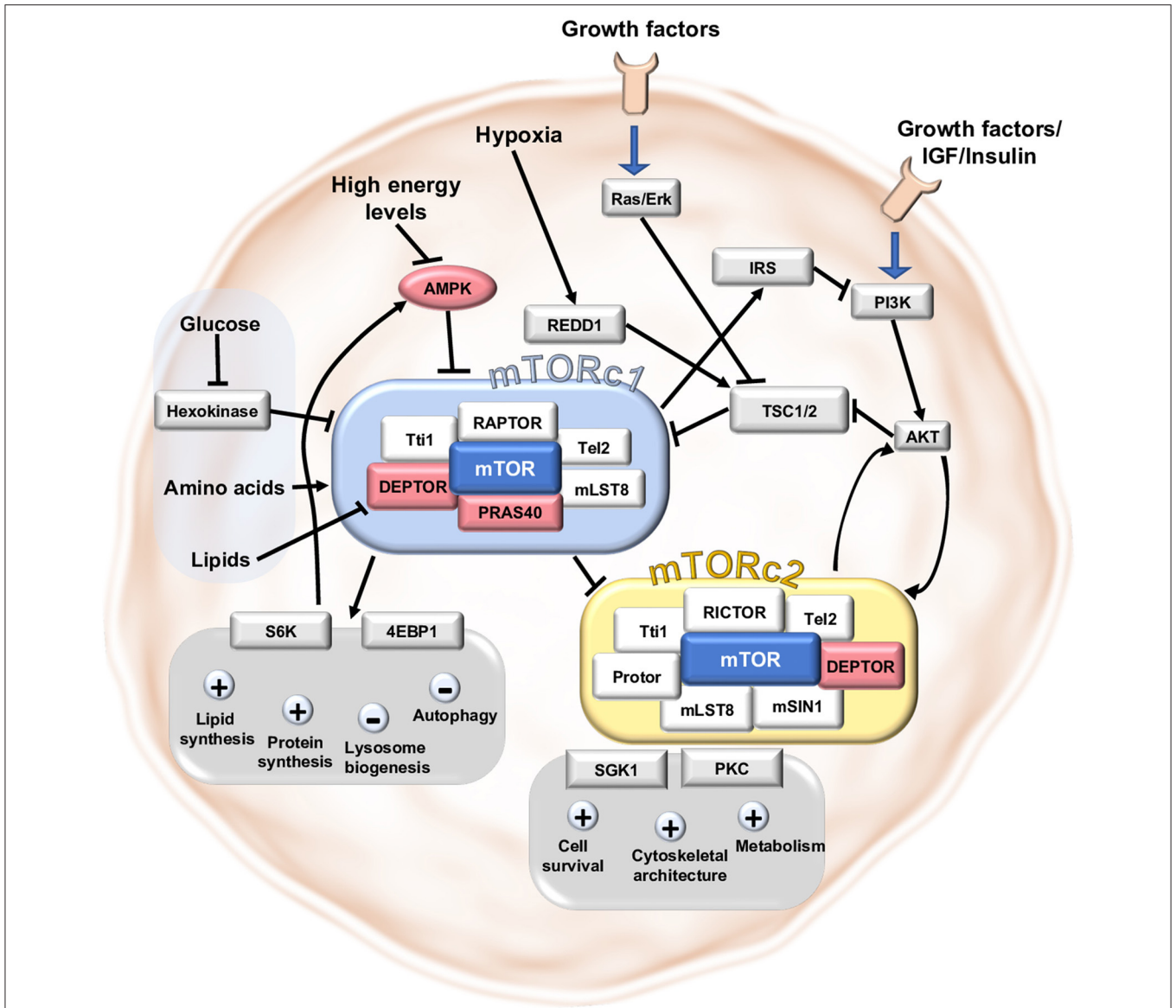

FIGURE 1 | Main signaling pathways in mTORc1 and mTORc2. The mTOR signaling pathway is a central mediator between nutrient abundance and growth stimuli on the one hand and proliferation, metabolism and cell survival on the other hand. mTORc1 and mTORc2 are both complexes consisting of the protein mTOR and further necessary proteins assembling around mTOR. DEPTOR and PRAS40 impede mTORc1-activity and DEPTOR negatively affects mTORc2-activity. Upstream activation is reached after stimulation by growth signals/IGF1 or insulin via PI3K and AKT or independent of AKT via Ras/Erk-pathway. High abundance of nutrients like amino acids, glucose, lipids and high energy levels stimulate mTORc1 directly or indirectly. Hindering circumstances like hypoxia can inhibit the mTORc1 complex by stimulation of the inhibitory protein REDD1. Downstream targets of mTORc1 involve the proteins 4EBP1 and S6K to induce lipid and protein synthesis and block autophagy and lysosome biogenesis. mTORc2 can regulate cell survival, cytoskeletal architecture and metabolism by modulating SGK1 and PKC.

cardiomyocytes damage-associated molecular patterns (DAMPs) are released (15). The activated resident immune cells increase the production of inflammatory cytokines and chemokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNFα), IL-6, and CC-chemokine ligand 2 (CCL2) lead to an increased invasion of neutrophil granulocytes and monocytes, which reinvigorate subsequent immune cell recruitment (15–18). To neutralize and opsonize necrotic cells, neutrophils release enzymes (matrix metalloproteinases (MMPs), myeloperoxidases (MPO), and elastases), complement proteins, and reactive oxygen species (ROS) (19, 20). Pro-inflammatory monocytes ($CD14^+$, $CD16^-$) and T cells enter the infarcted region at an early stage of the inflammatory response (15, 21–23). These early monocytes provide enzymes for tissue degradation and phagocyte debris, while also expressing additional pro-inflammatory cytokines such as interleukins, interferons (IFNs) and TNFα and present antigens on the MHC-II-complex, which promote further inflammation and involve adaptive immunity (22, 24, 25).

After a few days, the necrotic cells and debris is cleared by activity of resolving macrophages (23), which also produce anti-inflammatory cytokines such as IL-10, transforming growth factor beta (TGFß), and lipid-derivates (20). Perpetuating this anti-inflammatory response, regulatory T cells (Tregs) also produce IL-10 and TGFß while regulatory macrophages secrete growth factors (e.g., VEGF) that primarily activate anti-inflammatory, angiogenic, and reparative pathways (25–27). TGFß stimulates fibroblasts to differentiate into myofibroblasts and generate persistent scar tissue, which is accompanied by a fading immune response and results in a partially recovered myocardial function (15). However, resolving inflammation and reestablishing cellular homeostasis are required for long-term healing and preventing persistent unfavorable outcomes such as heart failure.

IMMUNE RESPONSE IN ATHEROSCLEROSIS

Atherosclerosis is the underlying vascular disease that–when occurring in coronary arteries -eventually leads to AMI. It is an artery intimal layer disease that develops over extended periods of time (28). The production of atheromata is strongly linked to excessive lipid levels and other risk factors (e.g. elevated blood pressure, smoking or metabolic diseases like diabetes mellitus) (29). Endothelial dysfunction is considered the beginning point for atherosclerotic plaques, involving decreased nitric oxide levels, increased oxidative stress, and pro-inflammatory signaling (30, 31). Early inflammation in the vessels occur after exposure to high lipoprotein levels within several days (32). Lipoprotein particle uptake and phagocytosis [e.g., oxidized Low Density Lipoprotein (LDL)] triggers the formation of "fatty streaks" that develop over time toward advanced plaques composed of a necrotic lipid core covered by a fibrous cap (33). Immune cells are active at all phases of atherogenesis. The occurrence of 14 unique cell types in human atherosclerotic plaques include diverse subtypes of macrophages, dendritic cells (DCs), T cells, B cells, natural killer T (NKT) cells, neutrophils, and mast cells (34). Activated monocytes and T cells express receptors that interact with the endothelium to trigger the further activated inflammatory phenotype and guide additional inflammatory cells to the damaged area (35). By presenting antigen and excreting cytokines, macrophages and DCs in atherosclerotic lesions contribute to T cell activation. The major subpopulation of T cells present in atheromata are $CD4^+$ T helper cells (36) while a minor number of cytotoxic $CD8^+$ T cells predominantly localize to the fibrous cap (37). Activity of $CD8^+$ T cells enables the release of enzymes that can additionally loosen the plaque structure and lead to plaque rupture worsening the prognosis in atherosclerosis (38). Recently, this concept is expanded by observations that not only plaque rupture but also superficial erosion of intimal layers of the endothelium can lead to thrombotic events and occlusion of the arterial vessel (39). Superficial erosion appears to lead through mechanical stress to a damaged and discontinuous endothelial layer that secondly involves generation of neutrophil extracellular traps (NETs) (40).

Analyzing the subgroups of the T helper cells, T helper cell type 1 (Th1 cells) represent the majority of T helper cells in atheromata and appear to be accountable for disease progression (41). Th1 cells produce IFNγ which specifically leads to enhanced lysis of collagen and reduced collagen-formation and therefore contributes to plaque instability (38). Th17 cells secrete IL-17 and foster plaque growth (42). Other cytokines like IL-6 can contribute to further foam cell accumulation (43) and TNFα further activates intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) in the vascular wall leading to further immune cell invasion (44). Tregs, on the other hand, boost anti-inflammatory monocytes through IL-10 release, limit antigen presentation in DCs (45), and reduce overall pro-inflammatory cytokines and T cell proliferation (26, 46). Neutrophil activity inside the atherosclerotic plaque attracts monocytes to the inflammatory site and increases inflammation by cytotoxic action (47).

The immune system plays an orchestrating role in all phases of plaque formation and complication and has been the focus of numerous experimental attempts to target atherosclerosis. Several clinical trials have recently demonstrated proof of concept for the feasibility of fighting CVD by modifying the chronic inflammatory response associated with atherosclerosis (48).

mTOR-PATHWAYS IN GENERAL AND CARDIOVASCULAR-RELATED IMMUNE RESPONSE

Currently, systemic mTOR inhibition is mostly utilized to treat cancer and for immunosuppression following organ transplantation. These clinical applications harness the major effects of decreased mTOR-signals: immunosuppression and decreased proliferation. Pivotal implications of mTOR-signaling are further identified for cardiovascular diseases like acute myocardial infarction, myocardial hypertrophy, atherosclerosis, and cardiomyopathies (49). Evidence for beneficial effects of mTOR-modulation in atherosclerosis comprises observation of reduced smooth muscle cell proliferation and macrophage influx as well as decreasing lipid metabolism and neo angiogenesis after mTOR inhibition (50). In daily clinical practice mTOR inhibition is used in drug-eluting stents to reduce restenosis rate (51) and after heart transplant to reduce cardiac allograft vasculopathy (51, 52).

Emerging evidence suggests that mTOR can also operate as a relevant target to alter the outcome of a myocardial infarction. Rapamycin, the prototypical mTOR inhibitor, was shown in hypertrophy experiments in mice to reduce damage following ischemia-reperfusion injury and myocardial infarction by modulating apoptosis, ERK, and NO signaling (53). Moreover, Buss et al. demonstrated that inhibiting mTOR in rats reduced infarct site, LV dimensions, and improved cardiac function (54). This observation was linked to an increase in autophagy, limited inflammation, and an overall decrease in proteasomal degradation. In the CANTOS study, reduction of inflammation was successful in reducing Major adverse cardiovascular events

(MACE) in patients with atherosclerosis (48). Reduction was managed through use of a monoclonal IL-1ß-antibody (48). To date, the CLEVER-ACS clinical trial (NCT01529554) is the only study that aims to translate the idea of targeting mTOR activity in myocardial infarction into the clinical setting while laying a specific focus on the immunomodulatory function of mTOR inhibition. In this randomized prospective study patients suffering from STEMI receive either treatment with the mTOR inhibitor everolimus or placebo to evaluate beneficial effects of mTOR inhibition on myocardial inflammation affecting infarct size and myocardial function (55).

Indeed, mTOR not only promotes immune cell proliferation by increasing cell division, but it also activates immune cells and controls cell destiny by guiding immune cell maturation. In general, mTOR activation in immune cells is triggered by an excess of nutrients and growth factors, chemokines, cytokines, toll-like receptors and their ligands, which leads to increased proliferation and more accessible energy in the course of an immune response (11). The most important mTOR downstream pathways are hypoxia-inducible factor 1 (HIF1α), peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulatory element-binding proteins (SREBPs), and MYC, which induce the synthesis of nucleic acids, proteins, and lipids, providing building blocks for growth, increased functional capacity, and multiplication. Elevated energy levels are maintained by a metabolic transition from mitochondrial respiration to glycolysis, which is fueled by AKT activation and inactivating phosphorylation of class IIa histone deacetylases (11, 56). This metabolic switch is observed in immune cells (57) and cardiomyocytes (58). Despite these broad effects, certain mTOR targets cause an essentially opposite response in various cell types. The effects of mTOR signaling in distinct subpopulations will be detailed in the following sections.

T CELLS

T cells primarily contribute to adaptive immune responses in inflammation by acting as cytotoxic or stimulating players for other cells (59). Of note, T cells can contribute to both pro-inflammatory and anti-inflammatory responses allowing classification into several subpopulations (60). Changes in mTOR pathway activation not only cause a proportionate rise or reduction in the proliferation and metabolic activity of all T cells, but also cause changes in the composition and size of the populations, as well as their activity and phenotype.

mTOR inhibition by active TSC1 is required to maintain naïve T cells in a quiescent state in the absence of inflammatory signals (61). In case of antigen binding via T cell receptor (TCR) and co-stimulation by the cell surface protein CD28, mTOR initiates T cell activation (61, 62). The source of energy after T lymphocyte activation switches from ß-oxidation to glycolysis, as seen in cancer cells, and is known as the "Warburg effect" (63).

Because T cell differentiation is prevalent and the effects of various subpopulations on inflammation vary greatly, crucial mTOR activity in these subpopulations is examined further. Reduced mTOR leads to an increase in the number of Tregs that have mitigating functions in atheromata and myocardial infarction as described (61, 64). The molecular mechanism behind this effect is linked to histone H3K4me2 and 3 methylation in proximity to the Foxp3 gene, which represents the lineage-determining transcription factor of this cell type (62). In general elevated Treg counts after mTOR-suppression support anti-inflammatory monocytes by IL-10 secretion (26), impair antigen presentation of DCs (45) and suppress overall pro-inflammatory cytokines (46). These processes mitigate overall inflammatory activity. In cardiac ischemia Tregs in particular lead to beneficial effects (65). The aforementioned generation of anti-inflammatory monocytes prevent an aggravation of the infarct size (66, 67). Tregs promote collagen synthesis by elevating levels of TGFβ leading to enhanced scar formation in myocardial infarction (65). The mechanism of elevated TGFß levels by Treg-activity also improves the course of the diesease in atherosclerosis by collagen formation and stabilization of the atherosclerotic plaque, too (68).

In the remaining $CD4^+$ T cell compartments mTOR inhibition limits polarization and activity of Th1, Th2 and Th17 cell subsets (61, 68). Th1 and Th17 cells depend on mTORC1 whereas Th2 cells require mTORc2 activity (69). Evidence for relevant implications of these cells in cardiovascular diseases exist in atherosclerosis, where Th1 and Th17 cells emerge in the development of atherosclerosis when autoreactive $CD4^+$ T cells are stimulated with ApoB peptides in an environment of inflammatory cytokines (42). The function of Th2 cells in atheromata remains indistinct but the abundance is also sparse (41). Suppression of mTOR could have a beneficial effect in impeding the development of the pro-atherogenic Th1 and Th17 cell subsets. However, recent single cell sequencing data question the separation of T helper cell-subsets that is performed to date in atherosclerotic T cells, transforming our understanding of T helper cell development in a more dynamic direction and generating questions about the implications of the mTOR pathway in the development (37).

In $CD8^+$ T cells mTOR inhibition shifts the cells from an effector phenotype into a memory cell status (70). This shift weakens the implicated pro-inflammatory effects of active $CD8^+$ cells, e.g. lowers IFNγ secretion and hence affects the healing process (71). In atherosclerosis, activity of $CD8^+$ T cells enables the release of enzymes that can additionally destabilize the plaque structure (38), suggesting that the decrease of effector cells could be beneficial in plaque stability. In myocardial infarction, it could be assumed, that a decrease of $CD8^+$ T cells by mTOR inhibition could lower apoptosis. However, deletion of $CD8^+$ cells was not beneficial for the outcome after myocardial infarction (66) and even induced a rupture of the ventricle due to inadequate clearing of necrotic material (72) indicating deleterious effects of overly inhibited mTOR signaling. In the subset of NKT cells, activation is critically controlled by IL-15 using mTOR pathways. Blocking this activation impedes augmentation of glycolysis and inhibits expression of inflammatory cytokines (11, 73). Since NKT cells release granzyme B, perforin, or FasL on targeted cells, it could be presumed that the injured cells enhance the inflammatory response by secreting IFNγ (74). Limiting NKT cell activity by mTOR inhibition could beneficially limit inflammatory response and cytotoxicity in both myocardial infarction and atheroma.

Altogether, mTOR inhibition dampens the activity of pro-inflammatory Th cells and NKT cells, shifts T helper

cell subpopulations in favor of Treg subpopulations with inflammation-resolving properties, and promotes conversion of cytotoxic effector T cells into a memory status. Besides a general decrease of inflammatory activity, Tregs specifically enhance a regenerative phenotype in monocytes/macrophages and block the activation of antigen-presenting cells (APC) and effector T cells (26, 45).

MONOCYTES/MACROPHAGES

Monocytes/macrophages are important cells of the innate immune system that remove debris from the site of inflammation. As described above, attraction of further immune cells as well as activation of immune cells and phagocytic function are important in both atherosclerosis and myocardial infarction.

In general, mTOR is involved in the differentiation and development of monocytes and macrophages after stimulation with granulocyte-macrophage colony-stimulating-factor (GM-CSF) (75). However, mTOR impairment does not diminish total cell numbers, demonstrating that mTOR alone does not regulate monocyte growth and differentiation entirely (76). Regardless of its overall influence on monocytes, mTOR modification can guide monocyte polarization toward either an inflammatory or anti-inflammatory phenotype. The specific pathways are still unknown, and inconsistent data on limiting mTOR activation have been obtained through diverse techniques (77). For instance an inflammatory phenotype linked to increased mTORc1 activity is observed in bone marrow-derived macrophages by TSC1 deficiency (78–80) whereas mTORc2 activity must be reduced to yield an enhanced inflammatory phenotype (77, 81). Further, modulation of the upstream activator AKT can induce contrary effects on the development of monocytes depending on the isoform of AKT (77, 82). Further regulators that influence polarization of monocytes/macrophages are upstream activators of mTOR, such as ketamine or endothelial growth factor (EGF) that enhance inflammatory monocytes/macrophages (83, 84). These observations indicate that polarization of monocytes/macrophages can be influenced by many components of the mTOR pathway with differential results, depending even on the isoform of the regulated component.

Focusing on myocardial infarction, high rates of inflammatory monocytes worsen the outcome (85). However, depletion of all monocytes with the aim to prevent tissue damage caused by inflammatory monocytes instead impairs myocardial wound healing after myocardial infarction (25) due to insufficient clearance of necrotic tissue in the absence of monocytes/macrophages. Therefore, polarization of monocytes/macrophages into a reparative phenotype could be an approach to ameliorate myocardial wound healing. As discussed above, immediate effects of mTOR modulation cause a variety of activating and polarizing consequences on monocyte/macrophage. However, mTOR inhibition could boost polarization of monocytes into a reparative phenotype through enhanced Treg generation (67).

Aside from the effect of mTOR on monocyte growth, it is also important to note that mTOR inhibition exerts relevant effects on chemoattraction by reducing the expression of MCP-1 (CCL2) (86), that is produced in various cell types like endothelial cells, smooth muscle cells (SMCs) and fibroblasts (87). This effect can be observed in both atherosclerosis and myocardial infarction. In atherosclerosis lower chemoattraction led to smaller atherosclerotic plaques with fewer macrophages (88–90).

mTOR modulation exerts various effects in monocyte development and polarization, that are not fully characterized and understood yet. Nevertheless, formation of reparative monocytes could be promoted indirectly by enhanced Treg generation after mTOR inhibition. Additionally, reduced CCL2 expression in the environment of monocytes/macrophages reduces the number of monocytes/macrophages present at the site of inflammation by limiting chemoattraction.

DENDRITIC CELLS

Dendritic cells are a subset of immune cells that differ from monocytes and macrophages in that they link the adaptive and innate immune systems by acting as professional APCs, i.e., presenting antigens to adaptive immune cells (91). Overall activation is mTOR-dependent and triggered by granulocyte-macrophage colony-stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (FLT3L), lipopolysaccharide (LPS), and TLR (11, 92). Systemic mTOR inhibition does not only repress proliferation and activation of all DCs but results in generation of tolerogenic DCs, a subgroup occurring after stimulation with IL-4 (93). This subset of DCs is involved in the generation of Tregs and, as a result, the downregulation of the inflammatory response (11). In myocardial infarction tolerogenic DCs regulate Tregs and macrophage polarization, preserving cardiac function and promoting functional remodeling (94). Similarly, in atherosclerosis hampered DC maturation disrupts Treg formation accelerating disease progression (41). Furthermore, DC-specific TSC1 activation reduces $CD8^+$ T cells (95), that enhance the risk for plaque rupture (38). However, contrary effects occur in different subsets of DCs after mTOR modulation, making it difficult to weigh up the consequences of the opposing effects. mTOR inhibitors increase pro-inflammatory cytokine expression in some myeloid DCs (mDC) and enhance antigen presentation to stimulate pro-atherosclerotic Th1 and Th17 cells (11) while suppressing inflammatory cytokines in monocyte-derived DCs (moDCs) (96).

As a conclusion, the action of mTOR inhibitors appears to be linked to the subpopulation of DCs as well as the stage of development (92). Suppression of the mTOR pathway could cause an advantage in myocardial wound healing due to enhanced amounts of tolerogenic DCs, as well as Tregs. In atherosclerosis plaque stability is rendered due to less $CD8^+$ T cells. On the other hand, secretion of pro-inflammatory cytokines as well as activation of T cells can be reinforced by augmented mTOR activity. Yet, several experimental models in atherosclerosis and myocardial infarction provide evidence that mTOR inhibition in DCs lead to a beneficial outcome.

NEUTROPHIL GRANULOCYTES

The mTOR pathway is involved in the metabolic stimulation of neutrophil granulocytes via GM-CSF. Activation of neutrophils

increases production of inflammatory proteins such as IL-6 and cyclooxygenase 2, which contribute to the inflammatory environment (11). By mTOR inhibition, proliferation and activation of neutrophils decline and chemokine-mediated attraction of neutrophils to the site of inflammation is hampered (97, 98). Mechanistically, specific inhibition of mTORc2 causes impaired migration by reducing pseudopod formation (99).

In myocardial infarction, neutrophils are essential to decompose the ischemic area, but secreted ROS and myeloperoxidases harm viable myocardium in the border zone (20). Blocking myeloperoxidase in particular has been proven to prevent heart dilation, resulting in a better cardiac prognosis (20). Even though the reduction of neutrophil count is beneficial, a complete elimination of neutrophils should be avoided since it resulted in increased fibrosis and heart failure following myocardial infarction (100). This observation indicates once again, that a strong mTOR inhibition could be harmful, whereas a moderate mTOR suppression could weaken the inflammation.

Furthermore, active mTOR facilitates neutrophil extracellular trap (NET) formation (101, 102). In addition to the essential role of NET formation in defense against bacteria, NETs are produced in sterile inflammation as well (103). A decline of mTOR activity could attenuate pro-inflammatory signals by limited NET formation.

Little is known about mTOR modulation in neutrophils in atherosclerosis. Risk factors, such as hypercholesterinemia and hyperglycemia, already increase neutrophil production (47). Neutrophils have an impact on the onset and growth of atherosclerotic plaques (104) and high neutrophil/lymphocyte ratios worsen the prognosis in coronary artery disease (105). Therefore, mTOR inhibition could be helpful to decelerate the progression and generation of atherosclerotic plaques by overall reduction of activity and number of circulating neutrophils.

In summary, the mTOR pathway is implicated in neutrophil granulocyte activation, migration and invasion, which together lead to an enhanced inflammation. mTOR inhibition is able to diminish neutrophil activity, generation and attraction. Lower neutrophil cell counts show improved post-myocardial infarction prognosis. Since neutrophils are involved in plaque development it is likely that mTOR inhibition could reduce atherosclerotic plaque burden by lowering neutrophil cell counts.

B CELLS

B cells are lymphocytes that are primarily responsible for the humoral immune response. Other important functions include antigen presentation to T cells and further cytokine production to influence overall immune response (106).

In the early development of B cells mTOR is highly activated (107). mTOR deficiency results in overall disruption of early B cell development in conjunction with insufficient metabolic capacity (107). In later maturation stages, B cells develop into subgroups of regulatory B cells (Breg), B1 and B2 cells (108). In a mature B cell ligation of the B cell-receptor (BCR) induces an mTOR-dependent B cell activation through NFKB, MAPK and PI3K (109). IL-4 from T cells, signals through TLR, and other chemokines and cytokines (like BAFF) work in a similar way with mTOR as a downstream-target that leads to survival and proliferation (8).

Overall depletion of B cells reduces the infarct size in models of myocardial infarction (110) which could be reached by mTOR inhibition to repress B cell development. Primed B cells with damage-associated molecular patterns (DAMPs) after myocardial infarction could be decreased by mTOR inhibition so that the progression of atherosclerotic plaques fueled by primed B cells is hampered (110–112).

In atherosclerosis, mTOR-dependent B2 cells are considered pro-atherosclerotic (8, 113, 114) B1 cells and B regs also depend on mTOR-activity (115). In contrast to B2 cells, however, these cells can diminish inflammation in atherosclerosis by antibody-secretion (116) blocking the uptake of oxLDL (109) and declining the number of associated $CD4^+$ T cells in case of B1 cells (117) and excretion of anti-inflammatory cytokines like IL-10, IL-35 and TGFß in B regs (118). Adoptive transfer of B1a cells, a constitutively IgM producing subgroup of B cells that expresses $CD5^+$ in mice (119), was able to decrease atherosclerotic plaque size (120, 121). Considering these soothing properties of B1 cells and B regs in inflammation in atherosclerotic plaques, mTOR inhibition could harm by suppression of B1 cell and B reg generation. Apart from the described knowledge of B cell polarization specific implication of mTOR in B cell fate decision is sparse.

In summary, during acute inflammation B cell-inhibition attenuates the pro-inflammatory effect of B cells whereas in case of chronic inflammation in atherosclerosis restraining of B cells via mTOR inhibition could hamper protective antibody expression against oxLDL. Additionally, division of B cells in smaller subpopulations is emerging and knowledge about specific effects of mTOR regarding the development and polarization of these subpopulations is lacking. Information about development of B cell subpopulations under mTOR modulation could open a whole new field of interactions that need to be further investigated regarding their relevance in myocardial infarction and atherosclerosis.

ENDOTHELIAL CELLS

Endothelial cells build a barrier between the blood and the inflammation site, that may lose its strict barrier function in the course of an inflammatory response. In acute myocardial injury, DAMPs and cytokines induce the expression of selectins on endothelial cells to bind leukocytes and facilitate their migration into the tissue (15). This process goes along with enhanced permeability, facilitating leukocyte transmigration (122). On the one hand, mTOR inhibition dampens cytokines preventing leukocyte recruitment due to downregulation of adhesion molecules (123). On the other hand, there is evidence that mTOR activation prevents endothelial disruption in infarction regions and preserves tight connections between cells (124). In accordance, mTOR activity is required to mediate growth and proliferation in a disrupted area in order to facilitate tissue regeneration (125). In the case of mTOR inhibition these observations could lead to limited immune cell recruitment in ECs but also to insufficient proliferation of ECs and wound healing in the necrotic area.

Mechanical stress transferred by blood flow initiates inflammatory activity and proliferation of ECs by mTOR-signaling with clinical importance in the development of atheroma. Laminar, pulsatile flow maintains a low level of proliferative and inflammatory signaling whereas turbulences and wall shear stress (e.g., at the branching point of an artery) have an pro-inflammatory and proliferative effect (126). This stimulus is conveyed via mechano-receptors involving phosphorylation of AKT and consequently activation of the mTOR pathway (127). Thus, chronic systemic mTOR inhibition could prevent emergence and growth of atheroma.

SMOOTH MUSCLE CELLS

Smooth muscle cells (SMC) contribute to plaque formation in atherosclerosis by proliferating and producing extracellular matrix (ECM). Initially, studies focused on SMCs proliferation as a major driver of atheroma development (38). Aside from anti-inflammatory benefits, Sirolimus, a mTOR inhibitor, decreased SMCs in atheroma and boosted collagen levels (128). In coronary artery disease, drug eluting stents (DES) containing antiproliferative drugs such as the mTOR inhibitor Everolimus are successfully utilized to prevent restenosis by inhibiting intimal proliferation (51). This approach delivers a high concentration of reactive chemicals to the atheroma while avoiding systemic negative effects. The supplied dose of Everolimus induces cell cycle arrest in smooth muscle cells without causing cytotoxicity (129). Everolimus-eluting stent-Implantation reduced major adverse events and the need for ischemic-driven target lesion revascularization in patients independent of indication (130).

These observations display the successful use of mTOR inhibitors in daily clinical practice to prevent further plaque growth in coronary artery disease.

CARDIOMYOCYTES

In ischemia necrotic cardiomyocytes release DAMPs, that set off an inflammatory cascade. Improved cardiomyocyte survival is, beyond its obvious benefit in preserving functional cells, one possible mechanism for reducing the pro-inflammatory signal. The effects of mTOR modulators on cardiomyocytes have been linked to autophagy and apoptosis. In the acute phase of ischemia activating mTOR by application of insulin reduced the number of apoptotic cells in the heart (131–133). On the other hand, in myocardial wound healing reduced mTOR signaling elevated autophagy, which improved left ventricular remodeling following myocardial infarction (134). In chronic heart failure helpful long-term effects of mTOR inhibition increased autophagy and decreased apoptosis, preventing additional cardiac functional decline (131). Generation of cardiomyocyte hypertrophy is dependent on mTOR as well (135) enabling prevention of hypertrophic remodeling by mTOR inhibition.

Apart from cardiomyocyte survival, viable cardiomyocytes in the border zone of an infarction assume an inflammatory role. After exposure to IL-1, TLR ligands, and ROS, viable cardiomyocytes contribute to cytokine and chemokine production, as well as the expression of intercellular adhesion molecule (ICAM)-1 (15). mTOR regulates the interaction between the immune system and cardiomyocytes, making it a potential target for future study.

In summary, whereas mTOR activation may protect cardiomyocytes initially after ischemia from cell death, mTOR inhibition and increased autophagy appears to have a better long-term impact in the wound healing process (136).

FIBROBLASTS

After myocardial infarction, fibroblasts are primarily involved in scar formation and the resolution of the inflammatory process. TGFß is important in myofibroblast transformation, which leads to proliferation and ECM synthesis and occurs in a higher abundance after mTOR inhibition (27). For ideal remodeling, a balance between critical scar tissue growth to avoid rupture and fibrosis is required (15). In both acute and chronic remodeling, mTOR inhibition can reduce cardiac fibrosis by repressing scar development into a mild phase (134).

POTENTIAL FUTURE CLINICAL IMPLICATIONS

In current clinical practice, mTOR-inhibiting compounds are essential in the treatments of malignancies, transplant patients and as coating in drug-eluting stents. Expansion of the indications to myocardial infarction could dampen overshooting inflammatory activity that damages viable cardiomyocytes in the border zone by reduced overall inflammation and fostering reparative mechanisms. In the setting of acute inflammation, a short intervention by mTOR inhibition may already mitigate the aggressive inflammatory reactions and potentially preserve myocardial function. In atherosclerotic plaques systemic mTOR inhibition could expand the beneficial effects on plaques on all vessels of the body and impede additional plaque development. To reach this aim a long-term treatment would be necessary to ensure persistent prevention of disease progression.

To translate this concept into clinical practice, side effects of these compunds have to be balanced against their benefit. Relevant side effects occur but differ depending on the dosage regimen. As mTOR is an energy and nutrition sensor, a powerful mTOR inhibitor can imitate a condition of acute food scarcity and hinder early cell growth (62). Side effects of mTOR-inhibiting therapies include severe infections as well as aplasia syndromes like anemia and thrombopenia (137) and metabolic side effects such as hyperlipidemia and hyperglycemia (138). Furthermore, poor wound healing, a risk of developing malignancies and pneumonitis must be considered when determining therapeutic dosages to treat cardiovascular disorders (139, 140). Of note, the therapy duration must be considered, as extended mTOR inhibition, for example, may increase fibrosis in myocardial infarction via persistent elevated levels of pro-fibrotic cytokines (e.g., TGFß).

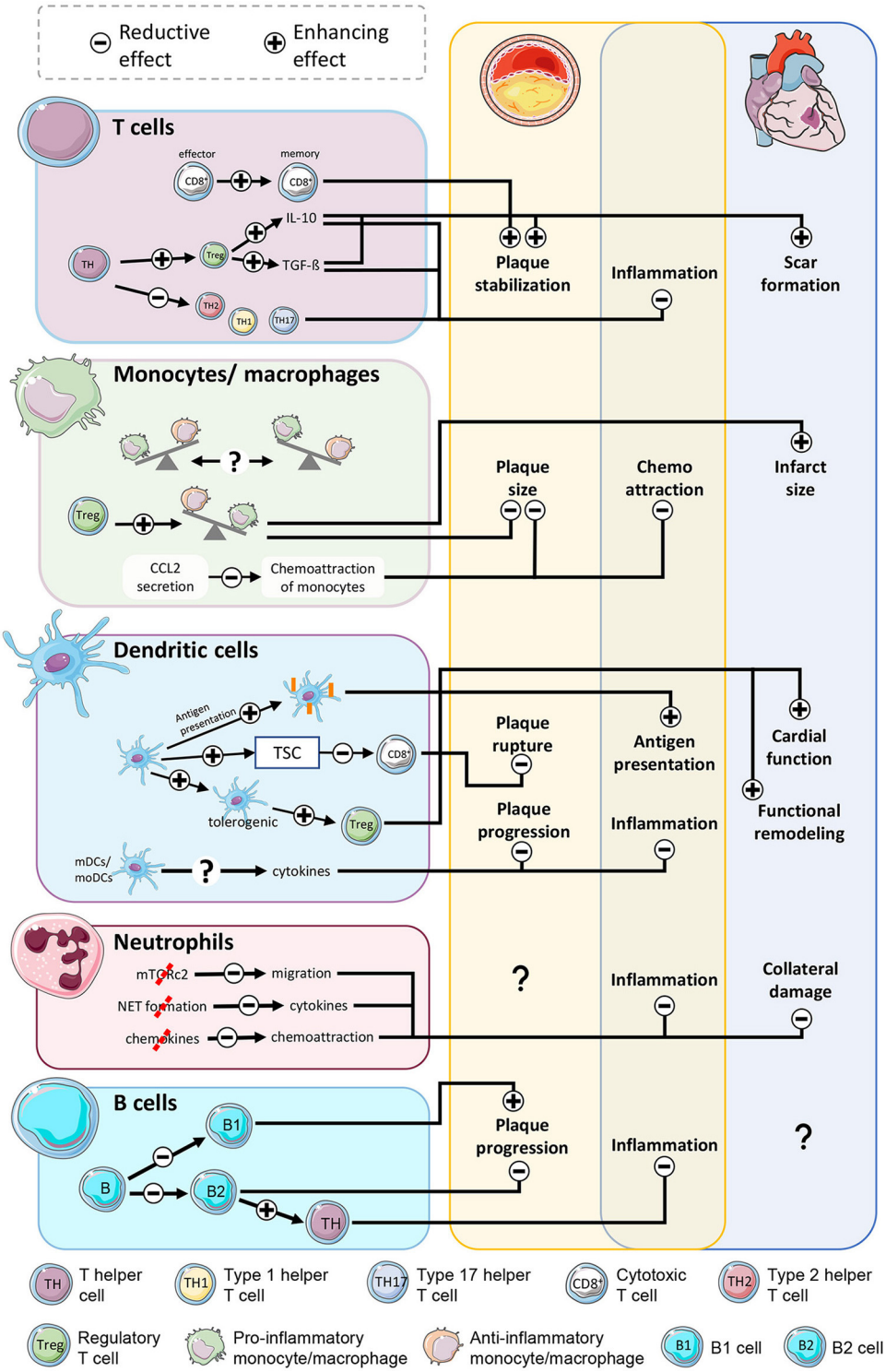

FIGURE 2 | mTOR-inhibition affects atherosclerosis and myocardial infarction by modulation of immune cells. Stabilization of plaques is accomplished by less active CD8+ T cells and a shift of T helper cells to an antiinflammatory phenotype. Reduced chemoattraction reduces the number of monocytes/macrophages at the inflammation site, ameliorating inflammatory activity in both diseases. The shift of monocytes into a reparative phenotype limits the scar size. Even though enhanced antigen presentation in dendritic cells increases inflammatory activity, the shift toward tolerogenic DCs and stimulation of Treg function soothes inflammation and benefits the outcome after AMI. Reduced neutrophil activity lessens the collateral damage to viable myocardium in the border zone. B cell subpopulations exert contrary effects on atherosclerosis with yet unclarified outcome.

Within the group of patients suffering from cardiovascular disease diabetic patients are a vulnerable subgroup prone to develop atherosclerotic plaques with a 2- to 4-fold chance to suffer from cardiovascular events (141) and higher rates in morbidity and mortality after myocardial infarction (142). This subgroup of patients could particularly take advantage from mTOR-inhibiting therapy. From the pathophysiological point of view, mTOR remains constantly activated because of persistent hyperglycemia (143). Negative feedback-loops implicating IRS (insulin receptor substrate) cause the deterioration of the insulin sensitivity (144). As described above, high levels of blood glucose led to higher inflammatory activity using the mTOR pathways. Therefore, higher baseline levels of inflammation are considered as a link between diabetes and accelerated plaque development in atherosclerosis (145).

After myocardial infarction diabetic patients also show higher inflammatory activity measured by elevated markers of inflammation (CRP and IL-6) (146, 147). Within this constellation, mTOR inhibition in myocardial infarction could interrupt the vicious cycle of constant mTOR activation and accelerated immune response to ameliorate myocardial wound healing in the critical days after the incident. Treatment of atherosclerosis would supposedly necessitate a long-term mTOR inhibition, that causes hyperglycemia itself and could deteriorate the deranged metabolism of diabetic patients (148).

SUMMARY

Inflammation is a substantial pathophysiological mechanism in myocardial infarction and atherosclerosis, two frequent and momentous examples for acute and chronic cardiovascular diseases. In myocardial infarction, a certain degree of inflammation is essential to decompose the necrosis, although overshooting inflammation may harm viable cells in the border zones of the affected myocardium, worsening the functional outcome. In atherosclerotic plaque development, chronic inflammation is the main process that sustains plaque growth, increasing the risk for plaque rupture and occlusion of the affected vessels. Thus, strategies that modulate inflammation may soothe aggressive metabolic processes in acute conditions, can cease chronic inflammation and build a basis for future therapies. mTOR inhibitors offer an enormous potential to

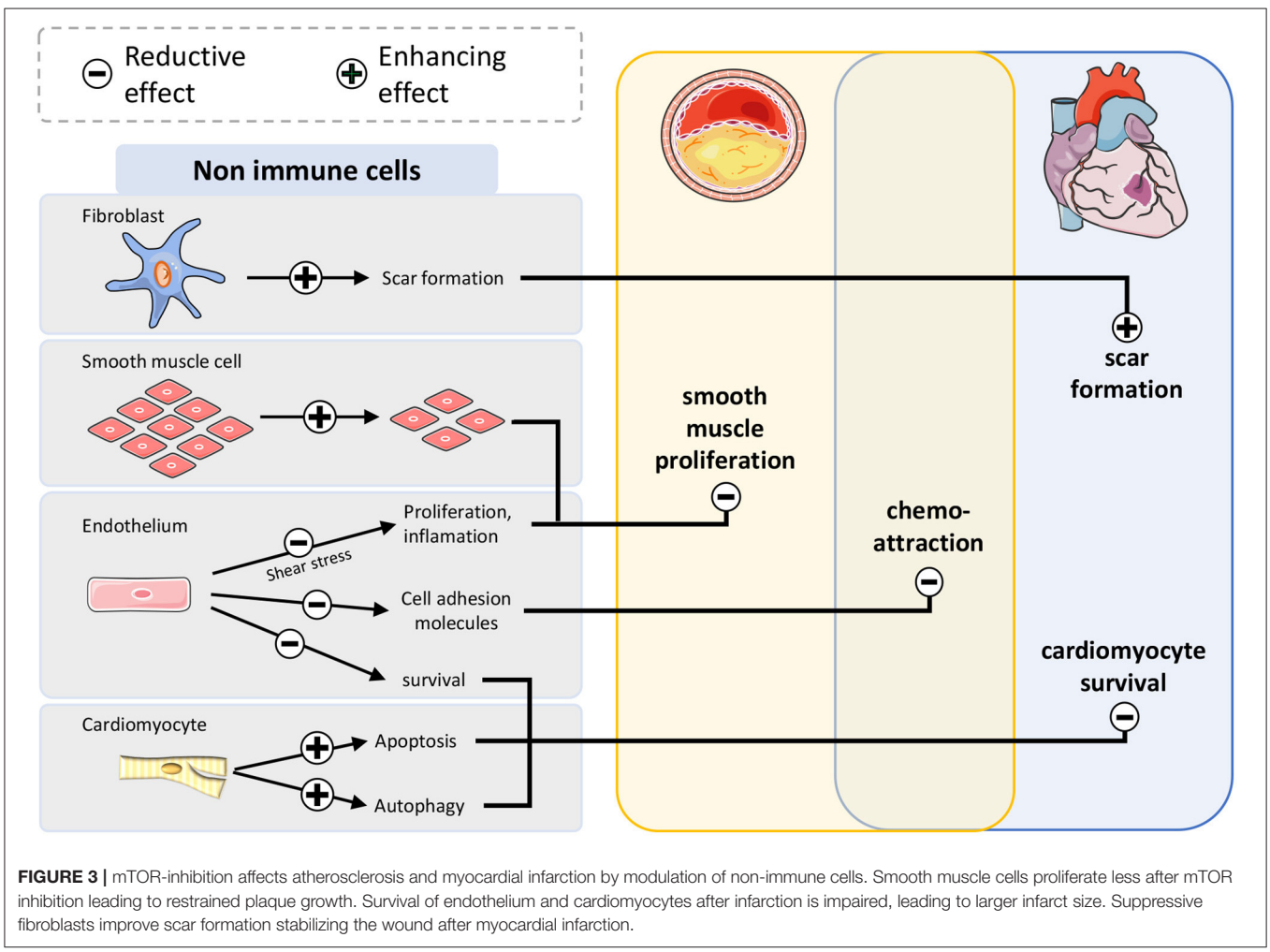

FIGURE 3 | mTOR-inhibition affects atherosclerosis and myocardial infarction by modulation of non-immune cells. Smooth muscle cells proliferate less after mTOR inhibition leading to restrained plaque growth. Survival of endothelium and cardiomyocytes after infarction is impaired, leading to larger infarct size. Suppressive fibroblasts improve scar formation stabilizing the wound after myocardial infarction.

interfere with the immune system because of their essential roles in central pathways of cellular proliferation, growth, and survival.

A large share of the beneficial effects of mTOR inhibition are based on decreased proliferation, activity and a dampening of inflammatory signals. Further, mTOR inhibition results in relevant developmental changes in specific subpopulations of immune cells relevant for the course of the disease in either atherosclerosis or myocardial infarction (**Figure 2**). In general, a large amount of practically every cell type is reduced, causing smaller amounts of secreted pro-inflammatory cytokines. Nonetheless, cytokines not only have a pro-inflammatory role, but they also act as messengers in a complex network with pleiotropic effects, like the anti-inflammatory cytokine IL-10. A certain number of these molecules is required to prevent negative consequences (149, 150). Chemoattraction is mostly reduced owing to lower production of inflammatory mediators, eventually leading to less-activated endothelium and reduced binding and recruitment of leukocytes. At the site of injury, this approach can already reduce numbers of neutrophils, T cells, NKT cells, B cells, monocytes/macrophages, and dendritic cells. As a consequence, clearance of necrotic/apoptotic cells is slowed, resulting in lower ROS production, degrading enzymes, and additional inflammatory cytokines, ameliorating the inflammation in both diseases. Furthermore, Tregs proliferate at a greater rate, causing monocytes and macrophages to adopt a reparative phenotype and scale down the infarct size after infarction and also stabilize the fibrotic cap of atherosclerotic plaques. Tolerogenic DCs, which are likewise boosted by mTOR inhibition may contribute to promotion of regulatory T cells and subsequently preserved cardiac function after infarction. Additionally, suppression of mTOR in mature effector cytotoxic T cells accelerated the transition of effector cells into memory cells. Reduction of the number of active cytotoxic T cells stabilizes atherosclerotic plaques. Further detailed identification of new cell types using modern methodology and ascribing these specific mTOR-regulated downstream effector function, like in B cells, will be instructive to understand the overall effect of inhibiting these pathways in CVD.

In non-immune cells (**Figure 3**), mTOR-inhibition disrupts survival signaling via mTOR in endothelial cells and cardiomyocytes at an early timepoint after infarction, enlarging the damaged area. In chronic inflammation mTOR inhibition prevents occlusion of coronary arteries caused by accelerated proliferation of smooth muscle cells. In fibroblasts scar formation improves when the mTOR pathway is suppressed.

To transfer mTOR inhibiting therapies into the clinical therapy of CVD further evidence is needed to titrate the correct dosing for a moderate mTOR inhibition without aplasia in all cells. Secondly, the duration and scheme of the therapy must be determined. While modulation of the immune response after infarction is needed most likely only for days to weeks after the incident, prevention of disease progression in atherosclerosis could require permanent therapy. Related to these treatment regimens the extent of side effects differs massively and ranges from high drug safety to an elevated burden in metabolic dysregulations including diabetes and hyperlipidemia, which are risk factors for atherosclerosis themselves.

In conclusion, mTOR inhibitors have a high potential to modulate inflammation in cardiovascular disorders by breaking the vicious cycle of autonomously maintained inflammation and boosting tolerance mechanisms. Even though some cells develop inflammatory properties under mTOR inhibition, anti-inflammatory and regulatory effects prevail. Apart from disease specific immune responses, this review demonstrates favorable effects of mTOR inhibition in both acute and chronic inflammation. For therapeutic usage, the dose and time range of the application must be explored further.

AUTHOR CONTRIBUTIONS

NG, AL, and MKa drafted the manuscript. MKa wrote the manuscript. NG, PF, and AL edited the manuscript. SP arranged the figures. Critical revision of the manuscript was made by NG, AL, PF, FB, and MKe. All authors read and approved the final manuscript.

FUNDING

This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)-Grant No. 236177352-CRC1116; projects B06 and B09 to MKe and NG and Grant No. 397484323-CRC/TRR259; projects A05 and B03 to NG and FB. MKa is supported by a GEROK stipend of CRC1116.

ACKNOWLEDGMENTS

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The reviewer FK is currently organizing a Research Topic with the author NG to the handling editor.

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frontiers | Frontiers in Cardiovascular Medicine

TYPE Mini Review
PUBLISHED 15 August 2022
DOI 10.3389/fcvm.2022.943214

Check for updates

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Atherosclerosis and Vascular Medicine, a section of the journal Frontiers in Cardiovascular Medicine

RECEIVED 13 May 2022
ACCEPTED 01 August 2022
PUBLISHED 15 August 2022

CITATION
Ganesh N, van der Vorst EPC, Spiesshöfer J, He S, Burgmaier M, Findeisen H, Lehrke M, Swirski FK, Marx N and Kahles F (2022) Gut immune cells—A novel therapeutical target for cardiovascular disease? *Front. Cardiovasc. Med.* 9:943214. doi: 10.3389/fcvm.2022.943214

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Gut immune cells—A novel therapeutical target for cardiovascular disease?

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Despite scientific and clinical advances during the last 50 years cardiovascular disease continues to be the main cause of death worldwide. Especially patients with diabetes display a massive increased cardiovascular risk compared to patients without diabetes. Over the last two decades we have learned that cardiometabolic and cardiovascular diseases are driven by inflammation. Despite the fact that the gastrointestinal tract is one of the largest leukocyte reservoirs of our bodies, the relevance of gut immune cells for cardiovascular disease is largely unknown. First experimental evidence suggests an important relevance of immune cells in the intestinal tract for the development of metabolic and cardiovascular disease in mice. Mice specifically lacking gut immune cells are protected against obesity, diabetes, hypertension and atherosclerosis. Importantly antibody mediated inhibition of leukocyte homing into the gut showed similar protective metabolic and cardiovascular effects. Targeting gut immune cells might open novel therapeutic approaches for the treatment of cardiometabolic and cardiovascular diseases.

KEYWORDS

gut immune cells, intraepithelial lymphocytes, integrin β7, cardiovascular disease, myocardial infarction, atherosclerosis, heart failure, GLP-1

Introduction

Scientific discoveries, advances in public health and innovations in medical care (including modern heart failure, lipid-lowering, and antiplatelet therapies) have contributed to a steady decline of mortality from cardiovascular disease in the last 50 years. Despite these improvements, cardiovascular diseases (such as coronary artery disease, myocardial infarction, or heart failure)

continue to be the main cause of death worldwide (1, 2). Especially patients with diabetes are more vulnerable compared to those without diabetes and face a very high risk to die from cardiovascular disease (3). Recent evidence showed that sodium glucose linked transporter 2 (SGLT-2) inhibitors (4) and glucagon-like peptide 1 (GLP-1) receptor agonists (5) improved cardiovascular prognosis of patients with diabetes and high cardiovascular risk. Importantly, SGLT-2 inhibitors reduced heart failure endpoints in patients with or without diabetes (6, 7). Thus, SGLT-2 inhibitors emerged as first-line heart failure therapy in current guidelines (8). Despite these recent advances in heart failure and diabetes therapy there is still an urgent unmet clinical need to further reduce cardiovascular morbidity and mortality. Novel therapeutic approaches especially targeting atherosclerosis development and plaque stability must be identified and translated into promising drug targets. Targeting the immune system might be a novel therapeutical approach to protect against cardiovascular disease and its life-threatening complications.

The role of inflammation in cardiovascular disease

Data from the last two decades have shown that leukocytes and inflammatory processes are important players during the development of cardiovascular diseases such as atherosclerosis, myocardial infarction, and heart failure (9–11). Elegant experimental studies have identified inflammatory organ cross talk networks between the heart, the bone marrow, the spleen, and the sympathetic nervous system which could explain how immune cells control and accelerate the development and progression of cardiovascular disease. These observations suggest that after myocardial infarction enhanced sympathetic nervous activity releases noradrenalin in the bone marrow niche, which results in increased hematopoietic stem cell (HSC) activity and emigration to extramedullary sites. Increased production of myeloid immune cells (myelopoiesis) in the bone marrow results in higher numbers of circulating myeloid immune cells that are recruited to atherosclerotic plaques in higher numbers, accelerating plaque growth and inflammation and decreasing plaque stability thus increasing the risk of re-infarction (12–14). This feedback loop and vicious cycle might be one of the explanations why patients after a myocardial infarction are at increased risk to experience re-infarction.

Immune cells play a crucial role in atherosclerosis formation and subsequent plaque growth. Following intimal lipid accumulation and endothelial dysfunction monocytes migrate into the vessel wall [reviewed in Swirski and Nahrendorf (12)]. Newly-infiltrated monocytes differentiate into macrophages which recognize and ingest lipids that have accumulated in the intima as a consequence of hypercholesterolemia. After lipid ingestion macrophages become lipid- rich "foam cells," thus activating various inflammatory pathways leading to activation of other immune (including T and B lymphocytes, neutrophils, and dendritic cells) and non-immune cells [including endothelial cells, platelets, smooth muscle cells; reviewed in Swirski and Nahrendorf (12)].

In line with these findings circulating leukocyte numbers and biomarkers of inflammation such as high-sensitivity C-reactive protein and interleukin-6 are associated with an increased risk of cardiovascular events in humans (15–17). In order to prove the inflammatory hypothesis of atherothrombosis several large cardiovascular outcome trials validating drugs which target inflammatory pathways have been conducted. In the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) canakinumab, a therapeutic monoclonal antibody targeting interleukin-1β, showed a 15% risk reduction of non-fatal myocardial infarction (MI), non-fatal stroke, or cardiovascular mortality in patients with previous myocardial infarction and hs-CRP above 2 mg/L (18). In this trial canakinumab was associated with a higher incidence of fatal infection compared to placebo. While this study for the first time demonstrated that an anti-inflammatory drug is able to improve cardiovascular outcomes, the Food and Drug Administration (FDA) rejected clinical approval of canakinumab for cardiovascular disease most likely due to safety concerns and only modest outcome effects. Colchicine, an NACHT, LRR, and PYD domains-containing protein 3 (NLRP3)-inflammasome inhibitor, was investigated in patients with a recent myocardial infarction [Colchicine Cardiovascular Outcomes Trial—COLCOT (19)] and in patients with chronic coronary disease [In the Low-Dose Colchicine 2—LoDoCo2 trial (20)]. In both studies low-dose colchicine significantly reduced cardiovascular events. However, the incidence of death from non-cardiovascular causes was numerically higher in the colchicine group than in the placebo group which requires further investigations. While these studies proved the inflammatory hypothesis of cardiovascular disease, interleukin-1β (canakinumab) or the NLRP3-inflammasome (colchicine) might not be the optimal drug candidates for this purpose. Thus, alternative therapeutical targets modulating inflammation need to be identified to improve prognosis in patients with cardiovascular disease.

Gut immune cells and cardiovascular disease

The gut microbiome and intestinal barrier dysfunction ("leaky gut") have emerged as potential contributors to the development of cardiovascular disease and are currently intensively studied (21–23). Despite the fact that the gastrointestinal tract (GI tract) is one of the largest leukocyte reservoirs of our bodies containing very high numbers of T lymphocytes, plasma cells, eosinophils, and macrophages

(24–26), the relevance of gut immune cells for cardiovascular disease is largely unknown. Integrin β7 (*Itgb7*) is expressed on circulating leukocytes and mediates immune cell migration selectively into the gut without affecting relevant chemotaxis into other tissues (27–31). Thus, *Itgb7*$^{-/-}$ (hereafter $\beta7^{-/-}$ mice) display a useful tool to study selective gut immune cell deficiency, since these mice show strongly reduced leukocyte numbers (especially intraepithelial lymphocytes: αβ and γδ T cells, B cells, and myeloid cells) in the gut. A recently published study investigated whether gut immune cells play a role in atherosclerosis development in mice (32). *β7* deficient mice on *Ldlr*$^{-/-}$ background fed a high cholesterol diet (HCD) showed no difference in weight gain, but had lower levels of plasma total cholesterol, a reduction in circulating Ly-6C^{high} and Ly-6C^{low} monocytes and smaller aortic lesions with an ∼50% reduction in plaque size and volume (32). To elucidate the question whether gut immune cells can be therapeutically targeted for cardiovascular disease the authors injected anti-integrin β7 antibodies (which block immune cell homing to the gut) into *Ldlr*$^{-/-}$ mice and found that these mice had attenuated atherosclerosis (32). These findings show that gut immune cells accelerate lesion growth while antibody mediated integrin β7 neutralization protects against atherosclerosis formation in mice.

Patients with diabetes and obesity show more severe atherosclerosis and higher cardiovascular risk than those without diabetes. Based on the promising findings on targeting intestinal leukocytes to treat atherosclerosis it is important to investigate the functional relevance of gut immune cells in cardiometabolic diseases. Several studies in obese mice and men demonstrated major changes to the intestinal immune cell landscape in the obese state compared with the lean state. During metabolic disease the gut contains increased numbers of γδ T cells, macrophages, dendritic cells, NK cells, CD8+ T cells (αβ TCR), and Th1 T cells and a reduction in Treg T cells, anti-inflammatory IgA+ antibody-secreting cells (ASCs) and eosinophils. These changes in immune compartments are associated with an inflammatory environment that is linked with intestinal barrier dysfunction, intestinal dysbiosis and a loss in bacterial diversity during diet-induced obesity (33–40). Interestingly, mice lacking gut immune cells ($\beta7^{-/-}$, Integrin alpha E knockout—*Itgae*$^{-/-}$ or C-C chemokine receptor type 9 knockout—*Ccr9*$^{-/-}$ mice) fed a diet high in fat, sodium and sugar showed improved glucose tolerance, less weight gain, and reduced white adipose tissue inflammation (reduced numbers of Ly-6C^{high} monocytes, neutrophils, and macrophages) (32, 35).

In contrast to wild-type control mice, $\beta7^{-/-}$ mice were also protected from hypertension (a key feature of the metabolic syndrome), indicating that gut immune cells aggravate adverse cardiometabolic consequences of high-fat diet (32). Mechanistically $\beta7^{-/-}$ mice on chow diet showed improved glucose tolerance by increased insulin secretion, expended more energy, produced more heat and had lower levels of fasting triglycerides without differences in hepatic secretion of triglycerides or fat absorption (32). These results suggest metabolic rate to be increased in the absence of gut immune cells. While diet-induced obesity is linked with intestinal barrier dysfunction and a loss in bacterial diversity (33–40), the beneficial metabolic effects in $\beta7^{-/-}$ mice were resistant to antibiotic treatment and not associated with gut permeability abnormalities (32). These findings support a direct effect of gut immune cells on metabolic and cardiovascular disease independent of gut barrier integrity or microbiome changes (Figure 1).

GLP-1: Mechanistic link connecting gut immune cells and cardiovascular disease?

In response to food intake the gut incretin hormone GLP-1 (glucagon-like peptide-1) is secreted by intestinal L cells leading to insulin secretion and glucose control (41). Pharmacological activation of the GLP-1 receptor is currently used for the treatment of patients with type 2 diabetes (42). Beyond their glucoregulatory function GLP-1 receptor agonists exert pleiotropic protective cardiovascular effects in different organ systems (43) and improved cardiovascular outcomes in diabetic patients at high cardiovascular risk (44–49). Besides food intake GLP-1 secretion is induced by acute inflammatory stimuli including LPS, IL-6, and IL1b. This was found to be mediated by IL-6 signaling in L-cells (50, 51). Consistently mice and patients with acute [sepsis (51) or myocardial infarction (52)] or chronic inflammatory cardiovascular diseases [coronary artery disease (53) or heart failure (54)] show increased circulating GLP-1 levels independent of food intake. Elevated GLP-1 levels were independently associated with mortality in patients with sepsis or myocardial infarction (55, 56). Mechanistic experimental studies suggest upregulation of GLP-1 secretion to be an endogenous protective counter-regulatory response circuit (52). Interestingly gut immune cell deficient mice ($\beta7^{-/-}$) which are protected from atherosclerosis, hypertension, obesity and diabetes have higher plasma GLP-1 levels (32, 35). Since gut immune cells (in particular intraepithelial αβ and γδ T lymphocytes) express high levels of the GLP-1 receptor (32, 57) it was elucidated whether these cells directly regulate GLP-1 secretion from intestinal L cells to modulate cardiometabolic disease. For this purpose the authors of a previous study generated mixed bone marrow chimeras (bmGlp1r$^{-/-}$β7$^{-/-}$) with selective GLP-1 receptor deficiency in β7^{+} gut immune cells and unaffected GLP-1 receptor expression on all other cell types. Mice with selective gut immune cell GLP-1 receptor deficiency showed higher plasma levels of GLP-1, were more glucose tolerant, presented with less hypercholesterolemia and developed smaller atherosclerotic plaques with fewer aortic leukocytes (32).

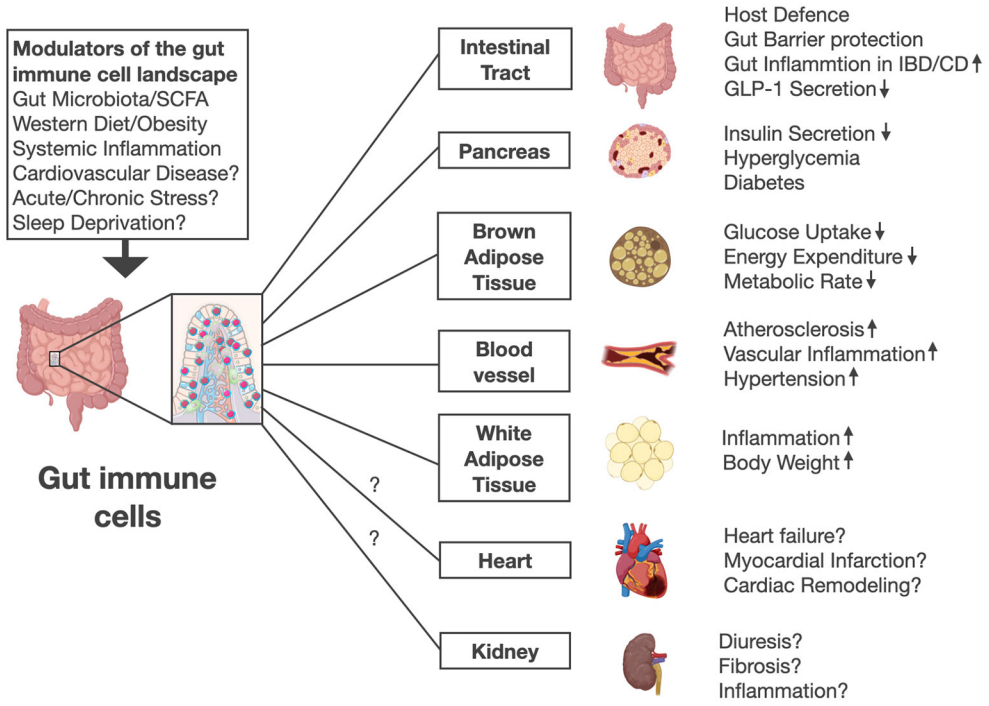

FIGURE 1
Gut immune cells are modulated by gut microbiota, short chain fatty acids (SCFA), diet and systemic inflammation in mice. Future work is needed to investigate whether other stimuli and lifestyle factors including stress or sleep disorders affect gut immune cell numbers and activation. Intestinal immune cells mediate pleiotropic effects in various organs beyond their role in host defense and gut barrier protection. Mice lacking gut immune cells show increased insulin secretion, improved glucose control, higher energy expenditure, lower body weight, less high fat diet-induced adipose tissue inflammation, and increased circulating GLP-1 levels. Thus, gut immune cell deficient mice were protected against diabetes, obesity, hypertension and atherosclerosis. Future work needs to address whether gut immune cells might affect other diseases including heart failure, myocardial infarction, and kidney function. The illustration was modified from Biorender (https://Biorender.com).

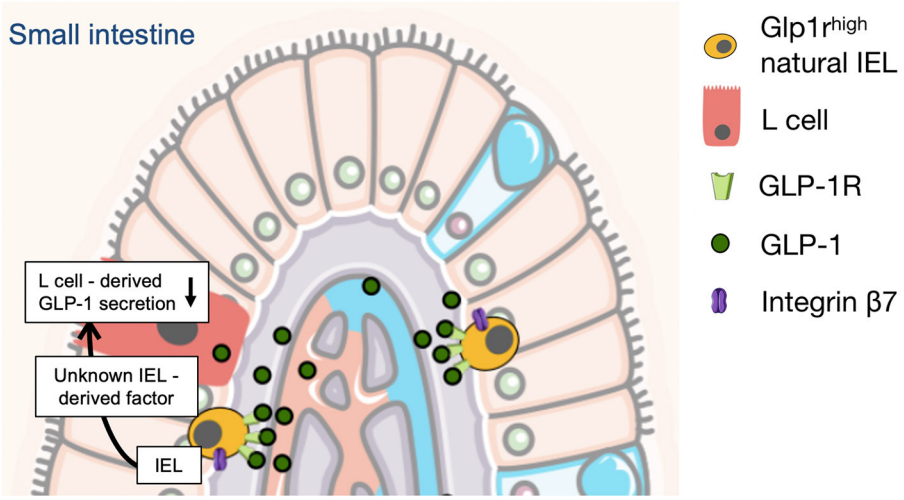

FIGURE 2
L-cell derived GLP-1 secretion is controlled and limited by gut intraepithelial (IEL) lymphocytes in mice. This is mediated through a negative feedback mechanism in which gut IEL GLP-1 receptor activation directly inhibits L cell derived GLP-1 secretion through a still unknown IEL-derived mediator (32). The illustration was modified from Servier Medical Art (https://smart.servier.com/).

Further mechanistic experiments identified a negative feedback mechanism in which gut immune cell GLP-1 receptor activation was found to directly inhibit L cell derived GLP-1 secretion through a still unknown gut immune cell derived mediator (32). These findings suggest that GLP-1 receptor deficiency in gut immune cells limits development of cardiovascular and cardiometabolic disease through upregulation of systemic GLP-1 availability (Figure 2). In other words GLP-1 might be one of the underlying mechanistic links connecting gut immune cells and cardiovascular disease.

Conclusion and future perspectives

Cardiometabolic diseases are characterized and driven by systemic inflammation. Recent data indicate that in cardiovascular and metabolic diseases inflammation is not limited to the vessel wall, bone marrow, spleen or adipose tissue. First experimental evidence suggests an important relevance of immune cells in the intestinal tract for the development of metabolic and cardiovascular disease in mice. Mice specifically lacking gut immune cells are protected against obesity, diabetes, hypertension and atherosclerosis. Importantly anti-Integrin-β7 antibody mediated inhibition of leukocyte homing into the gut showed similar protective metabolic and cardiovascular effects as genetic models. Based on these observations targeting gut immune cells and in particular Integrin-β7 might open novel therapeutic approaches for the treatment of cardiometabolic and cardiovascular disease. Therapeutic targeting of gut immune cell trafficking is well-established and safe in patients suffering from inflammatory bowel diseases (IBDs), including Crohn's disease (CD), and ulcerative colitis (UC). Blocking intestinal immune cell homing by Vedolizumab, an anti-α4β7 antibody, has become an important pillar of IBD therapy (58–61). Therefore, it is tempting to speculate that antibody-mediated blockage of intestinal leukocyte homing might be a promising drug target for patients with cardiovascular disease. Especially the recent identification of a previously unrecognized crosstalk between gut immune cells and the incretin system (increased GLP-1 secretion in the absence of gut immune cells) might open new therapeutic avenues for vulnerable high-risk patients with diabetes and cardiovascular disease. However, several key questions remain: Can we translate the experimental observations on gut immune cells and cardiovascular and cardiometabolic effects to patients? Is intestinal immune cell trafficking and activation relevant for human cardiovascular disease? How exactly do gut leukocytes accelerate atherosclerosis formation, hypertension, and diabetes? Is GLP-1 the only mechanistic link between gut immune cells and cardiovascular actions or do other incretin hormones such as GIP (glucose-dependent insulinotropic peptide) or GLP-2 or any other as of yet unknown factors play a role? Does gut immune cell trafficking or activation affect plaque inflammation by increasing circulating monocytes and neutrophils (due to upregulated hematopoiesis)? Do gut immune cells control local macrophage proliferation in atherosclerotic lesions? Beyond their role in atherogenesis what is the effect of blocking intestinal leukocyte homing on other manifestations of cardiovascular disease like cardiac remodeling after myocardial infarction or heart failure? What is the effect of cardiovascular events (i.e., myocardial infarction, stroke, or decompensated heart failure) on intestinal immune cell homing and activation? Are gut immune cell numbers and or activation status associated with cardiovascular risk in patients?

Answering these questions may be essential to better understand how intestinal leukocytes control development of cardiometabolic and cardiovascular disease and to evaluate whether the above mentioned promising experimental observations could the translated into clinical practice. Targeting gut immune cells might be a future therapeutical approach to prevent and suppress the devastating consequences of cardiovascular disease.

Author contributions

FK and NG drafted the manuscript, which was critically revised by all authors. All authors provided intellectual input and gave final approval.

Funding

This study was supported by grants from the European Research Area Network on Cardiovascular Diseases (ERA-CVD and BMBF, Grant No. JTC-2019, MyPenPath-01KL2004) and from The European Foundation for the Study of Diabetes (EFSD)/Novo Nordisk Foundation (NNF2OSA0066111) to FK and grants from the Deutsche Forschungsgemeinschaft (SFB TRR 219 M-03) and Interreg V-A grant EURlipids to ML. NM was supported by grants from the Deutsche Forschungsgemeinschaft (German Research Foundation; TRR 219; Project-ID 322900939 [M03, M05]) as well as the CORONA Stiftung, Germany. EV was supported by a grant from the Interdisciplinary Center for Clinical Research within the faculty of Medicine at the RWTH Aachen University and NWO-ZonMw Veni (91619053).

Conflict of interest

Author FK served as a consultant to Bayer and Novo Nordisk and served as a speaker for Novo Nordisk. Author NM has received support for clinical trial leadership from Boehringer Ingelheim, Novo Nordisk, served as a consultant to Bayer, Boehringer Ingelheim, Merck, Novo Nordisk, AstraZeneca, BMS, received grant support from Boehringer Ingelheim, Merck, Novo Nordisk, and served as a speaker for Bayer, Boehringer Ingelheim, Merck, Novo Nordisk, Lilly, BMS, and

AstraZeneca. Author ML received grants and personal fees from Boehringer Ingelheim, MSD, Novo Nordisk and personal fees from Amgen, Sanofi, Astra Zeneca, Bayer, Lilly, Daiichi Sankyo, Novarits, Amarin.

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frontiers | Frontiers in Cardiovascular Medicine

TYPE Review
PUBLISHED 21 September 2022
DOI 10.3389/fcvm.2022.929078

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Atherosclerosis and Vascular Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

RECEIVED 26 April 2022
ACCEPTED 17 August 2022
PUBLISHED 21 September 2022

CITATION
Wang Q, Wang Y and Xu D (2022)
Research progress on Th17 and T
regulatory cells and their cytokines in
regulating atherosclerosis.
Front. Cardiovasc. Med. 9:929078.
doi: 10.3389/fcvm.2022.929078

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Research progress on Th17 and T regulatory cells and their cytokines in regulating atherosclerosis

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Background: Coronary heart disease due to atherosclerosis is the leading cause of death worldwide. Atherosclerosis is considered a chronic inflammatory state in the arterial wall that promotes disease progression and outcome, and immune cells play an important role in the inflammatory process.

Purpose: We review the mechanisms of $CD4^+$ T subsets, i.e., helper T17 (Th17) cells and regulatory T cells (Tregs), in regulating atherosclerosis, focusing on the role of interleukin (IL)-17, IL-10, and other cytokines in this disease and the factors influencing the effects of these cytokines.

Results: IL-17 secreted by Th17 cells can promote atherosclerosis, but few studies have reported that IL-17 can also stabilize atherosclerotic plaques. Tregs play a protective role in atherosclerosis, and Th17/Treg imbalance also plays an important role in atherosclerosis.

Conclusion: The immune response is important in regulating atherosclerosis, and studying the mechanism of action of each immune cell on atherosclerosis presents directions for the treatment of atherosclerosis. Nevertheless, the current studies are insufficient for elucidating the mechanism of action, and further in-depth studies are needed to provide a theoretical basis for clinical drug development.

KEYWORDS

$CD4^+$ T cells, IL-17, Tregs, Th17/Treg balance, atherosclerosis

Cardiovascular disease due to atherosclerosis has become the leading cause of death worldwide (1). The formation of atherosclerosis is a complex process. low-density lipoprotein (LDL) facilitates lipid accumulation in the arterial wall, endothelial dysfunction allows lipoprotein particles penetrating into the subendothelial layer of the arterial wall, where plaque formation occurs, and local inflammatory response and misbalanced functioning of tissue macrophages contribute to the plaque growth and the formation of lipid core (2–4). Although lipid deposition has previously been considered a marker of atherosclerosis, an increasing number of studies have reported that immune inflammation also plays a crucial role in atherosclerosis occurrence, development, and clinical manifestations (5). The immune inflammatory response is caused by the activation of innate and adaptive immunity (6, 7) and T cell infiltration

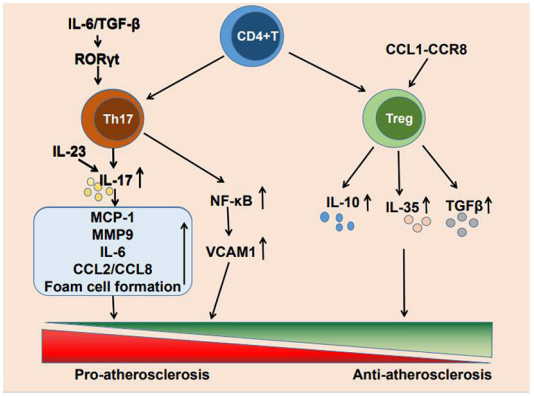

FIGURE 1
Mechanism of Th17 cells and Treg cells regulating atherosclerosis.

is present at all stages of atherosclerosis. $CD4^+$ T cells are activated by antigens and differentiate into helper T1 (Th1), Th2, Th17, and regulatory T cells (Tregs) (8). The main cytokine produced by Th17 cells is IL-17, It also produces cytokines such as IL-17A, IL-17F, IL-21 and IL-22 (9). And Tregs mainly secrete IL-10 and other cytokines such as IL-35 and TGF-β (10). These $CD4^+$ T cell subpopulations can secrete a variety of cytokines to promote or suppress the development of inflammation, thereby regulating atherosclerosis progression. Each of these numerous $CD4^+$ T cell subpopulations is involved in the development of immune inflammation. There is growing evidence that Th17 cells and Tregs are highly involved in atherogenesis and the progression of atherosclerosis (11) (Figure 1).

Here, we reviewed the mechanisms of Th17 cell- and Treg-mediated atherosclerosis in existing $CD4^+$ T cell subpopulations, thereby providing targets for atherosclerosis treatment and a feasible direction for research on the inhibition of atherosclerosis progression through immunomodulation.

Th17 cells

Th17 cells are characterized by the expression of interleukin (IL)-17A, IL-17F, IL-21, and IL-22. IL-17 is mainly secreted by Th17 cells and is considered their signature cytokine. Nevertheless, IL-17 can also be produced by various types of hematopoietic cells in addition to Th17 cells and by $CD8^+$ T cells, invariant natural killer T cells (iNKT), and other immune cells (12). As a major cytokine that is secreted by Th17 cells, IL-17 plays an important role in regulating the formation of atherosclerosis. The interaction between its pathway and cytokines is complex. Further exploration of its mechanism would provide a new direction for atherosclerosis treatment.

IL-17 promotes atherosclerosis development

There have been many studies on the role of IL-17 in atherosclerosis development and progression. Wang et al. (13) and Gao et al. (14) reported that ApoE-/- mice exhibited Th17 cells in atherosclerotic plaques and higher percentages of Th17 cells than wild-type (WT) mice, suggesting that Th17 cells are associated with atherosclerosis. Eid et al. (15) detected IL-17 in the serum of patients with coronary atherosclerosis and reported large Large amount of IL-17 production by Th17 cells infiltrating the arteries, suggesting an association of IL-17 with atherosclerosis. Gao et al. (14) injected IL-17 (2 mg/mouse) weekly into $ApoE^{-/-}$ mice. After 5 weeks, the IL-17 injection greatly exacerbated aortic root plaque formation, confirming that IL-17A exacerbated atherosclerosis development *in vivo*. Erbel et al. (16) reported that when co-cultured with atheromatous plaques, IL-17A induced upregulation of the mRNA of proinflammatory mediators, including the chemokine monocyte chemoattractant protein-1 (MCP-1) and the plaque-disrupting matrix metalloprotease 9 (MMP9). It also induced the expression of a proinflammatory transcriptome in macrophages, leading to the upregulation of proinflammatory mediators, including cytokines such as IL-1A and IL-6 or chemokines such as C-C motif chemokine ligand (CCL) 2 and CCL8, thereby promoting atherosclerotic plaque formation. Similarly, Shiotsugu et al. (17) stimulated human umbilical vein endothelial cells with IL-17A and reported that IL-17A induced adhesion molecule expression and promoted monocyte adhesion to vascular endothelial cells. Moreover, IL-17A stimulated oxidized low-density lipoprotein (oxLDL)-induced foam cell formation by upregulating LOX-1 in activated macrophages, thereby participating in atherosclerosis pathogenesis. In addition, IL-17 induced the release of chemokines such as CXCL1, CXCL2, and CXCL8 from endothelial cells and vascular smooth muscle cells (SMCs) (18), and the increase of these chemokines attracted other inflammatory cells to the inflammation site, exacerbating atheromatous plaque inflammation. Similar to a recent study, Zhang et al. (19) reported recently that IL-17A could bind to IL-17RA on the endothelial cell membrane and promote endothelial cell senescence, which is one of the main causes of structural changes and blood vessel dysfunction and the basis of atherosclerosis (20), by activating the nuclear factor kappa B (NF-κB)–p53–Rb signaling pathway.

In contrast, Erbel et al. (21) reported in 2009 that IL-17 antibody neutralization of IL-17A reduced the expression of vascular cell adhesion molecules (VCAM-1), immune cell infiltration, and the secretion of proinflammatory cytokines (IL-6 and tumor necrosis factor [TNF]-α) and chemokines (CCL5), significantly reducing the area of atherosclerotic lesions in $ApoE^{-/-}$ mice (IL-17A monoclonal antibody [mAb]-treated group: 1586 $\pm$ 723 μm^2; control group: 2716 $\pm$ 1187 μm^2),

maximum stenosis, and lesion vulnerability, suggesting a proatherosclerotic effect of IL-17. Similarly, Erbel et al. (16) reported that inhibiting IL-17A in ApoE$^{-/-}$ mice slowed the progression of advanced atherosclerotic lesions by reducing the necrotic core of atherosclerosis and that IL-17A mAb treatment slowed the progression of advanced atherosclerotic lesions by increasing fibrous cap thickness, collagen content, and connective tissue growth factor (CTGF) mRNA expression to exert a plaque-stabilizing effect. Recently, Wang et al. (13) injected anti-IL-17 mAb intraperitoneally into ApoE$^{-/-}$ mice for 4 weeks and observed reduced atherosclerotic plaque formation and decreased lesion size, collagen content, and necrotic core in the IL-17-neutralized ApoE$^{-/-}$ mice. These studies suggest that IL-17 acts as a proinflammatory cytokine to promote atherosclerosis development.

IL-17 mediates atherosclerosis-related mechanisms

IL-17 induces VCAM-1 expression in vascular SMCs to promote atherosclerosis

Vascular SMCs play a central role in atherosclerosis. VCAM-1 is expressed in endothelial cells and the intimal SMCs of atherosclerotic lesions and is required for leukocyte recruitment and vascular inflammation (22). VCAM-1$^{-/-}$ mice did not develop atherosclerosis, suggesting that VCAM-1 is required for atherogenesis (23). IL-17 mediates VCAM-1 expression in SMCs through multiple signaling pathways, thereby promoting atherosclerosis development. Zhang et al. (24) demonstrated that IL-17 was dependent on NF-κB to induce VCAM-1 expression in SMCs and that knockdown of p65 with small interfering RNA reversed IL-17-induced VCAM-1 expression, suggesting that NF-κB is indispensable in such expression in SMCs. TGF-β activated kinase 1 (TAK1) is considered an important IL-17-mediated signaling molecule (25), but Zhang et al. reported that knockdown of TAK1 did not reduce IL-17-induced VCAM-1 expression in SMCs, nor did it decrease NF-κB activation, suggesting that IL-17-activated NF-κB is independent of the TAK1 pathway. Similarly, they reported that IL-17-induced NF-κB activation was also not dependent on the Akt1 pathway. Furthermore, they noted that IL-17 promoted VCAM-1 expression in SMCs partly through the ERK1/2–MAPK signaling pathway. Therefore, further studies on the above mechanisms and therefore targeting the IL-17 signaling pathway will provide a new means of intervention for treating vascular diseases.

Multiple cytokines regulate IL-17 expression

IL-17A expression is regulated by a variety of factors, including cytokines and growth factors. These factors further regulate atherogenesis by regulating IL-17 expression through their downstream signaling pathways or in concert with other factors.

IL-6

The IL-6 receptor is a heterodimer composed of IL-6R and gp130. IL-6 binding to its receptor activates the downstream signal transducer and activator of transcription (STAT) 1 and STAT3 (26). IL-6 exerts direct proatherogenic effects (27, 28) but also exerts proinflammatory effects by inducing Th17 cell differentiation. Retinoid-related orphan receptor γ (RORγ) is a member of the nuclear receptor (NR) superfamily in the human genome, the thymus-specific RORγt plays vital roles in promoting T cell differentiation into the Th17 subtype and regulating IL-17A gene transcription in Th17 cells (29). It was found that IL-6 could acts synergistically with transforming growth factor beta (TGF-β) to induce RORγt expression, thereby promoting Th17 cell differentiation (30). Induction of RORγt under Th17 cell-polarizing conditions is dependent upon STAT3, which perceives and transduces signals from IL-6, IL-23 and other cytokines (29) and IL-6 can upregulate IL-21 expression through the STAT3 pathway, which in turn increases IL-23 receptor and RORγt expression, and the combination of RORγt and STAT3 further promotes IL-17 expression (31), which also inhibits forkhead transcription factor p3 (FOXP3) expression (32), thereby promoting the development of atherosclerosis. Blocking IL-6R led to a significant decrease in Th17 cells and an increase in Tregs (33), further demonstrating the important role of IL-6 in the induction of Th17 cell differentiation.

IL-23

IL-23 is a member of the IL-12 cytokine family and is a heterodimer composed of the p19 (specific to IL-23) and p40 (shared with IL-12) subunits. IL-23 is thought to be a major driver of the pathogenic human Th17 cellular response (34). The IL-23–IL-17 cytokine axis drives a variety of chronic inflammatory diseases, including arthritis, psoriasis, and inflammatory bowel disease (35). Abbas et al. (36) reported that IL-23 and IL-23R expression was increased in carotid atherosclerotic plaques and that IL-23 induced a significant increase in the release of IL-17 from the cells of patients with carotid atherosclerosis and accelerated their inflammatory state, suggesting that IL-23 may promote the development of atherosclerosis through the IL-23–IL-17 axis. The Th22 subset is a novel CD4+T-cell subset and the most important source of IL-22 in mice (37). In the middle and late stages of inflammation, IL-22 is pre-dominantly released by Th22 cells to exert immune effects (38). In addition, the study found that IL-23 can induce IL-22 production by CD4^{+} T cells, which may be a downstream signal for IL-23 to act (39). Shi et al. (40) reported that in ApoE$^{-/-}$ mice, plaque size was observed after treatment with recombinant (r)IL-22 and IL-22 mAb, which revealed that Th22 cell-derived

IL-22 activated IL-6/STAT3, increased dendritic cell-induced Th17-cell proliferation, stimulated SMC dedifferentiation to a synthetic phenotype, and ultimately promoted the development of atherosclerosis. This further suggests the importance of the IL-23–IL-17 axis in pro-atherosclerosis.

In contrast, it has been suggested that the IL-23–IL-17 axis may play a neutral role in atherosclerosis. The onset of inflammation in psoriasis and atherosclerosis shares the same cytokine pathway (41). The dysfunction in psoriasis includes systemic inflammation involving cytokines, such as IL-17, TNF-α and IFN-γ (42), as well as inflammatory transcripts such as CXCL10, IL-1β and VCAM-1.Psoriasis increases vascular stiffness and atherosclerosis tthrough the IL-17 pathway (43). Animal studies have demonstrated that the transfer of *Ldlr*$^{-/-}$ IL-23R$^{-/-}$ CD4^{+} T cells to *Ldlr*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice resulted in reduced the Th17 levels and IL-17^{+}IFN-γ^{+} Th17 cells, suggesting that IL-23 mediates IL-17 production. However, studies showed IL-23-dependent pathogenic Th17 effector cell differentiation exerted no significant effect on atherosclerosis in *Ldlr*$^{-/-}$ mice (44). Furthermore, a 5-year follow-up study of patients with severe psoriasis revealed the occurrence of cardiovascular events after IL-12–IL-23 blockade treatment and that IL-12–IL-23 blockade exerted no effect on cardiovascular events (45). Similarly, Marovt et al. (46) reported the significant efficacy of biologics targeting the IL-23–IL-17 axis in the treatment of psoriasis. However, the effect on vascular structure and function was neutral, suggesting that the IL-23–IL-17 axis may exert no effect on atherosclerosis development.

The above differences in the effects of the IL-23–IL-17 axis on atherosclerosis have not been elucidated and may lie in the models used in studies on the IL-23–IL-17 axis, the different periods in which atherosclerosis occurs, and the impact of other comorbid diseases in patients with atherosclerosis on the studies; or even the different types of IL-23 knockdown in the animal models. In addition, cytokines act on inflammation via complex pathways, and despite IL-23 knockout, whether IL-17 plays a role in atherosclerosis through other unknown pathways has not been ruled out. In conclusion, the existence of these differences suggests that there remain numerous unknown parts of the pathways of cytokine action, and further research to identify these pathways is expected to provide targets for atherosclerosis treatment.

Other cytokines

An isoform of the proinflammatory cytokine IL-1 (47), IL-1β is involved in atherogenesis. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) demonstrated significant reductions in recurrent cardiovascular events after selective neutralization of IL-1β with a monoclonal antibody (canakinumab) in patients who were at risk of residual inflammation (5). Inhibition of IL-1β reduced the sizes of formed atherosclerotic plaques and increased plasma IL-10 levels (48), thereby slowing the onset of atherosclerosis. In addition, Engelbertsen et al. (49) reported that knockdown of IL-1R1, the IL-1β receptor, reduced IL-17A production by stimulated splenocytes and plasma IL-17A levels and attenuated other proinflammatory cytokines secreted by Th17 cells, suggesting that IL-1R1 signaling affects atherosclerosis by promoting Th17 immune response development. In addition, receptor interacting protein 2 (RIP2) and tripartite motif containing (Trim) 21 are associated with Th17 cell differentiation. In *Rip2*$^{-/-}$ mice, T cells preferentially polarized toward pathogenic Th17 cells under pathogenic conditions, resulting in increased IL-17A production, which directly led to the significant enhancement in atherosclerosis (50). MMP expression was reduced in *Trim21*-deficient mice and human plaques (51), and *Trim21* deficiency in hematopoietic cell compartments led to plaque enlargement through increased T cell-mediated IL-17 responses, along with significant increases in plaque collagen content and thicker fibrous cap (52). These studies confirm the proinflammatory effects of multiple factors in inducing Th17 cell differentiation and increasing IL-17 production, but no studies have elucidated their mechanisms of action due to their intricacy. Therefore, further investigation of their mechanisms is of great importance for preventing and treating atherosclerosis.

IL-17 plays a protective role in atherosclerosis

Although a large number of studies have suggested the proatherogenic effects of IL-17, others have suggested that IL-17 inhibits the development of atherosclerosis. Suppressor of cytokine signaling (SOCS) protein is considered a key physiological regulator of innate and adaptive immunity and SOCS3 plays a protective role in atherosclerosis by inhibiting the STAT3 signaling pathway and suppressing proinflammatory responses (53). Taleb et al. (54) reported that neutralizing IL-17 in T cell-specific SOCS3-deficient mice completely eliminated the atherosclerotic protective effect of T cell-specific SOCS3 deletion and led to significant atheromatous plaque lesion formation. Furthermore, the administration of rIL-17 to *Ldlr*$^{-/-}$ mice significantly reduced the development of atherosclerotic lesions and reduced IL-1-induced endothelial VCAM-1 expression *in vitro*, suggesting that IL-17 inhibits the development of atherosclerosis. Subsequently, Danzaki et al. (55) found in ApoE$^{-/-}$, IL-17A$^{-/-}$ mice that IL-17A deficiency led to atherosclerotic lesions and the formation of unstable atherosclerotic plaques, whereas atherosclerotic plaque formation was reduced in ApoE$^{-/-}$ mice and ApoE$^{-/-}$, IL-17A$^{-/-}$ mice when treated with IL-17A. The authors also suggested that the lack of atheroprotective effects of IL-17A may have been due to increased interferon (IFN)-γ production and decreased IL-5 production in the splenocytes. Although such

studies on the anti-inflammatory effects of IL-17 are scarce, its role cannot be ignored, and the elucidation of its complex targets of action yields directions for the immune response to modulate atherosclerosis.

As a cytokine, IL-17 involves numerous pathways and has multiple interactions or influences. Therefore, studies on specific models or different stages of atherosclerosis and different IL-17 concentrations are expected to clarify its mechanism of action in atherosclerosis and provide a feasible and sustainable target for treating such diseases.

Tregs play a protective role in atherosclerosis

A growing body of evidence emphasizes the role of T cells as important drivers and regulators of atherogenesis (56). Tregs are a $CD4^+$ T cell subpopulation that suppress exacerbated inflammatory responses to enhance immune tolerance and homeostasis (57). Tregs can be subdivided into two major categories based on their developmental origin: thymic Tregs and peripherally induced Tregs. T cell differentiation to Tregs is driven by FOXP3 (58), which is essential for Tregs to maintain their immunosuppressive functions. Using an $ApoE^{-/-}$ mouse model, AitOufella et al. (59) demonstrated that $CD4^+CD25^+$ Treg deficiency was associated with a significant increase in atherosclerotic lesion size, demonstrating for the first time that endogenous $CD4^+CD25^+$ Tregs play a protective role in atherogenesis. In contrast, Gao et al. (60) performed selective transfer of $CD4^+FOXP3(GFP)^+$ Tregs into the aorta of $ApoE^{-/-}$ mice and observed that the Treg transmigration reduced the aortic facial atheroma plaque area by 54.3% ($P < 0.001$) and the aortic root plaque area by 34.0%, ($P < 0.001$) and significantly reduced macrophage infiltration (65.2%, $P < 0.001$), suggesting the important role of Tregs in atherosclerosis development.

Tregs maintain immune homeostasis and tolerance through the release of immunosuppressive cytokines (e.g., IL-10 and TGFβ), cell contact-dependent mechanisms, and the promotion of tissue repair (61). Further studies have revealed that Tregs could directly exert their anti-inflammatory effects on monocytes and macrophages. Coculturing monocytes and macrophages with Tregs significantly inhibited the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β, while the production of the anti-inflammatory cytokines IL-1RA and IL-10 was enhanced (62), suggesting that Tregs can inhibit proinflammatory cytokine production by acting on monocytes and macrophages. In addition, co-culture with Tregs rendered monocytes less capable of increasing the T cells that secreted harmful IL-17 by decreasing monocyte CD86 expression (63). When Tregs were transferred to $ApoE^{-/-}$ mice, there was a significant decrease in the relative level of macrophage and lipid content in plaques and significant increases in SMCs and collagen, thereby reducing the risk of plaque rupture (64). The mechanism by which Tregs affect various cells associated with atherogenesis is not well defined and exhaustive, and there are still many gaps to be filled.

Antiatherosclerotic effects of IL-10

IL-10 is an immunomodulatory cytokine mainly produced by Treg, as well as B cells, macrophages and other immune cells (65). The main cytokine secreted by Tregs, IL-10 plays an important role in regulating atherosclerosis.

IL-10 significantly affects the inflammatory response in atherosclerosis. Mallat et al. (66) studied $IL\text{-}10^{-/-}$ and $IL\text{-}10^{+/+}$ C57BL/6J mice and found that compared to the $IL\text{-}10^{+/+}$ mice, the $IL\text{-}10^{-/-}$ mice demonstrated a significant 3-fold increase in aortic sinus atherosclerotic lesions and lesions expressing higher levels of IFN-γ, increased activated T cell infiltration, and decreased collagen levels, which increased atherosclerosis while decreasing atheromatous plaque stability, suggesting an important atheroprotective role of IL-10. Subsequently, a study of $ApoE^{-/-}$ mice reported a significant increase in the proinflammatory Th1-cell response and lesion size in IL-10 deficiency, along with an increase in MMP and tissue factor activity in lesions (67), suggesting that IL-10 reduced atherogenesis. Similarly, Fourman et al. (68) measured IL-10 in serum samples with enzyme-linked immunosorbent assay and assessed coronary atherosclerosis using computed tomography angiography in an observational study of human immunodeficiency virus (HIV) patients and uninfected controls, and found that reduced IL-10 was associated with increased coronary plaque incidence and increased carotid intima-media thickness, suggesting a protective effect of IL-10 against atherosclerosis in HIV. Conversely, Liu et al. (69) used adeno-associated virus type 2 (AAV) vector transduction of IL-10 in $Ldlr^{-/-}$ mice to upregulate IL-10 gene and protein expression and observed reduced subintimal lipid accumulation and lower CD68 and reactive oxygen species levels, suggesting that systemic AAV vector transduction of the IL-10 gene could suppress inflammation and oxidative stress, thereby inhibiting atherosclerosis. Recently, Kim et al. (70) successfully reversed proinflammatory cytokine production by immune cells in lesions and reduced atherosclerotic plaque progression by targeting IL-10 delivery via nanocarriers to atheromatous plaque sites in $ApoE^{-/-}$ mice fed a high-cholesterol diet. The above studies bifurcated the protective role of IL-10 in atherosclerosis and explored how IL-10 could be more effectively transported to the target site to exert its anti-inflammatory effects. Although the study was successful, the experimental subjects were animals, and further research is needed to translate the results to atherosclerosis treatment in humans.

IL-35 regulates tregs to play a protective role in atherosclerosis

IL-35 is a cytokine secreted by Tregs and consists of the IL-12p35 and EBV-inducible gene 3 (EBI3) subunits. IL-35 is a reactive cytokine (71) induced by proinflammatory stimuli and is present in the early atherosclerosis development. IL-35 inhibits cardiovascular inflammation effectively through its important role in suppressing endothelial cell activation (72). Tao et al. (73) reported that exogenous human and mouse rIL-35 treatment reduced atherosclerosis in ApoE$^{-/-}$ mice, suggesting that IL-35 has atheroprotective effects. In addition, IL-35 is also involved in regulating Tregs. In an IL-35 treatment model, IL-35 increased splenic Tregs and inhibited atherosclerosis in ApoE$^{-/-}$ mice (73). Moreover, IL-35 promoted C-C motif chemokine receptor 5 (CCR5) expression in the Tregs of ApoE$^{-/-}$ mice (74), increased CCR5 enhance the immunosuppressive function of splenic Tregs in three ways, which including CCR5-mediated Treg migration (possibly from the spleen, which is a large Treg reservoir, to the aorta), inhibit ATK-mTOR signaling in the Tregs, and promote the immunosuppressive function of TIGIT and PD-1 in the Tregs (75). These studies suggested the importance of IL-35 in regulating Tregs to exert atheroprotective effects.

Other cytokines that regulate Treg function

The CCR8 ligand CCL1 is a cytokine expressed by activated T lymphocytes (76). In the cardiovascular system, CCL1 is expressed in endothelial cells, macrophages, and the extracellular regions of human atherosclerotic plaques (77). CCL1 stimulates vascular SMC migration (78) and activates endothelial cells in response to arterial wall injury (79). CCR8 is mainly expressed in Tregs and plays a key role in immunosuppression by activating Tregs through interactions with its ligand CCL1 (80). CCL1 knockdown exacerbated atherosclerosis in fat-fed ApoE-KO mice, and high-fat-fed ApoE/CCL1-DKO (double knockout) mice demonstrated a reduced percentage of Tregs in the aorta and spleen compared to ApoE-KO control mice, and significantly reduced IL-10 in the splenocytes and plasma, while the proliferative activity of CD4^{+} T cells isolated from the spleen and lymph nodes was not affected. Furthermore, treatment of adipose-fed Ldlr-KO mice with CCR8-blocking antibodies reduced Treg levels in the aorta and enhanced aortic atherosclerosis (81). These studies suggested that CCL1–CCR8 axis inactivation decreases Treg recruitment, leading to increased atherosclerosis. In addition, Tregs can also produce TGF-β, which inhibits effector T-cell differentiation; suppresses T- and B-cell proliferation; and inhibits macrophage, dendritic cell, and NK-cell activity to exert a stabilizing effect on atherosclerosis (10).

Studies showed that prolonged exposure to inflammatory cues can promote Treg functional plasticity or affect Tregs stability (82), leading to instability when Tregs lose Foxp3 expression and the loss impairs the suppressive capacity (functionality) of Treg cells. The animal study by Wolf et al. (83). Found that bulk FoxP3+ Tregs from 8-week-old Apoe$^{-/-}$ transferred into 24-week-old Apoe$^{-/-}$ mice mostly lost FoxP3 and started expressing RORγT and T-bet after 6 weeks. And in adoptive transfer experiments, converting ApoB+ Tregs failed to protect from atherosclerosis, suggesting that the instability of Treg cells deprives them of the ability to suppress inflammation. In contrast, the plasticity of Treg cells is beneficial, IFNγ+T-bet+CXCR3+Th1-like Tregs, IL4+IL5+IL13+GATA3+Th2-like Tregs and IL17A+RORγt+Th17-like Tregs have been identified currently (84–86). Studies found that in response to IFNγ or IL-27 Tregs acquire Th1 characteristics by expressing T-bet and CXCR3, preferentially accumulate in Th1 inflammatory niches, and render Th1 cells more susceptible to suppression (87), suggesting that the plasticity of Treg cells enhances their inhibitory ability. Although the mechanism remains unclear, the stability and plasticity of it provide a new idea to regulate atherosclerosis by intervening Treg.

Anti-inflammatory cells that regulate atherosclerosis, Tregs secrete a variety of cytokines that play important roles in all stages of atherosclerosis. Treg pathways and mechanisms of action have been increasingly described, further exploration based on existing studies presents the possibility of immunotherapies for atherosclerosis.

The role of the Th17/Treg balance in atherosclerosis

Tregs expressing FOXP3 exert anti-inflammatory effects through contact-dependent inhibition or the release of anti-inflammatory cytokines (IL-10 and TGF-β) (88). Proinflammatory Th17 cells expressing RORγt play an important role in the development of autoimmune and allergic reactions via the production of IL-17 and IL-6 (89). Th17-related cytokine (IL-17 and IL-6) and transcription factor (RORγt) levels were significantly higher and Treg numbers, Treg-related cytokine (TGF-β1) and transcription factor (Foxp3) levels were significantly lower in ApoE-/- mice than in age-matched C57BL/6J mice, suggesting that Th17/Treg balance is important (90). Furthermore, Zhu et al. (91) reported significantly higher expression of the Th17 transcription factor RORγt and IL-17 levels in the serum of patients with systemic lupus erythematosus (SLE) combined with atherosclerosis as compared with that in SLE-only patients and control groups, and significantly lower Treg numbers and the expression of the transcription factor Foxp3, further suggesting that Th17/Treg imbalance may play a role in atherosclerosis formation and development. Multiple signals, factors, epigenetic modifications,

metabolic pathways, and microbiota can regulate the balance between Tregs and Th17 cells (92).

Multiple factors regulate Th17/Treg homeostasis. CD69 is a C-type lectin, the earliest activation cell surface receptor on leukocytes, expressed by small subsets of T and B cells in peripheral lymphoid tissues (93). CD69+T lymphocytes are detected in cell infiltrates of various chronic inflammatory diseases (94). The lymphocyte activation antigen CD69 regulated Th17 cell and Treg differentiation, where CD69-deficient mice exhibited enhanced Th17 cell differentiation and defective Treg functions (95) and resulting in an increase in atherosclerotic plaque area (96). oxLDL was previously considered to be highly inflammatory and immunogenic (97), whereas recent studies have reported that its binding to CD69 on human T cells induces the upregulation of NR4A receptor expression, which downregulates the percentage of IL-17$^+$ cells produced by Th17 cell polarizing stimulation, promotes Treg differentiation, and exerts a protective effect against inflammatory responses (96). Huang et al. (98) reported that IL-12p35 deficiency decreased IL-35 levels and inhibited Treg production and function in ApoE$^{-/-}$ mice, while IL-12p35 deficiency was followed by increased Th17-cell levels and IL-17 levels, thereby exacerbating the Th17/Treg imbalance and promoting atherosclerosis. In contrast, treatment of ApoE$^{-/-}$ mice with human rIL-35 increased circulating and local Treg levels, inhibited the Th17 immune response, and reduced plaque size, suggesting that IL-35 alters the Th17/Treg balance by upregulating Treg immune responses, thereby attenuating atherosclerosis (99). In addition, studies have shown that indoleamine 2,3-dioxygenase (IDO) may play an important role in regulating Th17/Treg homeostasis. Tryptophan (Trp) is an essential amino acid for T-cell differentiation and its metabolite kynurenine (Kyn) inhibits Th17-cell differentiation and induces Treg proliferation. IDO is the rate-limiting enzyme for Trp degradation via the Kyn pathway, which controls Th17 and Tregs transformation by regulating Trp metabolism (100). Yang et al. (101) recently reported reduced IDO activity in P. gingivalis-infected atherosclerotic patients: the activity was negatively correlated with Th17-cell percentages and Th17/Treg ratios and positively correlated with Treg percentages; moreover, reduced IDO activity was accompanied by decreased serum IL-10 levels and increased IL-17 levels. This finding further suggests that IDO is important in regulating the Th17/Treg balance in atherosclerosis patients. Several studies have demonstrated that regulating the Th17/Treg balance by decreasing IL-17 and increasing Foxp3+ cells can reduce inflammatory cell infiltration, inhibit chronic inflammation, and stabilize atherosclerotic plaques (102, 103). In addition, remodeling the gut microbiota can prevent the development of atherosclerosis (104), and immune cells and cytokines can synergize with the microbiota and its metabolites to regulate atherosclerosis and plaque regression (105), moreover, altering the gut microbiota can re-establish the Th17/Treg balance (106). Although several studies have demonstrated that multiple cytokines affect the Th17/Treg balance, the specific mechanisms have not been elucidated, and further clarification of the mechanism would provide a new direction for delaying atherosclerosis by regulating Th17/Treg homeostasis.

Conclusions and prospects

Atherosclerosis is a major cause of mortality worldwide and its pathogenesis includes lipid infiltration, monocyte macrophage infiltration, injury responses, and the inflammatory responses. In recent years, the role of immune-mediated inflammatory responses in the progression of atherosclerosis has received increasing attention and different types of immune cells play anti-inflammatory or proinflammatory roles in atherosclerosis. Understanding the pathways by which these immune cells and their secreted cytokines act would yield therapeutic directions for preventing the onset and progression of atherosclerosis.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Ethics statement

Ethical review and approval was not required for this study in accordance with the local legislation and institutional requirements.

Author contributions

QW and YW initiated this article and wrote the manuscript. QW was the first author of this manuscript. DX revised our first draft and provided valuable comments. All authors contributed to the article and approved the submitted version.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 81672264 and 81871858).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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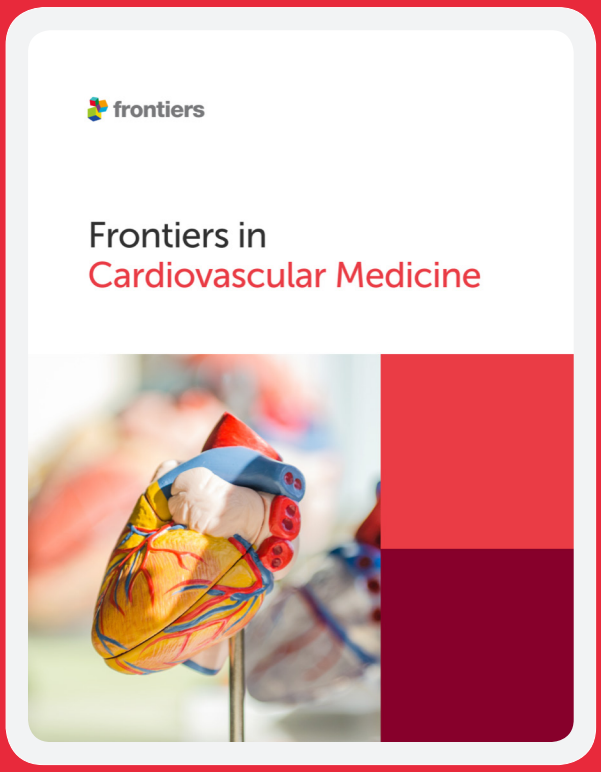

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