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Inflammation and immunomodulation in cardiovascular remodeling

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Inflammation and immunomodulation in cardiovascular remodeling

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Editorial: Inflammation and immunomodulation in cardiovascular remodeling

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Editorial on the Research Topic
Inflammation and immunomodulation in cardiovascular remodeling

This editorial features the collection of articles published in Frontiers in Cardiovascular Medicine: *Inflammation and Immunomodulation in Cardiovascular Remodeling*. The goal of this Research Topic is to focus on the different components of the immune system that can potentially influence the cardiovascular remodeling process. In addition, it is important how new insights such as gender differences and the influence of the microbiome may play a role. It is important to look for new therapies or strategies that exert their work through immunomodulation. And immunomodulation is an exciting field in regard to developing novel therapies and strategies. Following are the original research papers and (systematic) reviews published in this issue in 2021–2022 (Table 1).

Bioinformatics and Immune Infiltration Analyses Reveal Key Pathway and Immune Cells in The Pathogenesis of Hypertrophic Cardiomyopathy by Zhang et al. This study was designed to identify key pathway and immune cells for hypertrophic cardiomyopathy (HCM) *via* bioinformatics analyses of public datasets and evaluate the significance of immune infiltration in the pathogenesis of HCM. They found that the STAT3-related pathway and CD163+LYVE1+ macrophages were identified as the potential key pathway and immune cells in HCM and may serve as interesting targets for further deepening research.

Sex Differences in Inflammation during Venous Remodeling of Arteriovenous Fistulae by Chan et al. Vascular disorders frequently have differing clinical presentations among women and men. Sex differences exist in vascular access for hemodialysis; women have reduced rates of arteriovenous fistula (AVF) maturation as well as fistula utilization compared with men. Inflammation is increasingly implicated as a potent mechanism driving AVF maturation, especially in vessel dilation and wall thickening, allowing venous remodeling to support hemodialysis. Here, they review the current knowledge

and describe several crucial regulators of remodeling such as ephrins, T cells and macrophages and especially downstream genes are affected by sex differences and female hormones.

Molecular Interactions between Vascular Smooth Muscle Cells and Macrophages in Atherosclerosis by Beck-Joseph and Lehoux et al. Vascular smooth muscle cells and macrophages play dominant roles in atherosclerosis. A firm understanding of how these cells influence and modulate each other is pivotal for a better understanding of the disease and the development of novel therapeutics. Recent studies have investigated molecular interactions between both cell types and their impact on disease progression. Here, Intercellular communications through soluble factors, physical contact, and extracellular vesicles are discussed and their impact on atherosclerotic lesion formation is reviewed with respect to the current knowledge.

Stimulation of the PD-1 pathway decreases atherosclerotic lesion development in Ldlr deficient mice by Grievink et al. Signaling through the coinhibitory programmed death (PD)-1/PD-Ligand 1 pathway regulates T cell responses and can inhibit ongoing immune responses. Dampening the excessive immune response by promoting PD-1/PD-L1 signaling may have a high therapeutic potential to limit disease burden. With the current and increasing use of immune check point inhibition as cancer therapies, including inhibition of the PD-1/PD-L1 signaling, this is a very interesting point. This study aimed to assess whether an agonistic PD-1 antibody can diminish atherosclerosis development. They showed that stimulation of the coinhibitory PD-1 pathway inhibits atherosclerosis development by modulation of T- and B cell responses. These data support stimulation of coinhibitory pathways as a potential therapeutic strategy to combat atherosclerosis.

Association of neutrophil to lymphocyte ratio with plaque rupture in acute coronary syndrome patients with only intermediate coronary artery lesions assessed by optical coherence tomography by Jiang et al. Plaque vulnerability and rupture rather than plaque size are the major cause of clinical events in patients with intermediate coronary lesions. Therefore, the present study was aimed to explore potential markers associated with plaque rupture in acute coronary syndrome patients with intermediate coronary lesions. They found that the neutrophil to lymphocyte ratio (NLR) biomarker is closely associated with plaque rupture. Monitoring NLR may be useful in risk stratification and management for intermediate coronary artery lesions.

Interfering in the ALK1 pathway results in macrophage-driven outward remodeling of murine vein grafts by Jong et al. Vein grafts are frequently used to bypass coronary artery occlusions. Unfortunately, vein graft disease (VGD)

TABLE 1 Metrics (on October 22nd 2022) of the highlight's articles published in Frontiers in Cardiovascular Medicine, Inflammation, and Immunomodulation in cardiovascular remodeling, in 2021–2022.

Title	First author, country	Views	Downloads
Bioinformatics and Immune Infiltration Analyses Reveal Key Pathway and Immune Cells in The Pathogenesis of Hypertrophic Cardiomyopathy	Xu-Zhe Zhang, China	3,824	1,513
Sex Differences in Inflammation during Venous Remodeling of Arteriovenous Fistulae	Shin Mei Chan, United States	1,622	397
Molecular Interactions between Vascular Smooth Muscle Cells and Macrophages in Atherosclerosis	Stephanie Lehoux, Canada	2,395	598
Stimulation of the PD-1 pathway decreases atherosclerotic lesion development in Ldlr deficient mice	Hendrika W. Grievink, The Netherlands	1,337	474
Association of neutrophil to lymphocyte ratio with plaque rupture in acute coronary syndrome patients with only intermediate coronary artery lesions assessed by optical coherence tomography	Huili Zhang, China	1,477	187
Interfering in the ALK1 pathway results in macrophage-driven outward remodeling of murine vein grafts.	Alwin de Jong, The Netherlands	1,696	424
The intriguing role of TLR accessory molecules in cardiovascular health and disease	Taisiya Bezhaeva, The Netherlands	2,738	928
Inflammatory mediators in atherosclerotic vascular remodeling	Bryce Evans, Switzerland	1,825	350
Identifying novel mechanisms of abdominal aortic aneurysm *via* unbiased proteomics and systems biology	Stephanie Morgan, United States	1,454	319

causes impaired patency rates. ALK1 mediates signaling by TGF-β *via* TGFβR2 or BMP9/10 *via* BMPR2, which is an important pathway in fibrotic, inflammatory, and angiogenic processes in vascular diseases. A role of the TGF-β pathway in VGD is previously reported, however, the contribution of ALK1 signaling was unknown. They investigated ALK1 signaling in VGD in a mouse model for vein graft disease using either genetic or pharmacological inhibition of the Alk1 signaling and found that reduced ALK1 signaling in VGD promotes outward remodeling, increases macrophage influx and promotes an unstable plaque phenotype.

The intriguing role of TLR accessory molecules in cardiovascular health and disease by Bezhaeva et al. The present review outlines accessory molecules for membrane TLRs that are involved in cardiovascular disease progression. They summarize the up-to-date knowledge on TLR signaling focusing on membrane TLRs and their ligands that play a key role in cardiovascular system. They then survey the current evidence of the contribution of TLRs accessory molecules in vascular and cardiac remodeling including myocardial infarction, heart failure, stroke, atherosclerosis, vein graft disease and arterio-venous fistula failure.

Identifying novel mechanisms of abdominal aortic aneurysm via unbiased proteomics and systems biology by Morgan et al. Abdominal aortic aneurysm (AAA) leads to rupture if not surgically repaired. Mice aid the study of disease progression and its underlying mechanisms. The present study used unbiased proteomics and systems biology to understand the molecular relationship between the mouse models of AAA and the human disease. Aortic tissues of developing and established aneurysms produced by either angiotensin II (AngII) infusion in Apoe -/- and Ldlr -/- mice or intraluminal elastase incubation in wildtype C57BL/6J mice were examined as well as human samples of infrarenal aortic aneurysm tissues and aortic tissue collected from age-matched controls. They found that the aneurysmal tissue from both mouse and human had inflammation, coagulation, and protein processing signatures, but differed in the prevalence of neutrophil-associated pathways, and erythrocyte and oxidative stress-dominated networks in the human aneurysms. Moreover, the conclude that identifying changes unique to each mouse model will help to contextualize model-specific findings. Focusing on shared proteins between mouse experimental models or between mouse and human tissues may help to better understand the mechanisms for AAA and establish molecular bases for novel therapies.

Inflammatory mediators in atherosclerotic vascular remodeling by Evans et al. Atherosclerosis is initiated by endothelial dysfunction allowing the accumulation of intimal lipids and leukocytes. Inflammatory mediators such as cytokines, chemokines, and modified lipids further drive vascular remodeling ultimately leading to thrombus formation and/or vessel occlusion which can cause major cardiovascular events. Although it is clear that vascular wall remodeling is an elementary mechanism of atherosclerotic vascular disease, the diverse underlying pathophysiological mechanisms and its consequences are still insufficiently understood. Here, they review the current knowledge on the involvement of the various Chemokines and Cytokines, as well as other inflammatory and immune-modulating factors in the development and progression of atherosclerosis and what the role of these key inflammation driving factors in the various cell types in the vessel wall is.

The above presented collection of papers demonstrates the broad involvement of the immune system in cardiovascular remodeling. It is also highlights the importance of specific population differences such as gender. The selected reviews provide a clear structural overview of the current knowledge of the involvement of, for example, TLR accessory molecules, inflammatory mediators in atherosclerosis and interaction between macrophages and smooth muscle cells. In addition, there is innovative research that helps us understanding aneurysm formation and the influence of the chosen animal model on previously reported results. Other papers show new pathways in cardiovascular remodeling thereby exploring new potential therapeutic targets.

In our view, this collection of papers provides an interesting overview of how much the immune system is involved in the pathophysiological process of cardiovascular remodeling. This makes the immune system a potential therapeutic target for developing new interventions and clinical data seems to confirm this. Translational models are very useful but of course also have limitations. Knowledge and understanding of the involvement of the immune system is highly desirable given the rapid increase in specific immune modulation therapies in other fields such as rheumatology and cancer treatment. Without a doubt this has been a game changer for many patients, but we need to be vigilant about the potential adverse effects. Exploring pathophysiological mechanisms understanding involvement and interactions regarding immune processes is therefore crucial.

Author contributions

JK, MV, and PQ: writing–review and editing. ME: writing–original draft. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

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REVIEW
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Sex Differences in Inflammation During Venous Remodeling of Arteriovenous Fistulae

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Vascular disorders frequently have differing clinical presentations among women and men. Sex differences exist in vascular access for hemodialysis; women have reduced rates of arteriovenous fistula (AVF) maturation as well as fistula utilization compared with men. Inflammation is increasingly implicated in both clinical studies and animal models as a potent mechanism driving AVF maturation, especially in vessel dilation and wall thickening, that allows venous remodeling to the fistula environment to support hemodialysis. Sex differences have long been recognized in arterial remodeling and diseases, with men having increased cardiovascular events compared with pre-menopausal women. Many of these arterial diseases are driven by inflammation that is similar to the inflammation during AVF maturation. Improved understanding of sex differences in inflammation during vascular remodeling may suggest sex-specific vascular therapies to improve AVF success.

Keywords: vascular inflammation, arteriovenous fistulae, sex differences, estrogens, androgens sex differences in venous inflammation

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INTRODUCTION

Although sex differences exist in the epidemiology and clinical presentation of vascular pathologies, patient sex rarely plays a role in guiding medical or surgical management or specific therapeutic treatments. Sex differences in arterial pathologies have frequently been observed in clinical practice, with women presenting with symptoms of coronary artery disease at later ages and with different presentations compared with men; the later age of presentation is frequently after menopause when estrogen levels decrease (1). One explanation is that female hormones provide an anti-inflammatory effect; both estrogen and estrogen receptors exert cardioprotective effects by attenuating inflammatory cytokines, including interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) as well as recruitment of leukocytes (2).

A global rise in end-stage renal disease has resulted in increased placement of autogenous arteriovenous fistulae (AVF), particularly in light of the "Fistula First Breakthrough Initiative" published by the National Kidney Foundation in 2003 (3). This campaign pushed to attain a target of 40% of autologous AVF in the United States by 2006, and then 66% by 2009 (4). Despite these efforts to increase AVF use, up to 60% of AVF fail to mature by 5 months contributing to significant patient burden and healthcare cost (3, 5). There are several predictors of successful AVF adaptation

to the fistula environment and use as a successful conduit for hemodialysis, although none are perfect (6). Vein diameter is the most predictive factor in many studies, with larger preoperative vein diameters correlating with higher rates of maturation (7, 8). Other factors, such as diabetes, congestive heart failure, concomitant peripheral arterial disease, and older age may also play a role, as these are negatively correlated with successful AVF creation (5, 8).

Female sex also predicts poor fistula outcomes. Women are less likely to undergo fistula surgery, and when placed, AVF created in women take longer to mature and have higher rates of non-maturation compared with men (3, 9). In elderly patients, at 6 months post-surgery, women are less likely to be successfully dialyzed via their fistula, and at 1 year post-surgery, fistulae are more likely to be completely abandoned in women (10). In women, the time to fistula maturation may also be prolonged compared with men, and women also require more frequent salvage procedures (9, 11). Several hypotheses have been proposed to explain the sex discrepancy in rates of AVF maturation, including smaller mean vessel diameters, greater vascular reactivity following vascular injury, and decreased capacity for venous dilation in women (9). Given the worse clinical outcomes of AVF in women and growing evidence of sex differences in both venous and arterial inflammation, we review the evidence for sex differences in inflammation that occurs during venous remodeling that may contribute to discrepancies in AVF maturation.

VENOUS REMODELING

Surgical creation of an AVF results in remodeling of the venous outflow; successful hemodialysis depends on venous remodeling, that is venous dilation and wall thickening, to withstand the high flows required for efficient hemodialysis sessions and puncture with large bore needles 3 times a week (12). Venous remodeling has been studied frequently in the context of vein graft adaption; inflammation regulates vein graft adaptation and sex differences in inflammation may be a mechanism of the reduced vein graft patency among women (13, 14). Analysis of the Project of *Ex Vivo* Vein Graft Engineering via Transfection III (PREVENT III) clinical trial showed that both sex and race predict vein graft patency; black women are more likely to experience loss of vein graft patency and major amputation following bypass surgery (15). However, although AVF maturation and vein graft adaptation both reflect venous remodeling, these processes differ as they take place in different environments and result in different structures (12).

Animal models have shown that venous remodeling during AVF maturation is characterized by increased expression of the venous determinant Ephrin type B receptor 4 (Eph-B4) and the arterial determinant Ephrin-B2, as well as temporal regulation of expression of multiple components of the extracellular matrix (ECM), allowing venous adaptation to the fistula environment without loss of structural integrity (16, 17). Members of the ephrin family mediate cell-cell signaling to promote tissue development and remodeling. In embryogenic tissue, ephrins and their Eph receptors regulate angiogenesis and lymphangiogenesis; in adult endothelial tissues, expression of ephrins are associated with arterial and venous remodeling (16, 18). Expression of both Eph-B4 and Ephrin-B2 show plasticity in adults after surgical manipulation; in vein grafts placed in an arterial environment, Eph-B4 expression is decreased, but expression of arterial markers is not increased (19). However, in AVF, expression of both venous markers such as Eph-B4 and arterial markers such as Ephrin-B2 are increased, resulting in venous remodeling via an Akt1-mediated mechanism (16). During vascular remodeling, ephrins are upregulated by tumor necrosis factor alpha (TNF-α) and regulate nuclear factor -κB (NF-κB), a potential pathway regulating inflammatory processes in endothelial cells (20). In addition Ephrin-B2 promotes leukocyte extravasation and infiltration necessary for vascular remodeling associated with arteriosclerosis (21).

Sex differences involving Eph-B4 and Ephrin-B2 within the vascular system have not been described. However, sex differences have been described in downstream targets. One target of Eph-B4 is Akt, which phosphorylates endothelial NO synthase (eNOS), a critical mediator of venous dilation (22). Phosphorylated eNOS is increased in vein grafts, and absence of eNOS prevents thickening and remodeling of the venous wall (22). In an elegant analysis of a murine AVF model, eNOS mediates dilation of the remodeling vein exposed to the fistula environment; overexpression of eNOS was associated with larger diameter and less neointimal hyperplasia, and eNOS knockout was associated with small diameter and increased neointimal hyperplasia. eNOS overexpression was also associated with smoother blood flow streamlines, less shear stress at the vessel wall, luminal fluid vorticity, and radial wall thinning. These data suggest that eNOS increases NO release from endothelial cells to stimulate smooth muscle cell relaxation (23).

Interestingly, in ovariectomized mice, estrogen induces eNOS to produce and release NO from endothelial cells via the Akt pathway, leading to subsequent arterial dilation (24). In human endothelial cells, there is greater eNOS expression and activation in female-derived cells (25). After menopause there are reduced circulating estrogens, with subsequent reduced arterial NO, that likely contributes to the increased risk for cardiovascular events observed in post-menopausal women compared with pre-menopausal women (26–28). Thus, while sex differences in

Abbreviations: AAA, Abdominal aortic aneurysm; AMPK, 5′ adenosine monophosphate-activated protein kinase; AP-1, Activator protein-1; AVF, Arteriovenous fistula; BMP, Bone morphogenic protein; CC, Cysteine-cysteine; CD, Cluster of differentiation; CINC, Cytokine-induced neutrophil chemoattractant; CX3CR1, Cysteine-X3-cysteine motif chemokine receptor 1; ECM, Extracellular matrix; eNOS, Endothelial nitric oxide synthase; Eph-B4, Ephrin type B receptor 4; ER-α, Estrogen receptor-α; ICAM-1, Intercellular adhesion molecule 1; IL, Interleukin; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; KLF2, Kruppel-like factor 2; LPS, Lipopolysaccharide; M-CSF, Macrophage colony-stimulating factor; MCP-1, Monocyte chemoattractant protein-1; MMP, Matrix metalloproteinase; NF-κB, Nuclear factor-κB; PDGF, Platelet derived growth factor; PPAR, Peroxisome proliferator-activated receptor; SOC3, Suppressor of cytokine signaling; STAT, Signal transducer and activator of transcription; TAA, Thoracic abdominal aneurysm; TAK1, TGF-β activated kinase-1; TGF-β, Transforming growth factor-β; TIMP, Tissue inhibitor of metalloproteinases; TNF-α, Tumor necrosis factor-α; VCAM-1, Vascular cell adhesion molecule-1.

Eph-B4 and Ephrin-B2 expression have not yet been reported, components of the Eph signal transduction pathway shows sex differences, suggesting that this family of ligands and their receptors may play a large role in the observed differences in venous remodeling.

Transforming growth factor (TGF)-β and TGF-β activated kinase-1 (TAK1) are also important mediators of venous remodeling that promotes wall thickening and dilation by regulating ECM deposition, collagen, fibronectin, and lumen dilation necessary for AVF maturation (29, 30). In endothelial cells, TGF-β can stimulate inflammation and fibrosis via expression of cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), matrix metalloproteinases such as MMP-2, and fibronectins (31). Increased expression of TGF-β during early venous remodeling is likely required for initial maturation of fistulae, and may be a mechanism of compensation to hemodynamic changes, but sustained increased TGF-β and platelet derived growth factor (PDGF) expression likely leads to neointimal hyperplasia that contributes to late AVF failure (32). Several TGF-β polymorphisms alter TGF-β expression and may determine late AVF patency rates in human patients (33). However, sex differences in the TGF-β pathway and downstream effects on the ECM have not been adequately assessed during AVF maturation, despite strong evidence that sex hormones directly interact with the TGF-β superfamily (34). It is possible that sex differences in upstream regulatory pathways, such as in toll-like receptors, may also exist (35).

Since remodeling of the vein during AVF maturation results in expression of both arterial and venous identities in the remodeled venous wall, understanding of sex differences in arterial remodeling may suggest mechanisms relevant to venous remodeling (**Table 1**). Arterial remodeling involves multiple inflammatory processes. Inflammation and vasoactive peptides promote vessel remodeling by promoting the migration of monocytes and macrophages into the vascular wall, mediated by cell adhesion proteins such as ICAM-1 and VCAM (44, 45). Increased shear stress also promotes vascular remodeling by upregulating TNF-α and NF-κB, as well as activating cellular adhesion molecules to recruit leukocytes (46). Sex differences present during arterial remodeling may be relevant to venous remodeling.

In arterial remodeling, estradiol administration inhibits monocyte migration in a MCP-1-dependent manner (47). In human endothelial cells treated with lipopolysaccharide (LPS) to induce VCAM-1 expression, estradiol decreases VCAM-1 expression to a greater extent, compared with dexamethasone, by inhibiting NF-kB, activator protein-1 (AP-1), and GATA (48). Estradiol can also influence ICAM-1 expression; treatment of endothelial cells with estradiol leads to a shift from the NF-kB pathway to the c-Jun N-terminal kinase (JNK)/AP-1 pathway (49). Estradiol treatment inhibits TNF-α-dependent VCAM-1 and ICAM-1 expression, as well as inhibition of NF-kB via activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)-α (50). Conversely, administration of androgens to both female and male human endothelial cells shows increased TNF-α signaling and greater expression of inflammatory cytokines, and increased VCAM-1 expression (51). Since sex differences are present in inflammation-driven arterial remodeling, it is likely that similar sex differences exist in venous remodeling.

VENOUS DILATION

After an AVF is created, early outward remodeling of the vein is driven by Poiseuille's law, whereby contact with the higher arterial pressure, flow and oxygen content leads to increased venous diameter; this increased diameter, and thus increased volume of the vessel, is necessary for the vein to accommodate increased magnitudes of shear stress and volume flow (12). Preoperative vein diameter is a main predictor of clinical AVF success; in a retrospective review, Lauvao et al. showed that vein diameter was the only independent predictor of fistula maturation (odds ratio = 0.15) (8). Of note, women frequently have smaller diameter vessels compared with men; in a mouse AVF model, outflow veins in male mice were larger than female mice immediately after surgery, and remained larger postoperatively (8, 37, 52).

Outward remodeling is driven by immune and inflammatory processes, particularly CD4+ T cells (53). Rats devoid of mature T cells have decreased lumen sizes following AVF surgery and lower inflammatory cell counts at the fistula site; rescue with euthymic CD4+ T cells leads to increased blood flow through the fistula (54). Sex differences have yet to be assessed in the T cell-mediated venous dilation observed during AVF maturation. However, estrogen can influence Th responses, promote Treg cell populations, and mediate IL-17 release (55). Notably, estrogens can dampen inflammatory responses by modulating Th1 responses to Th2 responses (56). In addition, men have higher Th1:Th2 cytokine ratios (57). Future work is necessary to identify sex-associated differences in T cell-mediated outward remodeling in AVF.

MMP may also play sex-dependent roles in inflammatory venous remodeling. MMP are a family of zinc-dependent endopeptidases that degrade collagen and elastin in the ECM and have been implicated in dilatory venous and arterial diseases, including chronic venous insufficiency and aneurysmal disease (58–60). MMP are secreted by various cell types, particularly inflammatory cells (i.e., lymphocytes, macrophages, neutrophils), endothelial cells, and vascular smooth muscle cells (58). In chronic venous disease, multiple MMP have been implicated (58). During AVF maturation, serum levels of MMP-2, MMP-9, and metalloproteinase tissue inhibitors (TIMP) are associated with successful creation of AVF; higher MMP-2/TIMP-2 and MMP-9/TIMP-2 ratios were associated with better prognosis (61).

Notably, the relaxin family of hormones upregulates MMP-2 and MMP-9 expression to contribute to vessel remodeling (62, 63), suggesting that relaxin-relaxin receptor signaling might be a significant contributor to the sex differences present during AVF maturation as relaxin and its downstream molecules differ between sexes (64–68). Knockout of the relaxin receptor resulted in decreased outward remodeling in a murine model of AVF failure, accompanied by increased elastin content, reduced elastase activity, increased CD45+ leukocytes, and increased MCP-1 expression (69). Chronic administration

TABLE 1 | Observed sex differences in clinical studies and animal models.

Indication	Model	Inflammatory marker	Men:Women	Women:Men	References
AVF	Mice	KLF2, eNOS, VCAM-1	↑ in male mice following AVF creation		(36)
	Mice	TGF-β1, TGFβ-R1, α-smooth muscle actin, fibroblast-specific protein-1, CD68+		↑ in female mice following AVF creation	(37)
AAA	Human	Peripheral blood mononuclear cells (Monocytes, B-cells, T cells)	↑ in men with AAA compared with men without AAA (effect not seen in women)		(38)
	Human	MMP-9		↑, compared with men with AAA	(39)
	Rat	Neutrophils	↑ in elastase-perfused rat		(40)
				↓ in elastase-perfused rat	(41)
	Rat	Macrophages	↑ in elastase-perfused rat		
				↓ in elastase-perfused rats	(41)
	Rat	MMP-13	↑ in elastase-perfused rats		(40)
	Rat	BMP, TNF ligands		↓ in elastase-perfused rats	(41)
	Rat	TGF-β and VEGF		↓ in elastase-perfused rats	(41)
	Mice	JNK1 and downstream proMMP9, proMMP2, active MMP2	↑ in elastase-perfused mice		(42)
Atherosclerosis	Human	CD68+, CD3+, macrophage foam cells		↑ in carotid plaque caps	(43)

↑, *Increased and* ↓, *decreased.*

of recombinant relaxin reduces arterial load by decreasing systemic vascular resistance, reduces pulsatile arterial load by increasing compliance, and increases cardiac output in female mice (65); interestingly, there were no sex differences, despite relaxin being considered specific to female physiology (64, 66). Elastins may also be involved in the sex differences of vessel remodeling, as estrogen may decrease MMP-9 production, thus decreasing elastin degradation (70). Elastin haplodeficient mice show increased outward remodeling, without increased intimal hyperplasia, resulting in larger diameter venous outflow tracts (71). Although the data showing elastin regulates venous remodeling is strong in animal models, human clinical trials with recombinant elastase did not show clear improvements in AVF outcomes (72, 73).

The inflammatory process of venous outward remodeling may be similar to remodeling that occurs during the formation of arterial aneurysms (**Figure 1**). Sex differences in arterial aneurysms have been extensively described. Both thoracic aortic aneurysms (TAA) and abdominal aortic aneurysm (AAA) occur less commonly in women than in men (74). Women, however, have worse prognosis; in women, AAA and TAA grow at faster rates, rupture at smaller diameters, and are associated with higher mortality (75–81). Aortic stiffness predicts TAA growth in women but not men, further suggesting a potential sex difference in the pathophysiology (81). In addition, women develop AAA at older ages than men (82), which has been suggested to be associated with menopausal status and decreasing estrogen levels (39).

Similar to venous remodeling, AAA are driven by T cell-mediated inflammatory processes (**Table 2**); both $CD4^+$ and $CD8^+$ T-cells are highly activated in the aortic wall, and perivascular T cell counts correlate with aneurysm progression (89). Th2 cytokines, particularly IL-1, IL-4, and IL-10, simultaneously activates MMP, which promotes the outward remodeling of the aneurysmal wall (90–92). Estradiol attenuates inflammatory cytokines and chemokines necessary for MMP-2 and MMP-9 release and monocyte infiltration, which protect against AAA; this may be mediated through the JNK pathway (42, 70). Similarly, following aortic elastase perfusion in male rats there are increased numbers of infiltrating macrophages compared with female rats, and treatment with estrogen in male rats leads to smaller aneurysms accompanied by decreased macrophage infiltration (41, 70). Human aortic smooth muscle tissue incubated in testosterone showed increased MMP-3 expression, whereas incubation with estrogen and progesterone reduced collagen deposition and increased elastin deposition (83). In rats, MMP-13 expression is increased in male rats perfused with elastase compared with female rats (40). In mice, increased estrogen receptor-α (ER-α) in female mice was inversely correlated with MMP activity and aneurysm formation (84). Estrogen administration decreases MMP-2 and−9 expression, adhesion molecule expression and macrophage stimulators such as ICAM-1, VCAM-2, E-selectin, MCP-1, and macrophage colony-stimulating factor (M-CSF) (38, 70, 85). Since estrogen plays a protective mechanism in AAA formation via the inflammatory MMP pathway, further research is needed to understand the contribution of this mechanism to fistula maturation.

VENOUS WALL THICKENING

Vascular remodeling is composed of both changes in vessel diameter as well-changes in wall thickness. Excessive venous

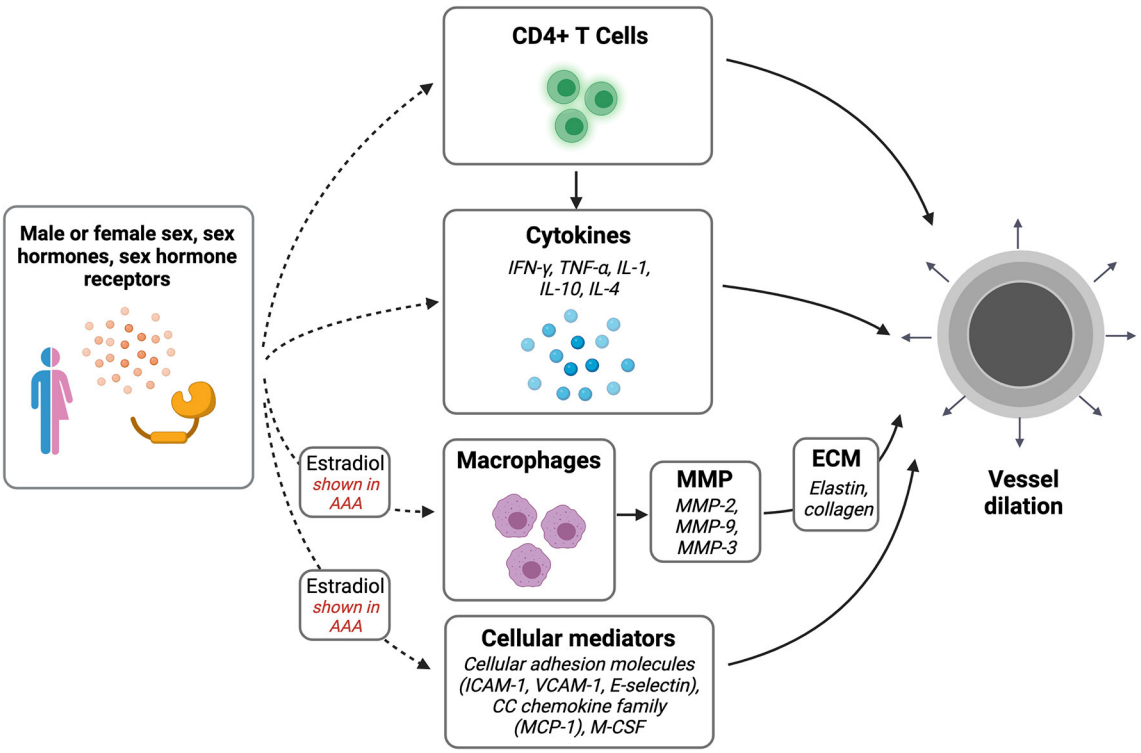

FIGURE 1 | Sex differences in vascular dilation. Potential pathways of interest in vessel wall dilation that may be affected by sex, sex hormones, and sex hormone receptors. Created with BioRender.com.

neointimal thickening leads to late AVF failure, observed more commonly in women than men (9). Venous neointimal thickening also depends on Eph function; in mouse models, decreased Eph-B4 signaling is associated with increased venous wall thickening (16, 93). Eph-B4 also regulates wall thickening in human veins, at least *in vitro* (94–96).

Interestingly, recent data using a mouse AVF model has shown that Kruppel-like factor 2 (KLF2), eNOS, and VCAM-1 increase in male but not female mice, despite being similar at baseline prior to AVF creation; in addition, female mice showed reduced laminar shear stress that was followed by reduced AVF patency at 42 days (36). Transcriptome RNA sequencing has also showed that inflammatory pathways are differentially upregulated in male mice following AVF creation; expression of proinflammatory cytokine IL-17 was less in females, but fibrotic markers TGF-β1 and TGF-β receptor 1 were increased, with correlation to negative vascular remodeling and increased medial fibrosis (36, 37). HIF-1α and heme oxygenase-1/2 are also inflammatory mediators of neointimal hyperplasia that forms during AVF maturation and that may differ between sexes (97–99). Although partial HIF-1α deficiency regulates differential changes in cardiac gene expression between female and male mice (100), and heme oxygenase expression differs in the setting of trauma and hemorrhage (101), sex differences in these mediators have not been studied in the setting of venous remodeling.

Macrophages and T cells contribute significantly to venous neointimal hyperplasia and may further contribute to sex differences observed during AVF maturation (53). Following angioplasty of fistulae, female mice had increased neointimal area-to-media ratio, accompanied by increased numbers of CD68+ cells, suggesting sex differences in macrophages during venous remodeling (102). M2-type macrophages are important for vascular wall thickening by secreting IL-10 that is necessary for wall thickening during AVF maturation (53, 103). Rapamycin, an immunosuppressant, decreases AVF wall thickness, ECM deposition, and smooth muscle cell proliferation via suppression of both M1-type and M2-type macrophages (104). The fractalkine receptor 1 (CX3CR1) reduces venous stenosis in AVF by decreasing proinflammatory signaling, including TNF- α, IL-1β, MCP-1, and NF-kB (105). In a murine AVF model, liposomal prednisolone inhibits venous inflammation and improves outward remodeling; inflammatory cytokine release from M1-type macrophages was reduced, suggesting conversion of macrophages into an anti-inflammatory profile (106). However, no significant effects on neointimal hyperplasia were observed, similar to the lack of efficacy observed in the human Liposomal Prednisolone to Improve Hemodialysis Fistula Maturation (LIPMAT) clinical trial (106, 107). These data suggest that the role of inflammatory signaling during AVF maturation is complex, and additional studies that separately examine the role of inflammation during early maturation and later patency are warranted.

In both atherosclerosis and arterial neointimal hyperplasia, macrophages have been implicated in sex differences (**Figure 2**) (108). Notably, men have more frequent atherosclerotic-related

TABLE 2 | Differences in vascular inflammation due to sex hormones in clinical studies and animal models.

Indication	Model	Inflammatory marker	Androgen-related	Estrogen-Related	References
AAA	Human	MMP-3	↑		(83)
	Mice	MMP-2, MMP-9		↓ (inversely correlated with estrogen receptor α)	(84)
				Ovariectomy resulted in higher levels of MMP-2 and MMP-9	(38)
				↓ in male rats given estradiol	(70)
	Mice	ICAM-1, VCAM-2, E-selectin, MCP-1, M-CSF		↓	(85)
	Mice	IL-1α mediated inflammatory response (MCP-1, MMP-2, MMP-9), TGF-β	↑ (knock-out of AR leads to ↓)		(86)
Atherosclerosis	Human	Monocyte migration		↓, in a MCP-1 dependent manner	(47)
	Human	VCAM-1	↑		(51)
				↓ via NF-kB, AP-1, GATA inhibition	(48)
				↓ via TNF- α, NF-kB inhibition	(50)
	Human	ICAM-1	No significant change		(51)
				↓ via TNF- α, NF-kB inhibition	(50)
				Causes shift in pathway (NF-kB JNK/AP-1)	(49)
	Human	E-selectin	No significant change		(51)
	Rats	Adhesion molecules (P-selectin, VCAM-1, ICAM-1), monocyte chemoattractant (CINC-2β, MCP-1), proinflammatory mediators (IL-1, IL-6)		↓	(87)
	Mice	SOCS3		↑	(88)
	Mice	JAK/STAT		↓	(88)

↑, *Increased and* ↓, *decreased.*

events and at younger ages compared with women, but cardiovascular diseases increase in women after menopause, suggesting significant sex hormone-dependent factors (109, 110). Sex differences are also present in the content of inflammatory cells in atherosclerotic lesions; for example, carotid plaque caps show more CD68+ and CD3+ inflammatory cells, including macrophage foam cells, in women compared with men (43). Macrophages express sex hormone receptors; higher levels of ER-α in premenopausal women are associated with a lower incidence of atherosclerosis (108). In addition, estradiol can attenuate macrophage foam cell formation within atherosclerotic plaques, via JAK/STAT/SOC3 pathway (88). Interestingly, administration of androgens to human male-derived macrophages showed increased expression of inflammatory genes associated with atherosclerosis, but not in female-derived macrophages, further suggesting differential susceptibilities to atherosclerosis based on sex (111). Thus, similar to sex differences observed in arterial neointimal hyperplasia, macrophages may contribute to sex-related differences in late AVF failure.

Vascular smooth muscle cells also play a dynamic role during AVF maturation. Differentiated, mature smooth muscle cells primarily contribute to medial wall thickening and dedifferentiated smooth muscle cells contribute to neointimal hyperplasia (112, 113). Sex differences in smooth muscle cell function have not been directly studied during AVF remodeling; however, it is reasonable that these differences exist, as studies in arterial neointimal hyperplasia have shown that smooth muscle cells function in a sex-dependent manner. In vein grafts implanted into arterial environments, Eph-B4 is found in both endothelial cells and smooth muscle cells, although Eph-B4 is preferentially distributed in endothelial cells (93). In cell culture, female-derived smooth muscle cells show more hypertrophic changes, whereas male-derived smooth muscle cells show more hyperplastic changes; additionally, female-derived cells were more adhesive, suggesting slower proliferation (114). In female rats, increased ER in smooth muscle cells was associated with reduced arterial contraction (115). Mineralocorticoid receptors in smooth muscle cells mediate later onset of aortic stiffening and vascular fibrosis in female mice compared with male mice (116). These studies suggest that sex differences in smooth muscle cell function may also be a mechanism of the sex differences observed during AVF maturation. However, the failed PREVENT trials

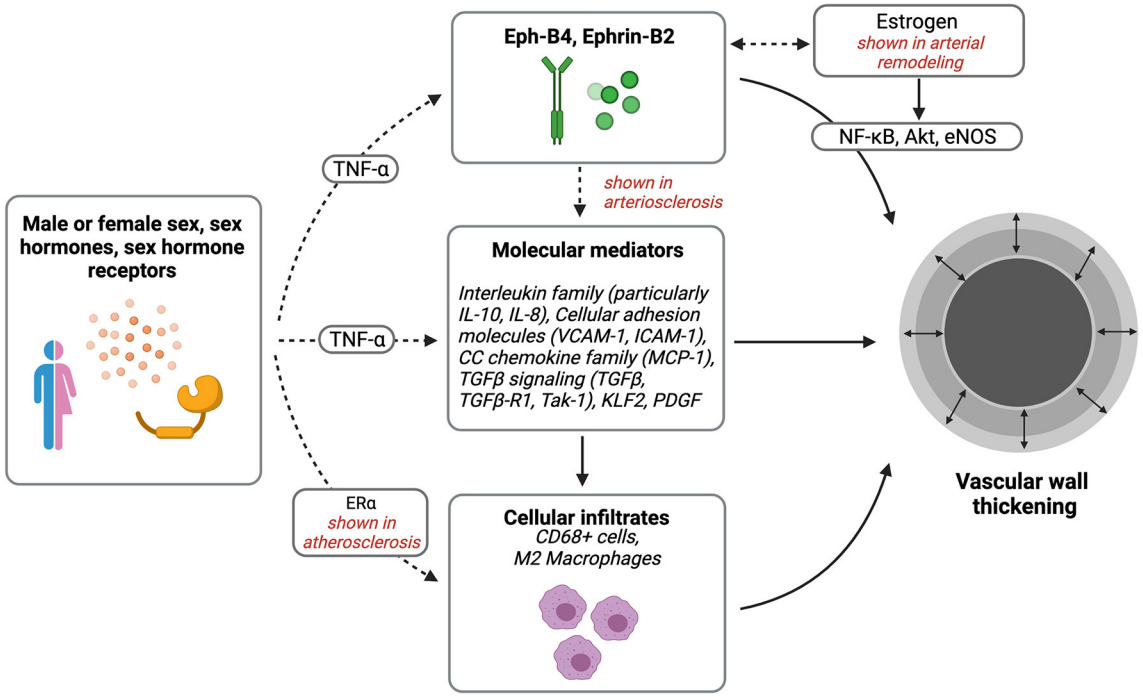

FIGURE 2 | Sex differences in vascular wall thickening. Potential pathways of interest in vessel wall thickening that may be affected by sex, sex hormones, and sex hormone receptors. Created with BioRender.com.

suggest that inhibition of smooth muscle cell proliferation during vein graft adaptation is unlikely to be a clinically successful strategy, and thus inhibition of smooth muscle cell function during AVF maturation would require another approach to optimize venous remodeling (117, 118).

CONCLUSION

AVF maturation and utilization remains poor, particularly in women. There is growing understanding of the inflammatory mechanisms that drive successful AVF maturation and may differ between men and women. Successful AVF creation depends on venous dilation and wall thickening, both of which involve inflammatory mechanisms. Outward remodeling is dependent on CD4+ T cells, and venous hyperplasia is dependent on distinct subsets of macrophages. In arterial aneurysms and atherosclerosis, where sex differences have long been recognized, there is a larger body of evidence supporting sex differences in inflammatory vessel remodeling. Mechanisms of arterial remodeling may help guide understanding of venous remodeling and may lead to improved clinical outcomes for women needing AVF.

AUTHOR CONTRIBUTIONS

SC and AD contributed to the conception and design of the study. SC, GW, JL, and AD performed the literature search, analyzed the data, synthesized the literature, and were responsible for writing the manuscript. DJ-W and AD provided critical expertise and expert revision. All authors contributed to manuscript revision, read, and approved the submitted version.

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Bioinformatics and Immune Infiltration Analyses Reveal the Key Pathway and Immune Cells in the Pathogenesis of Hypertrophic Cardiomyopathy

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Objective: This study was designed to identify the key pathway and immune cells for hypertrophic cardiomyopathy (HCM) via bioinformatics analyses of public datasets and evaluate the significance of immune infiltration in the pathogenesis of HCM.

Methods: Expressional profiling from two public datasets (GSE36961 and GSE141910) of human HCM and healthy control cardiac tissues was obtained from the GEO database. After data preprocessing, differentially expressed genes (DEGs) were then screened between HCM and healthy control cardiac tissues in parallel. Gene Ontology, pathway functional enrichment, and gene set enrichment analysis were performed using DAVID and GSEA application. The compositional patterns of immune and stromal cells in HCM and control cardiac tissues were estimated based on the merged data using xCell. Protein–protein interaction (PPI) network and module analyses were constructed by STRING and Cytoscape applications. Gender-based expressional differences analyses were also conducted to explore gender differences in HCM. GSE130036 and clinical samples were used for verification analyses.

Results: A total of 310 DEGs were identified. Upregulated DEGs were mainly enriched in "adhesion" and "apoptotic process" in the biological process. As for the downregulated DEGs, "inflammatory response," "innate immune response," "phagosome," and "JAK-STAT signaling pathway" were highly enriched. Immune infiltration analyses suggested that the scores of macrophages, monocytes, DC, Th1, Treg, and plasma cells in the HCM group were significantly decreased, while $CD8^+$ T cells, basophils, fibroblasts, and platelets were significantly enriched. Module analyses revealed that STAT3, as the hub genes in HCM together with $LYVE1^+CD163^+$ macrophages, may play a key role in the pathogenesis of HCM while there were no obvious gender differences in the HCM samples from selected datasets. Verification analyses performed on GSE130036 and clinical samples showed a strong positive correlation (Spearman correlation = 0.7646) and a good co-localization relationship between LYVE1 and CD163, suggesting the potential function of $LYVE1^+CD163^+$ macrophages in maintaining the homeostasis of cardiac tissue.

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Conclusion: STAT3-related pathway and CD163^{+}LYVE1^{+} macrophages were identified as the potential key pathway and immune cells in HCM and may serve as interesting targets for further in-depth research.

Keywords: hypertrophic cardiomyopathy, immune infiltration, biomarker, resident tissue macrophages, bioinformatics analyses

INTRODUCTION

Hypertrophic cardiomyopathy (HCM), as a complex genetic heterogeneous disease, is one of the main causes of sudden cardiac death in young adults (1). Epidemiological studies have shown that the prevalence rate of HCM in the general population is estimated to be 0.2% (2). The clinical characteristic of HCM is asymmetric cardiac hypertrophy. As for the cellular level, cardiac myocytes in HCM usually appear to be hypertrophied, disorganized, and separated by areas of fibrosis (3). Although MYH7 and MYBPC3, which encode beta-myosin heavy chain and myosin-binding protein C, respectively, are the two most common HCM-related mutant genes, a large number of underlying mutations and mechanisms are still undetermined (4).

Although regarded as a genetic disease, there is still a gap in how to fully understand the contribution of sarcomere gene mutation in the overall pathophysiological mechanism and clinical process of HCM. A wide range of opinions have suggested that the focus of HCM research should be shifted from single-gene hypotheses to alternative and complementary mechanisms, and novel determinants of HCM should be reviewed and identified in a comprehensive manner from the perspective of network medicine (5). The immune system can not only play the role of immune surveillance, maintaining normal physiological functions in the steady-state heart, but also mediate adverse inflammatory reactions and myocardial remodeling after injury (6–8). Studies have shown that the myocardium of HCM patients shows inflammatory cell infiltration and fibrosis (9). From the mechanism point of view, cardiac hypertrophy, as a pathological stimulus, will, in turn, cause inflammatory signal transduction and immune cell activation, thereby affecting heart function (7). It is still unclear whether this process will be independent or coordinated with the main pathogenic sarcomere mutation genes and thus interfere with the clinical course and prognosis of HCM patients. Above all, more extensive identification of key pathways and non-sarcomeric mutation-related pathophysiological mechanisms in HCM is very necessary and urgent.

Over the past decade, microarray, high-throughput sequencing techniques together with integrated bioinformatics analyses have provided tremendous assistance in identifying novel key genes and pathways in the pathological process of diseases. Here, we conducted full-sided bioinformatics analyses of public datasets to identify differentially expressed genes (DEGs) between HCM and healthy cardiac tissues, and explore the potential pathological mechanism of HCM via functional enrichment analysis including Gene Ontology (GO) and Kyoto Encyclopedia Genes and Genomes (KEGG) for evaluation, immune infiltration analysis, and protein–protein interaction (PPI) network construction. In addition, we explored the role of gender differences in HCM. Finally, we verified our conclusions through online dataset and clinical samples. We hope that our analyses can initially explore the potential role of signaling pathways and immune cells in HCM and provide useful targets for future in-depth research.

MATERIALS AND METHODS

GEO Datasets

Gene expression profile data were collected from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo), a public database of NCBI that contains a mass of datasets for gene expressional profiling by arrays and high-throughput sequencing. We screened potential workable datasets based on the following searching criteria: "hypertrophic cardiomyopathy" MeSH Terms AND "Homo sapiens" Organism. Qualified datasets had to meet the following inclusion criteria: (i) Microarray or high-throughput sequencing profiling studies on human patients with HCM together with non-HCM control/healthy samples for comparison; (ii) reports of adequate sample sizes for analyses; (iii) group label for each sample size; and (iv) availability of the raw data. Finally, GSE36961 and GSE141910 were selected for analyses.

HCM-affected myocardium ($n = 106$) from GSE36961 were collected from HCM patients undergoing therapeutic surgical septal myectomy for relief of obstructive symptom while control tissue ($n = 39$) consist of donor hearts without suitable transplant recipient. Clinical background for analyzed populations and detailed operation process in GSE36961 can refer to the original citation of this dataset (10) while a brief summary was listed in **Supplementary Table 1**. Data of GSE141910 came from the Myocardial Applied Genomics Network (www.med.upenn.edu/magnet). Left ventricular tissues in this series were harvested at the time of cardiac surgery from subjects with heart failure undergoing transplantation and control samples from unused donor hearts. Since the publication of GSE141910 has not yet been released, we can only use the information provided with the dataset and summarized the clinical background information of samples included as shown in **Supplementary Table 2**. **Figure 1** provides an overview of our analyses workflow.

Data Preprocessing and DEGs Identifying

The raw datasets were obtained from GEO and preprocessed by log2 transformation. Sample data were normalized by the limma package (11) in R software version 3.6.1 (https://www.r-project.org/) by plotting and adjusting the boxplot of each sample so

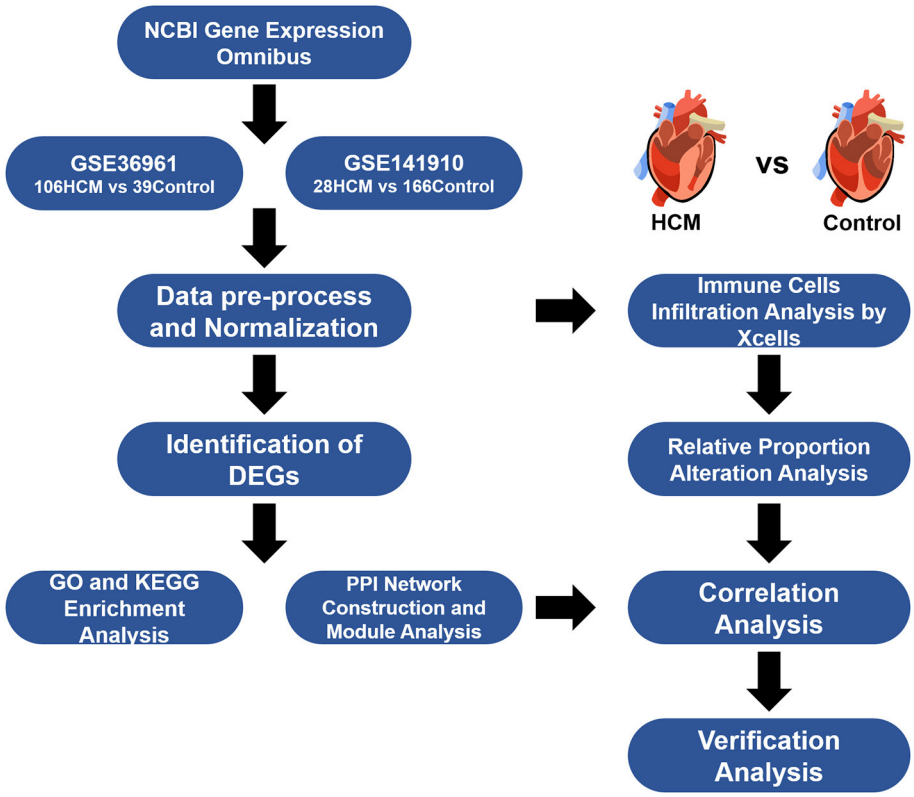

FIGURE 1 | Workflow of the study.

as to maintain the data from a parallel experiment at the same level. The R package limma was also used for the identification of DEGs between different groups. *p*-values were adjusted by the Benjamini–Hochberg method. An adjusted $p < 0.05$ and a |log2FC| value >0.5 were applied in screening significantly different expression levels of molecules. Volcano plots and heat maps for DEGs were plotted using R packages "ggplot" and "ComplexHeatmap" under the platform of R software. The gene symbols of DEGs were recognized and mapped by FunRich software version 3.1.3 and Venn diagrams were drawn by the online plot tool Sangerbox (http://www.sangerbox.com/tool).

GO, Pathway Functional Enrichment, and Gene Set Enrichment Analysis

GO and KEGG pathway enrichment analysis were performed using the DAVID (12) web service (https://david.ncifcrf.gov/) by inputting the DEGs. DAVID is an analytical platform that integrates multiple annotation datasets and could be used for the assignment of biological functions and potential pathways to the DEGs found to be up- and downregulated in HCM. The cutoff values for GO were set as $p < 0.05$ and KEGG with $p < 0.1$ analyses were set as $p < 0.05$. Dot plots for functional enrichment analyses were drawn by "enrichplot" packages under R software. Gene set enrichment analysis (GSEA) was conducted using the GSEA desktop application (13) in order to investigate enriched immune process-related signaling pathways. Visualization of GSEA results was performed using the Sangerbox tools.

Immune Cell Infiltration Analysis

xCell (14) is a novel gene signature-based strategy used to infer variety immune and stromal cell types and is validated using extensive *in silico* simulations and also cytometry immune-phenotyping. Applying xCell to the normalized data, portrayals of cellular heterogeneity landscape for cardiac tissue expression profiles can be acquired. Several cell types were picked out and categorized based on their properties into four categories, namely, "lymphoid cells," "myeloid cells," "stromal cells," and "stem cells and others." Group violin graphs were plotted and evaluated by *t*-test to compare the cell types from the HCM group to healthy control. Cutoff values for significance were $p < 0.05$. Correlation matrix of immune cell subtypes and hub genes were constructed by "corrplot" and "psych" package in R. Pearson correlation coefficients were calculated and used for evaluating the strength of correlation.

PPI Network Construction and Module Analysis

The identified DEGs were inputted and analyzed by the STRING (version 11.0) website service (15) with a minimum interaction score of 0.4. The networks were then exported and constructed by Cytoscape software (16), a platform for visualizing

complex PPI networks. By applying the available Apps including cytoHubba (17) and MCODE (18) in Cytoscape, we identified hub gene sets by degree and recognized highly interconnected clusters as potential functional molecular complexes of HCM. Plots were directly output from Cytoscape. Pearson correlation analyses were performed for correlation between immune cells and hub genes. Identified clusters were then input into the TRRUST (19) database (https://www.grnpedia.org/trrust/) for transcription factor–target interaction information, and $p < 0.05$ was considered of significance.

Verification of Crucial Genes and Cells in HCM

To verify the crucial genes in HCM, we downloaded another open strand-specific RNA-seq dataset GSE130036 from GEO containing expression profiles of myocardial tissues in 28 HCM patients and 9 healthy donors. HCM samples were harvested from septal myectomy while control hearts were collected from the left ventricle of unused healthy donated hearts. Clinical characteristics of patients could refer to the original publication (20) or **Supplementary Table 3**. Data were preprocessed based on the above criteria. Results were plotted by GraphPad Prism with *p*-value calculated through Welch's *t*-test, and the correlation plots were drawn using "ggplot2" package in R. Also, to examine the co-localization relationship between key gene and target immune cells in cardiac tissue, we conducted immunofluorescence staining on cardiac tissue harvested from multi-organ donors whose hearts were refused because of incipient coronary plaque formation in the left descending coronary artery. Heart tissues were then fixed in 4% paraformaldehyde at 25°C for 24 h, embedded in paraffin, and cut into 5-μm sections. A section from each heart was blocked with 1% BSA PBS buffer and stained with target fluorescent antibody. Finally, these sections were stained with DAPI to visualize the nuclei and viewed with confocal fluorescence microscopy. The protocol of the study was in accordance with the Declaration of Helsinki and was approved by the medical ethics committee of the Tongji Medical College of Huazhong University of Science and Technology (METC number: 2016S124).

Statistical Analysis

Public data preprocessing and analyses including sample data merging, ID conversion, and duplication removal

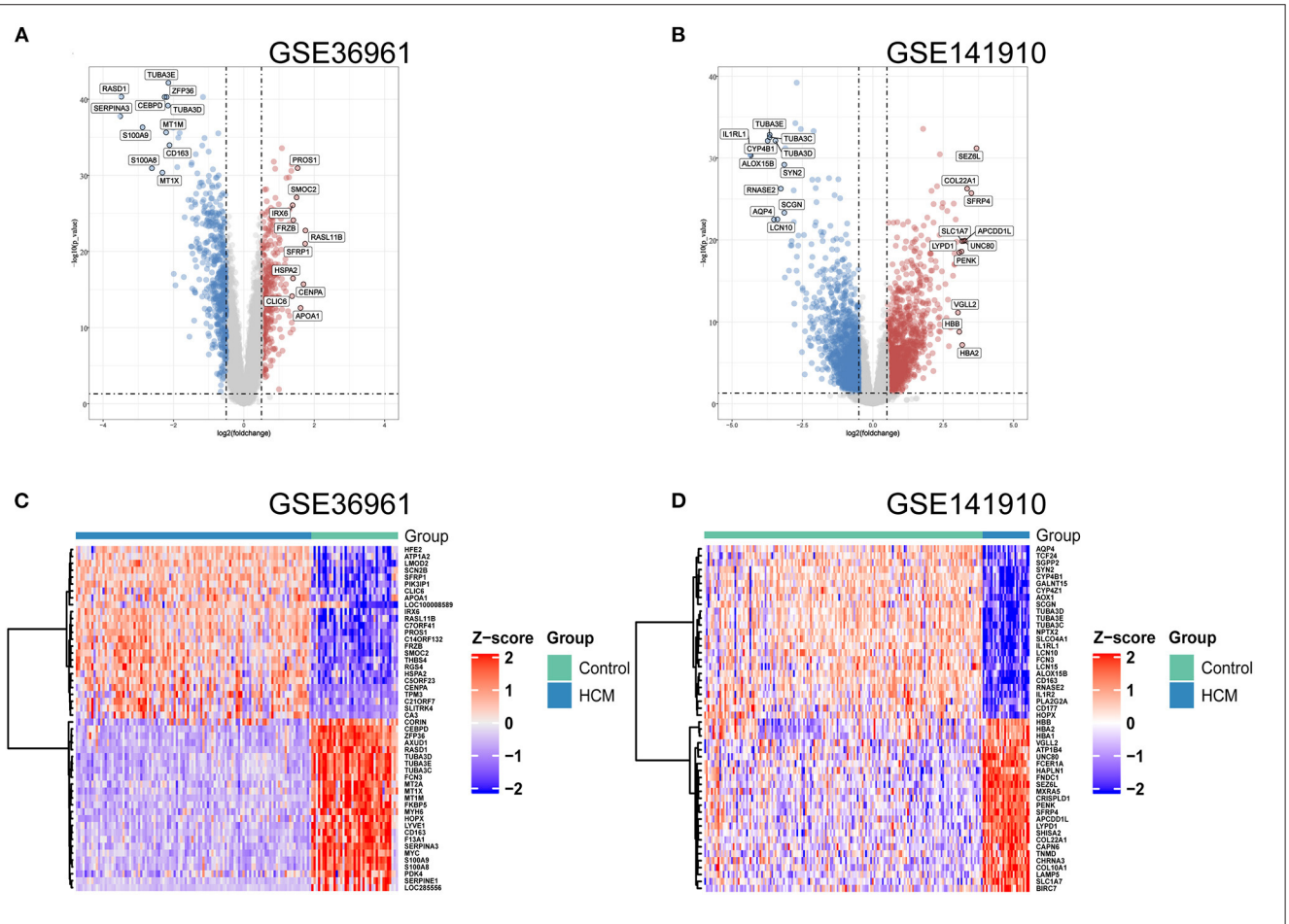

FIGURE 2 | Differentially expressed genes (DEGs) of used datasets. **(A,B)** Volcano plot of DEGs between HCM and healthy control cardiac tissues in GSE36961 and GSE141910. **(C,D)** Heat maps of potential DEGs between HCM and healthy control cardiac tissues in GSE36961 and GSE141910.

were performed using SPSS 22.0 and R software. $p < 0.05$ was considered statistically significant in all sections. Graphs were constructed with R software, GraphPad Prism, and online plot tools as described in the above sections in detail as well as the statistical methods used in this study.

RESULTS

Identification of DEGs Between HCM and Healthy Cardiac Tissue

GSE36961 and GSE141910 were included in our bioinformatics analyses. GSE36961 had 145 samples, namely, 106 HCM and 39 healthy control cardiac tissues. GSE141910 had 28 HCM and 166 healthy control cardiac tissues. The HCM samples of the above two datasets came from the ventricular septum of patients with obstructive symptoms that require surgical intervention and the left ventricle of patients with heart failure, both of which represent the period when pharmacological treatment cannot control the symptoms of HCM, while the control samples were derived from unused healthy donated hearts; therefore, we considered these two datasets to be comparable. Considering that the two datasets included are of different sequencing types, the identification of DEGs was performed separately.

As shown in the volcano plots and heat maps in **Figures 2A,C**, 870 DEGs (379 upregulated and 491 downregulated) were recognized in the GSE36961. In addition, a total of 2613 DEGs (1324 upregulated and 1289 downregulated) were obtained from the GSE141910 dataset (**Figures 2B,D**). By taking the intersections of the Venn diagram in **Figure 3A**, we obtained 159 DEGs that were upregulated in HCM together with 159 downregulated DEGs from both datasets.

Function and Pathway Annotations of DEGs

GO analyses were conducted to assess the consistency for functional annotations of the DEGs in the two datasets. Inputting all DEGs of the two datasets into DAVID, we obtained the functional annotation descriptions for DEGs of the two datasets. As shown in **Supplementary Figure 1**, two datasets shared a large degree of overlap in GO analysis including "calcium ion binding" and "heparin binding" in molecule function, "extracellular region" and "cell surface" in cellular component, and "inflammatory response" and "apoptotic

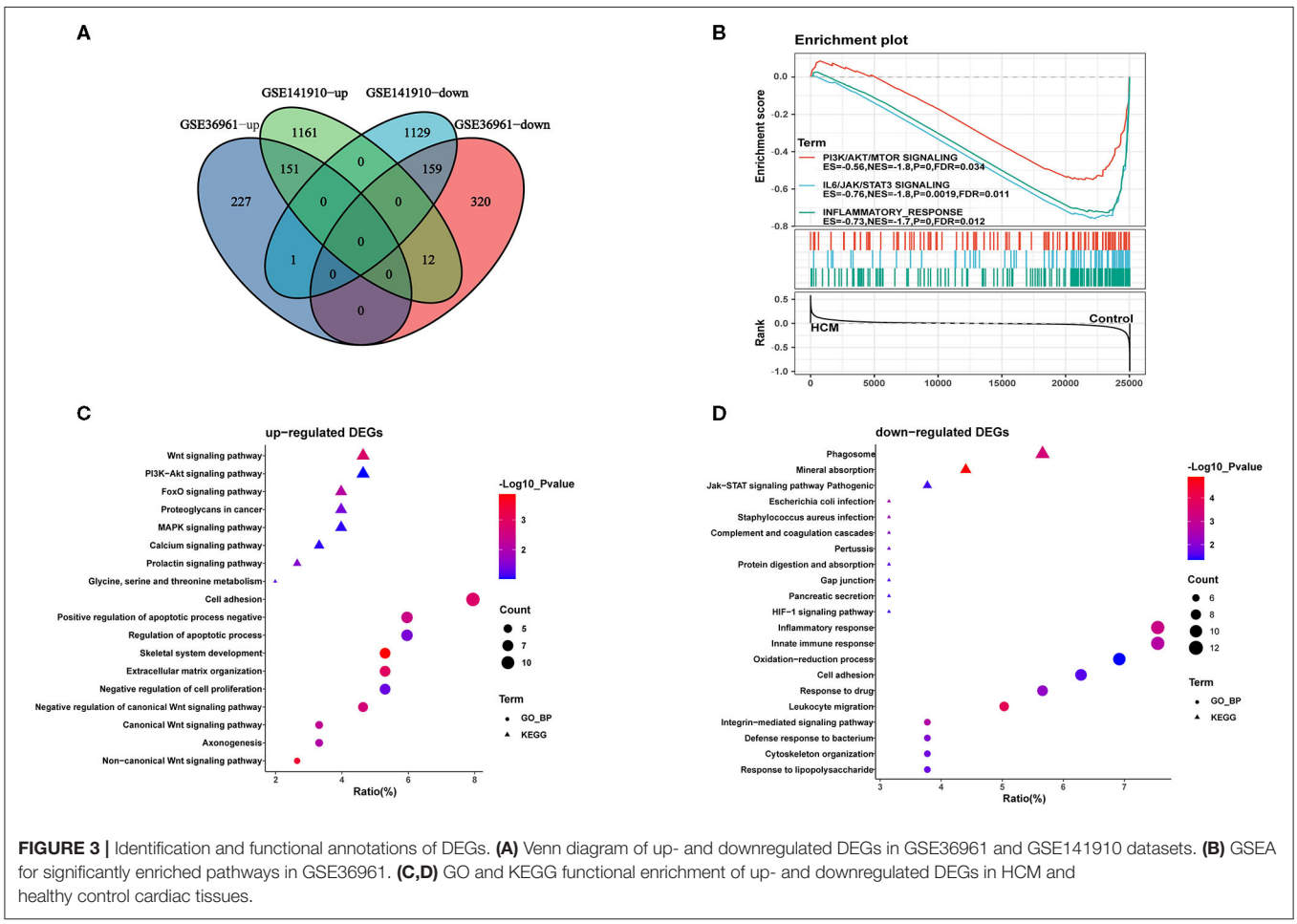

FIGURE 3 | Identification and functional annotations of DEGs. **(A)** Venn diagram of up- and downregulated DEGs in GSE36961 and GSE141910 datasets. **(B)** GSEA for significantly enriched pathways in GSE36961. **(C,D)** GO and KEGG functional enrichment of up- and downregulated DEGs in HCM and healthy control cardiac tissues.

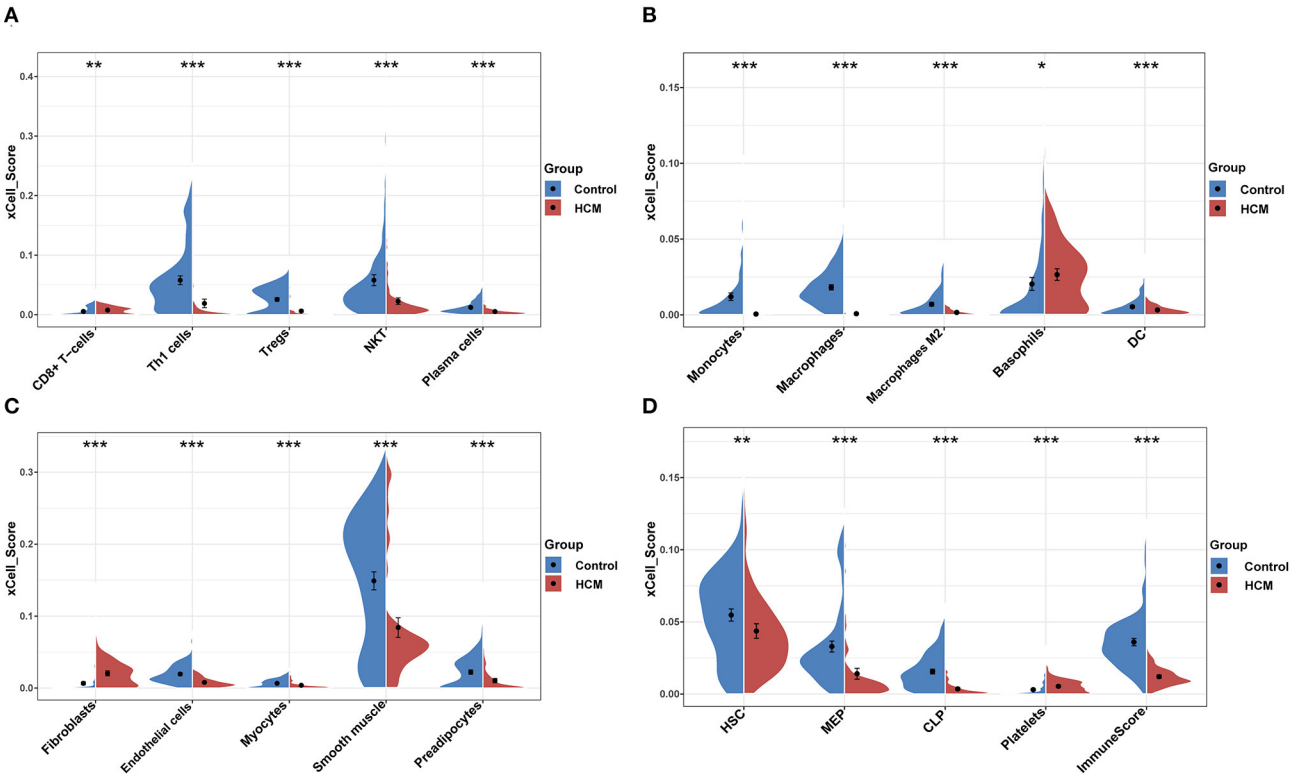

FIGURE 4 | xCell scores of immune or stromal cells between HCM and healthy control cardiac tissues in GSE36961 and GSE141910. **(A–D)** Violin charts of "lymphoid cells," "myeloid cells," "stromal cells," and "stem cells and others," respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

process" in biological process, indicating good homogeneity and reliability between the two datasets.

Furthermore, we conducted GO-biological process and KEGG pathway analyses by inputting the up- and downregulated DEGs in DAVID separately. As shown in **Figure 3C**, upregulated DEGs were mainly enriched in "adhesion" and "apoptotic process" in the biological process together with Wnt and PI3K-Akt signaling pathway, which were significantly enriched. As for the downregulated DEGs in **Figure 3D**, "inflammatory response" and "innate immune response" were highly enriched; in addition, "phagosome" and "JAK-STAT signaling pathway" were also ranked high in KEGG. We then took data in GSE36961 and conducted GSEA analysis, and the results shown in **Figure 3B** suggested significant enrichment of inflammatory response and JAK-STAT signaling pathway in the control group.

Immune and Stromal Cell Infiltration Analysis

xCell was used to identify immune or stromal cell types that may be involved in HCM. It generated enrichment scores of different cell types by using a large number of gene expression data. Because the xCell scores were not affected by different sequencing types, we combined the scores of GSE36961 and GSE141910 for further analysis. We divided 64 kinds of cells into four subsets, namely, "lymphoid," "myeloid," "stromal," and "stem cells and others." There were 26 cell types with significant differences in HCM cardiac tissue vs. control group in the merged dataset as partly shown in **Figures 4A–D**, among which the scores of macrophages, monocytes, DC, Th1, Treg, and plasma cells in HCM group were significantly decreased, while $CD8^{+}$ T cells, basophils, fibroblasts, and platelets were significantly enriched.

Furthermore, we calculated the correlation efficient between each immune cell to explore their relationship and possible interaction. As shown in **Figure 5**, among all immune cells, Th1 had the highest correlation with plasma cells (Pearson's correlation = 0.84), and the second strongest positive correlation was the correlation between macrophage M1 and aDC (Pearson's correlation = 0.77). In addition, macrophages as a whole showed strong positive correlation with both macrophages M1 and M2. On the other hand, $CD8^{+}$ T cells and aDC showed the strongest negative correlation (Pearson's correlation = −0.49).

PPI Network Construction and Module Analysis

To identify the significant pathways of HCM, we uploaded the filtered 308 DEGs into the STRING and obtained the exported TSV file for the interactions of multiple DEGs to Cytoscape for further network construction. CytoHubba identified 10 hub genes (**Figure 6A**), among which only BDNF, CCND1, and POSTN were upregulated in HCM; all the rest were downregulated. We then calculated the correlation efficient between 10 hub genes and interested immune cell types. As

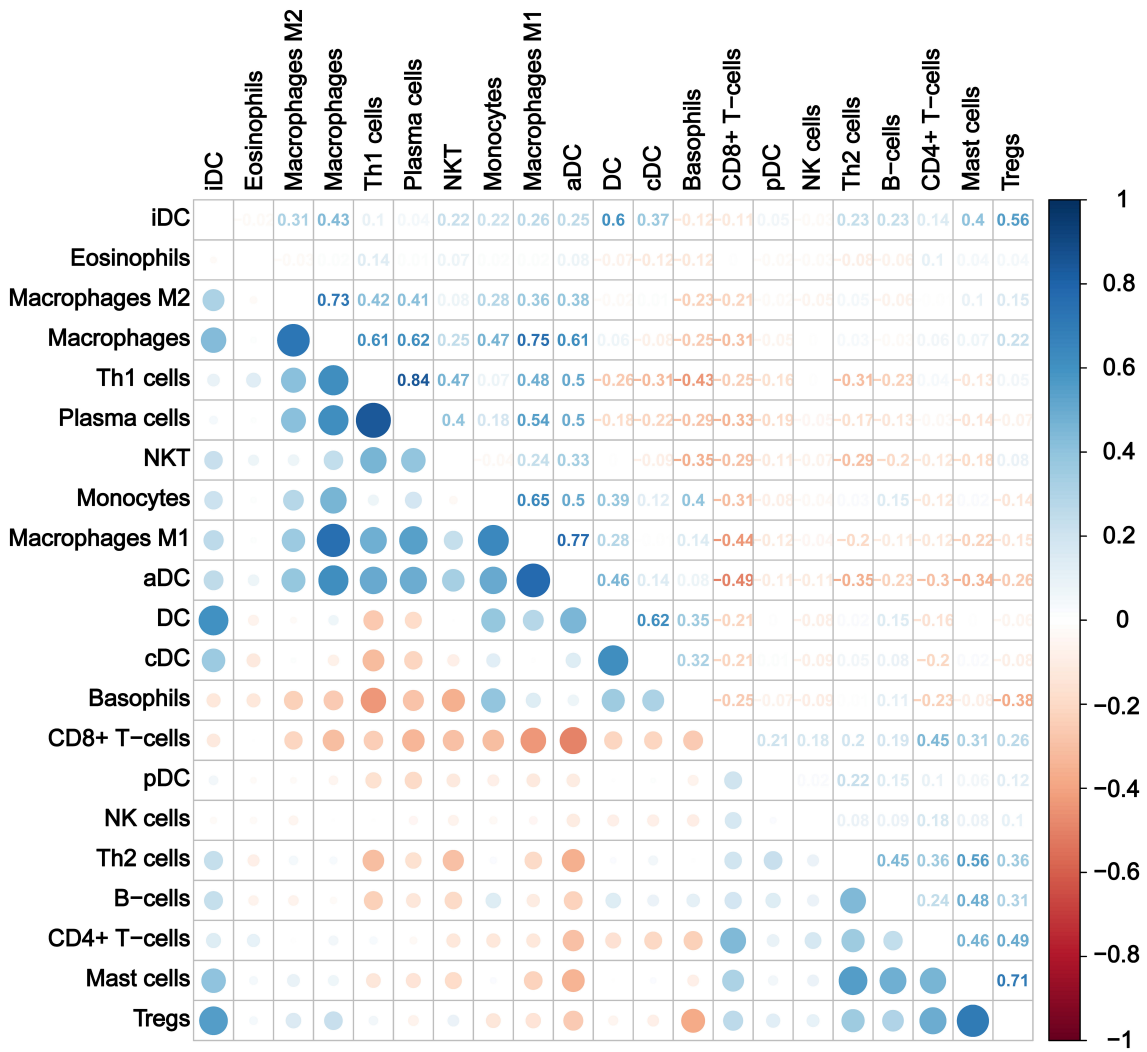

FIGURE 5 | Correlation matrix of immune cell subtypes.

shown in **Figure 6B**, macrophages M1, iDC, aDC, monocytes, and $CD8^+$ T cells presented a strong and reliable correlation to most of the hub genes, suggesting that the key genes in the DEGs had a great effect on the positive regulation of immune system. MCODE then recognized two highly interconnected clusters in DEGs as shown in **Figures 6C,D**. We used TRRUST to predict key candidate regulators for cluster 1 and cluster 2, and results are listed in **Supplementary Tables 4, 5**. For both clusters, STAT3 (Signal transducer and activator of transcription 3) was predicted to be the key regulator. In addition, for cluster1, HIF1A, and TP53 seemed to play a crucial role while ABL1 and NFYA ranked high in the prediction of cluster2.

To identify the key genes of HCM that were highly related to the function of immune cells, we filtered the 308 DEGs with |log2FC| value >1.0 and obtained 51 DEGs of significant difference. Further PPI network construction, hub genes, and clusters identification shown in **Figures 7A,B** suggested that CD163, regarded as a highly specific marker of macrophages M2, played the most important role in the downregulated DEGs while FMOD, the key gene in upregulated DEGs, was mainly related to the process of fibrosis. The CD163-related genes including FPR1, S100A9, FCER1G, LYVE1, and F13A1 were identified to be the candidate key genes highly related to the function of macrophages in HCM. Referring to the information on GeneCards, we summarized the relationships between candidate key gene with immune cells. As shown in **Table 1**, S100A9, FCER1G, and F13A1 were reported to be widely expressed in different kinds of immune cells while FPR1 was mainly expressed in neutrophils. We then focused on LYVE1 and its function in HCM together with its relationship with macrophages M2.

Gender Differences in HCM

To explore the potential role of gender differences in HCM, we selected samples of HCM patients from two datasets and performed differential expression analysis according to gender. Obtained DEGs between male and female HCM patients are

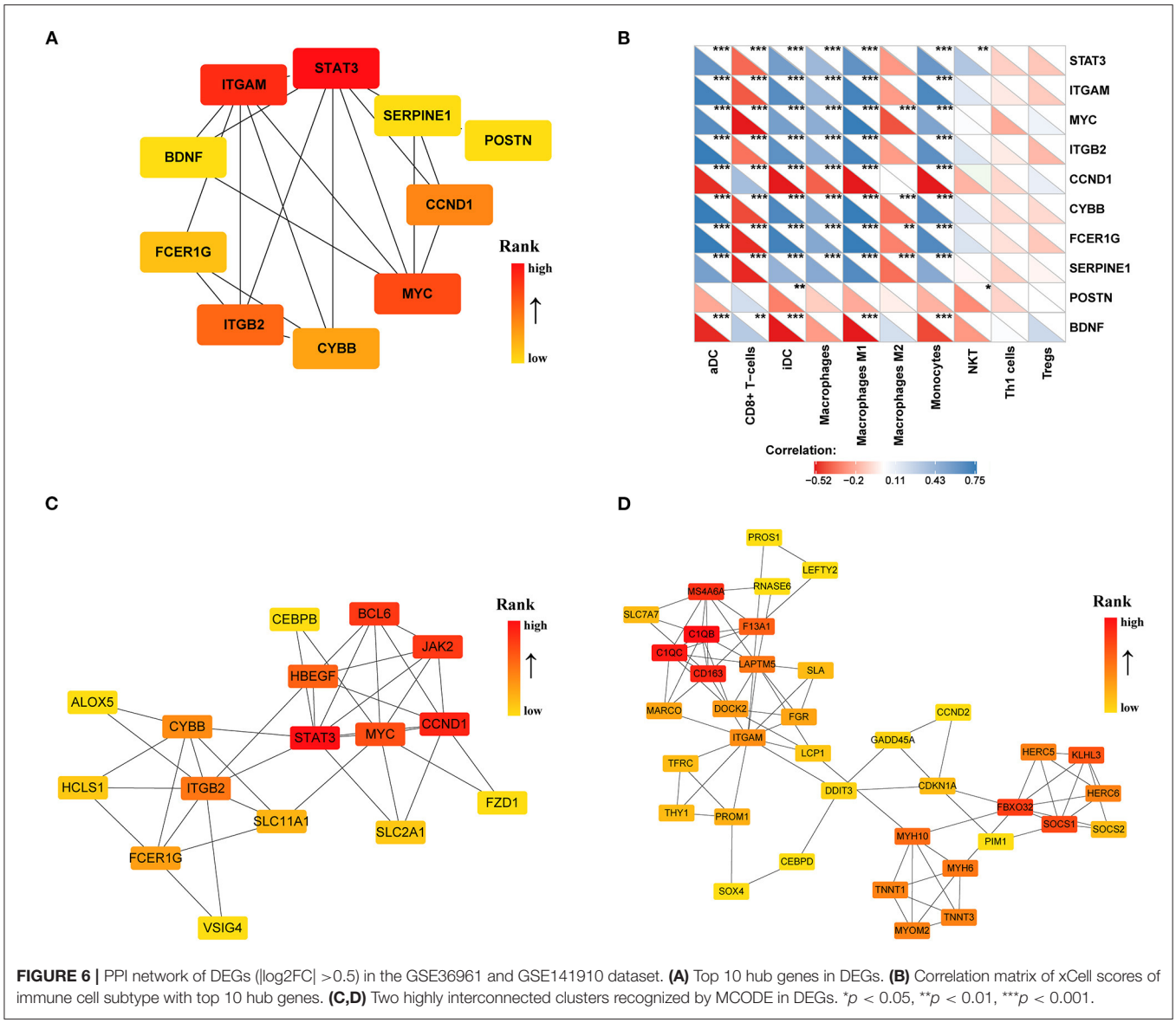

FIGURE 6 | PPI network of DEGs (|log2FC| >0.5) in the GSE36961 and GSE141910 dataset. **(A)** Top 10 hub genes in DEGs. **(B)** Correlation matrix of xCell scores of immune cell subtype with top 10 hub genes. **(C,D)** Two highly interconnected clusters recognized by MCODE in DEGs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

shown in **Supplementary Table 6**. Most DEGs are related or linked to sex chromosomes. In addition, MYL4 (myosin light chain 4) was found to be upregulated in male HCM patients from GSE36961, NPPA (Natriuretic Peptide A) was upregulated in female patients, and VIT was upregulated in male patients from GSE141910.

Furthermore, we examined whether gender differences would affect the expression of CD163 and LYVE1 in cardiac tissues of HCM patients. Still, we used the expressional matrix of GSE36961 and GSE141910 to calculate the relative expression differences of CD163 and LYVE1 between male and female. As shown in **Supplementary Figure 2**, there seems to be no gender difference in the expression of CD163 and LYVE1 in HCM patients.

Verification of Crucial Genes and Immune Cells in HCM

We chose GSE130036 as the validation dataset to verify the expression profiles of CD163 and LYVE1 between HCM and healthy control cardiac tissue. As shown in **Figures 8A,B**, CD163 and LYVE1 were significantly low expressed in HCM while the expression profiles of these two genes show a strong positive correlation (Spearman correlation = 0.7646). The immunofluorescence staining results in **Figure 8C** presented a good co-localization relationship between LYVE1 and macrophage M2. Above all, these results verified our previous analysis and suggested that the dysfunction of LYVE1^{+} CD163^{+} macrophages plays a vital role in the progression of HCM.

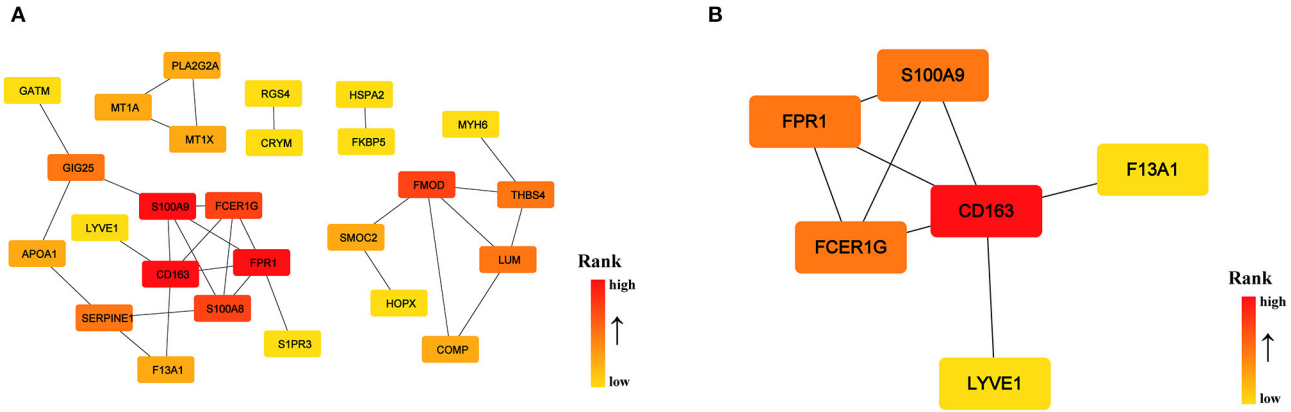

FIGURE 7 | PPI network of significant DEGs (|log2FC| >1.0) in the GSE36961 and GSE141910 dataset. **(A)** PPI network of significant DEGs. **(B)** Cluster recognized by MCODE in significant DEGs.

TABLE 1 | Relationships between candidate key genes with immune cells († Log_2FC from GSE36961/GSE141910).

Gene symbol	$Log_2FC^{\dagger}$	Adj. *p*-value	Related immune cells
CD163	−2.113/−2.704	<0.001	Monocytes
FPR1	−1.367/−1.313	<0.001	Neutrophil/Monocytes
S100A9	−2.879/−1.534	<0.001	Neutrophil/Monocytes/Lymphocytes
FCER1G	−1.482/−1.020	<0.001	Monocytes/Lymphocytes
LYVE1	−1.863/−2.002	<0.001	Monocytes
F13A1	−1.577/−1.179	<0.001	Neutrophil/Monocytes/Lymphocytes

† *represents the annotation in the title box.*

DISCUSSION

The specific underlying pathogenesis of HCM, one of the main causes of sudden death in young adult, is still unclear. More and more studies have shown that cardiovascular diseases, such as myocardial infarction (MI) and heart failure, are often accompanied with abnormal pro-inflammatory activation of the immune system and disorders of the cardiac-resident immune cells (21). Exploring the mechanism of the key immune cells and pathways in the pathogenesis of cardiomyopathy can clarify the specific role of the immune system in the maintenance and imbalance of cardiac function to a certain extent, so as to provide potential therapeutic targets on future cardiovascular immunotherapy.

It is well-known that macrophages have strong plasticity and will be polarized in response to different environmental stimuli during development (22). A widely used paradigm is defined as "classical" activated macrophages M1 and "alternative" activated macrophages M2 based on their function (23). Macrophages M1 are usually pro-inflammatory cells involved in the initial inflammatory reaction, while macrophages M2 have anti-inflammatory effects and are closer to the characteristics of steady-state resident tissue macrophages (RTM) (24, 25). Macrophage is the most important member of cardiac immune cells. In the healthy heart of adult mice, macrophage accounts for about 5–10% of the total number of non-cardiomyocytes, while in the resident $CD45^{+}$ immune cells, it accounts for up to 80% (26). With the deepening of research, the biological functions of cardiac RTM continue to expand. In a healthy heart, cardiac RTM is involved in promoting the development and maturation of coronary arteries in the embryonic stage (27), promoting atrioventricular node electrical conduction (28), and maintaining myocardial mitochondrial homeostasis (29). After the heart of newborn mice is injured, the RTM in it inhibits bad ventricular remodeling by promoting the proliferation of cardiomyocytes and the formation of blood vessels (30). In adult MI model mice, removal of cardiac RTM resulted in worsening of heart function and bad ventricular remodeling (31). Therefore, cardiac RTM not only participates in the maintenance of healthy heart tissue homeostasis but also participates in the repair of the heart after injury. Considering the significant number of macrophages in cardiac tissue together with its important organ-specific functions, it is necessary to further explore their phenotypes, functions, and dynamic changes between steady-state cardiac with disease-state.

LYVE1 (lymphatic vessel endothelial receptor-1) is one of the main receptors of hyaluronic acid in the body. Previous studies have found that LYVE1 is highly expressed on the basal surface and inner surface of lymphatic endothelial cells, and it participates in regulating the entry of immune cells from tissues into lymphatic vessels by combining with the hyaluronic acid coating on the surface of inflammatory cells (32). Deletion of Lyve1 in mice will prevent entering and traffic of leukocytes through the lymphatic endothelium, causing worsening of chronic inflammation and long-term deterioration of cardiac function (32). Recently, based on single-cell transcriptome analysis, Chakarov et al. found two cardiac RTM subgroups in mice identified by $LYVE1^{low}$ and $LYVE1^{high}$. $LYVE1^{low}$ cardiac RTM is mainly distributed around nerve fibers with the strong ability of antigen presentation, while $LYVE1^{high}$ cardiac RTM is

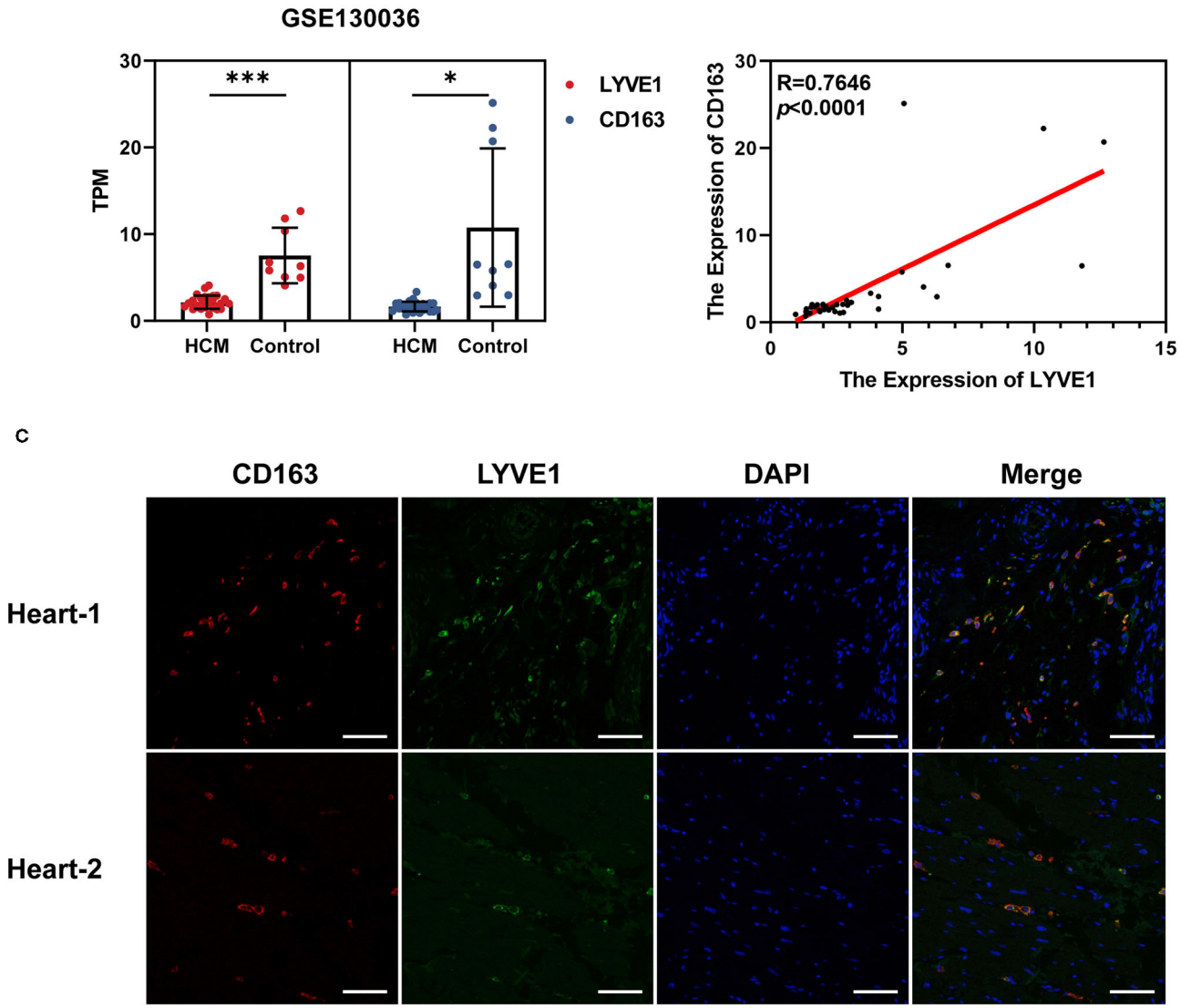

FIGURE 8 | Verification of crucial genes and immune cells in HCM. **(A)** Expression profiles of CD163 and LYVE1 in the verification dataset GSE130036. **(B)** Correlation plot of CD163 and LYVE1 in the verification dataset GSE130036. **(C)** Representative fluorescent staining images of heart-infiltrating $CD163^+$ and $LYVE1^+$ macrophages from cardiac tissues of two healthy donors (scale: 100 μm). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

mainly distributed around capillaries with possible anti-fibrosis function (33). Together, the existing research results have already hinted to us the possibility that $LYVE1^+$ macrophages may serve as homeostatic markers in cardiac tissue. Our results further suggest that the reduction of $CD163^+LYVE1^+$ macrophages may be an important process for the pathogenesis of HCM; it may act as a bridge between the heart's immune system and the lymphatic system to maintain the steady-state function of the heart. The process of pathological myocardial hypertrophy could mediate cardiac dysfunction and long-term damage by interfering with this connection, thereby affecting the clinical symptoms and prognosis of HCM patients.

STAT3 is one of the members of the STAT family and can be expressed in various tissues and organs, including myocardium (34). In recent years, a number of studies have found that STAT3 and its related signaling pathways play a key regulatory role in the pathophysiological processes of cardiovascular system, such as myocardial hypertrophy (35), ischemia–reperfusion injury (36), and myocardial fibrosis (37). Several lines of evidence have indicated that members of the IL-6 family are able to induce cardiomyocyte hypertrophy via gp130-mediated STAT3 activation while deletion of IL-6 weakened transverse aortic constriction-induced ventricle hypertrophy together with marked attenuation

of STAT3 activation (35). Our analysis results suggest that more in-depth research is needed to explore the relationship between STAT3-related pathways and the pathogenesis of HCM, so as to provide ideas to further search for related treatment strategies.

Gender difference in HCM has always been an attractive perspective. Studies have shown that female HCM patients are generally older, have more severe obstructive symptoms than male, and are at higher risk of progression to heart failure or death (38). However, the specific mechanism of this phenomenon is still unclear. We grouped HCM samples by gender and performed differential expression analysis and found that DEGs were mainly related or linked to sex chromosomes, while MYL4 and VIT were found to be slightly upregulated in the cardiac tissue of male patients, and NPPA was upregulated in female. It is reported that the percentage of MYL4-positive cells in male ventricular myocardium is higher, and it is also moderately related to HCM (39). However, there is still no evidence to prove the specific mechanism of gender regulating MYL4 expression in ventricular tissues. NPPA is often considered to be a marker of cardiac hypertrophy, and its elevated levels may be related to worsening cardiac function (40). The level of NPPA in the ventricular tissue of female HCM patients was significantly higher than that of males, suggesting that the female patients analyzed had a higher degree of deterioration of cardiac function, which was similar to the aforementioned clinical statistical results. VIT (vitrin) encodes an extracellular matrix (ECM) protein and its connection with HCM and gender has not yet been reported, and further exploration is needed. We also explored whether there is a difference in the expression of CD163 and LYVE1 in HCM patients of different genders, and the results showed that there is no significant difference in the expression of the two genes between male and female. Based on the above analysis, we temporarily believe that the differences in the clinical manifestations and prognosis of HCM between male and female may be more related to differences in diagnosis and clinical pathways caused by social, genetic, and endocrine factors, but still, more in-depth research on gender differences in HCM patients is needed.

In this study, we analyzed the GSE36961 and GSE141910 datasets related to HCM. A total of 339 samples are included to comprehensively analyze the state of immune cell infiltration in HCM and the correlation between immune cells. In addition, we built a network to screen for genes that play a key role in HCM and validated the results by verifying the dataset GSE130036 and the collected clinical samples for immunofluorescence analysis, which made our research results more reliable. In addition, the impact of gender differences in HCM was also explored, but we have not obtained any novel findings. In all, our results indicate the potential important roles of STAT3-related signaling pathway and $LYVE1^{+}CD163^{+}$ macrophages in the pathogenesis of HCM. It is worth noting that CD163, as a typical marker of macrophages M2, exhibits a good co-localization and a strong positive correlation with LYVE1. We speculate that the decrease in the proliferation of $CD163^{+}LYVE1^{+}$ macrophages or the decrease in the number caused by phenotypic conversion is a key part of the pathogenesis of HCM. It is necessary to study the specific mechanism of the role of this macrophage subset in cardiac hypertrophy, and the development of immunotherapy targeting RTM may help to alleviate heart injury and promote the recovery of heart function.

However, our study has certain limitations. The most important thing is that our study was based on bioinformatics immune infiltration analysis from the transcriptomic profiles of public datasets, which may be in discordance with actual scenarios. Secondly, whether there is a causal relationship between gene expression differences and the pathogenesis of HCM or whether it was just modified by compensatory mechanisms cannot be clearly determined. Finally, due to the difficulty of obtaining cardiac samples from HCM patients, we only verified the co-localization relationship between LYVE1 and CD163 in normal donated hearts, instead of comparing and quantifying the difference in the number of $CD163^{+}LYVE1^{+}$ macrophages in the cardiac tissue of HCM patients and healthy donors.

CONCLUSION

In summary, through bioinformatics analyses of public transcriptome data, STAT3-related pathway and $CD163^{+}LYVE1^{+}$ macrophages were identified as the potential key pathway and immune cells in HCM and may serve as interesting targets for further in-depth research.

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 in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by medical ethics committee of the Tongji Medical College of Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

X-ZZ and SZ conducted statistical analysis, carried out the experiments, and drafted the article. T-TT contributed to reviewing the article. XC edited and revised the article. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.696321/full#supplementary-material

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Molecular Interactions Between Vascular Smooth Muscle Cells and Macrophages in Atherosclerosis

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Atherosclerosis is the largest contributor toward life-threatening cardiovascular events. Cellular activity and cholesterol accumulation lead to vascular remodeling and the formation of fatty plaques. Complications arise from blood clots, forming at sites of plaque development, which may detach and result in thrombotic occlusions. Vascular smooth muscle cells and macrophages play dominant roles in atherosclerosis. A firm understanding of how these cells influence and modulate each other is pivotal for a better understanding of the disease and the development of novel therapeutics. Recent studies have investigated molecular interactions between both cell types and their impact on disease progression. Here we aim to review the current knowledge. Intercellular communications through soluble factors, physical contact, and extracellular vesicles are discussed. We also present relevant background on scientific methods used to study the disease, the general pathophysiology and intracellular factors involved in phenotypic modulation of vascular smooth muscle cells. We conclude this review with a discussion of the current state, shortcomings and potential future directions of the field.

Keywords: atherosclerosis, coronary artery disease, inflammation, cell crosstalk, smooth muscle cells, monocytes/macrophages, immunity

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INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide with atherosclerosis being the largest contributor toward cardiovascular events such as myocardial infarctions and strokes (1, 2). The disease is characterized by systemic inflammation and a buildup of fatty plaques in the arterial vessel wall (3). Medical complications arise through the restriction of blood flow due to lumen encroachment by the plaque or thrombosis occurring at sites of plaque rupture or erosion (4). Atherosclerosis develops over many years and progresses through a complex interplay between vascular cells, infiltrating leukocytes, endothelial shear stresses, and systemic factors such as liver-induced impairment of fibrinolysis or signaling from adipose tissue (4–6).

Elevated levels of cholesterol-associated apolipoprotein (apo) B are required for the development of atherosclerosis in humans and animal models (7). Intimal retention of these lipoproteins is facilitated by the production of proteoglycans from vascular smooth muscle cells (VSMCs). In response to sub-endothelial accumulation and oxidation of low-density lipoprotein (LDL), circulating leukocytes infiltrate into the arterial vessel wall. This critical event typically initiates the onset of the disease and the formation of early atherosclerotic lesions. Inside the vessel, monocytes differentiate into macrophages, engulf residential LDL, and adopt a pro-inflammatory phenotype (3, 8). Many

diverse cell-types play significant roles in disease progression. However, in numbers, macrophages and VSMCs dominate the landscape. Both cell types retain the capacity to engulf modified LDL and gradually transform into lipid-laden foam cells that amplify atherogenesis and aggravate the disease (4).

Hence, VSMCs and macrophages undergo remarkably diverse phenotypic transitions and their derived cell types play dominant roles in developing plaques. Furthermore, the fate of these cells is pivotal for disease progression and outcome (4, 9–11). Interactions between both cell types and their influence on the disease are still not well-understood. A better comprehension of the molecular changes they undergo might lead to new insights on how to prevent or revert disease progression; this may be particularly true at early stages where lesional complexity is low and cells might be amenable to reset toward a more reparative phenotype.

Experimental Approaches

Mouse models with genetic deletions such as ApoE–/– or LDL receptor–/–, which prime animals toward hypercholesterolemia and atherosclerosis, have been used extensively to explore the disease in live organisms. Additional gain- or loss-of-function mutations have been applied to investigate the impact of specific gene products or cell types on lesion progression. When applied to bone marrow transplants, they are useful for exploring the function of specific genes in hematopoietic vs. non-hematopoietic cells (12). Additionally, specific myeloid or lymphoid cells can be targeted at a chosen time using tamoxifen-inducible CreERT2 recombinase under the control of a cell-specific promoter such as LysM (macrophages and neutrophils), F4/80 (tissue macrophages), or CX3CR1 (certain monocytes and macrophages) (13). Alternatively, the loxP-Cre system can induce tissue-specific, cell-specific, or timed genetic modifications. Cre-recombinase activity results in targeted genetic excision around sequences sandwiched (floxed) between two loxP sites. Expression of the enzyme can be placed under a tissue/cell-specific or chemically inducible (e.g., tamoxifen) promoter, which allows for a wide range of spatial and temporal control (12, 14). The degree of exploratory freedom that genetic manipulations in live animals offer has played an important role in studying atherosclerosis. However, inter-species differences can limit the translatability of animal models to humans. This limitation is offset by the fact that human studies are expensive and restricted to post-intervention assessments without the power of *in vivo* genetic interventions (15).

The intricacy of atherosclerotic lesions complicates the interpretation of scientific observations. High-resolution techniques such as single-cell RNA-Seq in combination with computational data analysis have great potential to unravel this complexity. However, large amounts of pure cells are required, which can be challenging for small model organisms such as mice. The technology is further limited by technical drawbacks. Errors and biases are introduced at various steps such as during RNA amplification or cell lysis. Most notably, only 10-40% of all transcripts in a particular cell are captured and converted into cDNA (16). Additionally, inferring the function of a particular cell in its environment is currently challenging since metadata on cellular position is generally not available. This is a considerable drawback given that cells are expected to behave vastly differently depending on their position within the plaque and their immediate microenvironment (17). Lastly, the high price for sequencing on the single-cell level currently hampers widespread usage and high-throughput experimentation.

In vitro cultures of animal and human cells have been extensively used to abstract from the complex nature of the disease and focus on simpler, isolated aspects. Historically, three co-culture types have been used to address cell-cell interactions: (1) indirect contact with physically separated cell types, (2) direct cell contact, and (3) 3D scaffolds attempting to model features of the vasculature (18). While a lower level of complexity can be helpful, oversimplification of *in vivo* conditions can also be problematic. Factors relevant to the studied interaction might be missing. VSMCs in culture are known to undergo phenotypic switching with reduced contractility, enhanced proliferation, and susceptibility toward apoptosis (19–21). Cells with corrupted phenotypes may produce misleading results that are not transferable to live animals or humans.

PATHOPHYSIOLOGY

Pathological intimal thickenings (PITs) are believed to characterize the onset of atherosclerosis and develop from diffuse intimal thickenings (DITs) of the arterial vessel wall. DITs are characterized by their high proteoglycan content which facilitates the retention of apolipoproteins. Through continuous lipid retention, monocyte recruitment and foam-cell formation, DITs may progress toward sites of chronic inflammation with enhanced production of inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and macrophage colony-stimulating factor (M-CSF) (4, 22). Additional molecular processes occur that favor disease progression, such as accumulation of reactive oxygen species (ROS) and increased nitric oxide (NO) production (23, 24). Elevated levels of apolipoprotein B (apo B) containing lipoproteins such as LDL and VLDL are a prerequisite for atherosclerotic plaque development, independent of additional risk factors (7). Other critical events include enhanced endothelial permeability and increased monocyte recruitment from the circulation (23–25).

Atherogenic processes are often interconnected and may substitute or influence each other. For example, the transcription factor NF-κB is both redox-sensitive and a well-established master regulator of inflammation (26). Oxidative stress may, therefore, lead to inflammation and hence activation of endothelial cells (ECs). Both permeability and upregulation of surface binding proteins are enhanced in activated ECs and may facilitate monocyte infiltration (24, 27). Release of chemokines largely regulates the attraction of monocytes to the endothelial layer where binding occurs *via* surface proteins such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Infiltrating monocytes may differentiate into macrophages and further drive atherogenic processes including EC activation and inflammation.

Early Lesions

Within the intima, lipids are modified by resident oxygen radicals and enzymes, which precedes macrophage recruitment. Particularly, the production and retention of oxidized low-density lipoproteins (oxLDL) promote the differentiation of VSMCs into foam cells. Hence, early DITs contain mainly dedifferentiated VSMCs. These in turn synthesize proteoglycans that facilitate the retention of LDL. Proteoglycans play a critical role in early lesion formation but are not necessary for atherosclerosis to proceed (7, 28). Experiments showed no difference in advanced plaques from mice with proteoglycan binding deficient LDL and normal LDL (28). LDL engulfment also transforms intimal macrophages into lipid-laden foam cells, which are a hallmark of early pathogenesis (26, 29, 30). Once fully differentiated, foam cells are largely immobilized, sustain inflammation, and exhibit increased rates of apoptosis (23). Progression toward pathological intimal thickenings (PITs) is promoted through enhanced subendothelial LDL retention, LDL modification, inflammation, apoptosis, and phenotypic switching of VSMCs (4).

Advanced Lesions

The majority of life-threatening cardiovascular events arise from the rupture or erosion of advanced atherosclerotic plaques. Forming blood clots around the damaged vascular site may detach and form thrombotic occlusions further downstream in the vasculature. Eroded plaques tend to have an intact fibrous cap rich in ECM components and occur because of endothelial cell desquamation (31). In contrast to ruptured plaques, eroded lesions typically have lower lipid levels, fewer inflammatory cells such as macrophages and lymphocytes, and contain more VSMCs. The forming thrombus is also different between the two types. Thrombi formed during plaque erosion contain mainly platelets, whereas those from plaque rupture are rich in fibrin (31, 32).

Advanced atherosclerotic plaques are generally characterized by regions of enhanced cell death, calcification, necrotic core formation, and defective efferocytosis, which is the process by which apoptotic debris are cleared through phagocytic activity (4, 5, 33). VSMCs take on diverse roles as plaques evolve. Synthetic VSMCs near the endothelium produce a collagen-rich fibrous cap, which protects the plaque from rupture. Consequently, low VSMC counts and enhanced inflammation have long been associated with plaque vulnerability (5, 22, 33, 34). The presence of macrophages at the fibrous cap may further compromise plaque stability by initiating VSMC apoptosis, contributing toward inflammation, and secreting MMPs (22, 33–35). In addition, plaque VSMCs can differentiate into osteochondrogenic-like cells, releasing minerals and bone-generating factors (4). The impact of calcification on plaque stability seems largely a function of the pattern, location, and size of calcium aggregates. Spotty microcalcification with the formation of nodules near the fibrous cap appears to enhance vulnerability, whereas sheet-like macrocalcifications are associated with more stable plaques (36, 37). However, macrocalcification of the medial layer also confers arterial stiffness which may lead to systemic cardiovascular complications such as hypertension that may ultimately aggravate or initiate atherosclerosis (38). Chronic VSMC apoptosis in mice has also been linked to enhanced intimal calcification (39). In VSMC cultures, caspase targeted inhibition of apoptosis led to a 40% reduction of calcified nodules, while stimulation of death receptor Fas led to a 10-fold increase (40). These findings suggest VSMC death to be a significant factor in vascular calcification. Additionally, it was shown that apoptotic bodies, released during apoptosis, may serve as nucleation points for the formation of concentrated spotty calcium residues (40, 41). Mineral deposits, in turn, have been shown to induce cell death in VSMCs (21, 42). This may constitute a positive feedback loop *in vivo* where VSMC apoptosis and intimal calcification amplify each other. Thus, defective efferocytosis in combination with increased cellular death may be pivotal for plaque calcification. Furthermore, defective clearance leads to the aggregation of dead cell debris and lipids to form a growing necrotic core that weakens plaque integrity. Uncleared apoptotic cells may also undergo secondary necrosis and release potent proatherogenic cytokines that enhance inflammation and aggravate the disease (4, 19).

The use of single-cell sequencing in human and mice studies has recently identified lesional macrophage subsets that exceed the canonical surface marker-based paradigm. Furthermore, commonly used markers were expressed across distinct populations, suggesting that macrophage subtypes and functionalities may not be as clearly delimited as previously assumed. Interestingly, phenotypic diversity of lesional macrophages appeared to increase over time, which may be a direct consequence of the evolving plaque complexity (10, 43, 44). Given the high degree of macrophage diversity and their importance in atherosclerosis, cues that influence the balance of macrophage differentiation may have a critical role in disease progression and outcome.

Sex Differences

Significant age and sex differences exist in the incidence and constitution of plaques. Prior to menopause, women are relatively protected from cardiovascular disease with smaller necrotic core volumes and generally more stable plaques, which suggests a protective role for estrogen (45, 46). Estrogen in mice leads to a reduction of macrophage recruitment through two distinct mechanisms. Estrogen reduces endothelial expression of ICAM-1 and VCAM-1, which are critical for leukocyte binding, and it downregulates the synthesis of monocyte attracting chemokines such as CCL2. There is also evidence that estrogen regulates inflammation. Young women exhibit peak annexin A1 expression when serum estrogen levels are at their highest. Annexin A1 is an anti-inflammatory that plays a central role in the phagocytic removal of apoptotic leukocytes (efferocytosis). Furthermore, estrogen also acts on macrophages *via* estrogen receptors α and β. Receptor activation promotes macrophage polarization to an anti-inflammatory phenotype and the release of inflammation resolving factors such as IL-10 and TGF-β (46). Interestingly, a recent investigation of gene regulatory networks (GRNs) discovered differences between the sexes in the expression of key genes involved in atherosclerosis. Single-cell

RNA sequencing of mice plaques suggested that phenotypically modulated VSMCs were responsible for a majority of the detected GRN drivers in females. Consequently, sex differences in the regulation of VSMC phenotype modulation may also play a significant role in the observed differences in cardiovascular disease (47).

VSMC PHENOTYPIC MODULATION

VSMCs in atherosclerosis may accumulate in the tunica intima and transition to several distinct cell types. Signals from the micro-environment lead to transcriptional changes that result in phenotypic modulation. During this process, VSMC markers are lost, and genes typical of other cell types are up-regulated (48). Three major sub-populations have been identified. Synthetic VSMCs secrete extracellular matrix (ECM) components that increase LDL retention in early atherosclerotic lesions and stabilize the developing plaque at later stages. The osteochondrogenic-like cell type may enhance plaque vulnerability by excreting mineral deposits. Lastly, VSMCs may transition to a foam cell phenotype with surface markers indistinguishable from macrophages (4, 29).

Migration and De-differentiation

The majority of intimal VSMCs originate from vascular smooth muscle cells of the tunica media. Medial VSMCs detach and de-differentiate into a synthetic phenotype that produces and modulates extracellular matrix (ECM) components (4). Other cell populations such as myeloid, endothelial, and vascular resident stem cells may contribute to intimal VSMC accumulation by differentiating into an VSMC-like phenotype. Their relative abundance and impact on disease development remain, however, controversial (49–52). De-differentiation of medial cells is typically initiated in response to external cues such as vascular injury or inflammation. Agents known to promote VSMC migration are primarily growth factors and inflammatory cytokines such as angiotensin II (Ang II), vascular endothelial growth factors (VEGF), interleukin-6 (IL-6), and TNF-α but also extracellular matrix components including collagen I, IV, and VIII. Through the activation of receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), external signals are relayed and induce the remodeling of the cytoskeleton. Major pathways include the phosphorylation of various kinases such as Rho-, p21-, and mitogen-activated protein kinases. Downstream targets, such as actin-related protein 2/3 and heat shock protein 27, are involved in actin polymerization or participate in generating traction forces such as myosin II (53). Synthesized matrix metalloproteinases, such as MMP-2, -3, and -9 degrade cellular adhesion proteins and enable the mobilization of VSMCs into the intimal layer (54, 55).

MMP expression may influence plaque progression in various ways. Collagenases such as MMP-1, -8, and -13 have been associated with plaque vulnerability and may directly participate in degrading the collagen-rich fibrous cap (56, 57). MMPs have been implicated in a wide range of additional atherogenic processes such as enzymatic modification of LDL (MMP-2/MMP-9) (58), inflammation (MMP-14) (59), cellular proliferation (MMP-9/MMP-14) (59–61), apoptosis (MMP-7/MMP-12) (57), and vascular calcification (MMP-2) (62). Consequently, MMPs can have positive and negative influences on disease progression, depending on the surrounding context of MMP synthesis and which types are expressed.

Macrophage Marker Expression

Foam cell formation is a hallmark of early atherogenesis. These cells exhibit unbalanced uptake, esterification, and efflux of lipids, leading to an accumulation of cholesterol droplets and a foamy appearance (26). Foam cells were initially believed to originate exclusively from infiltrating monocytes due to the expression of surface markers typical for macrophages. The scavenger receptor Cluster of Differentiation 68 (CD68), for example, is expressed by foam cells and typically found on cells of monocyte lineage. However, recent studies have shown that VSMCs contribute significantly to CD68 positive foam cells in human and mouse atherosclerotic lesions. Through uptake of LDL, VSMCs initiate the transition toward a dysfunctional macrophage-like phenotype. In the process, smooth muscle-markers such as smooth muscle α-actin (SMA) and myosin heavy chain (MYH11) are either down-regulated or lost entirely. In contrast, macrophage-associated markers such as ABCA1, CD68, and LGALS (MAC-2) are up-regulated (9, 63). Studies in live mice have highlighted Kruppel-like factor 4 (KLF4) as a central agent in oxLDL-induced phenotypic switching of VSMCs. Furthermore, loss of KLF4 has been associated with a marked reduction in plaque size and increased fibrous cap thickness without affecting the overall number of lesional VSMCs (55). This suggests that phenotypic VSMC modulation and the relative abundance of different phenotypes have a stronger influence on plaque stability than total intimal VSMC count. Compared to foam cells derived from macrophages, smooth muscle-derived foam cells appear to exhibit lower expression of ABCA1, which may suggest a reduced capacity for cholesterol efflux (9). Therefore, VSMCs may have a higher propensity to transform into foam cells and to resist cholesterol-efflux-dependent plaque regression.

Calcification

Arterial intimal calcification (AIC) is positively correlated with plaque burden, progression, and vulnerability (36, 64). Fate mapping studies have identified VSMC-derived osteoblast- and chondrocyte-like cells as significant contributors toward AIC in tandem with bone marrow-derived cells (65, 66). In human atherosclerotic plaques, osteogenic VSMCs are associated with lipid-rich, calcified regions. Cholesterol accumulation in VSMC cultures is required for calcification and promotes the expression of factors involved in osteogenic differentiation, such as alkaline phosphatase and bone morphogenic protein (BMP-2) (41, 67). Particularly modified LDLs enhance calcification and phenotypic modulation (30). The master regulator runt-related transcription factor 2 (Runx2) is pivotal for osteochondrogenic differentiation of VSMCs and vascular calcification (38).

Oxidative stress in the form of accumulating ROS and inflammatory cytokines, such as IL-6 and TNF-α, have been linked to increased Runx2 expression and are believed to

play essential roles in vascular calcification (38, 68, 69). In a recent study on mice, vascular smooth muscle-specific KO of Runx2 significantly reduced AIC without affecting other pivotal aspects of the disease, such as atherosclerotic lesion formation or macrophage content. Different stages of chondrocyte differentiation were examined by measuring VSMC expression of Sox9 (prechondrogenic fate decision), Col II (early differentiation), and Col X together with MMP13 (maturation). Early markers were unaffected by the Runx2 deficiency, while MMP13 and Col X were significantly inhibited, suggesting that Runx2 is involved in the maturation process rather than the early stages of phenotypic switching (38). Further downstream, expression of the membrane-bound alkaline phosphatase TNAP ultimately enables extracellular phosphate capturing and mineralization (67). Recent evidence shows that the pattern of intimal calcification is relevant to plaque burden and vulnerability (36).

MOLECULAR INTERACTIONS BETWEEN VSMCs AND MACROPHAGES

Within the atherosclerotic micro-environment, macrophages and VSMC are found in close proximity. Intercellular interactions occur through direct contact or the exchange of soluble factors and are believed to have a significant impact on the disease (**Figure 1**) (3, 70, 71).

Exchange of Soluble Factors

VSMC to Macrophage Signaling

Macrophage recruitment is an important step in atherosclerotic lesion formation, and although monocyte adherence to endothelial cells is crucial, VSMCs also play a critical role in this process by releasing chemoattractants such as CCL2, CXCL1, and bone morphogenic proteins (BMPs) (72–74). The presence of oxLDL in the vessel wall stimulates the production of chemoattractants in both ECs and VSMCs (75). One study has implicated protease-activated receptor 2 (PAR2) in macrophage recruitment. PAR2 is involved in the inflammatory process and localizes to intimal VSMCs in atherosclerotic lesions (73, 74). Its activation in primary VSMCs of mice was shown to increase the secretion of CCL2 and CXCL1. *In vivo* knockout of PAR2 in vascular cells resulted in reduced expression of CCL2/CXCL1 and lower macrophage content in atherosclerotic lesions. Further, plaques were more stable, with increased smooth muscle ACTA-2 and collagen content and reduced inflammatory factors such as IL-1 and TNF-α (76).

VSMC-expressed BMP-2 and BMP-4 may also participate in the recruitment of macrophages to atherosclerotic lesions. Migration assays on primary mouse cells have shown that both BMP-2 and-4 attract monocytes through activation of BMPRII. The presence of antagonist Gremlin or siRNA significantly reduced migration (77). Furthermore, studies have shown that macrophages enhance BMP-2 expression in VSMCs by releasing proinflammatory cytokines such as TNF-α (78). Bone morphogenic proteins are implicated in a variety of atherogenic processes, including calcification (67), plaque instability (76), inflammation (77), and phenotypic modulation of VSMCs (65, 79). TNF-α has also been shown to increase alkaline phosphatase (ALP) activity and calcification in human VSMC cultures (80, 81). The BMP-TNF-α axis thus constitutes a link between macrophage recruitment and vascular calcification *in vivo*.

In the vessel wall, growth factors are generally considered to induce proliferation and amplification, but they also contribute to macrophage recruitment. In a model of angiotensin II (Ang II)-induced vascular remodeling, activation of the key regulatory transcription factor hypoxia inducible factor 1-alpha (HIF1α) was examined. Cre-recombinase-controlled HIF1α deficiency in VSMCs revealed a central role for macrophage recruitment through CCL7 signaling. Both VSMC HIF1α deficiency and CCL7 neutralization, respectively, suppressed the Ang-II induced recruitment of macrophages and subsequent vascular remodeling in mice. Further, HIF1α depletion disrupted only accumulation of CD206– macrophages consistent with a M1 phenotype. Infiltration of CD206+ macrophages, T-cells, and neutrophils were unaffected, suggesting a specific recruitment of inflammatory macrophages (82).

Macrophage to VSMC Signaling

A 2016 study employed transwell co-cultures of human aortic VSMCs and PMA-activated macrophages. Synthesis of Col I and Col III in VSMCs were demonstrated to be significantly reduced, while MMP1/9 expression was increased in both VSMCs and macrophages in response to co-culturing. This may decrease plaque stability from two angles. First, by reducing collagen synthesis and, second, by degrading ECM components of the fibrous cap. Furthermore, both cell types showed elevated expression levels of IL-1β, TLR-2, and VEGF-A, which may further reduce plaque stability through inflammation, neovascularization, and calcification (56, 70, 71, 83).

However, *in vivo* implications might be less straightforward. VEGF-A for example has also been demonstrated to be atheroprotective by promoting endothelial repair during vascular injury (84). Recently, macrophages were shown to attenuate VSMC apoptosis. IL-5 expression was found to be decreased in aortas from patients with acute aortic dissection (AAD) and localized to macrophages (35, 85). In membrane-separated co-cultures with primary mouse cells, macrophage IL-5 overexpression significantly reduced VSMC apoptosis by modulating the death regulators Bax and Bcl-2 (35). Macrophages exhibiting increased IL-5 expression may keep VSMC numbers high and consequently be beneficial for maintaining plaque stability. However, reduced ECM synthesis combined with increased VSMC survival may also lead to the accumulation of pathological phenotypes such as foam or calcifying cells. Such findings underline the importance of understanding the function of cellular subtypes and their roles in different pathological environments.

A recent study demonstrated nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome activation in VSMCs after co-culture with oxLDL-activated monocytes (34). NLRP has previously been demonstrated to play a significant role in atherosclerosis and was shown to transform VSMCs into a macrophage-like phenotype.

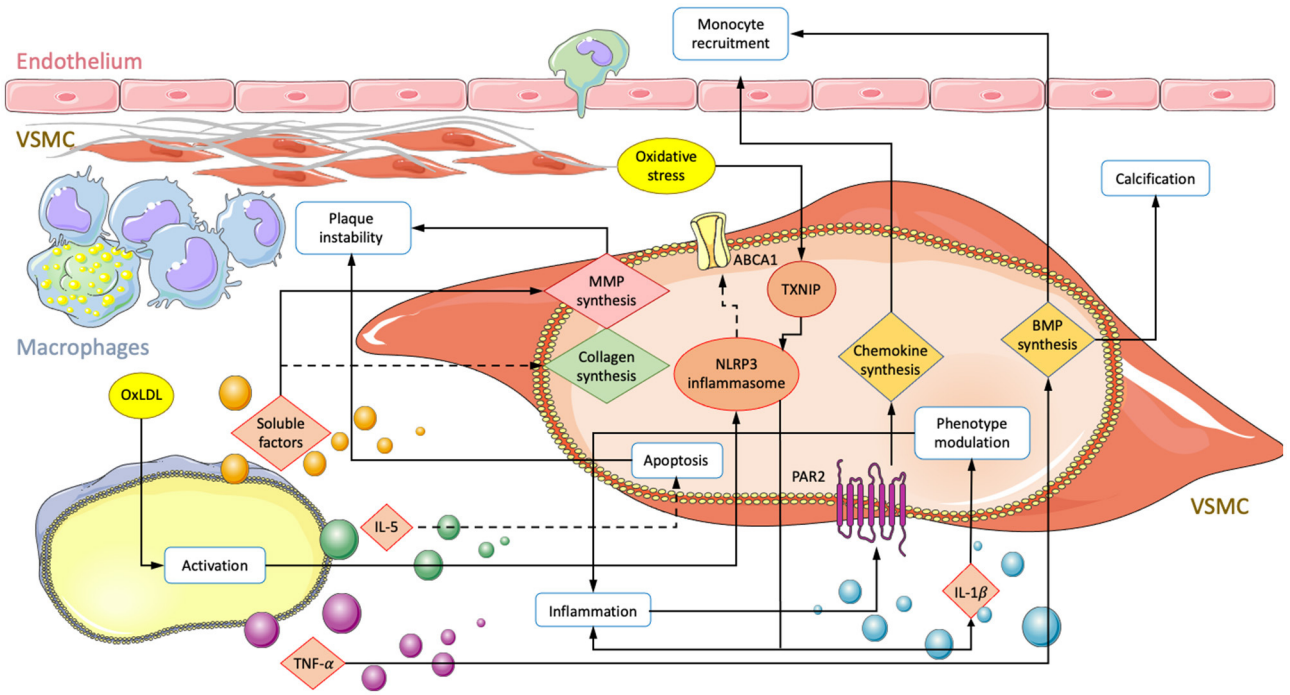

FIGURE 1 | Summary of interactions involving an exchange of soluble factors between macrophages and VSMCs in atherosclerosis. Depiction of a vessel with an endothelial layer on the top and fibrous cap producing VSMCs lying underneath. Macrophage secretion of unestablished soluble factors, TNF-α, IL-5, and oxLDL mediated activation lead to transcriptional changes in VSMC. Solid arrows correspond to enhanced activity and dotted lines represent down regulated processes. Modifications result in increased monocyte recruitment, plaque instability, subendothelial calcification, and inflammation.

NLRP3 induced VSMC proliferation and modulation to a foam cell-like phenotype was dependent on NF-κB activation (86–88). *In vivo*, Apoe−/− mice fed a high cholesterol diet were found to exhibit an increase in VSMCs expressing both CD68 and NLRP3 compared to control mice fed without cholesterol (88). Consequently, macrophages in combination with LDL may promote disease progression and VSMC differentiation to foam cells through NLRP3 inflammasome activation. Foam cell formation from LPS-stimulated primary human VSMCs was shown to depend on NLRP3-induced secretion of high mobility group box-1 protein (HMGB1) and subsequent intracellular lipid accumulation. Loss of function experiments that impaired HMGB1 binding to receptor for advanced glycation end product showed increases in ABCA1 expression and cholesterol accumulation (87). NLRP3 activation in VSMCs has also been shown to lead to maturation and secretion of IL-1β and IL-18 (89). Both cytokines play key roles in atherosclerosis and may participate in modulating VSMCs to a migratory phenotype with enhanced proliferation and expression of inflammatory markers (90–92). Lastly, NLRP has been linked to gasderminD-dependent pyroptosis in VSMCs, which occurs ubiquitously in atherosclerotic lesions and is a form of cell death that impacts atherogenesis, inflammation, and plaque instability (93).

During arterial injury-induced vascular remodeling, Cre-LoxP-directed deletion of dynamin-1-like protein (Drp1) in macrophages significantly suppressed intimal thickening and macrophage infiltration. VSMCs cultured indirectly with macrophages from macrophage-Drp1-KO mice showed reduced proliferation compared to control cells cultured with wild-type macrophages. Additionally, VSMC migration was found to be significantly reduced in media conditioned by macrophage-Drp1-KO rather than normal macrophages. Furthermore, Drp1 knockout and loss-of-function in activated macrophages led to decreases in inflammatory marker expression, including the macrophage attractant CCL2 and platelet-derived growth factor subunit B (PDGF-B); transient overexpression had opposite effects (94). In addition to proliferation and hyperplasia, PDGF-B overexpression in VSMCs has previously been reported to induce the expression of a wide range of monocyte attracting CC-chemokines and inflammatory cytokines. Expression changes were found to be primarily regulated through activation of signal transducer and activator of transcription 1 (STAT1). Increased PDGF-B expression was also shown to dedifferentiate VSMCs into a phenotype consistent with ECM-producing synthetic cells (95). These findings may suggest a positive feedback loop between VSMCs and macrophages that at least in part is regulated by macrophage Drp1 expression. During vascular remodeling, macrophages may initiate VSMC dedifferentiation, migration, proliferation, and expression of monocyte-attracting chemokines. The latter leads to further accumulation of macrophages and the closing of the loop. Additionally, the findings suggest that PDGF-B expression could prime incoming VSMCs toward a reparative phenotype.

Extracellular Vesicle Signaling

Extracellular vesicles (EVs) are a more recent paradigm of intercellular interactions and involve the transfer of complex protein, genetic, or lipid cocktails *via* endocytosis (**Figure 2**). The conferred content modulates gene expression in the recipient cells leading to physiological changes with vital implications in normal and pathological cardiovascular conditions (96–98). EVs are classified into three subtypes, exosomes, microvesicles, and apoptotic bodies. While exosomes are continuously formed, microvesicles and apoptotic bodies require cellular activation or apoptosis, respectively (98). Exosomes are packaged during endocytosis and usually assembled with CD9, CD63, CD81, and components required for endosomal transportation. Such intraluminal vesicles are 50-100 nm in diameter and are released by exocytosis. MVs are generally larger, having diameters of 100-1,000 nm. The formation of MVs is initiated by the transfer of phosphatidylserine (PS) from the inner to the outer membrane leaflet. Release occurs through plasma membrane budding which results in membrane constituents mirroring the parental cell. Having diameters of 1-5 μm, apoptotic bodies are the largest EV type. These vesicles are formed during the final stage of apoptosis and also exhibit a PS-positive phenotype but show greater morphological diversity (99).

Under pathological conditions, EVs have been implicated in various atherogenic processes such as endothelial dysfunction (100), vascular inflammation (101, 102), and oxidative stress (103). Using wound-healing and cell-adhesion assays, circulating EVs from atherosclerotic patients were shown to enhance VSMC migration by +28.6% and adhesion by +15.9% compared to EVs from healthy patients. Further, *in vitro* foam cell-derived EVs (FC-EVs) were investigated. Foam cells were formed from J774a.1 cells cultured with oxLDL and subsequently, EVs were isolated from the culture media. VSMC incubation with these synthetic FC-EVs led to comparable outcomes with +44.6% and +18.6% increases for migration and adhesion, respectively. EVs from normal macrophages (NM-EVs) showed no difference from control cells. Interestingly, FC conditioned media deprived of EVs showed results similar to isolated FC-EVs. The strongest effect was observed with complete FC media containing soluble factors and FC-EVs (97). This may suggest an additive effect of FC-EVs and other soluble factors secreted by foam cells. However, since the purification process is not perfect, it is also conceivable that instead two distinct fractions of soluble factors were isolated that were responsible for the observed effects. Proteomic analysis of FC-EV content revealed factors involved in "actin cytoskeleton regulation" and "focal adhesion" pathways.

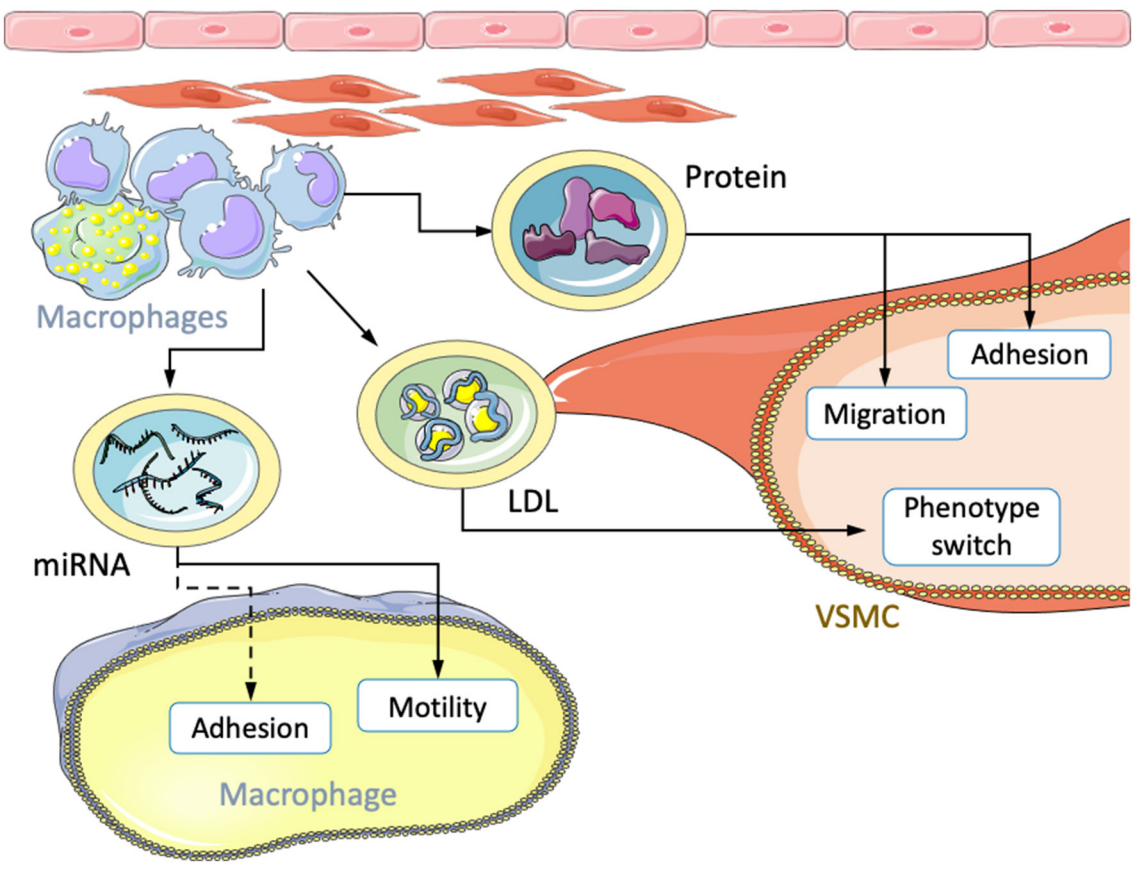

FIGURE 2 | Transfer of atherosclerotic factors *via* extracellular vesicles. Macrophages transfer miRNA, LDL, and protein cocktails to other macrophages and VSMCs. The received content leads to changes in migration, motility, adhesion and phenotypic modulation.

Furthermore, VSMCs cultured with FC-EVs showed increased expression of ERK and Akt, two protein kinases that are known to be involved in cell migration and proliferation (97). Therefore, EVs released by macrophage-derived foam cells may enhance the intimal accumulation of VSMCs through ERK/Akt, while other soluble factors further stimulate VSMCs.

In a similar study, the differential analysis of EVs derived from oxLDL-stimulated human and mouse macrophages revealed enrichment of several miRNAs involved in macrophage motility and adhesion. miR-146a was among the most differentially represented miRNAs. Its transcription was significantly increased in atherosclerotic plaques from mice and humans, suggesting an essential role in both organisms. In the presence of oxLDL-stimulated macrophage EVs, macrophage migration toward CCL2 was significantly inhibited *in vitro*. Further, *in vivo* emigration of peritoneal macrophages in response to LPS injection was also impaired. EVs derived from knockout or siRNA-mediated knockdown of miR-146a increased the migration of naive macrophages compared to EVs from wt mice (96). EVs derived from oxLDL activated macrophages may modify motility and inhibit migration in naive macrophages. Thus, newly infiltrated macrophages may be immobilized and entrapped in atherosclerotic lesions.

Finally, beyond transfer of soluble factors or miRNAs, transwell co-culture experiments with fluorescently labeled cholesterol demonstrated the transfer of oxLDL and acLDL from macrophages to VSMCs. VSMCs formed lipid droplets in response to the co-culture conditions and switched their phenotypes. Interestingly, LDL uptake was much more modest in VSMCs cultures in the absence of macrophages, suggesting a transport mechanism between both cell types (104). That being said, in contrast to macrophage-derived foam cells, those originating from VSMCs generally exhibit reduced phagocytic and efferocytic activity, which may entail accelerated necrotic core growth and inflammation *in vivo* (4). After 14 days of co-culture, VSMCs showed enhanced phagocytic activity and expression of typical macrophage markers, including *Cd68* and *Mac2*. Xenogenic co-cultures (rat VSMCs and human macrophages) demonstrated that no human smooth muscle marker transcripts were expressed during transwell co-culture, but that cell-cell contact was instead required. Endosomal transportation was ruled out; transfection of macrophages with the reporter LAMP1-mKate2 revealed that macrophage-derived lysosomes were responsible for the transfer of LDL (104). Given that cell-contact was required for cholesterol uptake, close proximity to lipid-laden macrophages may be crucial for switching VSMCs to foam cells, especially during early atherosclerosis. Other independent pathways are likely given the large degree of redundancy observed in atherogenic processes.

Communication Through Direct Contact

Communication between macrophages and VSMCs may also occur through direct contact between surface-expressed molecules such as ICAM-1, VCAM-1, and CX3CL1 with their corresponding receptors (**Figure 3**). Physical cell-cell interactions between both cell types have been implicated in various pro-atherogenic processes such as increased cytokine and metalloproteinase expression (71, 105), macrophage retention, and phenotypic modulation (106). CX3CL1 is a surface-bound chemokine that, together with its receptor CX3CR1, shows increased expression in atheroma resident VSMCs and macrophages. Binding through CX3CL1-CX3CR1 has been shown to upregulate the expression of inflammatory molecules in both cell types (29, 44, 71, 107). Conversely, disruption of the binding interaction significantly reduces the risk for atherosclerosis in humans and mice, suggesting an essential role (108, 109). Therefore, VSMCs and macrophages in physical contact may increase inflammation and promote atherogenesis. Also, CX3CL1 binding has been shown to favor VSMC proliferation and reduce apoptosis. Cellular binding may, therefore, also impact the number of VSMC-derived cells in atherosclerotic lesions. Since VSMCs can have both advantageous and detrimental effects, the impact of enhanced proliferation would likely depend on factors in the microenvironment that modulate VSMC phenotype (107).

Ablation of thioredoxin interacting protein (Txnip) in cultured VSMCs reduced NF-κB-mediated inflammation in response to oxidative stress. Expression of VCAM-1, ICAM-1, and production of CCL2 were significantly decreased. Macrophages from Txnip KO mice also showed lower CCL2 expression and a reduction of CX3CR1. For verification, assays were performed that showed a marked decrease of macrophage adhesion to VSMC in Txnip KO cells. Statistical significance was obtained from individual KOs, but the most substantial effect was obtained when Txnip was knocked out simultaneously in macrophages and VSMCs (25). This suggests a significant role of Txnip in the regulation of cellular interactions between macrophages and VSMCs during oxidative stress. However, validation from *in vivo* results is thus far missing. Additionally, Txnip has been demonstrated to be involved in NLRP3 activation, which may result in the modulation of VSMCs toward a macrophage-like phenotype with enhanced proliferation, migratory capabilities, and expression of inflammatory markers (25, 87).

Direct co-culture studies involving human cells also demonstrated macrophage induced VSMC apoptosis. Neutralization of Fas-L through antibodies and the use of NO inhibitors L-NAME and L-NMMA were shown to suppress cell death, suggesting that apoptosis is initiated *via* Fas ligand/receptor (Fas-L/Fas) interactions in a process that requires NO. NO may act by increasing the number of Fas receptors on VSCMs and Fas ligands on macrophages. Additionally, apoptosis was not observed in co-culture with monocytes, suggesting that macrophage maturation is required (110–112).

Further investigation showed similar results for TNF-α and its receptors TNF-R1 and -R2. At lower TNF-α concentrations (10 nmol/L), both Fas-L and TNF-α were required for VSMC apoptosis. Neither Fas-L nor TNF-α alone were sufficient to initiate cell death, suggesting a synergistic interaction. NO further amplified the process by increasing TNF-R1 expression in VSMCs, in addition to Fas. Antibody targeted neutralization of TNF-R1 and -R2 separately significantly reduced VSMC apoptosis in direct co-cultures with macrophages. Interestingly, selective TNF receptor neutralization on only macrophages

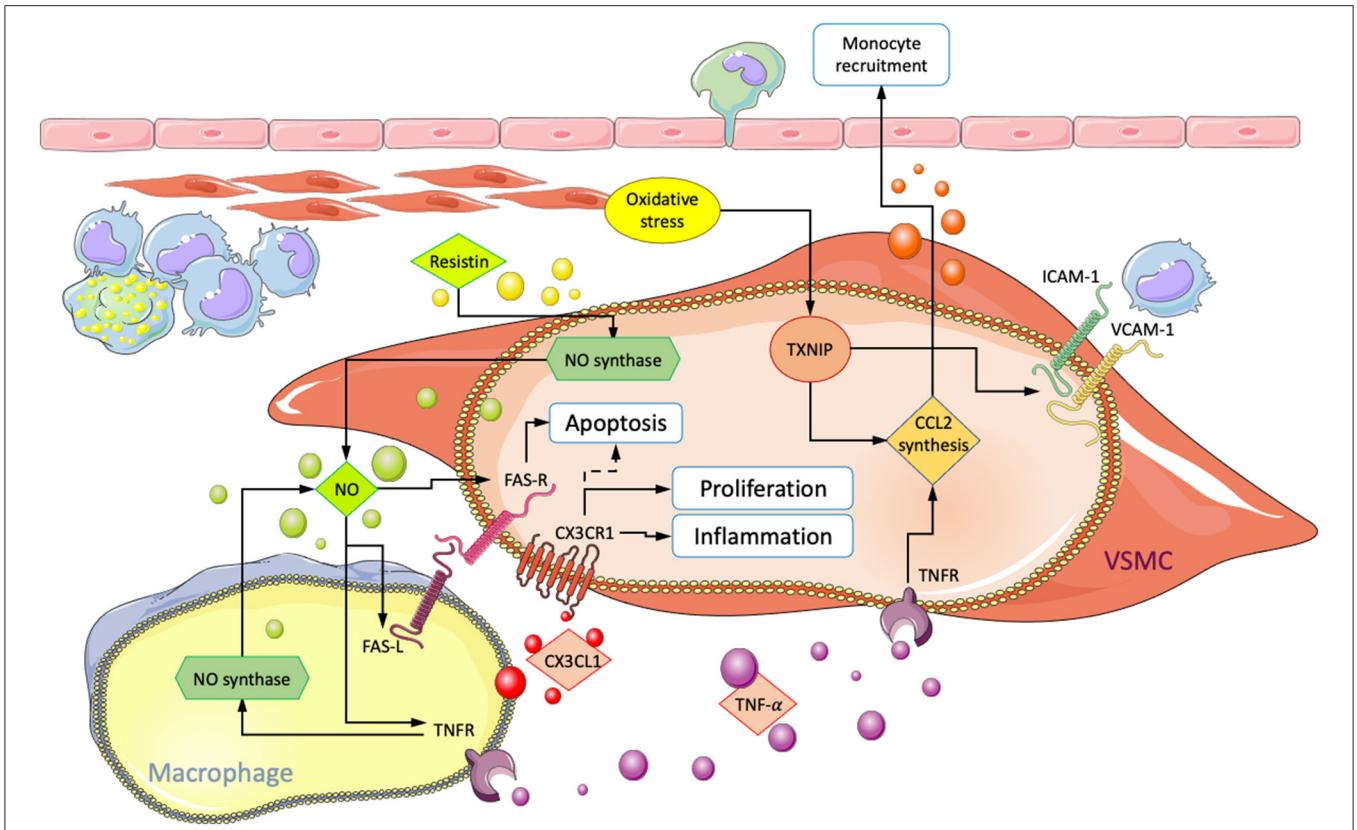

FIGURE 3 | Summary of interactions involving physical contact between macrophages and VSMCs in atherosclerosis. Arrows point in the direction of enhanced processes, and dotted lines indicate inhibition. CX3CL1/CX3CR1 interaction leads to increased VSMC accumulation and inflammation. ROS-mediated increase in VCAM1/ICAM1 and CCL2 result in enhanced subendothelial macrophage retention and monocyte recruitment, respectively. Fas-mediated VSMC-apoptosis and the influence of NO, TNF-α, and resistin on FAS-R/L surface expression.

also had an inhibitory influence. Macrophages with either of the two receptors neutralized exhibited reduced NO synthesis and abolished surface Fas-L expression. These findings suggest a synergistic mechanism involving TNF-α, Fas, and NO. TNF-α can directly induce apoptosis in VSMCs but also acts indirectly by increasing surface Fas-L and NO synthase activity in macrophages. NO, in turn, further sensitizes VSMCs toward macrophage-mediated apoptosis and TNF-α signaling by increasing both surface Fas and TNF-R1 (50). Additionally, incubation of VSMC cultures with TNF-α has been shown to induce production and release of the monocyte chemoattractant CCL2 (113). Exposure to NO and subsequent sensitization of VSMCs toward TNF-α could enhance macrophage recruitment and inflammation. Binding to VSMCs *via* VCAM-1 has also been shown to suppress macrophage apoptosis in culture and induce foam cell formation. *In vivo*, this could further amplify intimal inflammation (106).

Resistin, an inflammatory adipokine produced by macrophages, has been shown to have opposing influences on VSMCs. In indirect co-culture with macrophages, the addition of resistin enhanced VSMC proliferation *via* protein kinase C epsilon (PKCε). However, in culture conditions allowing for cell-cell interactions, resistin inhibited VSMC proliferation and enhanced apoptosis (114). It has previously been demonstrated that resistin activates NO synthesis *via* PKCε (115). Consequently, it is conceivable that resistin amplifies apoptosis through increased NO production and subsequent surface Fas-L/Fas upregulation on VSMCs.

By-Products of VSMC Death

Apoptotic VSMCs synthesize and release factors that can further impact various intimal cells, including other VSMCs and macrophages. Expression of cytokines in response to Fas-induced apoptosis was evaluated in a recent study. IL-6 and GM-CSF were found to be increased through a p38 dependent mechanism when VSMCs were stimulated by a Fas receptor agonist. Besides being mediators of inflammation, IL-6 acts as a mitogen in VSMCs while GM-CSF has diverse roles, including inhibition of proliferation and upregulation of ECM synthesis (19). Both molecules are also involved in the differentiation of monocytes to macrophages (116). By applying the transcription inhibitor actinomycin (ActD) prior to the induction of apoptosis, it was found that IL-6 and GM-CSF were synthesized after the onset of apoptosis. Stau and α-Fas-induced cell death showed increased expression profiles only in cells that were not treated with ActD, suggesting that IL-6 and GM-CSF were produced

during apoptosis and not released from previously established reserves. Furthermore, only cell populations undergoing cell death were found to exhibit enhanced IL-6 and GM-CSF expression. Consequently, VSMCs change their cytokine profile during apoptosis to communicate with local cells. In culture, VSMC death further exacerbated apoptosis in neighboring cells. Nevertheless, examinations in live animals led to contrary results. Selective induction of VSMC apoptosis was achieved using the diphtheria toxin receptor (DTR) and timed DT administrations. Targeted cell-death induced enhanced VSMC proliferation in both normal vessels and after ligation (19). Apoptosis might, therefore, work against further escalations of cell-death and establish a reparative phenotype in VSMCs during vascular injury. Previous studies using DTR for targeted apoptosis have shown that DT administration after ligation promoted vascular remodeling and VSMC proliferation. *In vitro* induction of apoptosis resulted in increased IL-6 expression, proliferation, migration, and collagen synthesis thus validating the *in vivo* results (117). These studies reveal responses to vascular injury of otherwise healthy vessel segments. The influence of synthetic VSMCs undergoing apoptosis within the atherosclerotic microenvironment or during chronic apoptosis was not addressed. Conflicting *in vitro* data and the complexity of the diseased microenvironment highlight the possibility of a markedly different response *in vivo* (19). A study contrasting DTR induced VSMC-death in healthy and atherosclerotic mice showed no inflammation, proliferation, or alteration of contractile markers in healthy vessels while plaque vulnerability was enhanced in diseased mice. Cap area and collagen content were reduced while percent necrotic core area was increased (39).

Finally, defective efferocytosis in atherosclerotic plaques may lead to apoptotic cells undergoing secondary necrosis. During this process, potent inflammation-promoting factors such as IL-1 and HMGB1 are released (118, 119). HMGB1 is also released by activated macrophages and acts as an autocrine stimulant of inflammation (119). Apoptotic VSMCs undergoing secondary necrosis were shown to be particularly inflammatory through the combined release of IL-1α and IL-1β rather than IL-1α alone (118). *In vivo*, IL-1α acts primarily in early lesion, while IL-1β promotes plaque growth at later stages (120). Further, media conditioned with IL-1α induced the production of CCL2 in VSMCs, which may lead to amplified recruitment of macrophages *in vivo* (118).

CONCLUSION

The early atherosclerotic microenvironment enhances detachment, migration, and dedifferentiation of VSMC from the tunica media. During this process, contractile protein expression is either reduced or lost and replaced by other markers (48). Transcriptional modulation may eventually switch VSMCs into distinct phenotypes. Over the course of the disease, some become foam cells and aggravate the disease through inflammation and necrotic core formation. Others migrate to the inner sub-endothelial layer to synthesize matrix components that stabilize the plaque. VSMCs can also adopt an osteogenic-like phenotype leading to calcification and plaque vulnerability. Negative consequences on plaque stability appear to mostly depend on the topological structure of calcification (9, 29). VSMCs are phenotypically highly malleable in atherosclerosis and capable of influencing disease progression in profound ways. Knowledge of different cellular subtypes and their functions in varying atherosclerotic plaque environments is critical to advancing our understanding of the disease and the development of novel therapeutics.

Past studies have established diverse means of communication between VSMCs and macrophages. Interactions between both cell types were shown to significantly impact disease progression, as demonstrated by the interruption of relevant signal transducers such as CX3CR1. The consequences of macrophage-to-VSMC communication vary considerably among different models and cellular contexts. For example, studies have demonstrated VSMC foam cell induction from oxLDL while during vascular injury-induced remodeling, macrophages evoked a phenotype consistent with ECM-producing synthetic cells. Changes in the ratio of synthetic to foam cells may considerably impact plaque stability and disease progression. Therefore, understanding VSMC and macrophage subtypes and their relative functions in different atherogenic environments is pivotal for advancing our understanding of the disease and developing novel therapeutic approaches.

In vitro transdifferentiation of VSMCs was generally achieved without physical cell-contact, suggesting a significant role for soluble factors; either directly released into the media or packaged into EVs. The current literature suggests that physical contact to macrophages predominantly modulates VSMC apoptosis and inflammation. NO, and TNF-α act in synergy to sensitize cells toward apoptotic signaling. Binding *via* VCAM-1 may additionally enhance macrophage survival and foam cell formation, thus potentially amplifying interactions. Diminishing VSMC populations and increasing inflammatory cell accumulation should translate *in vivo* into decreasing plaque integrity. However, VCAM-1, NO, and TNF-α are critical factors that could also tilt disease progression into different directions depending on their relative concentrations. This ambiguity further highlights the need for a more comprehensive understanding of cellular and molecular functions in diverging disease environments.

The high complexity and variety of atherosclerotic plaques constitute significant hurdles for deriving meaningful data from studies. Animal models offer a high degree of exploratory freedom within the context of a live organism but are hampered by substantial cross-species and even intra-species differences. Genetic and dietary modifications are applied to align the animal pathophysiology more closely to that of humans but may themselves introduce confounding variables (121). Murine models have been most extensively used for studying atherosclerosis owing to the ease of genetic manipulations, low overall cost, and short breeding time frames. The current literature indicates significant overlap of relevant risk factors, genes, and pathways in atherosclerosis between mice and humans. However, important differences can also not be ignored (122). C-reactive protein (CRP), for example, has

been established to be central to human inflammation and atherosclerosis but not to mice. Plaque instability and rupture are also not accurately represented by mouse models (123). Importantly, classically (M1) and alternatively (M2) activated macrophages are less clearly defined in humans (124). Differences in cellular functions may significantly alter the composition of atherosclerotic plaques and compromise translatability to humans.

Culture systems abstract from the complexity of animal models and allow focusing on specific aspects of cellular functions. However, findings are only meaningful if disease conditions can be reasonably replicated, which is challenging and has revealed inconsistencies. Cells in culture have undergone phenotypic transition and exhibit enhanced proliferation and susceptibility toward apoptosis which may explain some of the conflicting findings between *in vitro* models and live animals. Additionally, distinct molecular environments can radically influence cellular functions, as evident from the diverse modes of interaction between macrophages and VSMCs. Simple co-culture systems may lack potentially relevant signals from excluded cell types and ECM constituents. ECs, for example, continuously communicate with intimal cells and are often neglected. Multi-cell culture systems are useful if each cell type exhibits a phenotype reasonably consistent with what would be expected *in vivo*. However, the issue of distorted phenotypes may compound in cultures with multiple cells. Promising findings may result from novel models that mimic human disease conditions more accurately and permit higher throughput experimentation. New models could emerge from 3D cultures that mirror specific plaque environments including different cellular subtypes and ECM components.

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.

AUTHOR CONTRIBUTIONS

JB-J and SL have been extensively involved in the preparation, writing, and editing of this review. All authors contributed to the article and approved the submitted version.

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Stimulation of the PD-1 Pathway Decreases Atherosclerotic Lesion Development in Ldlr Deficient Mice

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Aim: Signaling through the coinhibitory programmed death (PD)-1/PD-L1 pathway regulates T cell responses and can inhibit ongoing immune responses. Inflammation is a key process in the development of atherosclerosis, the underlying cause for the majority of cardiovascular diseases. Dampening the excessive immune response that occurs during atherosclerosis progression by promoting PD-1/PD-L1 signaling may have a high therapeutic potential to limit disease burden. In this study we therefore aimed to assess whether an agonistic PD-1 antibody can diminish atherosclerosis development.

Methods and Results: Ldlr$^{-/-}$ mice were fed a western-type diet (WTD) while receiving 100 μg of an agonistic PD-1 antibody or control vehicle twice a week. Stimulation of the PD-1 pathway delayed the WTD-induced monocyte increase in the circulation up to 3 weeks and reduced T cell activation and proliferation. CD4$^+$ T cell numbers in the atherosclerotic plaque were reduced upon PD-1 treatment. More specifically, we observed a 23% decrease in atherogenic IFNγ-producing splenic CD4$^+$ T cells and a 20% decrease in cytotoxic CD8$^+$ T cells, whereas atheroprotective IL-10 producing CD4$^+$ T cells were increased with 47%. Furthermore, we found an increase in regulatory B cells, B1 cells and associated atheroprotective circulating oxLDL-specific IgM levels in agonistic PD-1-treated mice. This dampened immune activation following agonistic PD-1 treatment resulted in reduced atherosclerosis development ($p < 0.05$).

Conclusions: Our data show that stimulation of the coinhibitory PD-1 pathway inhibits atherosclerosis development by modulation of T- and B cell responses. These data support stimulation of coinhibitory pathways as a potential therapeutic strategy to combat atherosclerosis.

Keywords: atherosclerosis, immunology, T cells, coinhibitory pathways, immunotherapy

INTRODUCTION

Atherosclerosis is a chronic autoimmune disease characterized by the accumulation of lipids and immune cells, such as macrophages and pro-atherogenic IFNγ-producing Th1 cells, in the atherosclerotic plaque (1, 2). During disease progression, immune cells respond to atherosclerosis-specific antigens, such as apoB100, the primary protein in low-density lipoprotein (LDL), which are presented via MHC molecules on the surface of antigen-presenting cells (APCs). Subsequent activation of immune cells is regulated by a network of costimulatory and coinhibitory molecules present on both T cells and APCs. The most familiar costimulatory network is the B7/CD28 family, which has proven to be detrimental for Th1-driven atherosclerosis (3). In the past two decades, interference in other costimulatory networks, including the CD40-CD40L and OX40-OX40L pathways, confirmed its potential to inhibit experimental atherosclerosis and to target a broad range of immune responses involved in this disease process (4, 5). Whereas costimulatory molecules need to be suppressed to dampen the overactive immune system in atherosclerosis, signaling through coinhibitory pathways must be promoted. The interaction of programmed death (PD)-1 with PD-L1/2 forms such a coinhibitory pathway, and can inhibit proliferation, cytokine production, cytolytic function, and survival of T cells (6). PD-1 expression is not restricted to activated T cells (7, 8), but can also be upregulated on B cells and certain dendritic cells upon antigen stimulation (7, 9). PD-L1 is expressed by a large variety of cell types, including T cells, macrophages, dendritic cells and endothelial cells (10–12). Previously it has been shown that the PD-1/PD-L1 pathway is a key regulator of many autoimmune diseases, including rheumatoid arthritis (13), multiple sclerosis (14), and cardiac inflammation (15).

In cardiovascular disease patients, altered levels of circulating PD-1 and PD-L1 expressing cells have been reported (16, 17). For example, PD-1 expression on circulating T cells was decreased compared to healthy control individuals and concomitantly, decreased PD-L1 expression on APCs was reported, which corresponded to increased T cell responses (18). Recent proteomics analysis of human atherosclerotic plaques revealed the presence of PD-1 expressing T cell populations inside the advanced atherosclerotic plaque as well (19). Moreover, PD-1/PD-L1 deficiency aggravates experimental atherosclerosis in LDL receptor deficient ($Ldlr^{-/-}$) mice (20–22), with increased numbers of pro-atherogenic $CD4^+$ and $CD8^+$ T cells in the plaque. It is however unknown whether stimulation of PD-1 signaling can inhibit atherosclerosis. Previous studies have shown that stimulation of coinhibitory molecules, such as CTLA-4 and BTLA, suppressed pro-atherogenic T- and B cell immunity and decreased atherosclerosis development in $ApoE^{-/-}$ and $Ldlr^{-/-}$ mice, respectively (23, 24). In addition, Seko et al. showed that treatment of C3H/He mice with an agonistic PD-1 antibody protected against virus-induced myocarditis (25).

Together, these findings fuel the hypothesis that stimulation of the PD-1 pathway can limit the overactive immune system during atherosclerosis development and thus inhibit plaque progression. Therefore, $Ldlr^{-/-}$ mice fed a western-type diet (WTD) were treated with a stimulatory PD-1 antibody for either 2 or 6 weeks to determine the effects on atherosclerosis development and the atherosclerosis-related immune response.

METHODS

Animals

$Ldlr^{-/-}$ mice on a C57Bl/6J background were purchased from Jackson Laboratory (Sacramento, CA, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided *ad libitum*. In order to develop atherosclerotic lesions, female mice (8–12 weeks old) were fed a western-type diet [WTD, 0.25% cholesterol, 15% cocoa butter (SDS, Essex, UK)] for 2 or 6 weeks. The agonistic PD-1 antibody (25) (clone: PIM-2, 100 μg/mouse), isotype control (Tebu-Bio, Heerhugowaard, The Netherlands) or PBS were injected intravenously twice a week in 100 μl volumes. Mice were randomized over the groups using baseline age, weight and cholesterol levels. During the experiments, 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 followed by perfusion with phosphate-buffered saline (PBS) through the left cardiac ventricle. Total white blood cell count and monocyte content in blood were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

Cell Preparation

Upon sacrifice, K_2EDTA anti-coagulated blood, serum, spleens and hearts were harvested. Single-cell suspensions of spleens were obtained using a 70-μm cell strainer (Greiner Bio-one, Kremsmunster, Austria). WBCs were obtained by lysing the blood and splenocytes with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3). Hearts were embedded in OCT compound (Sakura, Tokyo, Japan) and stored at −80°C until further processing.

Flow Cytometry

Cell suspensions were stained using fluorochrome-labeled antibodies. A complete antibody list is provided in **Supplementary Table 1**. Intracellular cytokine staining was performed after stimulation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 200 ng/mL ionomycin (both Sigma-Aldrich, Deisenhofen, Germany) for 4 h in the presence of brefeldin A (Thermo Fisher Scientific, Waltham, MA, USA). Samples were fixed and permeabilized using the fixation and permeabilization kit (BD Biosciences) prior to intracellular staining. Flow cytometry analyses were performed on a Cytoflex S (Beckman Coulter, Brea, CA, USA) or MACSQuant 16 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and FlowJo software (Treestar, San Carlos, CA, USA) or Flowlogics software (Inivai, Mentone, Australia).

Immunohistochemistry

To determine plaque size, 10 μm sections of the aortic root were prepared and collected. Mean plaque size was calculated using 5 sequential sections stained with Oil-Red-O (ORO) and hematoxylin (both Sigma Aldrich). Intraplaque collagen and necrotic core content were quantified after staining with Masson's Trichrome staining kit (Sigma Aldrich) according to the manufacturer's protocol. Corresponding sections on separate slides were stained for monocyte/macrophage content with a MOMA-2 antibody (1:1000, rat IgG2b, Serotec Ltd.) followed by a goat anti-rat IgG-alkaline phosphatase antibody (1:100, Sigma-Aldrich). $CD4^+$ and $CD8^+$ T cells were stained using CD4 (RM4-5, 1:90, ThermoFisher) and CD8a (Ly-2, 1:100, eBioscience) antibodies, and a secondary rabbit anti-rat IgG antibody (BA-4001, Vector), followed by the Vectastain ABC kit (PK-4000, Vector). Color development was achieved using novaRED peroxidase (Vector laboratories) as enzyme substrate. T cells were scored manually. The relative amount of collagen and that of macrophages in the lesions is expressed as the percentage collagen- or MOMA-2-positive area of the total lesion surface area. Spleens were sectioned at a 10 μm thickness and were stained with hematoxylin & eosin (Sigma Aldrich) for white pulp quantification and with ORO and hematoxylin to analyze lipid content. All morphometric analyses were performed in a blinded fashion on a Leica CTR6000B microscope with mikroCam II 20 mp (Bresser) using Leica QWin software (Leica Imaging Systems, UK) or ImageJ (FIJI).

Serum Measurements

Total cholesterol levels were assessed in serum using an enzymatic colorimetric assay (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as internal standard. Total serum levels of IgM and oxLDL-specific IgM were determined by ELISA as described previously (26).

Proliferation Assay

Splenocytes were cultured in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well) in RPMI1640 + 10% FCS + 100 U/ml streptomycin/penicillin. Cells were stimulated with anti-CD3 and anti-CD28 (2 μg/ml) for 48 h. Proliferation was measured by addition of 3H-thymidine (0.5 μCi/well, Amersham Biosciences, the Netherlands) for the last 16 h. The amount of 3H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R, Perkin Elmer). Responses are expressed as stimulation index (SI): ratio of mean counts per minute of triplicate cultures with stimulation to triplicate cultures without stimulation.

Statistical Analysis

Data are reported as mean ± standard error of mean (SEM). Differences between groups were calculated using a Student's *t*-test, or one way ANOVA with Dunnet's *post-hoc* analysis when 3 groups were compared. When 3 groups were compared, the control chow group and PD-1 agonist group receiving WTD were both compared to the control WTD group. Statistical analyses were performed using Graphpad Prism version 8 (Graphpad, San Diego, CA, USA). One mouse in the control group did not develop any atherosclerosis and was therefore excluded as an outlier (ROUT method) from the data.

RESULTS

PD-1 Stimulation Promotes Anti-atherogenic Immunity

To assess the short-term effects of PD-1 stimulation on the immune system, $Ldlr^{-/-}$ mice were treated for 2 weeks with a PD-1 agonist or control vehicle while receiving a WTD diet. Additionally, a control group was kept on chow diet to be able the assess the direct effects of the high cholesterol diet on the immune system (**Figure 1A**). As shown in **Figure 1B**, cholesterol levels increased upon WTD administration and no differences in body weight were observed between the experimental groups. Administration of a WTD significantly increased the relative amount of monocytes in peripheral blood (chow: 9.6 ± 1.1% vs. WTD: 15.4 ± 1%, $p = 0.004$, **Figure 1C**), which was less pronounced in the PD-1 stimulated WTD-fed mice (12.4 ± 1.3%). Similar patterns were seen for both the patrolling ($Ly6C^{int}$) and inflammatory monocyte ($Ly6C^{hi}$) subsets, suggesting that agonistic PD-1 treatment did not affect a specific monocyte subset. Absolute total monocyte numbers as measured by automated hematology analysis revealed a similar trend, albeit these data did not reach significance ($P = 0.15$, **Supplementary Figure 1**). An additional experiment shows that the relative monocyte effect is PD-1 specific, since monocyte levels upon isotype control treatment (**Supplementary Figure 2A**) correspond to those of the PBS treated mice (**Figure 1C**). In contrast, the percentage of splenic monocytes was increased in PD-1 stimulated mice (3.5 ± 0.3%) compared to control WTD (2.2 ± 0.2%, $p = 0.004$) and control chow (0.6 ± 0.1%, **Figure 1D**). Despite enlarged spleens in mice treated with the PD-1 agonist (**Supplementary Figure 3A**), we did not observe significant differences in T- or B cell percentages compared to the control vehicle treated mice (**Supplementary Figure 3B**). As the PD-1/PD-L1 pathway inhibits proliferation of activated T cells (6), the proliferative capacity of splenocytes isolated from either control or PD-1 stimulated mice was measured after stimulation with anti-CD3/CD28 antibodies. As shown in **Figure 1E**, PD-1 stimulation resulted in a 75% decrease in T cell proliferation compared to the controls (PD-1: 12.0 ± 6.8 S.I. vs. control: 47.8 ± 4.9 S.I., $p = 0.0003$). PD-1 stimulation did not alter the percentages of $CD4^+$, $CD8^+$ or total $CD19^+$ in the periphery (**Supplementary Figure 3C**). Interestingly, we did observe increased circulating regulatory B cells (Bregs), defined as $CD19^+CD5^+CD1d^{hi}$ cells, upon 2 weeks of PD-1 stimulation (PD-1: 1.0 ± 0.1% vs. control WTD: 0.6 ± 0.1%, $p = 0.02$) (**Figure 1F**).

Reduced T Cell Activation and Pro-atherogenic IFNγ-Producing T Cells Upon Agonistic PD-1 Treatment

Since PD-1 stimulation promoted anti-atherogenic responses in our short-term experiment, we next investigated the

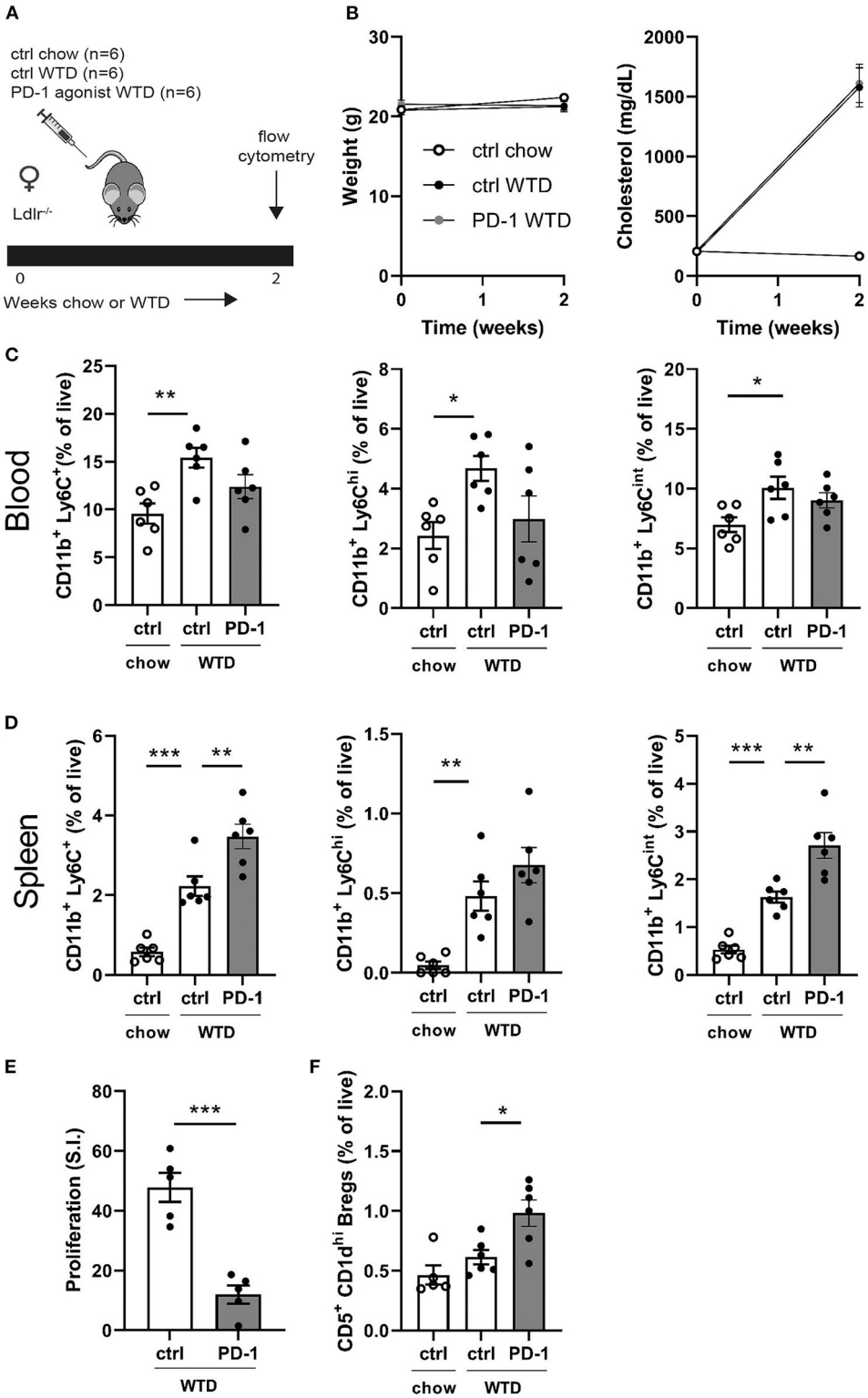

FIGURE 1 | Short term PD-1 stimulation alters monocyte levels and inhibits T cell proliferation. **(A)** Experimental setup. Ldlr$^{-/-}$ mice were fed a WTD or chow diet for 2 weeks while receiving an agonistic PD-1 antibody or control vehicle. **(B)** The weight and serum cholesterol levels were assessed before and after treatment. **(C)** Peripheral blood and **(D)** splenic monocyte percentages were measured by flow cytometry. **(E)** Proliferation of splenocytes after 3 days of stimulation with anti-CD3 and anti-CD28 measured by [3H]thymidine labeling ($n = 5$/group). **(F)** Regulatory B cells in peripheral blood were measured by flow cytometry. Data are displayed as mean ± SEM. Statistics was performed using one-way ANOVA, with *post-hoc* comparison using Dunnett's multiple comparisons test, comparing control chow and PD-1 WTD groups to the control WTD group. $P \leq 0.05$ are considered significant. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

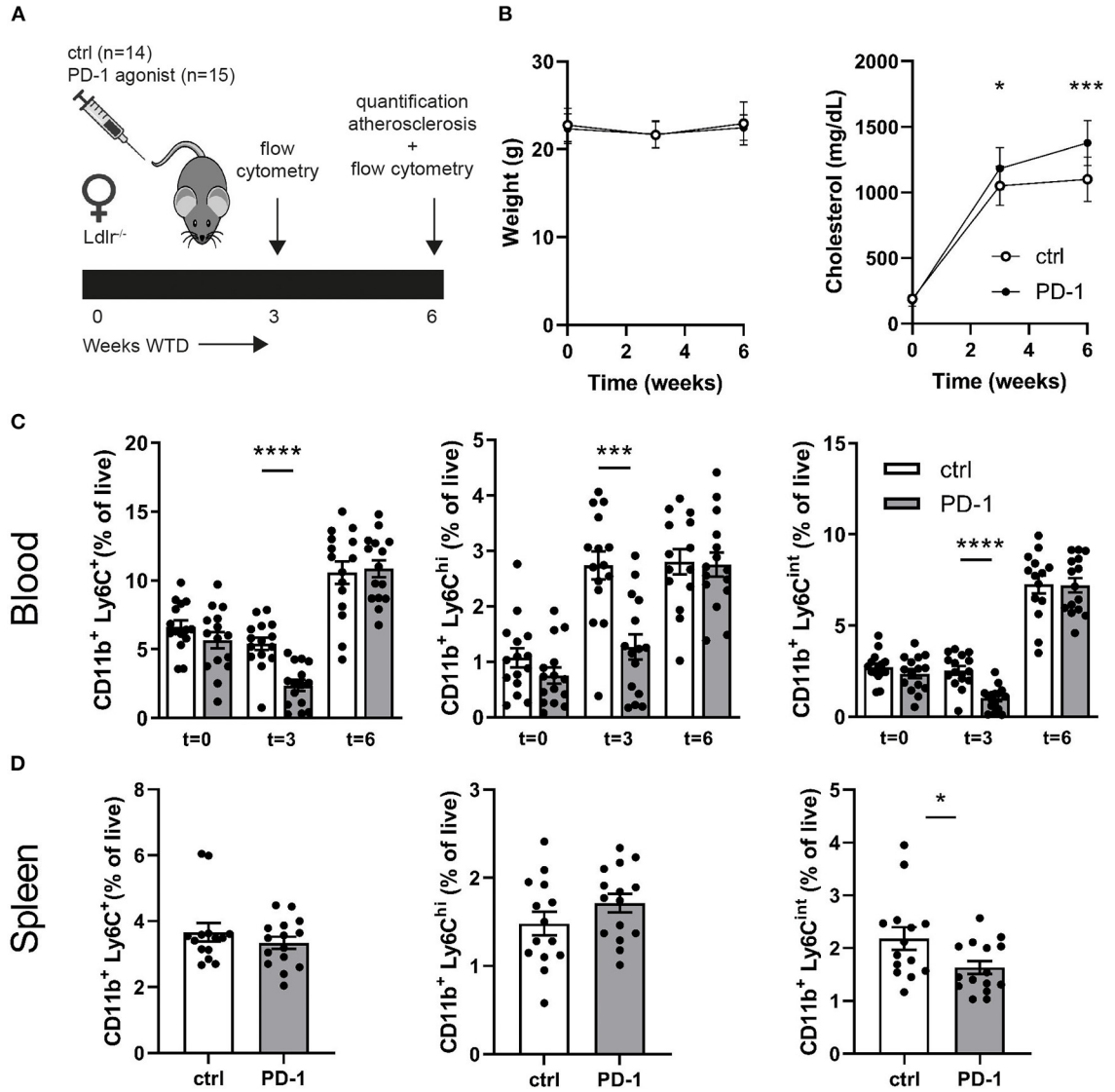

FIGURE 2 | Agonistic PD-1 treatment inhibits WTD-induced increase in circulating monocytes. **(A)** Experimental setup. Ldlr$^{-/-}$ mice were fed a WTD for 6 weeks while receiving an agonistic PD-1 antibody ($n = 15$) or control vehicle ($n = 14$). **(B)** Weight and serum cholesterol levels were assessed before, during and after treatment. Total monocytes (CD11b^{+}Ly6G^{-}Ly6C^{+}), inflammatory monocytes (CD11b^{+}Ly6G^{-}Ly6C^{high}) and patrolling monocytes (CD11b^{+}Ly6G^{-}Ly6C^{int}) were measured by flow cytometry in peripheral blood **(C)** and spleen **(D)**. Mean ± SEM are shown. Statistics was performed using unpaired *T*-test. $P \leq 0.05$ are considered significant. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

immunomodulatory effect of PD-1 stimulation during atherosclerosis development. Ldlr$^{-/-}$ mice were fed a WTD for 6 weeks while receiving an agonistic PD-1 antibody or control vehicle (**Figure 2A**). During the experiment we observed an increase in serum cholesterol in PD-1 stimulated mice, while no difference in weight was observed between the groups (**Figure 2B**). In line with our previous findings (**Figure 1C**), PD-1 stimulated mice show a decrease in circulating monocytes (2.4 ± 0.4%) compared to control mice (5.4 ± 0.5%, $p = 0.001$, **Figure 2C**) after 3 weeks. At sacrifice, circulating monocyte percentages did not differ anymore between the groups. No differences in relative monocyte content was observed in the spleen after 6 weeks of treatment, except for a significant decrease in patrolling monocytes (PD-1: 1.6 ± 0.1% vs. control: 2.2 ± 0.2%, $p = 0.03$) (**Figure 2D**). Again, spleen weight was increased in PD-1 stimulated mice, however no differences were found in the relative white pulp content of the spleen (PD-1: 21.6 ± 1.0% vs. control: 23.4 ± 1.0%) and no excess fat depositions were found in spleens of PD-1 agonistic treated mice compared to spleens of control mice (**Supplementary Figure 4A**). Percentages and absolute values of total CD4^{+} and CD8^{+} T cells were not affected in the spleen (**Figure 3A** and **Supplementary Figure 4B**). Similarly, circulating CD4^{+} and CD8^{+} T cells and their activation status was unchanged (**Supplementary Figure 5**).

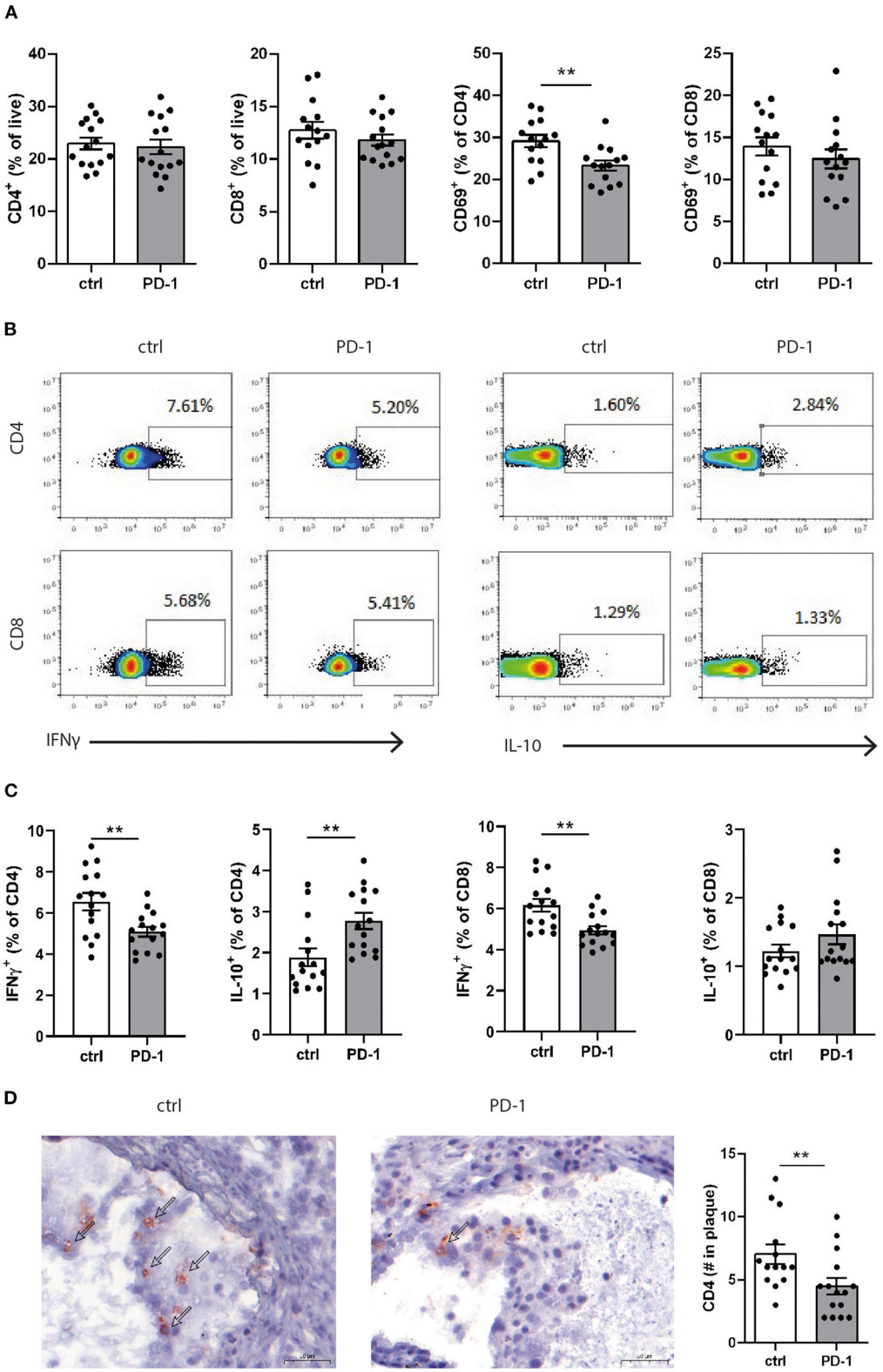

FIGURE 3 | PD-1 stimulation promotes an anti-atherogenic T cell phenotype. **(A)** The amount of $CD4^+$ and $CD8^+$ T cells and their expression of CD69 was measured in splenocytes by flow cytometry. To measure intracellular cytokines, splenocytes were stimulated for 4 h with PMA/ionomycin and BrefA. **(B)** Representative dotplots and **(C)** data graphs of intracellular IFNγ and IL-10 within $CD4^+$ and $CD8^+$ T cells are shown. **(D)** $CD4^+$ T cell numbers in the atherosclerotic plaque were manually scored. Scale bars indicate 50 μm. Mean ± SEM are shown. Statistics was performed using unpaired *T*-test. $P \leq 0.05$ are considered significant. $^{**}p \leq 0.01$.

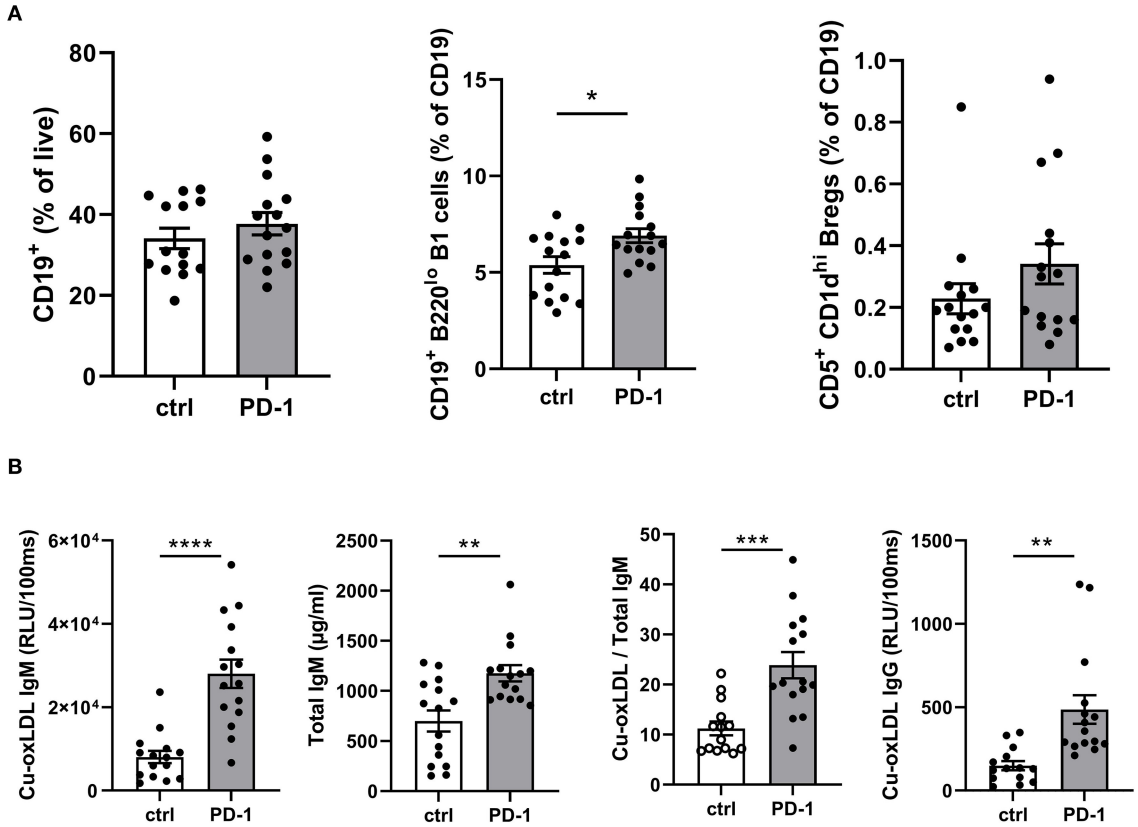

FIGURE 4 | Elevated B1 cells and oxLDL-IgM levels in agonistic PD-1 treated mice. Total $CD19^+$ B cells, B1 cells ($CD19^+B220^{lo}$) and regulatory B cells ($CD19^+CD5^+CD1d^{hi}$) were measured by flow cytometry in blood **(A)**. Serum Cu-oxLDL IgM (relative light units (RLU)/100 ms), total IgM (μg/ml), Cu-oxLDL IgM/total IgM ratio, and Cu-oxLDL IgG (RLU/100ms) were measured by ELISA **(B)**. Mean ± SEM are shown. Statistics was performed using unpaired *T*-test. $P \leq 0.05$ are considered significant. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

However, the activation status of splenic T cells, as measured by the expression of activation marker CD69, was decreased in PD-1 agonist treated mice (PD-1: 23.3 ± 4.6% vs. control: 29.2 ± 5.4%, $p = 0.005$) (**Figure 3A**). Moreover, a significant decrease in pro-atherogenic IFNγ-producing cells was seen in both $CD4^+$ and $CD8^+$ populations upon PD-1 stimulation ($CD4^+$ T cells: PD-1: 5.1 ± 0.9% vs. control: 6.6 ± 1.6% $p = 0.005$; $CD8^+$ T cells: PD-1: 4.9 ± 0.8% vs. control: 6.2 ± 1.2% for control, $p = 0.006$) (**Figures 3B,C** and **Supplementary Figure 6**), whereas a significant increase in atheroprotective $CD4^+$ IL-10-producing cells was found after PD-1 stimulation (PD-1: 2.8 ± 0.8% vs. control: 1.9 ± 0.8%, $p = 0.003$). In the lymph nodes draining from the heart, we did not observe differences in T cell numbers or activation status (data not shown). Locally in the atherosclerotic plaque, a decrease in the number of $CD4^+$ T cells after PD-1 stimulation was observed (PD-1: 4.5 ± 0.6 vs. control: 7.0 ± 0.8, $p = 0.008$) (**Figure 3D**), while the number of $CD8^+$ T cells was relatively low and did not significantly differ between the groups (PD-1: 1.2 ± 0.4 vs. control: 0.8 ± 0.2, $p = 0.61$) (**Supplementary Figure 5C**).

Increased Circulating B1 Cells and oxLDL-Specific IgM in Agonistic PD-1 Treated Mice

As T cells play an important role in B cell activation, and PD-1 is also expressed on activated B cells, we anticipated PD-1 stimulation may also affect humoral immunity during atherosclerosis development. Although total circulating B cells were unaltered after 6 weeks of PD-1 stimulation, we did observe a significant increase in B1 cells (PD-1: 6.9 ± 1.4% vs. control: 5.4 ± 1.7%, $p = 0.01$, **Figure 4A**). In line with this finding, total serum IgM levels as well as oxLDL-specific IgM levels were increased by PD-1 stimulation (**Figure 4B**). Notably, the levels of oxLDL IgM were increased to a greater extent than total IgM, indicating also a relative increase of atheroprotective IgM. Total oxLDL-specific IgG were also increased after PD-1 stimulation. Similarly to the short-term experiment, an increase of circulating Bregs was observed, although not statistically significant (PD-1: 0.34 ± 0.07% vs. control: 0.23 ± 0.05%, **Figure 4A**).

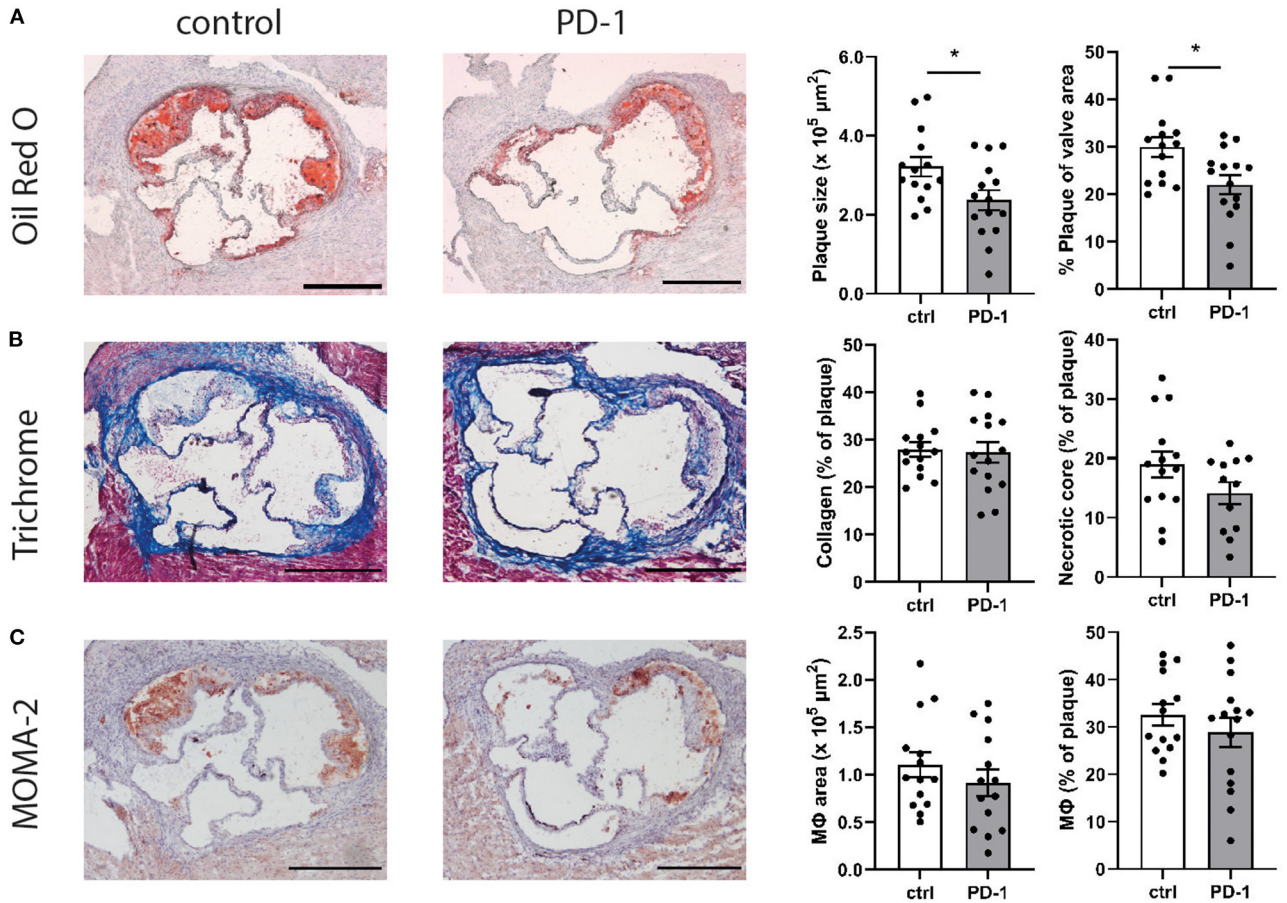

FIGURE 5 | PD-1 stimulation inhibits atherosclerosis development. Aortic root plaque size and composition was determined using an Oil-Red-O staining and representative images of the stainings are shown. Data is shown as total plaque size, and as percentage of total valve area **(A)**. Collagen content and necrotic core size were determined using trichrome staining, and are showed as percentage of the total plaque **(B)**. Macrophage content was determined using MOMA-2 antibody staining, and is shown as total MOMA-2 positive area, as well as the percentage of total plaque **(C)**. Scale bars indicate 250 μm. Mean ± SEM are shown. Statistics was performed using unpaired *T*-test. $P \leq 0.05$ are considered significant. $^{*}p \leq 0.05$.

PD-1 Stimulation Reduces Plaque Development in the Aortic Root

Given the immunosuppressive effect of PD-1 stimulation, we investigated the effect of PD-1 stimulation on atherosclerotic plaque development. Ldlr$^{-/-}$ mice treated with an agonistic PD-1 antibody showed a 26.4% smaller plaque size compared to the control group (PD-1: 2.40 ± 0.25 × 10^5 μm^2 vs. control: 3.22 ± 0.25 × 10^5 μm^2, $p = 0.04$) (**Figure 5A**). Similarly, when plaque size was calculated as a percentage of the total lumen area a significant decrease was seen in PD-1 stimulated mice (PD-1: 23.0 ± 1.8% vs. control: 30.0 ± 2.1%, $p = 0.02$). The percentage of collagen in the plaque was similar in both groups (PD-1: 27.3 ± 2.2% vs. control: 28.0 ± 1.5%, **Figure 5B**). Likewise, the necrotic core content of the plaque (PD-1: 14.1 ± 1.9% vs. control: 19.0 ± 2.2%) and the macrophage content in the plaque as assessed by MOMA-2 staining (PD-1: 30.5 ± 2.8% vs. control: 32.6 ± 2.3%) did not differ between the groups (**Figure 5C**).

DISCUSSION

Immune checkpoint proteins are extremely potent targets to modulate immunity in autoimmune diseases such as cardiovascular disease (23, 24, 27). In this study, we show that stimulation of signaling through the immune checkpoint protein PD-1 inhibits atherosclerotic lesion development in WTD-fed Ldlr$^{-/-}$ mice despite elevated serum cholesterol levels. This is accompanied by a decrease in inflammatory monocytes in peripheral blood in the early stages and a decrease in IFNγ-producing T cells, while atheroprotective IL-10 producing T cells, Bregs, B1 cells and oxLDL IgM levels were increased (20–22).

Previously, it was shown that absence of PD-1/PD-L1/2 signaling can aggravate atherosclerosis by enhancing T cell proliferation, activation of both CD4$^+$ as CD8$^+$ T cells, and more specifically by increasing pro-atherogenic IFNγ production by T cells (20, 22). In line with these findings,

we show that agonistic PD-1 treatment resulted in a strongly impaired proliferative capacity of T cells, with a concomitant shift from Th1-associated IFNγ-producing $CD4^+$ cells toward anti-inflammatory IL-10 producing $CD4^+$ T cells. This was accompanied by a significant decrease in $CD4^+$ T cell numbers in the plaque. This is not surprising, as PD-1 signaling is known to suppress T cell activation and proliferation (28, 29). Reduced IFNγ-producing T cells in our agonistic PD-1 treated mice likely contributed to diminished atherosclerosis, as administration of exogenous IFNγ to $ApoE^{-/-}$ mice resulted in an increase plaque formation (30), while $Ldlr^{-/-}IFN\gamma^{-/-}$ mice developed smaller plaques compared to control (31). In contrast, IL-10 has well-described anti-atherogenic properties (32) and is often associated with regulatory T cells, which can suppress activation and proliferation of immune cells during atherosclerosis, including IFNγ-producing $CD4^+$ T cells (33). Despite our observed reduction in T cell proliferation and IFNγ-producing T cells in agonistic PD-1 treated mice, we did not observe a difference in the percentage of $Foxp3^+$ $CD4^+$ T cells in the circulation (**Supplementary Figure 5A**). That PD-1 directly acts on pro-atherogenic T cells without affecting Treg levels, was also described by Bu et al. who showed that PD-1 deficiency in $Ldlr^{-/-}$ mice did not alter $CD4^+Foxp3^+$ T cells (21).

Furthermore, we also observed a decrease in IFNγ-producing $CD8^+$ T cells, which are considered pro-atherogenic due to their cytotoxic capacity and inflammatory cytokine production (34). This is in line with previous studies in which $PD\text{-}1^{-/-}Ldlr^{-/-}$ mice showed an increase in pro-inflammatory cytokine expression, including IFNγ by $CD8^+$ T cells, and in which PD-1 blockade induced IFNγ production by both $CD4^+$ and $CD8^+$ T cells (20). Moreover, PD-1 expressing $CD8^+$ T cells from patients with atherosclerosis produced more anti-atherogenic IL-10 and less pro-atherogenic cytokines (IFNγ, TNFα) compared to $PD\text{-}1^-CD8^+$ T cells, further supporting a protective role for PD-1 in T cell-mediated immunity. Interestingly, $CD8^+$ T cells can also control monopoiesis and circulating monocyte levels in atherosclerosis (35), which may have contributed to our observed reduction in circulating monocytes. The latter can also result from reduced T cell activation in PD-1 agonist treated mice as it has been shown that activated T cells can induce pro-inflammatory cytokine secretion by monocytes (36). Lack of this monocyte activation upon PD-1 stimulation possibly prevents influx of new monocytes into the circulation. We show this reduction in monocytes is directly associated with PD-1 stimulation, as treatment with an isotype control resulted in comparable monocyte levels to PBS treatment. Due to limited statistical power in that particular experiment, no concrete conclusion can be drawn on atherosclerosis development. Agonistic PD-1 treatment was only able to delay WTD-induced monocytosis, as relative monocyte levels did not differ between control and PD-1 agonist treated mice after 6 weeks of treatment. Although we have no supportive evidence, the comparable levels of monocytes at sacrifice could be attributed to enhanced monocyte release from the bone marrow in PD-1 agonist treated mice to compensate for the reduced monocytes in the first few weeks of the treatment. As we also observe decreased monocyte levels in the spleen after 3 weeks of treatment, it is also a possibility that monocytes from the splenic reservoir (37) are suppressed during the initial weeks of PD-1 agonist treatment, resulting in reduced circulating monocytes. Notably, we did not observe a difference in macrophage content in the plaque, rendering it unlikely that the observed reduction in monocyte levels during the 1st weeks of the treatment is solely responsible for the observed plaque size reduction upon PD-1 agonism. However, a more detailed analysis regarding macrophage subsets in the plaque may shed more light on the underlying mechanisms involved.

The PD-1/PD-L1/L2 pathway is not only an important negative regulator of T cell responses but can also impact B cell immunity (9, 38). Although PD-L1 expressing B cells have been more extensively investigated, PD-1 is also upregulated on activated B cells and these $PD\text{-}1^+$ B cells have been linked to CD4 and CD8 T cell suppression previously (9, 38, 39). Within 2 weeks upon PD-1 stimulation, we already observed elevated circulating $CD5^+CD1d^{hi}$ regulatory B cells, which could possibly exert an atheroprotective role by suppressing T cells. Previously, decreased amount of circulating Bregs were shown in patients with coronary atherosclerosis compared to healthy controls (40) and we showed that adoptive transfer of $IL\text{-}10^+$ Bregs in $Ldlr^{-/-}$ mice reduced total leukocyte counts, lymphocytes, monocytes and activated T cells in circulation (41). Notably, this adoptive transfer of $IL\text{-}10^+$ Bregs as well as lack of IL-10 producing B cells in $Ldlr^{-/-}$ mice (42) did not affect plaque size, suggesting our observed increase in Bregs upon PD-1 agonism mainly contributes to reduced inflammation. Finally, we also found elevated levels of B1 cells in the circulation after PD-1 stimulation. B1 cells are considered atheroprotective via their production of primarily IgM natural antibodies directed at amongst others to oxLDL, which can prevent foam cell formation, and facilitate the clearance of apoptotic cells (43). Corresponding to the increase in B1 cells, we observed elevated serum IgM levels and more specifically an absolute and relative increase in oxLDL-specific IgM, which has been shown to inversely relate to the incidence of CVD (44).

Finally, we would like to address a few findings that warrant further research. First of all, our study was performed in female mice. Previous studies investigating the PD-1/PD-L1 pathway were performed in either male (22) or female mice (20), and in both sexes PD-1 or PD-L1 deficiency aggravated atherosclerosis development. Therefore, we do not anticipate sex differences upon PD-1 stimulation in our atherosclerosis model, although further research would be necessary to confirm this. Moreover, we also observed an enlargement of the spleen after PD-1 stimulation, while the amount of splenocytes, white pulp content and fat deposits is similar to those in control mice after 6 weeks of treatment. Previous studies using radioactively labeled monoclonal antibodies show that the spleen is a preferential site of accumulation for therapeutic antibodies (45), which could possibly explain this observation. Finally, despite decreased atherosclerosis, we did observe elevated cholesterol levels in our PD-1 agonist treated

mice. This is in contrast to Cochain et al. who found increased cholesterol levels upon PD-1 deficiency in Ldlr$^{-/-}$ mice (22), while cholesterol levels remained unchanged in other studies investigating PD-1/PD-L1 deficiency in atherosclerosis (20, 21). It thus remains to be elucidated whether the observed effect on serum cholesterol levels in our study is directly related to PD-1 agonism or whether this is a secondary effect. Despite the increase in cholesterol levels, we did observe a decrease in atherosclerosis development, suggesting that the atheroprotective effects that we observe on the immune system upon PD-1 treatment are effective in counteracting the increase in serum cholesterol levels. Further research investigating the direct effects on PD-1 agonism on cholesterol metabolism may provide mechanistic insights in this regard.

Given the recent advances in immune modulation as treatment for atherosclerosis (46), our data show that PD-1 stimulation is a possible treatment to reduce atherosclerosis development. Although our study provides novel insights in the role of the PD-1 pathway in atherosclerosis, caution is warranted when using this target for treatment. While dampening the immune system and thus stimulation of the PD-1/PD-L1 axis is a potential pathway to reduce progression of atherosclerosis, activation of the immune system by blockade of the PD-1/PD-L1 pathway has shown promising results in the treatment of several cancers (47). Indeed, a number of patients treated with anti-PD-1 or PD-L1 show cardiotoxicity, mostly myocarditis, especially in combination with anti-CTLA4 treatment (48). Although more research is needed to enhance our knowledge regarding this ambivalence, recently it was reported that melanoma patients receiving anti-PD-1 and anti-CTLA4 treatment did not show a direct effect on atherosclerosis after 10 weeks of treatment (49).

In conclusion, our data show that stimulation of the co-inhibitory PD-1 pathway inhibits atherosclerosis development via modulation of the immune response and supports that stimulation of co-inhibitory molecules can be a potential therapeutic strategy to limit atherosclerosis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

AUTHOR CONTRIBUTIONS

HG, JK, IB, and AF designed the research. HG, RV, VS, MB, DS, IB, and AF acquired the data. HG, MB, IB, and AF analyzed the data. HG and AF drafted the manuscript. IB and AF provided critical feedback on the manuscript. All authors provided feedback on the research, analyses, and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.740531/full#supplementary-material

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Interfering in the ALK1 Pathway Results in Macrophage-Driven Outward Remodeling of Murine Vein Grafts

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Aims: Vein grafts are frequently used to bypass coronary artery occlusions. Unfortunately, vein graft disease (VGD) causes impaired patency rates. ALK1 mediates signaling by TGF-β *via* TGFβR2 or BMP9/10 *via* BMPR2, which is an important pathway in fibrotic, inflammatory, and angiogenic processes in vascular diseases. The role of the TGF-β pathway in VGD is previously reported, however, the contribution of ALK1 signaling is not known. Therefore, we investigated ALK1 signaling in VGD in a mouse model for vein graft disease using either genetic or pharmacological inhibition of the Alk1 signaling.

Methods and Results: Male ALK1 heterozygous ($ALK1^{+/-}$), control C57BL/6, as well as hypercholesterolemic ApoE3*Leiden mice, underwent vein graft surgery. Histologic analyses of $ALK1^{+/-}$ vein grafts demonstrated increased outward remodeling and macrophage accumulation after 28 days. In hypercholesterolemic ApoE3*Leiden mice receiving weekly ALK1-Fc injections, ultrasound imaging showed 3-fold increased outward remodeling compared to controls treated with control-Fc, which was confirmed histologically. Moreover, ALK1-Fc treatment reduced collagen and smooth muscle cell accumulation, increased macrophages by 1.5-fold, and resulted in more plaque dissections. No difference was observed in intraplaque neovessel density. Flow cytometric analysis showed increased systemic levels of $Ly6C^{High}$ monocytes in ALK1-Fc treated mice, supported by *in vitro* increased MCP-1 and IL-6 production of LPS-stimulated and ALK1-Fc-treated murine monocytes and macrophages.

Conclusion: Reduced ALK1 signaling in VGD promotes outward remodeling, increases macrophage influx, and promotes an unstable plaque phenotype.

Translational Perspective: Vein graft disease (VGD) severely hampers patency rates of vein grafts, necessitating research of key disease-driving pathways like TGF-β. The three-dimensional nature of VGD together with the multitude of disease driving factors ask for a comprehensive approach. Here, we combined *in vivo* ultrasound imaging, histological analyses, and conventional *in vitro* analyses, identifying the ambiguous role of reduced ALK1 signaling in vein graft disease. Reduced ALK1 signaling promotes

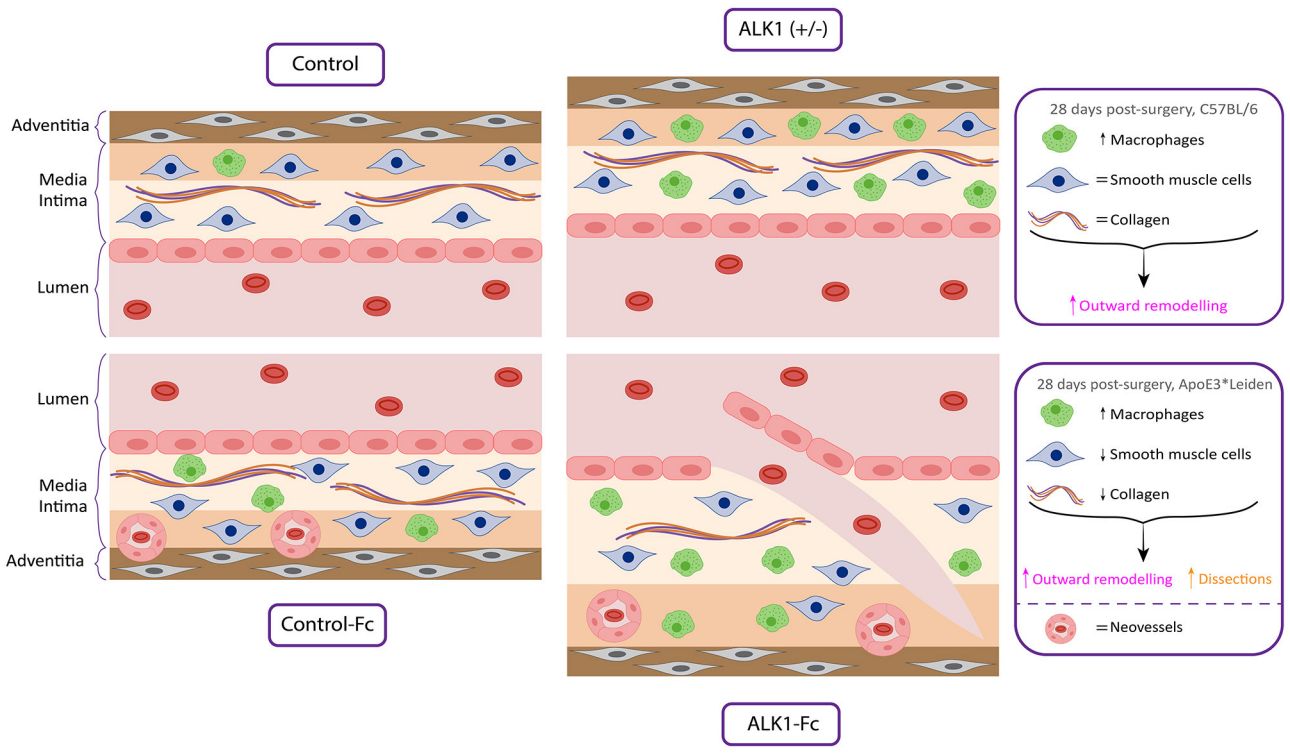
outward remodeling, increases macrophage influx, and promotes an unstable plaque phenotype in murine vein grafts. Characterization of *in vivo* vascular remodeling over time is imperative to monitor VGD development and identify new therapies.

Keywords: vascular remodeling, ALK1 signaling, vein graft disease, ultrasound, macrophage

INTRODUCTION

The vena saphena magna is the commonly used vein for bypass surgery in coronary or peripheral arterial circulation in patients with cardiovascular disease. After surgery, the vein graft requires arterialization to adapt to the pressure of the arterial circulation. Intimal hyperplasia develops through the proliferation and migration of vascular smooth muscle cells, ingrowth of neovessels, macrophage accumulation, and collagen deposition (1, 2). Critical processes for arterialization and successful long-term patency of the grafts are moderate intimal hyperplasia (IH) combined with outward remodeling (2). The beneficial process of proportional outward remodeling is often accompanied by moderate inflammation and angiogenesis (1, 3, 4). Excessive forms and overshooting of these mechanisms may induce disproportionate IH, causing inward, frequently partially occluding, vascular remodeling, and subsequent blood flow reduction. These processes together are indicated as vein graft disease (VGD). To reduce VGD, new therapeutic strategies are needed.

Members of the Transforming Growth Factor-β (TGF-β) superfamily are involved in the pathophysiology of VGD. Accordingly, TGF-β has been described as a driver for intimal hyperplasia, and fibrosis in vein grafts (5, 6). The TGF-β type I receptor activin receptor-like kinase (ALK)1 mediates signaling by TGF-β1 and TGF-β3 in the presence of TGFβR2 or high-affinity ligands BMP9 and BMP10 in the presence of BMPR2 that initiate downstream signaling *via* pSMADs. This is an important pathway in the fibrotic, inflammatory, and angiogenic processes in vascular disease (7–9). Considering that inflammation and angiogenesis play an important role in VGD, the ALK1 signaling pathway may be an interesting target in VGD. Neovessels facilitate both (i) the diapedesis of immune cells (10), and especially in the case of immature leaky neovessels, (ii) erythrocyte efflux, and subsequent intraplaque hemorrhage formation (11). Targeting ALK1 has been shown to reduce endothelial cell sprouting, impair neovessel ingrowth into Matrigel plugs, and decrease tumor angiogenesis (12, 13). Moreover, modulation of the ALK1 signaling pathway



GRAPHICAL ABSTRACT | A reduction in ALK1 signaling induces outward remodeling, macrophage influx and promotes an unstable plaque phenotype in vein graft disease.

is suggested to regulate macrophage-mediated inflammatory processes and monocyte-macrophage differentiation (14).

In the current study, ALK1 heterozygous mice ($ALK1^{+/-}$), as well as ALK1-Fc-treated hypercholesterolemic ApoE3*Leiden mice, were used to study the effects of interfering with ALK1 signaling *via* two approaches, a genetic and a pharmacologic approach. Homozygosity of ALK1 is embryonic lethal at mid-gestation due to severe vascular abnormalities associated with an increased expression of angiogenesis-related factors and reduced number of smooth muscle cells (8). With the background of ALK1 signaling in angiogenesis and inflammation, we aimed to elucidate the mechanism through which ALK1 affects vein graft remodeling. ALK1-Fc, consisting of the extracellular domain of ALK1 coupled to an Fc tail, acts as a scavenger receptor for BMP9 and BMP10. Specifically, ALK1-Fc sequesters BMP9/10, increasing their retention in the circulation and inhibiting BMP9/10-induced Smad-1 phosphorylation (pSMAD) and encompassing downstream transcription processes (15). Our study shows that in a mouse vein graft model (1), both ALK1 heterozygosity and ALK1-Fc ligand trapping were found to induce outward remodeling. Further investigations demonstrated that plaque angiogenesis was not affected by ALK1-Fc treatment, but an increased influx of inflammatory macrophages and a disbalance toward vessel wall destabilizing factors were observed.

MATERIALS AND METHODS

Study Approval and Mice

This study was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. The Institutional Committee of the Leiden University Medical Centre approved all the animal experiments licensed under project numbers (11045, 116002016645). Written informed consent was obtained from the owners for the participation of their animals in this study. Heterozygous mice ($ALK1^{+/-}$) and corresponding littermates were bred in our institute (16). Male C57BL/6 ($ALK1^{+/+}$) and ALK1 heterozygous mice ($ALK1^{+/-}$) were fed with a chow diet and ApoE3*Leiden (ApoE3*Leiden) mice were fed with a western type diet containing 1.0% cholesterol and 0.5% cholate (HFD) (Sniff Spezialdiäten, GMBH, Soest, Germany) (17). These mice were bred in our institute. In both breeding colonies, the mice were genotyped to confirm gene heterozygosity of *ALK1* or *ApoE3*Leiden*. Wild-type littermates were used as controls and all mice had free access to food and water.

Experimental Design

Caval veins were obtained from male donor littermates with the same age (10–16 weeks) as the recipient mice. Both donor and recipient mice were anesthetized with an intraperitoneal (i.p.) injection consisting of a combination of midazolam (5 mg/kg, Roche), medetomidine (0.5 mg/kg, Orion), and fentanyl (0.05 mg/kg, Janssen). The depth of anesthesia was assessed by the pedal reflex. These caval veins were interpositioned in the carotid arteries of $ALK1^{+/+}$, and $ALK1^{+/-}$ mice, as previously described (18). After surgery and on the indication, buprenorphine (0.1 mg/kg, MSD Animal Health) was given as an analgesic. After 28 days, mice were anesthetized, pressure perfused with phosphate-buffered saline (PBS, Braun), and vein grafts were harvested, fixated in 3.7% formalin, and subsequently processed for paraffin embedding.

Pharmacological targeting of ALK1 signaling was performed in ApoE3*Leiden mice. These ApoE3*Leiden mice were fed with the HFD for 4 weeks before vein engraftment. Mice were randomized based on their plasma cholesterol values, measured enzymatically by using the Roche Diagnostic kit (Kit 1489437). Mice with plasma cholesterol values of >35 mM were excluded. Treatments consisted of 10 mg/kg bodyweight ALK1-Fc (RAP-041, an ALK1 extracellular domain/Fc fusion protein) or Fc control protein (12), which was administered via i.p. injection twice a week for 4 weeks in total.

Histological and Immunohistochemical Analysis of Vein Grafts

Vein grafts were sectioned in sequential cross-sections of 5 μm thick made throughout the embedded vein grafts. The total vein was analyzed in equally spaced section. The plastic cuff was the starting point for mounting sections onto glass slides. HPS staining was performed using Hematoxylin, Phloxin 0.25%, and Saffron 0.3% to analyze the morphometrics of the vein grafts with ImageJ. The total vessel area and lumen area were measured. Vessel wall thickening was defined as the area between lumen and adventitia and determined by subtracting the lumen area from the total vessel area. The differences in the composition of the vein grafts were visualized by a Sirius red staining (Klinipath 80,115) to quantify the amount of collagen present. Anti-smooth muscle cell actin antibodies (1A4, 1:1,000, Dako) stained the vascular smooth muscle cells or anti-MAC3 (BD) (Clone M3/84, 550292 BD Biosciences) stained the macrophages, which were visualized by the DAB substrate complex. The images were loaded in ImageJ (FIJI), which calculated the percentage DAB isolated with the IHC toolbox FIJI plugin in the vein graft wall determined as a region of interest. The histological measurements were performed by a blinded experimenter. The macrophages were semi-quantitatively scored based on 0: none, 1: 10%, 2: 20–40% or 3: >40%.

Ultrasound Measurements and Analyses

The animals were anesthetized with isoflurane and placed on the mouse imaging platform of the Vevo '3100' LAZR-X system (VisualSonics, FUJIFILM), where temperature, heart rate, and respiration rate were monitored in real-time. During the ultrasound acquisitions, anesthesia was maintained using a vaporized isoflurane gas system (1 L/min of oxygen 0.3 L/min air and 2.5% isoflurane). The concentration of isoflurane was adjusted accordingly to the pedal reflex and respiration rate to ensure adequate anesthesia. The region ranging from the salivary gland to the sternum was shaved and covered with ultrasound gel. Ultrasound imaging of vein grafts were performed with the MX550S transducer. Short-axis scanning 3D B-mode, and 3D color Doppler images were acquired weekly.

Image visualization, reconstruction, and processing were realized with VevoLAB 3.2.6 software (FUJIFILM, VisualSonics). Both the cranial and caudal plastic cuff acted as landmarks to indicate the area for lumen and wall measurements. These lumen and wall measurements in 2D, as well as 3D, were performed by two persons, blinded to the grouping of mice in the two-study arms. Lumen and total vein graft area measurements were calculated using a software-corrected, freehand tracing of the region of interest on the short axis. The wall area was defined as the area in between the traced lumen and total vein graft area. Color doppler and EKV modes were used as supporting tools to clearly distinguish the vein graft from surrounding tissues (**Supplementary Figure 1**). Detailed analyses were performed using a 3D, short-axis ultrasound method with which lumen, wall, and outward remodeling area were calculated based on the mean of three measurement sites (caudal, medial, and cranial).

Immunofluorescence

Neovessels were visualized by staining CD31 (1A4, sc376764 SantaCruz Biotechnology) with secondary goat anti-mouse Alexa Fluor 488 (A-11001, Thermo Fischer Scientific) and Hoechst 34580 1:1,000 (63493 Sigma-Aldrich). Images were obtained *via* laser scanning microscopy (LSM700, Zeiss). Polarization of macrophages was evaluated by staining CCR2 (CD192 Alexa luor 647, clone SA203G11, Biolegend) or CD206 (Alexa fluor 647, clone C068C2, Biolegend) in conjunction with CD107b (CD107b Alexa fluor 488, clone M3/84, Biolegend) into CD107b (Alexa Fluor 488, clone M3/84, Biolegend). The antibodies were diluted 1:100 in PBS supplemented with 1% normal goat serum and 1% BSA and incubated overnight at 4 degrees Celsius. The next day, the slides were washed, and incubated with Hoechst 34580 for 5 min. Prolong gold was used as a mounting medium. The integrated density of the CD107b, CCR2, and CD206 signal was quantified *via* a custom integrated density analysis script, by utilizing macro IJM (FIJI). Subsequent determination of double-positive cells (CD107b & CCR2 or CD107b & CD206) was performed by using a custom Python script run in Jupyter Notebook (Anaconda 3) and was represented as a percentage of the total CD107b positive cell count. The first percentile of the integrated density distribution was set as a threshold.

Flow Cytometric Analysis

Flow cytometry was performed on peripheral blood mononuclear cells (PBMC) from ApoE3*Leiden mice treated with ALK1-Fc or control-Fc. Blood was obtained from the tail vein with EDTA as an anticoagulant. Erythrocytes were lysed in ACK lysis buffer (A1049201, Thermo Fischer) and washed twice with PBS supplemented with 0.1% heat-inactivated fetal bovine serum and 0.5 mM EDTA. Conjugated monoclonal antibodies to mouse CD11b PerCP Cy5.5 (M1/70, 1:100, BD Biosciences) and Ly6C APC (RB6-8C5, 1:250, BD Biosciences) were incubated for 20 min on ice. Flow cytometric acquisition was performed on a BD LSR II flow cytometer (BD Biosciences). Flow cytometric data were analyzed by using FlowJo V10.1 software (BD).

Bone Marrow-Derived Cell Culture and ELISAs

In total, 250.000 murine bone marrow-derived monocytes were plated in 100 μl/well, and 200.000 M-CSF differentiated macrophages were plated in 500 μl/well. Both cell cultures were pre-treated with ALK1-Fc (0, 10, 100 ng/mL) or control-Fc (0, 10, 100 ng/mL) for 4 h and subsequently stimulated with 10 or 100 ng/ml lipopolysaccharide. After 24 h, the supernatant was stored at −20°C. The IL-6 and MCP-1 (BD Biosciences 550950, BD Biosciences 555260) concentrations were determined by ELISA according to the protocol (BD Biosciences) in the supernatant of these macrophages and monocytes.

mRNA Expression Analysis

Total RNA was isolated from paraffin-embedded vein grafts using the FFPE RNA isolation kit (Qiagen). Total RNA was quantitated using a NanoDrop 1,000 Spectrophotometer (Thermo Scientific). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. qPCR was performed on ABI7500 Fast system using Taqman gene expression assays for Hprt1, TNFα, MCP-1, MMP2/9/14, TIMP1, BMP9, ID3, PAI, ELN, Col3A1, NLRP1/3, and IL-1β.

Statistical Analysis

Differences in continuous variables between experimental groups were statistically assessed by using the unpaired parametric *T*-test in Graph Pad Prism 8 software. Data are represented as means ± SD unless stated otherwise. Significance was set at $P<0.05$. Significant differences are graphically represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Reduced ALK1 Signaling Shows an Increase in Outward Remodeling but Induces an Increase in Macrophage Presence

To examine the contribution of the ALK1 signaling pathway to vein graft remodeling in mice, vein grafts from ALK1 heterozygous mice ($ALK1^{+/-}$, $n = 7$) were compared to control mice ($ALK1^{+/+}$, $n = 10$) mice since $ALK1^{-/-}$ homozygosity is embryonic lethal (16) (**Figures 1A,B**). Notably, heterozygosity of ALK1 significantly increased vein graft lumen area by 38% ($p = 0.03$) (**Figure 1C**) and showed a 30% increased outward remodeling of the vein grafts ($p = 0.04$) (**Figure 1E**) while not affecting the vein graft wall area (**Figure 1D**). A significant increase in plaque macrophages was found in the $ALK1^{+/-}$ mice in comparison to the $ALK1^{+/+}$ animals ($p = 0.03$) (**Figures 1F,I**). In contrast, collagen (**Figures 1G–M**) and vascular smooth muscle cell content (**Figures 1H–N**), two factors that indicate stability, were unaffected by ALK1 heterozygosity. Thus, heterozygosity of ALK1 contributes to

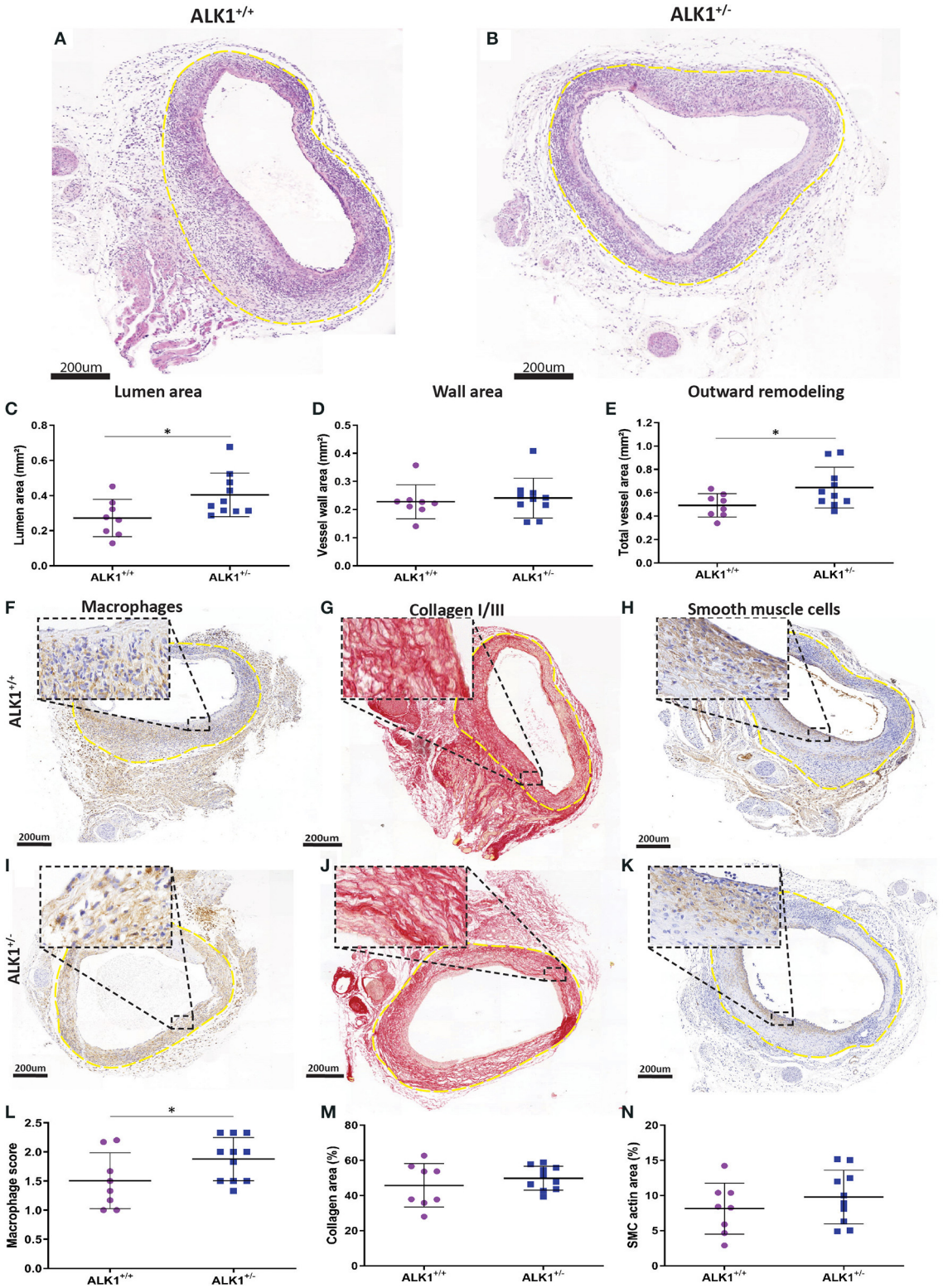


FIGURE 1 | Morphometric and compositional analysis of ALK1$^{+/-}$ and ALK1$^{+/+}$ vein grafts. Vein grafts from ALK1 heterozygous mice (ALK1$^{+/-}$, $n = 7$) were compared to vein grafts from ALK1$^{+/+}$ ($n = 10$) mice. Typical examples of a vein graft from an ALK1$^{+/+}$ mouse **(A)** and a vein graft from an ALK1$^{+/-}$ mouse **(B)**. The outer adventitial layer is highlighted by the dotted yellow line. The morphometric parameters in mm^2 represent the vein graft lumen area **(C)**, the vein graft wall area **(D)**, and the total vein graft area **(E)**. The composition of the vein grafts was evaluated by visualizing the macrophages **(F,I)** collagen **(G,J)** and the vascular smooth muscle cells **(H,K)** in vein grafts from ALK1$^{+/+}$ and ALK1$^{+/-}$ mice. Quantification of the macrophage score **(L)**, collagen area **(M)**, and smooth muscle cell positive area **(N)**. Statistical evaluation was performed with the unpaired parametric *T*-test, $^*p < 0.05$.

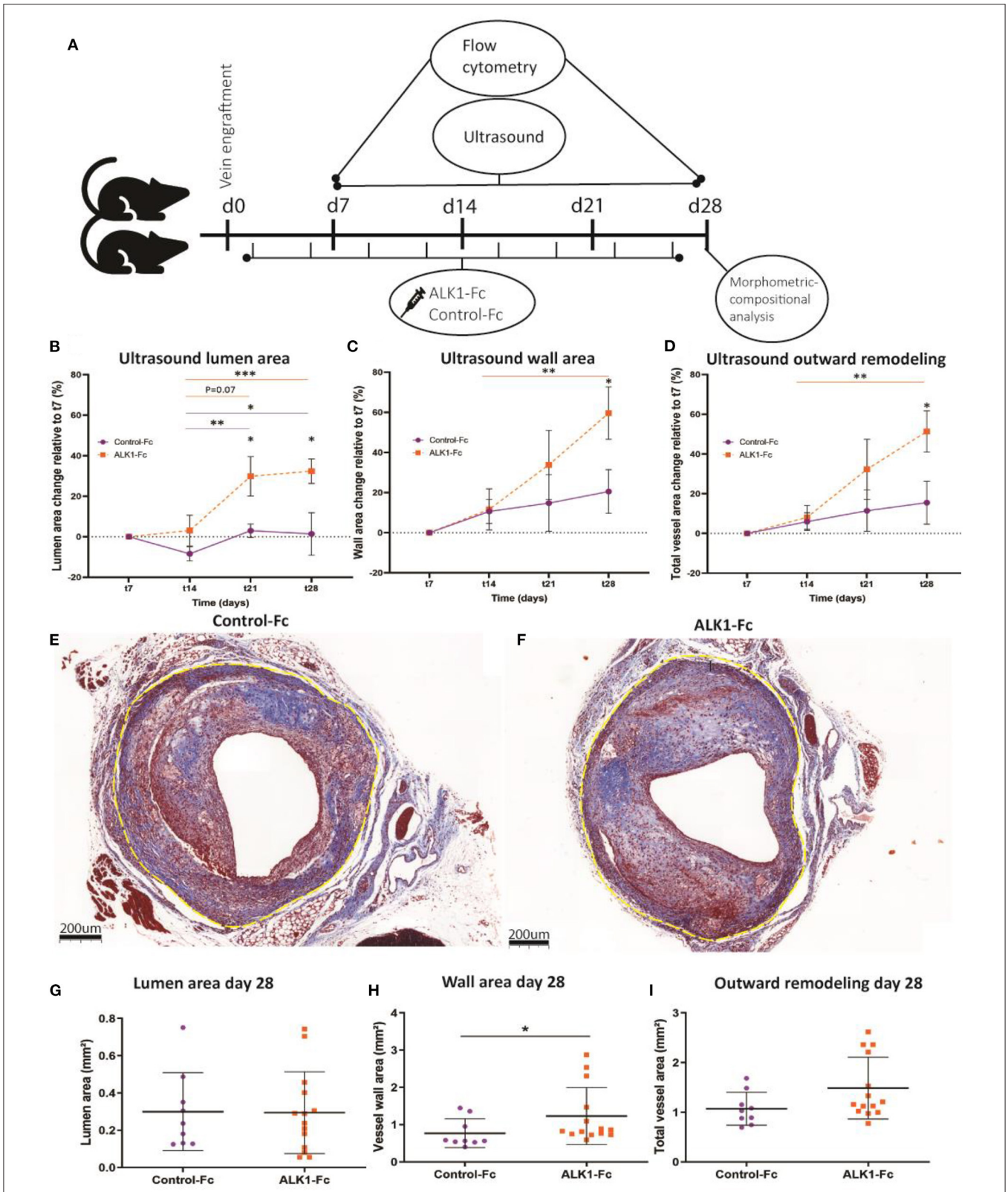


FIGURE 2 | Non-invasive ultrasound analysis and histology analysis of control-Fc and Alk1-Fc treated vein grafts. High fat/cholesterol-fed ApoE3*Leiden mice were treated with ALK1-1Fc or control-Fc (10 mg/kg) twice a week for 28 days. **(A)** Ultrasound measurements were performed in vein grafted ApoE3*Leiden mice treated

(Continued)

FIGURE 2 | with control-Fc ($n = 5$) or ALK1-Fc ($n = 6$) at three separate locations in the vein graft, in which the vein graft lumen area **(B)**, vein graft vessel wall area **(C)**, and the total vein graft area **(D)** were quantified, represented as percentage change relative to day 7. For histology analysis, ALK1-Fc treated mice ($n = 13$) were compared to control-Fc treated mice ($n = 9$). Representative examples of a control-Fc treated vein graft **(E)**, and an ALK1-Fc treated vein graft **(F)** stained with Masson's trichrome. Lumen area **(G)**, wall area **(H)**, and outward remodeling **(I)** were measured 28 days after engraftment. Repeated measures were statistically tested by ANOVA and non-repeated measures by an unpaired *T*-test, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

an increase in outward remodeling of the vein graft and induces an increase in macrophages present in the vein graft wall.

ALK1-Fc Treatment of Vein Grafted ApoE3*Leiden Mice Results in Increased Outward Remodeling

Ultrasound imaging provides real-time, quantitative anatomical and physiological information (19). To interfere with the ALK1 signaling pathway, hypercholesterolemic ApoE3*Leiden mice were treated with either ALK1-Fc or control-Fc and vein graft remodeling was quantified using both ultrasound imaging and endpoint histology (in two different cohorts) according to the set-up indicated in **Figure 2A**. Ultrasound imaging demonstrated that in the control-Fc treated group ($n = 5$) the lumen area only slightly differed in time with a small dip at the 14-day time point. Treatment with ALK1-Fc ($n = 6$) resulted in a gradual increase in lumen area from day 14 to a 10-fold non-significant increase at 21 days and resulting in a significant increase at 28 days compared to 7 and 14 days ($p = 0.01$, $p = 0.004$, **Figure 2B**). When comparing ALK1-Fc treatment to control-Fc at the individual time points, significant differences could be observed at the endpoint of 28 days (32 fold-increase by ALK1-Fc, $p = 0.04$) (**Figure 2B**). The vessel wall of both groups gradually thickened in time. In the control-Fc group, this resulted in a 1.9-fold increase between 14 and 28 days, although non-significant. Upon ALK1-Fc treatment, the observed gradual thickening was much stronger, demonstrated by a 5-fold increase in vessel wall growth at 28 days relative to 14 days after surgery ($p = 0.04$) (**Figure 2C**). This resulted in a 1.5-fold increased enlargement in size between the control-Fc and ALK1-Fc treated mice ($P = 0.04$, **Figure 2C**). Total vein graft area, which is a measure for outward remodeling, can be calculated based on adding lumen and vessel wall area together. In time, the vein grafts display an increase in outward remodeling. Although the control-Fc treated mice showed a small, non-significant increase in total vein graft growth, the vein grafts of the ALK1-Fc treated group demonstrated a steep incline in outward remodeling (6-fold increase at 28 days compared to 14 days, $p = 0.02$, **Figure 2D**). Comparing both groups, this resulted in a significant 3-fold increase upon ALK1-Fc treatment compared to control-Fc at 28 days post vein graft surgery ($p = 0.03$, **Figure 2D**). Histological analysis at 28 days (**Figures 2E,F**) revealed no differences in lumen area between both groups (**Figure 2G**). However, a significant 1.6-fold increase in vessel area can be observed ($p = 0.03$, **Figure 2H**), resulting in a non-significant ($p = 0.08$) trend toward outward remodeling (**Figure 2I**).

Taken together, ALK1-Fc treatment contributes to an increase in outward remodeling of vein grafts.

Vein Graft Lesions Show a Destabilizing Phenotype Upon Treatment With ALK1-Fc

Treatment with ALK1-Fc has been shown to reduce angiogenesis in murine tumor models (12). Since neovessels aggravate vein graft disease (11), the effect of ALK1-Fc treatment on vein graft angiogenesis was investigated. Neovessel density was not different between the two groups (**Figures 3A,D,G**), indicating that ALK1-Fc treatment did not affect angiogenesis in this vein graft model. Next, other lesion composition elements were investigated. Examination of smooth muscle actin expression showed that ALK1-Fc treatment reduced the percentage area of vascular smooth muscle cells significantly by 24% compared to controls ($p = 0.04$) (**Figures 3B,E,H**). In addition, the presence of collagen was significantly decreased by 30% in the ALK1-Fc treated group ($p = 0.005$) (**Figures 3C,F,I**). A phenomenon that is observed in unstable lesions is plaque dissections which are characterized by blood-filled gaps within the intimal hyperplasia, that reach from the lumen to the adventitia (20) (**Figure 3J**). The ALK1-Fc treated group showed an increase in plaque dissections compared to the control-Fc treated group (**Figures 3J–L**).

Vein Graft Vulnerability Is Increased Due to the Influx of Pro-inflammatory Macrophages Upon ALK1-FC Treatment

Vein graft distension upregulates the expression of endothelial adhesion molecules. This triggers the influx of monocytes that subsequently differentiate into macrophages and promote vein graft disease (11). A 1.5-fold increase in macrophages was present in ALK1-Fc treated vein grafts as compared to control-Fc treated vein grafts ($p = 0.001$) (**Figures 4A,D,G**). Macrophages are key modulators and effector cells of the immune response. They may differentiate into pro-inflammatory, $CCR2^{+}$ (CD192, formerly called M1) macrophages or anti-inflammatory mannose $receptor^{+}$ (CD206, formerly called M2) macrophages. Both macrophage types were detected in the vein graft wall in both treatment groups, but no differences in the number of $CCR2^{+}$ macrophages were observed between both treatment groups (**Figures 4B,C,H**). And also no differences in the mannose $receptor^{+}$- macrophages (**Figures E,F,I**), indicating that ALK1-Fc treatment increased the total amount of macrophages in the vein grafts, but not in the specific subsets of $CD192^{+}$ or $CCR2^{+}$ macrophages. Regarding the presence of monocytes systemically in the blood, the ratio of $Ly6C^{high/low}$ monocytes was significantly increased by 2.3-fold after the ALK1-Fc treatment compared to control-Fc treated mice at 7 days after engraftment ($p = 0.008$)

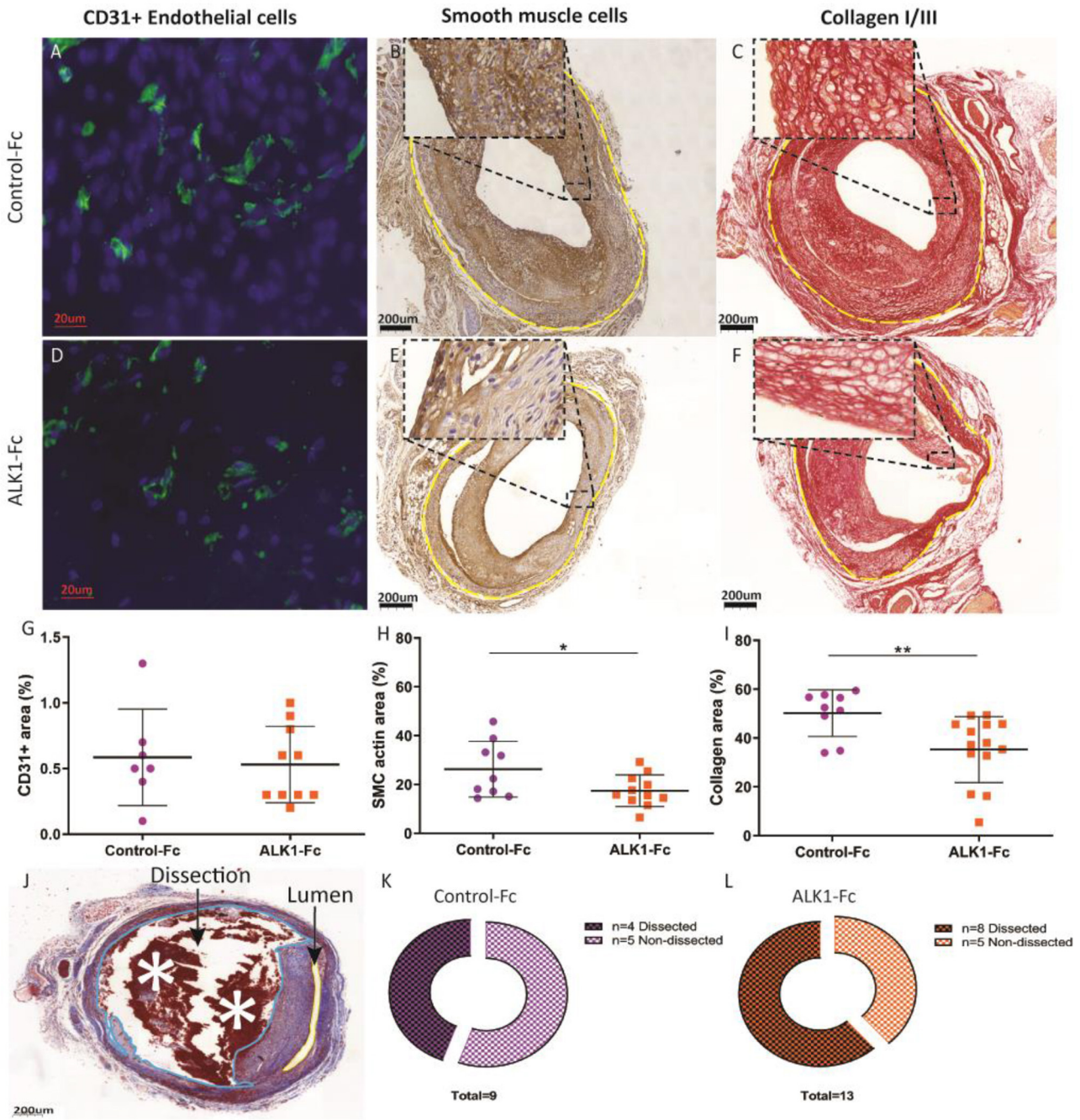


FIGURE 3 | Compositional analysis of ALK1-Fc or control-Fc treated mice. ALK1-Fc treated mice ($n = 11$) were compared to control-Fc treated mice ($n = 8$). Representative examples of CD31 staining **(A)**, vascular smooth muscle cells **(B)**, and collagen **(C)** from a control-Fc treated mouse and examples of CD31 staining **(D)**, vascular smooth muscle cells **(E)**, and collagen **(F)** from an ALK1-Fc treated mouse. The vein graft is highlighted by the yellow dotted line. The relative presence of the CD31 staining, vascular smooth muscle cells, and collagen was identified in the vessel wall *via* calculation of a percentage area **(G–I)**. Destabilizing plaque dissections characterized by blood-filled gaps within the intimal hyperplasia reaching from the lumen to the adventitia **(J)**, dissections (dissection blue, lumen yellow) in control-Fc treated mice **(K)**, and ALK1-Fc treated mice **(L)**. Statistical evaluation was performed with the unpaired parametric *T*-test $^{*}p < 0.05$, $^{**}p < 0.01$.

(**Figures 4J,K**). However, no differences between the two groups of mice (**Figure 2A**) were observed in the $Ly6C^{high/low}$ ratio after 28 days (**Figures 4J,K**), suggesting that ALK1-Fc induced an early systemic response in the pathogenesis of vein graft disease.

In short, compositional analyses of murine vein grafts showed a decrease in stabilizing factors (collagen and smooth muscle cells) and an increase in destabilizing factors (monocytes and macrophages) and dissections upon treatment with ALK1-Fc.

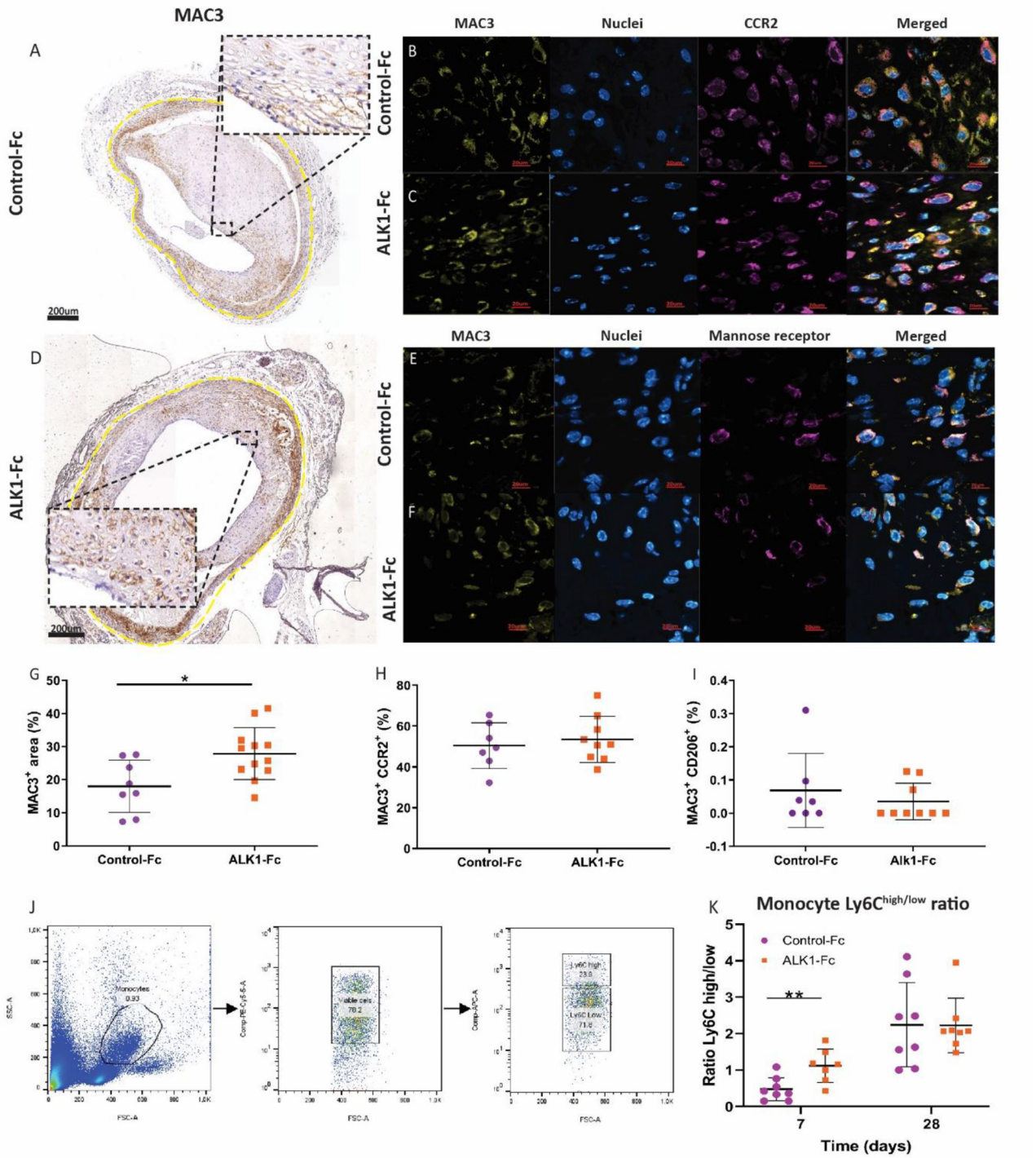


FIGURE 4 | Macrophage content and polarization in vein grafts from ALK1-Fc or control-Fc treated mice. ALK1-Fc treated mice ($n = 11$) were compared to control-Fc treated mice ($n = 8$). Representative examples of a MAC3 (CD107b) **(A)**, MAC3, and CCR2 (CD192) from a control-Fc treated mouse **(B)** and an ALK1-Fc treated mouse **(C,D)**. MAC3 and the mannose receptor (CD206) from a control-Fc treated mouse **(E)** and an ALK1-Fc treated mouse **(F)**. The relative presence of the macrophages was identified in the vessel wall *via* calculation of a percentage area **(G)**. Double positive integrated densities of MAC3 and CCR2 positive macrophages **(H)** and MAC3 and mannose receptor-positive macrophages **(I)** areas were calculated. Monocytes in the blood that expressed Ly6C represented as a high/low ratio **(J,K)**. Statistical evaluation was performed with the unpaired parametric *T*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

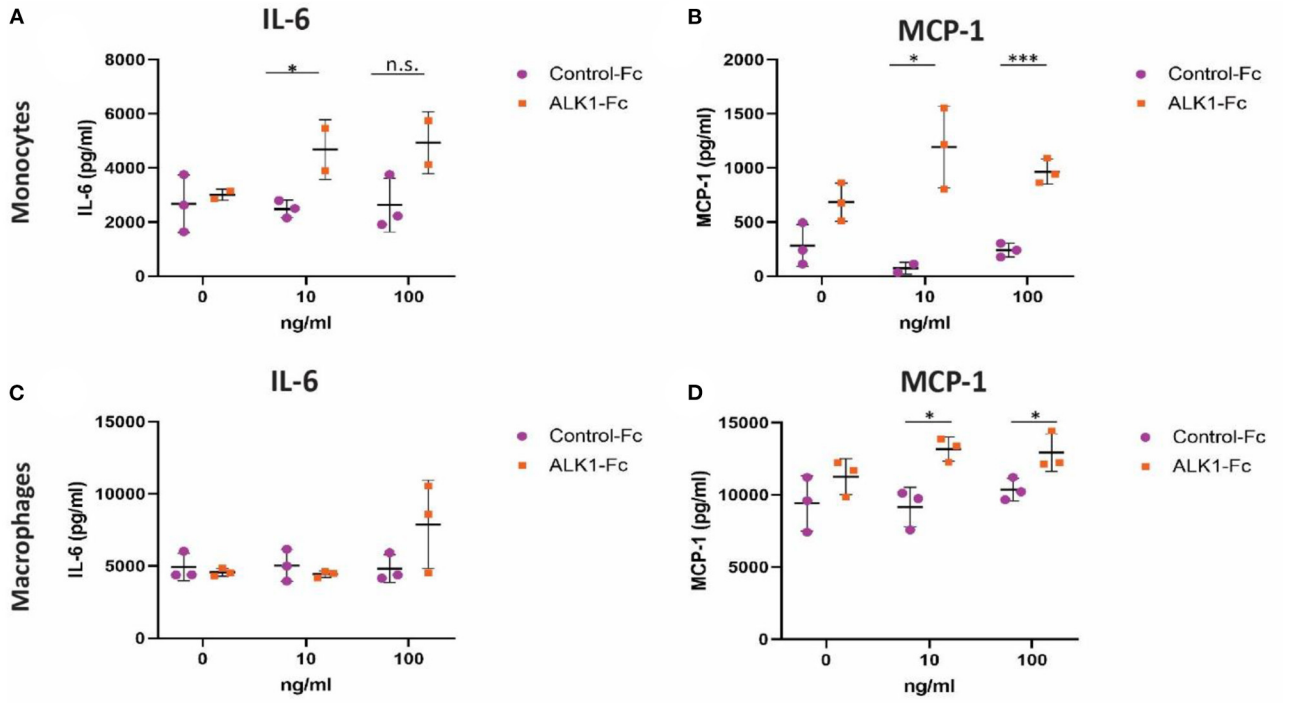


FIGURE 5 | Production of pro-inflammatory cytokine IL-6 and chemokine MCP-1 by LPS-stimulated macrophages and monocytes upon treatment with ALK1-Fc or control-Fc. Monocytes ($n = 3$ experiments) and macrophages ($n = 3$) treated with 0, 10, or 100 ng/ml ALK1-Fc or control-Fc subsequently stimulated with 10 ng/ml LPS. The IL-6 **(A,C)** and MCP-1 **(B,D)** concentrations were determined in the supernatant. Statistical evaluation was performed with the unpaired parametric *T*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n.s. = non-significant.

Monocytes and Macrophages Treated With ALK1-Fc Produce More Pro-inflammatory Factors

Since ALK1 heterozygosity and ALK1-Fc treatment had a profound effect on macrophages *in vivo*, we studied the effects of ALK1 on monocytes and macrophages in more detail, in particular, zooming in on pro-inflammatory cytokines. The production of IL-6 by monocytes *in vitro* was significantly increased by 1.8-fold in the 10 ng/mL ALK1-Fc treated monocytes compared to the control-Fc treated monocytes (**Figure 5A**). Furthermore, the production of MCP-1 by monocytes *in vitro* significantly increased by 16-fold 10 ng/mL ALK1-Fc ($p = 0.03$) and 4-fold after 100ng/mL ALK1-Fc treatment ($P = 0.0007$) (**Figure 5B**).

Although the concentrations of IL-6 released by macrophages were higher compared to monocytes, IL-6 release was not significantly affected by ALK1-Fc treatment in the macrophages (**Figure 5C**). However, the MCP-1 release by macrophages *in vitro* significantly increased by 1.5-fold 10 ng/mL ALK1-Fc ($p = 0.01$) and 1.2-fold after 100 ng/mL ALK1-Fc treatment ($P = 0.04$) (**Figure 5D**). These results indicate that ALK1-Fc treatment increases the inflammatory response in both monocytes and macrophages as was seen *in vivo*.

DISCUSSION

In the current study, we show that inhibition of ALK1 signaling either genetically or pharmaceutically impacts vein graft remodeling by inducing outward remodeling as well as increasing macrophage influx.

Histopathological studies and experimental vein graft models have shown that the inflammatory components of the innate immune system are present in all stages of vein graft adaptation (21). Crucial for a long-term good vein graft patency is the proper amount of vascular remodeling. On the one hand, the vein graft wall needs to thicken to cope with the arterial pressure resulting in inward remodeling, and on the other hand, the lumen should increase due to outward remodeling. A certain degree of inflammation is beneficial in the process of remodeling (22). Inflammatory cytokines and growth factors excreted by leukocytes invading the vessel wall contribute to the reendothelialization of the, during the surgery, damaged endothelium and activate smooth muscle cell migration and activation (1, 21, 22). This inflammatory trigger needs to dampen after the initial period to drive the remodeling process toward the positive outward remodeling. Our work has demonstrated that dampening the ALK1 signaling by half using the heterozygous $ALK1^{+/-}$ mice induced outward remodeling toward the ideal situation without affecting the vein graft composition. A positive

outward remodeling effect was also observed after ALK1-Fc treatment. When monitoring with *in vivo* ultrasound analysis this effect on outward remodeling seemed to be stronger than observed in histological sections, which might be due to tissue processing for histological analysis.

Pro-inflammatory cytokines modulate vein grafts (21), not only by stimulating the initial VSMC proliferation (23) but also by creating a positive loop of monocyte recruitment to the intima thereby increasing macrophage content in the vein graft wall (24). This is substantiated by our data showing the increased macrophage content in the $Alk1^{+/-}$ mice. Moreover, upon treatment of cultured bone marrow-derived monocytes and macrophages with ALK1-Fc after a pro-inflammatory boost (stimulation with LPS), an increase in the production of the inflammatory chemokine MCP-1 is found. *In vivo,* upon ALK1-Fc treatment, positive outward remodeling was also accompanied by an increase in monocyte-mediated inflammation. This was also highlighted by the increase in Ly6C positive monocytes in the blood of ALK1-Fc treated mice.

It has already been shown that ALK1 plays important functions in EC and SMC regulation (25), as indicated by its expression during vasculogenesis and angiogenesis in early development (26). Although repressed in adult blood vessels, ALK1 is re-expressed upon processes of wound healing, tumorigenesis, and angiogenesis (27). ALK1-Fc treatment showed in a murine tumor model a reduction in tumor size and reduced angiogenesis. Vein grafts in mice on a non-atherogenic background such as the $ALK1^{+/+}$ and $ALK1^{+/-}$ mice do not display angiogenesis in thickened vein graft vessel walls. To study the effect of ALK1-Fc treatment on plaque angiogenesis we used ApoE3*Leiden mice that display larger lesions with lipid-rich cores and plaque angiogenesis in vein grafts (4). Our data showed no differences, however, in neovessels present in the vein graft wall. This can be explained that plaque angiogenesis is composed of heterogeneous processes and differs from tumor angiogenesis and therefore requires a different treatment strategy. This is what we demonstrated previously using VEGFR2 blocking antibodies that inhibit tumor angiogenesis that is unresponsive for plaque angiogenesis (28), and this could also be the case for ALK1-Fc. Alternatively, this unexpected lack of effect of ALK1-Fc treatment on intraplaque angiogenesis may be explained that ALK1 can be seen as a factor that mediates the maturation phase of angiogenesis, rather than the activation phase because *in vitro* transduction of endothelial cells with a constitutively active form of ALK1 leads to inhibition of proliferation, migration, and adhesion of these cells (29). But in the end, we can conclude that ALK1-Fc treatment with this concentration is not affecting intraplaque angiogenesis in vein graft disease.

There is some evidence for the involvement of ALK1 signaling in extracellular matrix (ECM) regulation (30). It has been described that the ratio of ALK1–ALK5 regulates ECM protein degradation *via* matrix metalloproteinases (31). Our data has shown that ALK1 heterozygosity did not affect the collagen content in the vein grafts, whereas the pharmacological approach by ALK1-Fc reduced the collagen content in the vein grafts, where it is not clear whether this is due to effects on collagen synthesis or turn-over. Furthermore, ALK1-Fc treatment reduced the number of VSMCs in the vein grafts, This is in agreement with the study of Seki et al., which showed overexpression of ALK1 increases proliferation of VSMCs and induced expression of alpha-smooth muscle actin (25).

Increasing evidence supports a role for ALK1 signaling in modulating monocyte differentiation *via* TGF-β binding to the TGF-βRII/ALK5 complex and exerting inhibitory signals for immune cells (7, 32). In the vein grafts of ALK1 heterozygous mice, an increased macrophage score was found as compared to wild-type mice. Moreover, similar macrophage activation and polarization toward a more inflammatory phenotype was found upon ALK1-Fc treatment. However, there is little information on the effects of ALK1 signaling in macrophages in general. A possible mechanism behind the observed macrophage accumulation upon ALK1 reduction and ensuing plaque destabilization upon ALK1-Fc treatment can be the antagonistic functions of ALK1 and ALK5. Upon ligand trapping with ALK1-FC, the Smad1/5/8 pathway is downregulated in macrophages. Subsequent upregulation of the ALK5 Smad2/3 pathway may result in macrophage activation and release of pro-inflammatory cytokines (33).

Vein grafts and macrophages are not likely to produce the ALK1 ligand BMP9, as underscored by two mechanisms. Firstly, ALK1-Fc acts directly *via* BMP9-ligand trapping, resulting in low BMP9-pSmad1 signaling and endoglin-dependent favoring of TGFβ-SMAD2 signaling in macrophages (34, 35). Secondly, the indirect sequestration of BMP9 by ALK1-Fc from the circulation reduces Smad1 production in the vein graft itself (36). These mechanisms are reflected by the increase in $Ly6C^{high}$ monocytes in the blood of ALK1-Fc treated mice. Monocytes represent a systemic inflammatory response, while macrophages locally affect vein graft remodeling and inflammation, both of which are increased upon ALK1-Fc treatment. Reduced Smad1 signaling in ECs and VSMCs recruits macrophages to promote their pro-inflammatory status *via* the upregulation of ICAM and VCAM (12). In our experiments, an increase in macrophages present in the vein graft wall of both ALK1 heterozygous mice and ALK1-Fc treated mice was observed, suggesting that ALK1-Fc treatment exacerbates inflammation and masks the effect of ALK1-Fc on angiogenesis.

In conclusion, this study shows that inhibition of ALK1 signaling, either *via* a genetic approach or a pharmacological approach, in murine vein grafts promotes outward remodeling, increases macrophage influx, and promotes an unstable plaque phenotype, demonstrating a balancing role for ALK1 signaling in vein graft remodeling and disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Committee of the Leiden University Medical Centre (licensed under project numbers 11045 and 116002016645). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AJ, VS, NS, HP, and MV performed the experiments for the article. PQ and JJ provided funding. MG and MV did the first conceptualization. AJ, VS, MV, JJ, MG, and PQ wrote the manuscript. All authors contributed substantially to the discussion of content, reviewed, and edited the manuscript before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.784980/full#supplementary-material

Supplementary Figure 1 | mRNA expression levels of genes involved in TGF-β/BMP signaling. Total RNA was isolated from ALK1-Fc treated ApoE3*Leiden mice ($n = 6$) and control-Fc treated ApoE3*Leiden mice ($n = 6$). The mRNA expression levels of genes involved in TGF-β/BMP signaling were assessed by qPCR. Statistical evaluation was performed with the unpaired parametric *T*-test, $*p < 0.05$, $**p < 0.01$.

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REVIEW
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The Intriguing Role of TLR Accessory Molecules in Cardiovascular Health and Disease

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Activation of Toll like receptors (TLR) plays an important role in cardiovascular disease development, progression and outcomes. Complex TLR mediated signaling affects vascular and cardiac function including tissue remodeling and repair. Being central components of both innate and adaptive arms of the immune system, TLRs interact as pattern recognition receptors with a series of exogenous ligands and endogenous molecules or so-called danger associated molecular patterns (DAMPs) that are released upon tissue injury and cellular stress. Besides immune cells, a number of structural cells within the cardiovascular system, including endothelial cells, smooth muscle cells, fibroblasts and cardiac myocytes express TLRs and are able to release or sense DAMPs. Local activation of TLR-mediated signaling cascade induces cardiovascular tissue repair but in a presence of constant stimuli can overshoot and cause chronic inflammation and tissue damage. TLR accessory molecules are essential in guiding and dampening these responses toward an adequate reaction. Furthermore, accessory molecules assure specific and exclusive TLR-mediated signal transduction for distinct cells and pathways involved in the pathogenesis of cardiovascular diseases. Although much has been learned about TLRs activation in cardiovascular remodeling, the exact role of TLR accessory molecules is not entirely understood. Deeper understanding of the role of TLR accessory molecules in cardiovascular system may open therapeutic avenues aiming at manipulation of inflammatory response in cardiovascular disease. The present review outlines accessory molecules for membrane TLRs that are involved in cardiovascular disease progression. We first summarize the up-to-date knowledge on TLR signaling focusing on membrane TLRs and their ligands that play a key role in cardiovascular system. We then survey the current evidence of the contribution of TLRs accessory molecules in vascular and cardiac remodeling including myocardial infarction, heart failure, stroke, atherosclerosis, vein graft disease and arterio-venous fistula failure.

Keywords: vascular remodeling, TLR signaling, TLR accessory molecules, inflammation, cardiovascular disease, myocardial infarction, NF-kappa B

PATTERN RECOGNITION RECEPTORS IN CARDIOVASCULAR DISEASE

Upon cardiovascular tissue injury a number of self-derived immunomodulatory molecules are released into the systemic circulation and the interstitial space where they act as damage associated molecular patterns (DAMPs) also called alarmins. Together with exogenous pathogen-associated molecular patterns (PAMPs), DAMPs are recognized by highly specific germline encoded pattern recognition receptors (PRRs) to activate various immune signaling cascades (1).

Depending on cell localization PRRs can be divided into two major groups, the transmembrane protein families—Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and the cytoplasmic protein families—nucleotide-binding oligomerization domain (NOD-like) receptors (NLRs), retinoic acid-inducible gene-I-like receptors (RLRs) and absent in melanoma-2 (AIM2)-like receptors (ALRs) (2). There are several classes of PRRs known to accelerate the inflammatory response in cardiovascular disease (CVD) particularly when the heart or vessel wall respond to ischemia or mechanical stress (3–6). The classic PRRs, TLRs and the more recently discovered NLRs, interact with each other to facilitate progression of several CVDs (e.g., atherosclerosis and heart failure) (4, 7).

Although mainly expressed on immune cells, PRRs are present on cardiovascular cells including endothelial cells, cardiomyocytes, smooth muscle cells (SMCs) and fibroblasts where they trigger a wide array of immune responses against cell damage (8, 9).

TLRs are the first discovered and most essential PRR (10). Initially it was described as a receptor with similarity to the Drosophila Toll protein, which was originally identified in fly embryonic development. Toll was shown to be critical for Drosophila immune defense against pathogens via induction of pathways homologous to those activating the transcription factor Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) (11). TLRs can also recognize non-microbial endogenous molecules that are released upon cell death or present in the extracellular matrix (12, 13). TLR-ligand interaction leads to the activation of both innate and adaptive immune responses, culminating in activation of transcription factors and subsequent production of pro-inflammatory cytokines and type I interferons. These downstream pathways include positive feedback loops which can culminate in a strong response that can induce repair of tissue damage but can overshoot and with that cause acute and chronic inflammation such as atherosclerosis. Especially, accessory molecules can guide and dampen these responses toward an adequate response.

NLRs are intracellular sensors of DAMPs that can be divided into 4 subfamilies depending on the configuration of N-terminal domain. They orchestrate a number of pathways including NF-κB signaling, retinoic acid–inducible gene-I–like receptor signaling, autophagy, major histocompatibility complex gene regulation, reproduction, and development. NLRP3 and NOD1 gathered specific attention in the field of CVDs due to their association with inflammasomes (14, 15). NLRP3 and NOD1 inflammasomes play an essential role in atherogenesis (16–19), aortic aneurysm formation (20), cardiac inflammation and fibrosis (21–24). Recently, AIM2 inflammasome was shown to recognize cytoplasmic self-double-stranded DNA which might point toward a role in sterile inflammation (25).

CLRs are increasingly recognized as PRRs that are not only important in host defense against pathogens but also can recognize number of DAMPs in the progression of cancer, CVD and autoimmune diseases (26, 27). It is a large family of more than 1,000 proteins that have been placed into 17 groups based on their structure and/or function. One of the subgroups of CLRs is Dectin-1 cluster that is comprised of seven receptors and draw particular attention to its role in CVD. For instance, LOX-1 has been extensively studied in atherosclerosis progression and associated hypertension and stroke (28, 29). For the scope of this review it is important to mention that CLR signaling pathways in some cases can synergize with TLR signaling pathways to upregulate cytokine and chemokine production.

In further sections of this review, we will discuss key characteristics of TLRs, with a particular focus of the role of TLRs and their accessory molecules in the development of cardiovascular-related pathologies.

TLR FAMILY AND PATHWAY SIGNALING

The TLR family is a key component of the innate immune system that constitutes of 13 different receptors that are evolutionarily conserved between species. Humans express 10 functional TLRs (TLR1 to TLR10) whereas mice express 12 (TLR1–TLR9, 11, 12, and 13). Except for speculative ligands for TLR10, most of other TLRs-ligand pairs are known. Ligands recognized by TLRs include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of exogenous and endogenous sources.

TLRs are type I transmembrane receptors that contain a N-terminal ligand recognition ectodomain with signature leucine-rich repeats (LRRs), a single transmembrane helix, and a Toll/interleukin-1 receptor-like (TIR) signaling domain. For a proper signal transduction TLRs form homo- or heterodimers. Subsequently, they can interact with various adaptor proteins, including myeloid differentiation primary response protein

Abbreviations: AAA, abdominal aortic aneurism; AF, atrial fibrillation; AGEs, advanced glycation end products; ALRs, Absent in melanoma-2 (AIM2)-like receptors; AngII, angiotensin II; CAD, coronary artery disease; CLRs, C-type lectin receptors; CVD, Cardiovascular disease; DAMPs, Damage associated molecular patterns; DCM, dilated cardiomyopathy; ECM, Extracellular matrix; EndMT, endothelial-to-mesenchymal transition; ER, Endoplasmic reticulum; FFA, free fatty acids; HMGB-1, High Mobility Group Box-1; HSPs, Heat Shock Proteins; IFN-I, Type I interferon; IRFs, Interferon regulatory factors; IRI, Ischemia reperfusion injury; LBP, LPS-binding protein; LDL, low density lipoprotein; LPS, Lipopolysaccharide; LRRs, Leucine-rich repeats; MD1, Myeloid differentiation factor 1; MD2, Myeloid differentiation factor 2; MI, Myocardial infarction; MIRI, myocardial ischemia-reperfusion injury; MyD88, Myeloid differentiation primary response protein 88; NLRs, Nucleotide-binding oligomerization domain-like receptors; NT-proBNP, N-terminal pro b-type natriuretic peptide; PAMPs, Pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; RLRs, Retinoic acid-inducible gene-I-like receptors; ROS, reactive oxygen species; RP105, Radioprotective protein 105; PRRs, Pattern recognition receptors; SMCs, Smooth muscle cells; TIR, Toll/interleukin-1 receptor-like; TLRs, Toll-like receptors; TRIL, TLR4 interactor with leucine-rich repeats.

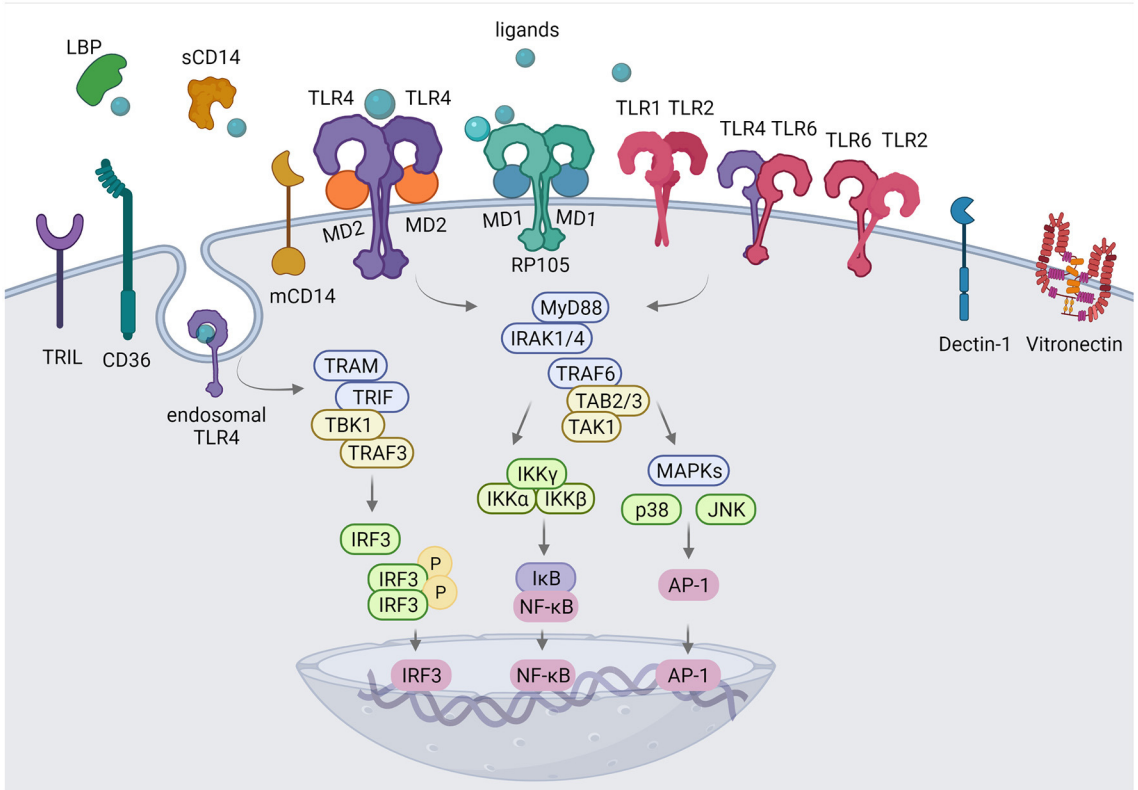

FIGURE 1 | Membrane TLRs and their accessory molecules. Cell surface TLR1, TLR2, TLR4, and TLR6 are essential for the recognition of exogenous and endogenous ligands. TLR1/2, TLR2/6 heterodimers and TLR4/TLR4 homodimer utilize MyD88-dependent pathway to control inflammatory responses *via* activation of NF-κB and AP-1 transcription factors, endosomal TLR4 activates TRAM/TRIF-dependent pathway resulting in type I IFN responses. CD36 induces the assembly of the TLR4/6 and TLR2/6 heterodimers. CD14 can be secreted as a soluble molecule (sCD14) or a membrane bound protein (mCD14) and is involved in ligand delivery to several TLRs. LPS-binding protein (LBP) binds to lipopolysaccharide (LPS) and presents it to CD14. MD2 is necessary for TLR4 to bind to LPS and homodimerize. RP105-MD1 complex has a structural similarity to TLR4-MD2 and exerts dual regulatory activity on TLR4 and TLR2 -regulated inflammatory response. Dectin-1 facilitates TLR2 signaling whereas TRIL interacts with ligands to activate TLR4 signaling. Vitronectin enhances TLR2 and TLR4-mediated responses. Created with BioRender.com.

88 (MyD88) and TIR domain containing adaptor protein-inducing interferon IFN-β (TRIF), which leads to downstream activation of MAPKs and activation of transcription factors such as NF-κB, activator protein-1 (AP-1) and interferon regulatory factors (IRFs) (**Figure 1**). After translocation to the nucleus the transcription factors can induce transcription of proinflammatory genes and interferons.

Depending on the cellular localization and respective ligands TLRs are divided into two subclasses:

(i) Cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) that recognize exogenous microbial membrane components such as lipids, lipoproteins and proteins and number of endogenous molecules amongst others cell-derived proteins, components and degradation products of the extracellular matrix (ECM), lipoproteins and free fatty acids.

(ii) Intracellular TLRs (TLR3, TLR7, TLR8 and TLR9) that are expressed within the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, and recognize components of nucleic acids (**Table 1**).

Upon internalization the cell surface TLR4 can also localize intracellularly into endosomes. Accessory molecule CD14 assures its proper internalization and switch in signaling pathway toward activation of TRIF-dependent signaling and type-I interferons production (30).

There is a wide variety of ligands recognized by TLRs. But how TLRs are able to discriminate between ligands, self-vs. non-self and orchestrate proper cellular response? The LRRs within ectodomain that are composed of just 20 to 30 amino acids (AA) determine structural confirmation of TLRs for a specific ligand interaction, yet variations in AA sequences are limited (31). TLR accessory molecules serve as another important mechanism to ensure proper detection of DAMPs by specific TLRs.

The classical example of the importance of accessory molecules is TLR4 mediated response to bacterial lipopolysaccharide (LPS). There are a number of key-accessory molecules [LPS-binding protein (LBP), CD14, Myeloid differentiation factor 2 (MD2)] that are required for successful propagation of TLR4 signaling. In the presence of bacterial infection first, soluble plasma protein LBP binds to LPS whereupon it is recognized by CD14—a glycosylphosphatidylinositol-linked, LRRs-containing protein that delivers LBP-LPS complex to the cell surface. On the cell surface TLR4 forms a complex with MD2 that serves as the main LPS-binding site. The resultant formation of a receptor

TABLE 1 | TLRs and their ligands.

TLR	Exogenous Ligands	Endogenous Ligands
Localization: Cell surface		
TLR1	Cooperates with TLR2 to recognize bacterial lipopeptides	Cooperates with TLR2 to recognize amyloids
TLR2	Bacterial lipoproteins, peptidoglycans, lipoteichoic acid, yeast mannans	HSP60, 70, 96; biglycan; HMGB1; hyaluronic acid fragments; human β-defensins; acute serum amyloid A; histones; ECM; serum amyloid A, snapin A; endoplasmin; monosodium urate crystals
TLR4	Bacterial LPS, plant taxol, viral fusian protein	HSP20, 60, 70, 72, 96; biglycan, HMGB1, hyaluronic acid fragments; oxidized LDL; mmLDL, fetuin-A; Ang II; serum amyloid A; histones; S100 proteins; fibronectin; fibrinogen; heparan sulfate; syndecan-1; resistin; β-defensin; surfactant protein A
TLR5	Bacterial flagellin	Flagellin from gut microbiota
TLR6	Cooperates with TLR2 to recognize bacterial lipopeptides, fungal zymozan, modulin	Cooperates with TLR2 and TLR4 to recognize HMGB1; HSPs; ECM; HSP60, −70, −96
TLR10	NA	NA
Localization: Intracellular		
TLR3	Viral dsRNA, polyinosine-polycytidylic acid	mRNA
TLR7	Viral ssRNA	Self-ssRNA
TLR8	Viral ssRNA	Self-ssRNA
TLR9	Bacterial and viral DNA	Self-dsDNA; histones; mitochondrial DNA; chromatin immune complexes

multimer, composed of two copies of the TLR4-MD2-LPS complex, initiates signal transduction by recruiting intracellular adaptor molecules.

FROM TLR LIGAND RECOGNITION TO SIGNAL TRANSDUCTION IN CVD

TLRs and their accessory molecules are present on most of the cells of cardiovascular system including endothelial cells, ***smooth muscle cells***, cardiomyocytes, fibroblasts and resident tissue macrophages (32). A number of DAMPs that are released upon cardiovascular tissue injury serve as TLR ligands to induce nuclear translocation of various transcription factors (e.g., NF-κB) and promote pro-inflammatory cytokine release. TLR-DAMP interactions may exhibit both protective and detrimental effects. A short-term TLR mediated inflammatory response is required for a proper cardiovascular adaptation to stress, and is essential for tissue repair and regeneration, whereas long term or excessive TLR activation induces a chronic inflammatory state resulting in adverse cardiac and vascular remodeling. Thorough data supports the importance of TLRs in the pathogenesis of atherosclerosis (33–35), vein graft disease (9, 36), myocardial infarction (MI) (37, 38), ischemia reperfusion injury (IRI) (39), and cardiac allograft rejection (40).

TLR LIGANDS

Amongst the TLRs, membrane bound TLR1, TLR2, TLR4, and TLR6 can be activated by numerous intracellular proteins that are released upon cell damage and cell death [e.g., Heat Shock Proteins (HSPs), High Mobility Group Box-1 (HMGB-1), ATP, mtDNA, RNA and histones]. The intracellular TLR3, TLR7, TLR8, and TLR9 on the other hand are activated by endogenous nucleic acids in endosomes. The role of cell surface TLR5 that recognizes bacterial flagellin was recently depicted in obesity and metabolic syndrome, as it can sense components from gut microbiota to drive systemic inflammation (41, 42). Unlike other TLRs, TLR10 is the unique receptor with anti-inflammatory properties, yet its ligands and functions are not well-defined (43, 44). Besides intracellular proteins, TLRs recognize a number of ECM components [e.g., biglycan, hyaluronic acid, versican, extradomain A of fibronectin (EDA), fibrinogen fragments and surfactant protein A, amyloid-b] and other fragments amongst others including oxidized low density lipoprotein (LDL), free fatty acids (FFA), angiotensin II (AngII), mtDNA, circular RNA, extracellular ATPs and immune complex-containing self-antigens.

A number of DAMPs contribute to cardiovascular remodeling and have been extensively studied in both experimental models and in humans. Following ischemia-reperfusion injury 3 weeks after left coronary artery ligation in rats, administration of HMGB-1 resulted in modulation of inflammation via reduction in dendritic cells, attenuated fibrosis and overall improvement of cardiac function (45). In a similar model, inhibition of HMGB-1 by neutralizing antibodies resulted in enlarged infarct size, increase the plasma troponin-T and norepinephrine content in the heart as compared to untreated animals (46). HMGB-1 also plays a pivotal role in ischemic stroke (47). High levels of systemic HGMB-1 were measured in serums of patients with cerebral ischemia (48). In another study in 338 patients, plasma levels of HMGB-1 was an independent predictor of 1-year clinical outcomes of ischemic stroke (49). A role of HSP60-induced apoptosis via TLR4 in myocyte loss in heart failure was demonstrated on rat primary isolated cardiomyocytes *in vitro* (50). In a prospective study with 251 patients admitted for ST-segment elevation myocardial infarction (STEMI) increase in HSP70 levels were associated with larger infarcts, increased LV dilation and worse clinical outcome post MI (51). Levels of extracellular matrix components, fibronectin, hyaluronic acid and proteoglycans associate with adverse cardiac remodeling post MI. Permanent ligation of the left anterior coronary artery in fibronectin-EDA deficient mice was characterized by reduced inflammation, fibrosis and MMP-2,−9 activity as compared to the WT animals. Together with the reduced recruitment of monocytes and decrease in monocytic TLR2 and CD49 mRNA expression levels after infarction, fibronectin-EDA plays a critical role in adverse cardiac remodeling (52). Another component of ECM—hyaluronan—serves as a promising biomarker for myocardial damage among patients with acute myocardial infarction (AMI). In 56 patients plasma levels of hyaluronan were significantly elevated 30 day after AMI (53). Both fibronectin and hyaluronan were

linked to cardiac allograft rejection as demonstrated in heart allografts in rats. Fibronectin protein levels were upregulated in the vessels exhibiting cardiac allograft vasculopathy and in fibrotic areas whereas increased accumulation of hyaluronan was evident in the edematous interstitial tissue in the heart that was infiltrated with lymphocytes (54, 55). During vascular remodeling in vein graft disease events of distension injury lead to endothelial and smooth muscle cell damage, and degradation of ECM in the media and adventitia. Furthermore, the ischemia-reperfusion injury during and after surgery will also result in the production of DAMPs. Release of hyaluronic acid, proteoglycans and fibronectin that act as endogenous TLRs ligands prime proinflammatory responses which further damage vascular cells (56, 57). Another well-described set of DAMPs – lipoproteins and FFA are well-known factors contributing to atherogenesis and are widely used in clinic as prognostic biomarkers for coronary artery disease (CAD). Levels of oxidized low-density lipoprotein were strongly associated with CAD in a cohort of 504 patients whereas in a prospective cohort of 3,315 participants levels of FFA independently predicted all-cause mortality (58, 59). Mitochondrial DNA and circulating extracellular RNA that are released as a consequence of cell death during myocardial IRI can act as DAMPs to induce pro-coagulation and pro-inflammatory responses (60).

Targeting the DAMP/TLR mediated inflammatory response was proven to be successful in small and large animal models, yet clinical translation remains to be very challenging, since the most investigated systemic therapies can give detrimental side effects. In this light fine-tuning of TLR signaling via accessory molecules might provide better therapeutic outcomes.

TLR SIGNALING

Despite the wide range of DAMPs recognized by TLRs, their structural organization with extracellular and ligand-binding domains is very similar. Following ligand recognition TLRs will form dimers to trigger recruitment of adaptor proteins MyD88 and TRIF to initiate intracellular signaling. TLR2 will form heterodimers with either TLR1 or TLR6, TLR4 will interact with its accessory molecule MD2 to form homo or heterodimers with TLR6, whereas TLR3 forms homodimers upon dsRNA binding (61).

Activation of the downstream MAPKs and IκB kinase (IKK), resulting in activation of the transcription factors AP-1 and NF-κB, respectively culminates in inflammatory cytokine release. In addition, TRIF recruits another cellular kinase, TANK binding kinase 1 (TBK1), to activate the IRF3 and IFN-I production (62) (**Figure 1**).

Although the ligand-induced dimerization of TLRs has many common features, the nature of the interactions of the TLR extracellular domains with their ligands varies markedly between TLR paralogs. Accessory molecules play essential role not only in assuring proper TLR/ligand interaction but governing complex TLR signaling.

CELL SURFACE ACCESSORY MOLECULES IN CARDIOVASCULAR DISEASE PROGRESSION

As discussed earlier in this review, accessory molecules provide mechanisms that can support complexity and diversity of TLR ligand composition. They contribute not only to TLRs signal propagation but facilitate the crosstalk between different TLRs and serve as cofactors.

Delivery of DAMPs by accessory molecules to specific TLRs will assure its proper dimerization, folding, cell localization, and protein processing, all of which guarantee that TLR/ligand interaction will initiate signaling cascades.

Depending on cellular localization, properties and functions TLRs accessory molecules form different groups.

1. TLR accessory molecules that act as cofactors required for cell surface ligand recognition and delivery (LBP, MD2, CD36, CD14, TRIL)
2. TLR accessory molecules that are required for endosomal ligand recognition and delivery (Granulin, HMGB1, LL37)
3. TLR accessory molecules that act as chaperones (Gp96, PRAT4A), trafficking proteins (UNC93B1, AP3) and processing factors (cathepsins, AEP)
4. Adaptor proteins that are required for TLR signaling (MYD88, TRIF, MAL, TRAM)
5. Cross-talk molecules that facilitate inflammatory signal transduction (NOD1, NOD2, NALPs)
6. Proteins with both TLR crosstalk and cofactor function (RP105-MD1, Dectin-1, Vimentin)
7. Receptors that interact with TLRs and passively modulate TLR functions: B cell receptor, RAGE

To restrict the focus of this review, in this review we will only discuss accessory molecules that are required for TLR ligand recognition and delivery on cell surface or involved in crosstalk of signaling pathways (**Figure 1**). Factors that serve as scaffolding or adaptor proteins required for signaling are excellently reviewed elsewhere (10).

The contribution of inflammation in vascular remodeling is well-accepted although a number of questions on what initiates and maintains inflammatory state remains not completely understood. Here, we summarize the current knowledge on the contribution of TLR accessory molecules and downstream signaling in the course of cardiovascular remodeling.

TLR ACCESSORY MOLECULES THAT ACT AS COFACTORS REQUIRED FOR CELL SURFACE LIGAND RECOGNITION AND DELIVERY

Myeloid Differentiation Factor 2—MD2

Myeloid differentiation factor 2 (MD2 also known as Ly96 or ESOP-1) is a small 18–25-kDa protein that binds to ectodomain of TLR4 and can also be secreted as a soluble molecule. TLR4-MD2 interactions are extremely important for proper signaling

as no physiological role of TLR4 has been demonstrated in the absence of MD2. MD2 is responsible for LPS binding, TLR4 glycosylation and cell trafficking (63). LPS interacts with a hydrophobic residue in MD2 and directly bridges the two copies of the TLR4-MD2 multimer. The crystal structure of TLR4-MD2-LPS complex demonstrates how five of the six lipid chains of LPS embedded inside the MD2 pocket whereas the remaining chain is exposed to the surface of MD2. Formation of MD2-LPS complexes are essential for bridging the two TLR4 molecules and propagation of intracellular signaling (64).

Even though MD2 is known to be an important modulator of innate immune system, our knowledge on its role in cardiovascular remodeling is still limited.

Number of studies investigated the role of MD2 as biomarker for several CVDs. The clinical study by Riad et al. assessed the predictive value of MD2 in dilated cardiomyopathy (DCM) (65). DCM is characterized by ventricular chamber enlargement and systolic dysfunction, with most cases being idiopathic. Furthermore, a limited number of studies suggests that DCM associated with a chronic inflammatory state (66). MD2 is highly expressed within the myocardium of DCM patients as well as in murine cardiac tissue suggesting that MD2 can have local cardiac regulation of inflammatory responses. *In vitro* stimulation of cardiomyocytes with MD2 showed dose-depended negative ionotropic effect as demonstrated by cardiomyocyte cell shortening. In 174 patients diagnosed with DCM elevated MD2 blood levels served as additional biomarker to the gold-standard NT-pro-BNP for mortality risk prediction (67, 68).

Genome-wide association study in 304 individuals undergoing coronary artery bypass graft surgery identified potential positive association between MD2 and postoperative atrial fibrillation (AF). The same study identified genetic polymorphisms in the MD2 gene that were associated with decreased risk for postoperative AF (69). Further randomized trials should confirm the role of MD2 as a useful diagnostic and/or therapeutic target in DCM and postoperative AF.

Interesting data on the role of MD2 in atherosclerosis-driven cardiovascular diseases comes from a recent study by Chen et al. Here, the authors investigated to what extend MD2 participates in ox-LDL-induced TLR4 activation during atherogenesis. MD2 was highly present in atherosclerotic lesions from $ApoE^{-/-}$ mice and in peripheral blood mononuclear cells (PBMC) from patients with coronary artery disease. In monocytes and macrophages MD2 is essential for ox-LDL-induced TLR4 dimerization, downstream activation of NF-kB and subsequent production of proinflammatory cytokines. Deficiency of MD2 reduced atherosclerotic plaques through reduced lesional macrophage content and expression of inflammatory cytokines (70).

Besides circulating immune cells, MD2 has local expression within vasculature including SMCs and endothelial cells, where it may regulate cell phenotypic switching (71, 72).

In an angiotensin II (Ang II) induced pathological model of aneurism formation, deficiency of MD2 resulted in the phenotypic switch of SMCs toward a proliferative phenotype and increased fibrosis (71).

Besides its well-known effect on arterial blood pressure, Ang II can directly induce cardiac remodeling through induction of TLR4 mediated cardiac inflammation (73). Han et al. demonstrated that MD2 mediates Ang II induced cardiac remodeling by direct binding to Ang II and the subsequent activation of the TLR4/MyD88/NF-kB signaling cascade (72). Moreover, in Ang II induced aortic remodeling MD2 was identified as a critical point in the Ang II induced endothelial-to-mesenchymal transition (EndMT) – an important mechanism of pathological vascular and cardiac remodeling. Pharmacological inhibition of MD2 reduced Ang II-induced EndMT changes, including increased levels of endothelial marker VE-cadherin, and reduction of mesenchymal markers alpha smooth muscle actin (α-SMA) and vimentin.

In obesity induced cardiac remodeling, both pharmacological inhibition and genetic deletion of MD2 resulted in attenuated cardiac inflammation and fibrosis via reduction of JNK, ERK and NF-kB signaling and reduced expression of cell adhesion molecules ICAM-1, VCAM-1, and CD68 (74).

Within the cardiovascular system MD2 was shown to interact with number of endogenous molecules that are released in response to stress, including free fatty acids (75, 76). In diabetic cardiomyopathy, advanced glycation end products (AGEs) bind to MD2 resulting in TLR4 activation and myocardial injury (77). Interestingly Huang et al. demonstrated the role of MD2 in vascular oxidative stress via SIRT1/MAPKs and reactive oxygen species (ROS) generation.

Taken together MD2-mediated chronic inflammation occurs in diverse cells and MD2 deficiency and pharmacological inhibition may alter a number of parallel pathways in vascular tissues (78). Promising results on the therapeutic benefits of MD2 inhibition comes from a recent study by Fang et al. Treatment with a small peptide Tat-CIRP which can pass through the blood brain barrier and competitively bind to MD2 was shown to induce long-lasting neuroprotection against ischemic and hemorrhagic stroke in rodents and non-human primates (79).

LPS-BINDING PROTEIN—LBP

LPS-binding protein (LBP) is a 53 kDa protein that facilitates delivery of LPS to membrane bound and soluble CD14 to induce TLR4 signal transduction (80). Even though the crystal structure of LBP has not been reported, emerging state-of-the-art computer tools that utilize artificial intelligence allow to predict protein's 3D structure from its amino acid sequence that would help to foster our understanding of molecular interactions between LBP-LPS in the near future[1].

Besides LPS, LBP can bind other PAMPs derived from gram-negative and gram-positive bacteria including lipopeptides and peptidoglycans. In addition to TLR4, upon ligand binding LPB can also activate other TLRs, including TLR1, TLR2, and TLR6 (81).

Recent attention to the contribution of microbiome in CVD supports the notion of endogenous PAMPs act as drivers of systemic CVD. A state of dysbiosis triggers production of endogenous PAMPs by the gut microbiota which in turn activates LBP-dependent low grade systemic inflammation. In this light

[1] Available online at: https://alphafold.ebi.ac.uk/.

proinflammatory action of LBP might contribute to vascular remodeling and development of cardiovascular complications suggesting its role as potential biomarker (82).

In two consecutive studies the LBP concentration was significantly elevated in patients with coronary artery disease and was associated with all cause and cardiovascular mortality (83, 84). In type 2 diabetes, increased levels of LBP correlated to diabetic retinopathy and arterial stiffness suggesting its role in the activation of local inflammatory response within the vasculature (85, 86).

In an exploratory study on 72 individuals LBP was reversely associated with CVD risk in older adults (87). Potentially counterintuitive, higher levels of cholesterol in elderly coupled with higher LBP may promote faster clearance of bacterial toxins from the circulation resulting in reduction of systemic inflammation (88).

Traditionally, LBP promotes TLR4 signaling shuttling to CD14 to interact with LPS. An elegant study by Han et al. explored the opposite effect of LBP on TLR4 cascade in the presence of intestine specific form of high density lipoprotein (HDL)—HDL3 (89). LBP was required to inhibit LPS-TLR4 signaling on liver macrophages via interaction with HDL3 particles. In this way, HDL3 interacts with LBP to mask LPS from detection by TLR4 signaling platform, resulting in an anti-inflammatory and anti-fibrotic mode of action. This finding might be relevant in cardiovascular disease as HDL3 levels correlate with better health outcomes (90).

These studies clearly indicate that there is a link between the accessory molecule LBP and CVD, however, more studies are needed to elucidate the role of LBP in various comorbidities and risk statuses.

CLUSTER OF DIFFERENTIATION 36—CD36

CD36 is an 88 kDa membrane glycoprotein that belongs to the class B family of scavenger receptors. It is expressed on various cell types including monocytes/macrophages, platelets, dendritic cells, microglia, cardiovascular cells and adipocytes (91). Recent studies demonstrated that CD36 is involved in inflammation, angiogenesis, lipid metabolism and atherosclerosis progression. In CVD, CD36 is largely known as the receptor for oxLDL that accounts for 60 to 70% of cholesterol ester accumulation in macrophages. Solid number of literature on the role of CD36 in lipid trafficking and atherogenesis are available, see this excellent review (92), but will not be discussed here.

CD36 can interact with number of exogenous and endogenous ligands to facilitate downstream TLRs signaling. Recent studies revealed that CD36 induces the assembly of the TLR4/6 and TLR2/6 heterodimers underlining its role as TLR accessory molecule (93). Mediation of TLR4-TLR6 heterodimerization occurs from the C-terminus of CD36 within the cell. Point mutation at tyrosine 463 of CD36 resulted in inability of TLR4-TLR6 dimerization and NF-κB activation in response to oxLDL. In addition, a functional study reported the importance of CD36 interaction with Lyn kinase to assure TLR4 and/or TLR6 phosphorylation, TLR4-TLR6 association and signal transduction (93).

Interaction with microbial PAMPs mainly diacylglycerides LTA and R-MALP2 enhances TLR2-TLR6 mediated immune response (94) whereas binding to DAMPs including oxLDL, amyloid-b fibrils and apoptotic cells mediate TLR4-TLR6 inflammatory responses (93, 95).

In a hyperlipidemic ApoE$^{-/-}$ model of atherosclerosis CD36 was shown to trigger TLR4-TLR6 dependent accumulation of TRIF-dependent chemokine RANTES and an overall increase in ROS production by macrophages. Such response amplifies oxidative stress in the artery wall, DAMPs generation and chronic macrophage activation (93).

Despite its apparent detrimental role in ischemic damage through activation of inflammation and ROS, CD36 may also exert a beneficial role during post-stroke and post-MI resolution phase of inflammation by mediating phagocytosis (96, 97). Interestingly, in the absence of elevated circulating lipids cardiomyocyte-specific deletion of CD36 accelerated the progression of pressure overload-induced cardiac hypertrophy to cardiac dysfunction (98). Contrary, CD36 can promote neovascularization and scar formation worsening post-stroke recovery (99, 100).

Clearly, CD36 is a multifunctional receptor that may play different roles in the CVD pathogenesis/repair. Further mechanistical studies on the role of CD36 as TLR accessory molecule will help to determine its potential as a therapeutic target.

CLUSTER OF DIFFERENTIATION CD14—CD14

CD14 is a pattern recognition receptor that has long been known as co-receptor for several TLRs. It is a 375 amino acid LRR-containing glycoprotein that can be secreted into the serum as a soluble molecule (sCD14) or expressed as a glycosylphosphatidylinositol (GPI)-linked membrane bound protein on the surface of cells (mCD14) (101). mCD14 is highly expressed on myeloid cells, whereas sCD14 is present in different body fluids to transduce LPS-responsiveness to cells not expressing CD14 (102, 103). Before the discovery of TLR in 1997 (104), CD14 was known as a cell differentiation marker for human monocytes and classical dendritic cells (105, 106). The crystal structure demonstrates CD14 to form a dimer with hydrophobic pocket located at its N-terminus that is essential for recognition of LPS and other microbial peptides. Formation of CD14-LPS complex is important to enhance the detection of LPS by the TLR4–MD2 complex by monomerizing LPS before its presentation to TLR4–MD2 (107).

CD14 has a wide range of exogenous and endogenous ligands, including bacterial PAMPs, heat shock proteins, phospholipids and amyloid. CD14 is involved in ligand delivery and functions as an adaptor and coreceptor of TLRs to increase ligand affinity to TLR and augment signal transduction (108–110). CD14, together with TLR4 and MD-2, forms the multi-receptor complex that recognizes LPS on the cell membrane resulting in activation

of MyD88-dependent signaling (111). Upon low doses of LPS CD14 allows the activation of the intracellular TLR4-TRAM-TRIF pathway resulting in TLR4 internalization and endocytosis (112). As a coreceptor CD14 is necessary for the TLR7- and TLR9-dependent induction of proinflammatory cytokines *in vitro* and for TLR9-dependent innate immune responses in mice (113, 114).

Uncontrolled inflammation with TLR overactivation on $CD14^+$ monocytes has long been recognized as a driving mechanism for atherosclerotic disease (115, 116). Population of intermediate monocytes with increased mCD14 and CD16 can independently predict cardiovascular events in patients with coronary artery disease (117, 118). CD14 is also known to regulate function of endothelial and smooth muscle cells. The interaction of LPS with CD14 on the surface of endothelial cells results in expression of proinflammatory cytokines and adhesion molecules (119). Local expression of CD14 by human coronary artery smooth muscle cells can potentially increase tissue sensitivity to pro-atherogenic risk factors (120).

Upregulation of CD14 in adventitial macrophages in a murine model of abdominal aortic aneurism (AAA) and both locally and systemically in human AAA underscore its role in the pathogenesis of AAA (121).

In the central nervous system CD14 deficiency causes attenuated monocyte influx to the brain and aggravation of tissue injury after ischemic stroke (122).

sCD14 has gained interest as a risk factor for different CVD. Important to mention are racial difference in sCD14 levels. Elevated plasma sCD14 was an independent risk factor for heart failure, coronary heart disease and stroke in cohorts of African Americans whereas was not linked to increase incidence of CVD in Caucasians (123–125). Activation of endothelial cells through sCD14 results in upregulation of adhesion molecules and procoagulant activity (108).

A number of recent studies unraveled the contribution of disturbed intestinal barrier toward chronic low-grade endotoxemia. Bacterial LPS binds and stimulates systemic secretion of CD14 which is required for propagation of TLR signaling. CD14-LPS complex is implicated in LPS-induced myocardial dysfunction (126).

In summary, as mCD14 but also as sCD14, CD14 exerts significant impacts in the pathogenesis of cardiovascular diseases. It can both promote and diminish TLR signaling which makes it an important regulator of the inflammatory response.

TLR4 INTERACTOR WITH LEUCINE-RICH REPEATS – TRIL

TRIL (TLR4 interactor with leucine-rich repeats) is a 811 amino acid leucine-rich repeat protein that plays an important role in TLR3 and TLR4 signaling. TRIL is primarily expressed in the brain where it interacts with LPS or poly(I:C) ligands to activate TLR4 and TLR3 signaling, respectively (127, 128).

TRIL induction helps to prolong LPS signaling and is structurally similar to CD14 which makes it a functional homolog of CD14. As LRR- containing protein without signaling domain TRIL could also have a role similar to RP105 positively or negatively regulating TLR4 signaling in a cell-type-dependent manner. Whether it can bind to MD1 and/or MD2 as well as its role beyond the brain is not defined yet.

Some preliminary data indicates that TRIL might be required to control lipid-based ligands for TLR2 signaling.

Recently Jia et al. were the first to demonstrate a role of TRIL-TLR4 signaling in the progression of spinal cord ischemia reperfusion injury (129). Due to its localized expression to the brain contribution of TRIL to systemic CVD remains obscure.

NON-CLASSICAL ACCESSORY MOLECULES WITH TLR SIGNALING AND CROSSTALK FUNCTION

Radioprotective 105—RP105

RP105 (radioprotective 105, CD180) is a TLR-like accessory molecule that has a striking structural similarity to TLR4 with LLR domain and association with MD1, an MD2 homolog. Unlike the TLRs, however, RP105 lacks an intracellular signaling TIR domain. The crystal structure of RP105-MD1 bound to LPS shares a similar overall architecture to its homolog TLR4–MD2. Interestingly, assembly of RP105-MD1 homodimer occurs in a head-to-head orientation with N-termini interacting in the middle which is different from TLR4-MD2 complex with the tail-to-tail configuration of C-terminal cytoplasmic signaling domain. Such unique mode of assembly in RP105–MD1 suggesting a potential molecular mechanism for regulating LPS responses and regulation of TLR4-MD2 signaling complex (130). Depending on the cell type RP105-MD1 exerts dual regulatory activity on TLR-regulated inflammatory response. On B-cells, where it was originally discovered, it stimulates cell proliferation and antibody production (131, 132). On myeloid cells, including monocytes, macrophages and dendritic cells RP105-MD1 complex acts mainly as TLR4 antagonist. Importantly, RP105-MD1 is also expressed locally within cells of the CV system, including arterial and venous SMCs, endothelial cells and cardiomyocytes (133). A number of studies indicate a role for RP105 in several CVD pathologies as both physiological inhibitor and agonist of TLR4 signaling.

RP105 was shown to play an important role in IRI. In a model of hind limb ischemia RP105 deficiency resulted in an uncontrolled inflammatory response, impaired blood flow recovery and reduced arteriogenesis. These outcomes were linked with premature systemic activation of pro-inflammatory monocytes ($Ly6C^{hi}$) in RP105 knockout animals that resulted in accumulation of $Ly6C^{hi}$ monocytes in the bone marrow and spleen rather than in the ischemic tissues where the reparative response was needed (134).

Several studies explored the role of RP105 in myocardial infarction. In a model of a short-term myocardial ischemia-reperfusion injury (MIRI) adenoviral delivery of RP105 protected myocardium against IRI via inhibition of TLR4/TLR2 inflammatory cascade and regulation of cardiomyocyte apoptosis and autophagy via Bcl-2/ Beclin1 complex (135–137). *In vitro*, overexpression of RP105 was effective to protect cardiac

microvascular endothelial cells against hypoxia induced reoxygenation injury (133). As expected, knockdown of RP105 resulted in increased myocardial infarction size during MIRI that was associated with TLR2/4 activation and increase in myocardial cell apoptosis (138).

Louwe et al. examined the effects of RP105 on cardiac function post-MI using permanent ligation of the left anterior descending coronary artery model that closely mimics MI in humans. RP105 deficiency hampered repair processes and resulted in the right shifted PV-loop similar to what is seen in dilated cardiomyopathy. Interestingly, even though RP105 deficiency resulted in enhanced inflammatory status and more pronounced cardiac dilation, there was no difference in the infract size between RP105$^{-/-}$ and WT animals (139).

RP105 was shown to contribute to vascular remodeling in vein graft disease and failure of hemodialysis vascular access conduit. Vein graft disease progression is linked to intimal hyperplasia, inflammation and superimposed atherosclerosis development. It is known that TLR4 signaling plays an important role in vein graft disease progression (56). In a study by Wezel et al. RP105 deletion exacerbated vein graft disease in the presence of hypercholesterolemia. Increase in lesional CCL2 expression resulted in macrophage and mast cell accumulation accompanied by lesion destabilization and intraplaque hemorrhage (140).

Similar to vein graft disease, arteriovenous fistula failure is associated with increased inflammation and intimal hyperplasia. Impaired outward remodeling is another important parameter of successful vascular remodeling assuring sufficient blood flow during dialysis treatment. RP105 deficiency resulted in an impaired outward remodeling of murine arteriovenous fistula. Interestingly, the importance of RP105 in the balance between pro-inflammatory and regenerative response in macrophages and SMCs was demonstrated (141). Specifically, accumulation of anti-inflammatory macrophages in vascular lesions from RP105 deficient mice and decrease in proliferation and migration of *in vitro* cultured venous and arterial SMCs, respectively.

Therapeutic delivery of soluble RP105 by electroporation mediated gene transfer was shown to be an effective strategy to dampen vascular remodeling and intimal hyperplasia in a mouse model of post-interventional restenosis (142), but only when solRP105 was co-expressed with MD1.

Contrary, in atherosclerosis RP105 appears to play the role of a negative regulator. RNA levels of RP105 are upregulated early during atherogenesis. Deletion of RP105 was linked to decrease in plaque development and CCR2 dependent inhibition of monocyte influx (143). Wezel et al. very elegantly showed an altered migratory capacity of monocytes upon deletion of RP105, and that *in vitro* stimulation of monocytes with LPS induced a downregulation of CCR2, a chemokine receptor crucially involved in monocyte influx to atherosclerotic lesions, which was more pronounced in RP105$^{-/-}$ monocytes. In a different model of atherosclerosis, bone marrow transplantation from RP105$^{-/-}$ mice into hyperlipidemic LDLR$^{-/-}$ recipients resulted in plaque size decrease. Such effect was explained by reduction in activation and proliferation of proinflammatory subset of B-cells and diminished production of immunoglobulin IgG2c (144).

From the literature it is clear that RP105 is involved in the complex regulation of vascular remodeling and control of TLR4-mediated inflammatory response in different cardiovascular pathologies. To design a successful therapeutic strategy targeting TLR4/RP105 axis cell specific targeting and time of the application should be taken into consideration in view of these complex pathophysiological roles.

Myeloid Differentiation 1—MD1

Myeloid differentiation 1 (MD1, also known as lymphocyte antigen Ly86) is a secreted glycoprotein that interacts with RP105 to assure sufficient RP105 cell surface expression whereas MD1 acts as an MD2 homolog (145). MD1 has a similar structure to MD2 with a hydrophobic cavity that accommodates LPS or related microbial peptides (146). As we previously described, the RP105-MD1 complex plays an important role in TLR4 signaling. Expression of MD1 is not limited to the cells of the immune system but is highly expressed within cardiac tissue[2]. A number of studies elaborated on the role of MD1 in pressure induced cardiac remodeling and post IRI myocardial adaptation amongst others, hypertrophy, fibrosis, arrythmias and heart failure.

Cardiac hypertrophy is a compensatory mechanism in response to biomechanical wall stress wall that is associated with increases in cardiomyocyte size, increased ***extracellular matrix*** synthesis and a higher organization of sarcomere (147). Even though pathways that promote hypertrophic response are well-defined, little is known on key molecular players underlying these pathways that can serve as effective therapeutic targets. Xiong et al. were the first to demonstrate the importance of MD1 in pathological cardiac remodeling (148). Constitutive cardiac overexpression of MD1 in mice had a prominent effect against cardiac hypertrophy and fibrosis via inhibition of TLR4 downstream signaling molecules MEK-ERK1/2 and NF-κB, whereas loss of MD1 caused chronic pressure overload induced cardiac remodeling. Hyperactivation of TLR4 signaling in the absence of MD1 triggers Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) signaling, resulting in alteration of Ca^{2+} handling and K^+ and Na^+ channels in stressed myocardial tissues (149). Consistent with previous findings, in a model of myocardial infarction MD1 depletion resulted in elevation of MI-induced fibrosis, inflammation and electrical remodeling via upregulation of TLR4/CaMKII signaling that was linked to increased vulnerability to ventricular arrhythmias (150). The link between inflammatory TLR4 signaling and regulation of ion channels in LV structural and electrical remodeling further support importance of accessory molecule MD1 as potential therapeutic strategy in heart failure.

Interestingly, in a model of post-interventional vascular remodeling overexpression of MD1 alone did not have any effect on vascular remodeling whereas simultaneous expression of soluble RP105 and MD1 resulted in a significant reduction in intimal hyperplasia (134). In line, Divanovic et al. showed that RP105 can only act as inhibitor for TLR4 signaling if MD1 is sufficiently present (151). Similar to MD2 dependent TLR4

[2]Available online at: https://www.proteinatlas.org/. (2003).

surface expression, RP105 surface expression was shown to be dependent on MD1 (145).

Expression of MD1 was reported to be decreased in hearts from obese patients. Obesity is a known risk factor in metabolic syndrome development that is associated with cardiac remodeling and impairment of left ventricular function. When mimicking obesity in a mouse model, deletion of MD1 aggravated high-fat diet-fed induced maladaptive left ventricular hypertrophy *via* TLR4/MyD88/CaMKII (152) and TLR4/NF-kB signaling (153). The role of MD1 seems to be of particular importance in obesity-related structural and electrical remodeling as it triggers not only ventricular but also atrial remodeling via the earlier described TLR4/MyD88/CaMKII signaling pathway (154).

A recent study demonstrated the importance of MD1 in myocardial ischemia-reperfusion injury and IRI related arrhythmia (155). Loss of MD1 led to a larger infarct size, increase in pro-inflammatory TNF, IL1β, and IL6 plasma levels, induction of myocardial apoptosis and accumulation of neutrophils and macrophages. In a concomitant study MD1 overexpression protected myocardial function against high-fat induced hypertrophic cardiomyopathy, further supporting the importance of TLR4 inflammatory signaling in the mechanism of myocardial injury and therapeutic potential of MD1 in cardiac remodeling (156).

Dectin-1

Dectin-1 is a pattern recognition receptor that belongs to the class of C-type lectin receptors and is mainly expressed on activated myeloid cells (157). When bound to its ligand, β-glucans, dectin-1 initiates recruitment and phosphorylation of spleen tyrosine kinase (SYK), thereby activating the NF-κB dependent inflammatory cascade. Traditionally, dectin-1 has been associated with the recognition of fungi, but recent discoveries underlined its role in non-infectious diseases including those related to the cardiovascular system. Dectin-1 aggravates cardiac remodeling after myocardial infarction and worsens inflammatory response after ischemic stroke (158, 159). In patients, increase in circulating dectin-1^{+} monocytes correlates with the severity of cardiac dysfunction (160).

One study indicates that there is a crosstalk between dectin-1 and TLR2 signaling in response to fungal pathogens suggesting that dectin-1 is involved in crosstalk of TLR-dependent signaling pathways. Contribution of dectin-1 and its therapeutic potential in the context of TLRs mediated responses in the pathogenesis of cardiovascular requires further investigation (161).

Vitronectin

Vitronectin is a 75 kDa glycoprotein that is present in plasma, ECM, and in alpha-granules of blood platelets. It has a major impact on cell adhesion, migration and vascular remodeling via interaction with integrin receptors. Similar to dectin-1, via interaction with its receptor integrin beta3 vitronectin was shown to enhance TLR2-mediated responses to microbial lipopeptides (162). In addition, vitronectin was reported to enhance TLR4 mediated signaling by recruitment of the adaptor protein TIRAP to the plasma membrane (163).

Vitronectin plays role in vascular remodeling via activation of adhesion and migration of SMCs contributing to intimal hyperplasia (164) and it is highly expressed in atherosclerotic plaques (165). Several integrins expressed on vascular SMCs and platelets, including vitronectin receptor αvβ3, recognize the Arg-Gly-Asp (RGD) sequence present on many adhesion molecules, to participate in the cell adhesion to vitronectin and migration of the cells toward fibronectin, laminin, and collagen types I and IV. Thus, SMCs adhesion to vitronectin can be inhibited by RGD-containing peptides to prevent formation of intimal hyperplasia (166). In a hamster model of intimal hyperplasia induced by surgical damage of the carotid arteries and continued administration of G4120, a cyclic RGD-containing peptide, reduced the platelet activation and the SMCs content, preventing intimal hyperplasia formation as compared to untreated animals (167). In a rat model of arterial injury caused by balloon angioplasty, administration of abciximab—monoclonal antibodies involved in inhibition of integrin signaling, prevented formation of intimal hyperplasia. Abciximab interacts with αIIbβ3 (glycoprotein IIb/IIIa complex) integrin on platelets that inhibits platelets adhesion in injured vessels and it can also bind to αvβ3—vitronectin receptor present of SMCs hindering cell migration and proliferation (168).

Vitronectin contributes to regulation of vascular homeostasis at sites of vascular injury. It stabilizes PAI-1—a central physiological inhibitor of plasminogen activation (169, 170) and can bind to platelet glycoproteins mediating platelet adhesion and aggregation (171).

Interestingly, elevated circulating levels of vitronectin correlated with female specific increase in inflammation after ischemic stroke in mice which points toward its prognostic value for stroke outcomes in women (172).

Even though vitronectin was shown to contribute to vascular remodeling, the mechanistic link between vitronectin and TLR signaling is not yet well defined. Clearly, further studies are required to elucidate the mechanism of vitronectin-TLR mediated vascular remodeling.

SARS-COV-2 AND TLR SIGNALING

In light of the current pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for the scope of this review it is important to mention vascular complications that are linked to TLRs immune response (173). SARS-CoV-2 induces release of proinflammatory cytokines and activation of procoagulant factors that activate coagulation cascades leading to thrombosis, rupture of atherosclerotic plaques and ischemic events (174, 175). Clinical studies demonstrated that hypercoagulability and vascular complications are utmost important predictors of disease outcomes (174, 176, 177). Recent work by Zheng et al., demonstrated contribution of TLR signaling in response to SARS-CoV-2 that results in proinflammatory cytokine production and disturbed immune response as seen in patients with severe form of COVID-19 (178). Interestingly, increase in mRNA expression levels of adaptor protein Myd88 as

well as various TLRs that signal through it were positively correlated with disease severity. Further mechanistic study in Myd88$^{-/-}$ mice infected with mouse hepatitis virus (MHV), a laboratory analog for SARS-CoV-2 resulted in reduction in TNF as compared to WT controls. Subsequent *in vitro* experiments with SARS-CoV-2 infected peripheral blood mononuclear cells (PBMCs) demonstrated that inhibition of TLR2, but not TLR4, resulted in downregulation of cytokine and chemokine production. This study elegantly demonstrates importance of TLR2 and Myd88 in sensing SARS-CoV-2 by triggering inflammatory response and release of proinflammatory cytokines such as TNF and IFN-γ. Therapeutic targeting of the immune response such as inhibition of TLR2 might be a promising therapeutic target for mitigating COVID-19 severity. Besides induction of pro-inflammatory signaling, TLRs are also programmed to activate negative feedback loops to exert anti-inflammatory and tissue repair signals. COVID-19 disease is highly associated with the development of the cytokine storm due to uncontrolled TLR response – a devastating inflammatory reaction leading to multi-organ failure and death. It is not known how the TLR-induced negative regulatory mechanisms fail to inhibit exaggerated activation of TLRs and associated cytokine storm. Administration of MD2-TLR4 antagonist Eritoran has shown effective downregulation of TLR4 in animal model of sepsis (179), but did not proof to be effective in patients (180). During acute influenza virus infection Eritoran has successfully targeted the cytokine storm in experimental studies (181). The protective action of Eritoran also involves CD14 and TLR2. The Eritoran directly binds to the CD14 preventing the ligand binding to MD2 or lymphocyte antigen 96 (181). Hence, further studies on the role of TLR accessory molecules are relevant to design effective therapeutics to target

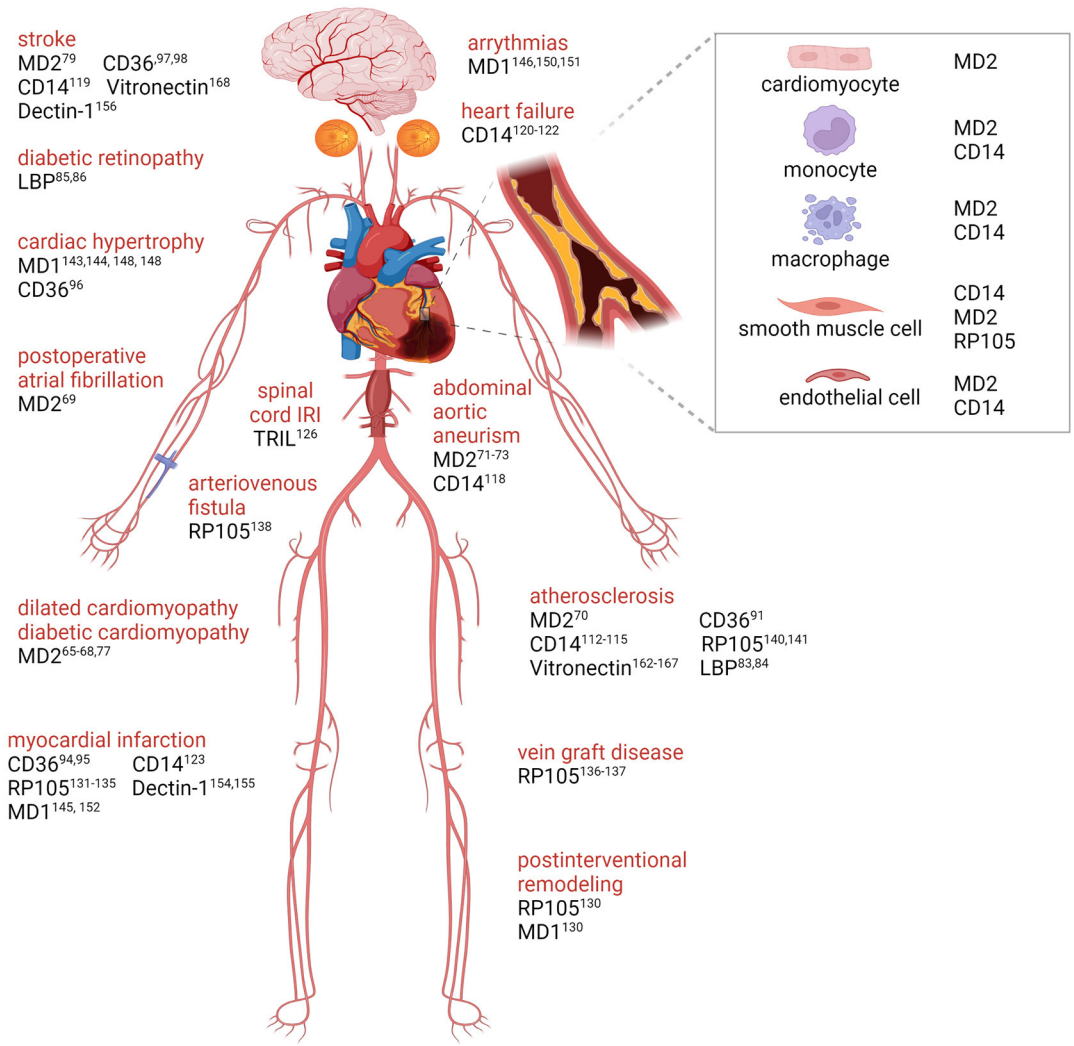

FIGURE 2 | TLRs accessory molecules in cardiovascular disease. Graphical overview with supporting literature references underlying the role of accessory molecules for membrane TLRs that are involved in cardiovascular disease progression. Created with BioRender.com.

inflammatory diseases, including cytokine storm associated with SARS-CoV2 infection-induced severe COVID-19.

CONCLUDING REMARKS AND THERAPEUTIC PERSPECTIVES

Not even three decades have passed since the discovery of TLR, nonetheless this field has rapidly evolved and its importance was underscored by the Nobel Prize in Medicine in 2011. TLR can recognize a broad repertoire of PAMPs and DAMPs yet contain structurally conserved ectodomains. Accessory molecules provide an important tool to ligand discrimination and receptor signaling to assure proper TLR specificity, signal transduction and tissue response. In CVD, inflammation plays an important role in both disease progression and resolution as has been showing elegantly the successes of the CANTOS trial (17) and the COLCOT and LoDoCo2 trials (182, 183). Single cell analysis of various cardiovascular diseases further confirmed the involvement of downstream TLR signaling inflammatory cytokines further illustrating the importance of these pathways. In this review we reported on the complex regulatory mechanisms that are involved in the regulation of the TLR signaling pathways triggered by the broad range of ligands, both exogenous and endogenous. As indicated the various accessory molecules are key regulators in the disease specific or cell type specific responses of TLR activation (**Figure 2**). This of course suggests a new line of potential therapeutic approaches based on manipulating TLRs and their downstream signaling as has been described for the use of soluble coreceptors like soluble RP105 (142). Overexpression of the soluble RP105 was shown to dampen the TLR4 mediated inflammatory response in vascular remodeling, by acting as a decoy receptor of the TLR4 binding DAMPS. However, these experiments directly demonstrated the complexity of such an approach since solRP105 was only functionally active when co-expressed with the cofactor MD1. The latter is required for the stability of the solRP105 protein (142). This is illustrative for the lack of knowledge in this field to translate these findings toward actual therapeutic application. Better understanding of the regulation of TLR signaling via accessory molecules in vascular and cardiac remodeling could help to develop new therapies aiming at guiding beneficial TLR response.

AUTHOR CONTRIBUTIONS

TB, JK, MV, and PQ conceptualized and formulated review aims. TB and MV developed methodology, prepared original draft, and collected data and evidence. JK and PQ performed reviewing and editing. TB visualization. PQ and MV supervised the research. All authors discussed the results and contributed to the final manuscript.

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Association of Neutrophil to Lymphocyte Ratio With Plaque Rupture in Acute Coronary Syndrome Patients With Only Intermediate Coronary Artery Lesions Assessed by Optical Coherence Tomography

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Objectives: Plaque vulnerability and rupture rather than plaque size are the major cause of clinical events in patients with intermediate coronary lesions. Therefore, the present study was aimed to explore potential markers associated with plaque rupture in acute coronary syndrome (ACS) patients with intermediate coronary lesions.

Methods: A total of 82 ACS patients presenting with only intermediate coronary lesions (40–70% stenosis demonstrated by quantitative coronary angiography) and no severe stenosis in other main coronary arteries [median age 63 years, 53 male and 29 female] were enrolled. Plaque morphology were assessed by optical coherence tomography (OCT). Hematological indices were assayed by automated hematological analyzer.

Results: Plaque rupture was identified in 14 patients by OCT. Neutrophil to lymphocyte ratio (NLR) in patients with plaque rupture (n = 14) was significantly higher than that in patients with non-plaque rupture (n = 68) [3.85 (3.28, 4.77) vs. 2.13 (1.40, 2.81), $p < 0.001$]. Multivariate logistic regression analysis revealed that NLR was one of the independent risk factors for plaque rupture in intermediate coronary artery lesions (odds ratio 1.64, 95% confidence intervals 1.18–2.29, p = 0.003). ROC curve analysis found a cutoff point of NLR > 2.94 for plaque rupture with 93.8% sensitivity and 77.9% specificity.

Conclusion: NLR, an inflammatory biomarker, is closely associated with plaque rupture in intermediate coronary artery lesions. Monitoring NLR may be useful in risk stratification and management for intermediate coronary artery lesions.

Keywords: intermediate coronary artery lesion, plaque rupture, optical coherence tomography, neutrophil to lymphocyte ratio (NLR), atherosclerosis

INTRODUCTION

Intermediate coronary artery stenosis is defined as an angiographic stenosis of 40–70% (1, 2). It may account for up to 25% of patients undergoing coronary angiography (3). Optimal assessment and interventional strategy for intermediate lesions continues to be a clinical challenge for cardiologists. Therefore, it is meaningful to explore potential biomarkers which can provide useful information on pathophysiology, risk stratification and management for this condition.

Numerous studies have shown that plaque vulnerability and rupture rather than plaque size and stenosis severity are the major cause of cardiovascular events in patients with coronary artery disease (CAD) (4, 5). The process of plaque rupture may be attributed to neutrophil infiltration and subsequent neutrophil-platelet adhesion. Neutrophil infiltration in disrupted plaques and tissue damage caused by neutrophil elastase were directly visualized by immunohistochemistry in the arteries of the circle of Willis from human autopsy cases (6). Animal studies suggest that infiltrated neutrophils make plaques prone to rupture by releasing proteolytic enzymes, superoxide radicals and inflammatory mediators (7, 8).

Neutrophil to lymphocyte ratio (NLR), reflecting the combination of neutrophil and lymphocyte alterations is currently proposed as an accurate biomarker with predictive power for cardiovascular adverse events in patients with acute coronary syndrome (ACS), stable CAD patients and patients undergoing coronary artery bypass graft (CABG) (9–13). Unfortunately, to the best of our knowledge, there is a paucity of the association of NLR with plaque vulnerability in CAD patients, especially in patients with intermediate lesions. Given this background, the present study was aimed to investigate the possible link between NLR and plaque rupture in patients with only intermediate coronary lesions in the acute phase of ACS.

Optical coherence tomography (OCT) is a catheter-based imaging modality that provides detailed visualization of intraluminal coronary artery structures. By OCT imaging, cardiologists are able to observe the plaque morphology and composition *in vivo*, such as thin-cap fibroatheroma, plaque erosion, plaque rupture and calcified nodule (14, 15). Therefore, we used OCT to identify plaque rupture in ACS patients with only intermediate coronary lesions and then compared blood cell counts, hematological indices and inflammatory response between patients with and without plaque rupture.

MATERIALS AND METHODS

Study Population

This is a prospective observational study conducted at Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine. Our study complied with the declaration of Helsinki and was approved by the hospital ethnics review board (No. 2016-256-T191). Informed consent was obtained from all the participants. A total number of 186 consecutive ACS patients were evaluated for enrollment in this study during the period from September 2016 to December 2020. All the patients received elective coronary angiography and OCT within 72 hours after admission. A total number of 82 ACS patients with only intermediate coronary lesions were finally enrolled and evaluated. One hundred and four ACS patients were excluded from the study because they also had severe stenosis (>70%) in other main coronary arteries. ACS was clinically diagnosed according to the elevated cardiac biomarkers, abnormal electrocardiogram indicating myocardial ischemia, clinical symptoms of angina pectoris or cardiac wall motion abnormalities by echocardiography. ACS included ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI), and unstable angina pectoris (UA). Patients with severe valvular heart disease, severe heart failure, infectious disease, autoimmune diseases, serious lung diseases, sever liver and kidney disease, malignant tumor, hemorrhage, and hematologic disorders were excluded from the study.

All patients were routinely treated with anti-platelet drugs, statins, β-blocker or angiotensin converting enzyme inhibitor or angiotensin II receptor blocker. Demographic information, clinical data and laboratory findings were collected. All the patients were followed up every 3 months for a median of 35.0 months [18.0, 45.5]. Major adverse cardiac events (MACE) were recorded during the period of follow-up. MACE was defined as the composite of all cause death, cardiac death, MI, coronary revascularization and stroke.

Coronary Angiography

All participants underwent elective coronary angiography by the standard Judkin's technique. Coronary angiograms were assessed and quantified separately by two independent interventional physicians blind to the clinical data. An intermediate coronary lesion was defined as a luminal narrowing with a diameter stenosis of 40–70% in any of the main coronary arteries, including the left main artery (LM), left anterior descending artery (LAD), left circumflex coronary artery (LCX), and right coronary artery (RCA), or main branches of the vascular systems (the diameter of the target vessel is $\geq$ 2.5 mm).

Optical Coherence Tomography Procedure and Imaging Analysis

All the intermediate lesions were assessed by OCT. OCT images were acquired using ILUMIEN OPTIS System with Dragonfly OPTIS imaging Catheter (Abbott Vascular, Santa Clara, CA, United States) and analyzed by two independent investigators who were blinded to the clinical presentations and angiographic findings. Morphological evaluation of intermediate lesions included plaque composition, plaque rupture, erosion, calcified nodule, vasospasm, and thrombus (14, 15). Plaque rupture was defined as the presence of a fibrous cap discontinuity and cavity formation in the plaque (**Figure 1A**).

Blood Examination

Blood samples for evaluating hematological parameters and biomarkers of cardiac injury and inflammation were collected from a large antecubital vein of each patient immediately after

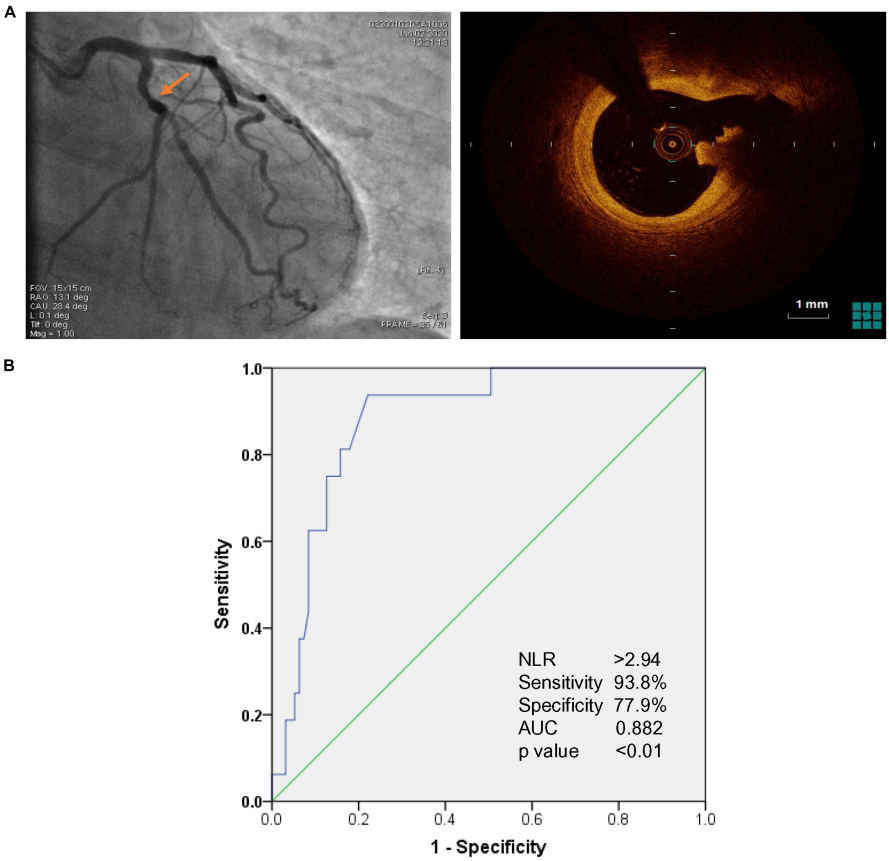

FIGURE 1 | (A) Representative image of optical coherence tomography (OCT)-identified plaque rupture (right panel) in angiographically intermediate coronary lesions (arrowhead in left panel). **(B)** Receiver Operating Characteristic (ROC) for evaluation of Neutrophil to lymphocyte ratio (NLR) associated with plaque rupture in acute coronary syndrome (ACS) patients with intermediate coronary artery lesions. The cutoff value of NLR associated with plaque rupture was NLR > 2.94 with 93.8% sensitivity and 77.9% specificity [Area Under Curve (AUC): 0.882, CI 95% 0.809–0.955, $p < 0.01$].

admission. Blood samples for assaying biochemical parameters were collected after a 12-h overnight fast usually within 24 hours after admission. Blood cell counts and hematological indices were determined using an automated blood cell counter, the Coulter LH780 Hematology Analyzer (Beckman Coulter Ireland Inc.). Serum levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were measured by biochemical auto-analyzer (Siemens Advia 2400). NLR was calculated by dividing neutrophil count to lymphocyte count. Platelet to lymphocyte ratio (PLR) was calculated by dividing platelet count to lymphocyte count.

Statistical Analysis

Statistical analysis was performed with SPSS version 21.0. Continuous variables were presented as median [first quartile, third quartile] and compared using the Mann-Whitney *U* test. Categorical variables were expressed as percentage and compared using the chi-square-test. Clinical features and hematological indices with p-value < 0.10 in univariate analysis were included in multivariate logistic regression analysis. Multivariate logistic regression analysis with forward stepwise was performed to evaluate the independent factors associated with the risk of plaque rupture. The odds ratios (OR) and 95% confidence intervals (CI) were calculated. Areas under the receiver-operator characteristic (ROC) curve for NLR related to plaque rupture were conducted. A two-tailed p-value of < 0.05 was considered statistically significant.

RESULTS

A total of 82 ACS patients (median age 63 years [57, 70], 53 male and 29 female) with only intermediate coronary lesion were enrolled and investigated. Sixty-six patients were diagnosed with UA. Fourteen and two patients were diagnosed with NSTEMI and STEMI, respectively. A total of 124 lesions (2 lesions in LM, 69 lesions in LAD, 18 lesions in LCX, and 35 lesions in RCA) were assessed by OCT. The OCT findings were described as follow: 35.7% (45/124) fibrous plaques, 22.6% (28/124) fibroatheroma, 8.1% (10/124) fibrocalcific plaques, 16.9% (21/124) mixed plaque, 11.3% (14/124) plaque rupture, 1.6% (2/124) spasm, 1.6% (2/124) dissection, 0.81% (1/124) intimal ulceration/erosion, and 0.81% (1/124) calcified nodule.

Patients were divided into rupture group (n = 14) and non-rupture group (n = 68) based on the OCT findings. Their demographic and clinical characteristics as well as laboratory data were summarized in **Table 1**. As shown in **Table 1**, patients in rupture group were significantly younger than non-rupture group. They also had higher proportions of male gender and current smokers as well as an increased level of Troponin I in comparison with non-rupture group. No substantial difference was observed between the two groups in terms of BNP, CRP, lipid profiles, hypertension, diabetes, and CAD family history. After follow-up for a median of 35 months [18.0, 45.5], an obvious difference was observed in the incidence of MACE between rupture and non-rupture group (3/14 vs. 3/68, p = 0.026). Three patients (1 NSTEMI and 2 recurrent UA) in non-rupture group underwent revascularization because of exacerbation of stenosis severity in intermediate coronary lesions (>70% stenosis). In rupture group, one recurrent NSTEMI and one recurrent UA occurred. These two patients received drug-eluted stent implantation as the diameter stenosis caused by intermediate lesions dramatically increased to > 70%. One patient in rupture group got ischemic stroke at 2-year follow-up.

Table 2 showed the comparison of hematological parameters between rupture and non-rupture groups. Total number of WBC and differential count of neutrophils in rupture group were significantly higher than those in non-rupture group (both $p < 0.01$) whereas the lymphocyte count in rupture group was much lower than non-rupture group ($p < 0.01$). Median NLR was 3.85 [3.28, 4.77] and 2.13 [1.40, 2.81] in rupture and non-rupture group, respectively. NLR in rupture group was significantly higher than that in non-rupture group ($p < 0.001$). Median PLR was 169.45 [127.81, 210.21] and 121.62 [92.72, 160.04] in rupture and non-rupture groups, respectively and the difference between two groups was statistically significant (p = 0.002). Additionally, the two groups had similar levels of red blood cell count, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red blood cell distribution width (RDW), monocyte count, platelet count, mean platelet volume (MPV), and platelet distribution width (PDW).

Logistic regression analysis was performed to identify potential risk factors of plaque rupture in ACS patients with only intermediate coronary lesions (**Table 3**). Multivariate logistic regression analysis showed that NLR (OR1.64, 95% CI 1.18–2.29, p = 0.003) and status of current smoking (OR 6.74, 95% CI 1.61–28.21, p = 0.009) were independent factors associated with the risk of plaque rupture in intermediate lesions. Moreover, ROC curve analysis identified that NLR of 2.94 was an optimal cutoff value associated with the risk of plaque rupture in ACS patients with only intermediate lesions. NLR > 2.94 had 93.8% sensitivity and 77.9% specificity (AUC 0.882, CI 95% 0.809–0.955, $p < 0.01$) for predicting plaque rupture (**Figure 1B**).

In addition, sex differences in inflammatory markers were analyzed. As shown in **Supplementary Table 1**, male patients in rupture group had more WBC and neutrophils, less lymphocytes as well as higher NLR and PLR in comparison with male patients in non-rupture group. In contrast, female patients in non-rupture and rupture group had similar levels of WBC, leukocyte differential count, NLR and PLR. No sex difference in inflammatory markers was obtained within rupture or non-rupture group.

TABLE 1 | Baseline demographic and clinical characteristics of patients with or without plaque rupture.

	Plaque rupture (n = 14)	Non-plaque rupture (n = 68)	p-value
Age (years)	57 [51, 65]	64 [60, 70]	0.005
Male	12 (85.7%)	39 (57.4%)	0.046
Current smoking	10 (71.2%)	18 (26.5%)	0.001
Hypertension	9 (64.3%)	42 (61.7%)	0.859
Diabetes mellitus	2 (14.3%)	11 (16.2%)	0.860
Family history of CAD	3 (21.4%)	10 (14.7%)	0.531
Aspirin and/or thienopyridine	12 (85.7%)	60 (88.2%)	0.793
Beta blocker	4 (28.6%)	16 (23.5%)	0.689
ACEI/ARB	6 (42.9%)	26 (38.2%)	0.859
Statin	8 (57.1%)	35 (51.5%)	0.699
BMI (kg/m^2)	23.49 [21.34, 27.09]	24.56 [23.25, 26.65]	0.346
TG (mmol/L)	1.63 [0.97, 2.12]	1.63 [1.18, 2.15]	0.810
TC (mmol/L)	3.91 [3.47, 4.28]	4.01 [3.52, 4.81]	0.820
LDL-C (mmol/L)	2.73 [2.18, 3.21]	2.70 [2.12, 3.47]	0.844
HDL-C (mmol/L)	0.99 [0.84, 1.13]	1.01 [0.90, 1.18]	0.824
Troponin I (ng/ml)	0.07 [0.00, 0.71]	0.01 [0.00, 0.03]	0.003
BNP (pg/ml)	50.00 [25.75, 109.25]	55.00 [25.00, 106.00]	0.900
CRP (mg/L)	1.99 [1.28, 3.89]	1.28 [1.28, 3.45]	0.074
eGFR (ml/min)	79.54 [52.08, 99.28]	64.30 [50.68, 78.40]	0.068

Values are expressed as n (%) or median [first quartile, third quartile]. CAD, coronary artery disease; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

DISCUSSION

Plaque rupture has been shown to be associated with the risk of MACE in patients with angiographically intermediate coronary artery lesions (16, 17). Exploring potential biomarkers associated with plaque rupture will be helpful in the follow-up of intermediate lesions and risk monitoring. NLR is a well-established and easily accessible inflammatory marker. Its prognostic value in CVD and predictive power for MACE have been highlighted by numerous epidemiological studies (9–13). Therefore, the present study assessed the plaque features in intermediate lesions by OCT and then explored the association of NLR with plaque rupture. We found that inflammation is more active in plaque rupture and elevated NLR is one of the independent risk factors for plaque rupture in intermediate lesions in the acute phase of ACS. The optimal cut-off point of NLR for plaque rupture in ACS patients with only intermediate lesions is >2.94 with 93.8% sensitivity and 77.9% specificity. Our study suggests that NLR, an inexpensive and readily available marker may provide useful information on cardiovascular risk assessment and management in intermediate coronary lesions. To the best of our knowledge, it is first study to investigate the relationship between NLR

TABLE 2 | Hematological indices of patients with or without plaque rupture.

	Plaque rupture (*n* = 14)	Non-plaque rupture (*n* = 68)	*p*-value
Red blood cells ($\times 10^{12}$)	4.54 [4.26, 4.80]	4.33 [3.99, 4.68]	0.530
Hemoglobin (g/L)	140.50 [128.00, 146.75]	134.00 [124.00, 141.00]	0.345
MCH (pg)	30.40 [29.70, 31.58]	30.30 [29.22, 31.40]	0.426
MCV (fL)	89.85 [88.53, 93.35]	90.10 [87.40, 92.70]	0.786
RDW (%)	13.10 [12.80, 13.50]	13.00 [12.40, 13.50]	0.286
White blood cells ($\times 10^9$)	7.50 [6.73, 8.15]	6.10 [5.10, 6.90]	0.006
Neutrophils ($\times 10^9$)	4.90 [4.00, 5.90]	3.50 [2.60, 4.40]	0.001
Lymphocytes ($\times 10^9$)	1.30 [1.10, 1.50]	1.70 [1.30, 2.00]	0.002
Monocytes ($\times 10^9$)	0.45 [0.39, 0.80]	0.44 [0.34, 0.60]	0.232
NLR	3.85 [3.28, 4.77]	2.13 [1.40, 2.81]	0.000
Platelets ($\times 10^9$)	228.00 [143.75, 262.00]	206.00 [178.50, 224.75]	0.080
MPV (fL)	10.05 [9.93, 10.88]	10.40 [9.90, 10.80]	0.887
PDW (%)	11.85 [10.60, 13.18]	12.30 [11.20, 13.20]	0.707
PLR	169.45 [127.81, 210.21]	121.62 [92.72, 160.04]	0.002

Values are expressed as n (%) or median [first quartile, third quartile]. MCH, Mean corpuscular hemoglobin; MCV, Mean corpuscular volume; MPV, Mean platelet volume; NLR, Neutrophil to lymphocyte ratio; PLR, Platelet to lymphocyte ratio; PDW, Plate distribution width; RDW, Red blood cell distribution width.

TABLE 3 | Logistic regression analysis for plaque rupture in patients with intermediate coronary artery lesions.

	Univariate			Multivariate		
	OR	95%CI	*p*-value	OR	95%CI	*p*-value
Age < 63 years*	3.62	0.96–11.80	0.058			
Male	4.46	0.93–21.49	0.062			
Current smoking	6.94	1.93–24.94	0.001	6.74	1.61–28.21	0.009
Troponin I	1.95	0.88–4.33	0.101			
WBC	1.41	1.03–1.94	0.031			
Neutrophils	1.65	1.14–2.37	0.007			
Lymphocytes	0.10	0.02–0.50	0.005			
NLR	1.73	1.21–2.46	0.002	1.64	1.18–2.29	0.003
PLR	1.02	1.00–1.03	0.002			

**Age was categorized according to the median of age distribution (<63 years or ≥63 years).*

and acute atherosclerotic events, for instance plaque rupture in intermediate lesions.

Since chronic inflammation in arterial walls plays an essential role in every stage of atherosclerosis, the present study investigated the profiles of circulating leukocytes in patients with only intermediate lesion. We found an obvious elevation in neutrophil count and a pronounced decrease in lymphocyte count in plaque rupture group although their total WBC count was still within the normal range. As a results, NLR significantly raised in plaque rupture group. The close correlation between increased neutrophils and acute cardiovascular events is well-established because the presence of neutrophil infiltration has been identified at the site of plaque rupture in both animal models and human autopsy cases (6–8). Moreover, lymphopenia is another common hematological finding observed in ACS or MI (18–20). Lymphopenia has recently been suggested to be associated with increased risk of long-term mortality in patients undergoing CAG, regardless of the coronary presentation (21). Several possible mechanisms may account for the reduction of circulating lymphocytes in acute cardiovascular events (22, 23). During the acute setting of coronary artery disease, activation of immune system increased lymphocyte apoptosis in atherosclerotic lesions, resulting in the destabilization of atherosclerotic plaques (22). In response to physiological stress, a release of cortisol, catecholamines, and proinflammatory cytokines may lead to lymphopenia (23). Acute stress also caused the redistribution of lymphocytes to lymphoid organs and the inversion of $CD4^+/CD8^+$ T lymphocyte ratio (23).

Of note, NLR is a better marker of inflammation associated with plaque rupture than a single white blood cell count. Univariate logistic regression showed that neutrophil and lymphocyte differential counts as well as NLR were associated with plaque rupture. Nevertheless, by multiple logistic regression analysis, only NLR was identified as one of the independent factors associated with plaque rupture. Thus, NLR is likely to be a more accurate and reliable biomarker for evaluating inflammatory status and the risk of plaque rupture in intermediate lesions, as it reflects the combination of neutrophil and lymphocyte alterations in atherosclerotic events. In another word, ACS patients with only intermediate lesions should be cautiously monitored and followed-up when they have increased NLR > 2.94 (NLR cut-off value of 2.94 was observed in our study). Unfortunately, we did not analyze the predictive value of NLR to the long-term outcome of intermediate lesions in ACS patients because of the limited number of MACE observed during the follow-up. It warrants further investigation to elucidate whether NLR is a prognostic factor in patients with intermediate lesions.

As platelets contribute to the pathophysiology of ACS, the present study assessed the alterations of platelet indices (PLR, PDW, and MPV) in intermediate lesions. PLR dramatically increased in plaque rupture group while PDW and MPV remained unchanged. Unfortunately, PLR was not closely related to plaque rupture in intermediate lesions analyzed by multivariate analysis although it has been suggested to have a prognostic value in ACS patients (24, 25). On the other hand, it is worth emphasizing that EDTA, an anticoagulant used in blood sample collection may induce platelet swelling and thus increasing the value of PDW and MPV (26). Some medications, such as statin also have effect on the variability in platelet size in CAD (27). Thus, the link between platelet indices and plaque rupture still warrants further investigation and these factors affecting platelet features should be taken into account in our future study. Additionally, other hematological parameters, such as monocyte count and RDW did not alter in plaque rupture group. Taken together, our findings show that platelet indices and RDW are not powerful indicators for plaque rupture in intermediate lesions.

In addition to NLR, we found that the status of current smoking is another attributor to the plaque rupture in intermediate lesions [OR 6.74, 95%CI 1.61–28.21]. It has been well-documented that cigarette smoke destabilizes plaques and promotes plaque rupture by enhancing inflammation, activating matrix metalloproteinases, stimulating platelet activation and shifting the balance of hemostasis toward thrombus formation (28, 29). Smoke quitting causes an exponential reduction in acute cardiovascular events, particularly in the first year after cessation (30). Therefore, smoke cessation is highly recommended in ACS patients although they have only intermediate coronary lesions, especially those whose NLR is more than 2.94.

Limitations of the Study

We recognized that there were several limitations in our study. Firstly, this prospective and observational study was conducted in a single center with a relatively small sample size. NLR was assayed in ACS patients with only intermediate lesions at admission. It is difficult to discriminate that NLR is upregulated before or after the event of plaque rupture. Thus, it is insufficient to provide a causative explanation for the association between NLR and plaque rupture in intermediate lesions. Secondly, it is difficult to analyze the prognostic value of NLR in intermediate coronary lesions because of the small sample size and the limited number of MACE. Thirdly, our findings may not reflect the alterations in female patients since the majority of the study population was male, especially in plaque rupture group. Large prospective cohort studies are required to obtain a better understanding between NLR and plaque rupture in intermediate lesions.

CONCLUSION

Increased NLR is associated with OCT identified-plaque rupture in ACS patients with only intermediate coronary artery lesions. NLR may be used as a potential biomarker for plaque rupture in risk stratification and management for intermediate coronary artery lesions.

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 Ethnics Review Board of Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JJ and HZe performed data collection and analysis and wrote the manuscript. YZ and CW performed CAG and OCT. JG contributed to data interpretation and assisted in conducting the study. HZh and JZ were the principal investigators and provided study design, interpretation, manuscript drafting and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.770760/full#supplementary-material

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REVIEW
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Inflammatory Mediators in Atherosclerotic Vascular Remodeling

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Atherosclerotic vascular disease remains the most common cause of ischemia, myocardial infarction, and stroke. Vascular function is determined by structural and functional properties of the arterial vessel wall, which consists of three layers, namely the adventitia, media, and intima. Key cells in shaping the vascular wall architecture and warranting proper vessel function are vascular smooth muscle cells in the arterial media and endothelial cells lining the intima. Pathological alterations of this vessel wall architecture called vascular remodeling can lead to insufficient vascular function and subsequent ischemia and organ damage. One major pathomechanism driving this detrimental vascular remodeling is atherosclerosis, which is initiated by endothelial dysfunction allowing the accumulation of intimal lipids and leukocytes. Inflammatory mediators such as cytokines, chemokines, and modified lipids further drive vascular remodeling ultimately leading to thrombus formation and/or vessel occlusion which can cause major cardiovascular events. Although it is clear that vascular wall remodeling is an elementary mechanism of atherosclerotic vascular disease, the diverse underlying pathomechanisms and its consequences are still insufficiently understood.

Keywords: atherosclerosis, remodeling, inflammatory mediator, chemokines, cytokines

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INTRODUCTION

Inflammatory mediators such as chemokines and cytokines are quickly released by a multitude of cell types during inflammation and trauma and thereby orchestrate a vital immune response and vascular remodeling. These interactions can occur in either an autocrine or paracrine fashion resulting in structural and functional changes of the vascular wall. However, during chronic inflammatory conditions these mediators can also cause tissue damage or (irreversible) tissue remodeling, a term referring to structural and functional changes of the arterial vessel wall (1). A chronic disease that is characterized by such arterial vessel remodeling is atherosclerotic vascular disease, which is the most common cause of cardiovascular disease (CVD) (2). Mechanisms involved in atherosclerotic arterial remodeling include hyperplasia of the arterial intima and media, changes in vascular collagen and elastin, endothelial dysfunction, and arterial calcification. Identifying the cross talk between cells of the vasculature which maintain or disrupt vascular homeostasis and result in vascular remodeling may offer strategic insight for CVD prevention.

Atherosclerosis is initiated by endothelial dysfunction allowing lipids to accumulate in the intima, which subsequently results in intimal inflammation driving (persistent) vascular changes (3). Various inflammatory mediators comprising of modified lipids, such as *oxidized low-density lipoprotein* (oxLDL), but also chemokines, cytokines and lipid mediators further foster the endothelial dysfunction and increase vascular permeability. This increased permeability further stimulates the influx and accumulation of lipids and immune cells in the intimal layer of the vascular wall, resulting in a vicious circle (3). The immune cell infiltration into the vessel wall is further increased by the upregulation of adhesion molecules on the endothelium, stimulated by the inflammatory environment. This further induces the arrest of monocytes and other leukocytes onto the vessel wall which subsequently transmigrate into the intima (4, 5). Infiltrated monocytes subsequently differentiate into macrophages which will engulf excess lipids and develop into lipid laden foam cells. Due to the excess uptake of lipids, these foam cells will eventually undergo apoptosis and necrosis, resulting in the formation of a necrotic core in atherosclerotic lesions (6). Accumulation of such necrotic debris leads to the continued release of toxic and pro-inflammatory stimuli in the intima, further promoting inflammation, remodeling and vulnerability of the plaque.

Key cells in shaping the vascular architecture are *vascular smooth muscle cells* (VSMCs), which are normally present in the arterial media and express a range of "SMC markers" including *smooth muscle cell myosin heavy chain* (MYH11), *smooth muscle cell actin* (SM-α), smoothelin and others. These VSMCs are considered to have a contractile phenotype, which is important to maintain the vascular tone. However, during atherosclerosis formation a phenotype switch is induced by inflammatory mediators resulting in the transition from a contractile phenotype to a synthetic phenotype. Additionally, VSMCs will proliferate and migrate from the media into the intima where they will produce *extracellular matrix* (ECM) to form a fibrous cap and stabilize the atherosclerotic lesions. Moreover, VSMCs with a synthetic phenotype adopt macrophage-like characteristics and can also develop into SMC-derived foam cells (2). Additionally and especially in later stages of lesion development, synthetic VSMCs also produce and secrete matrix metalloproteinases (MMPs) resulting in greater proteolytic activity toward elastin and collagen, which destabilizes the plaque and increases the risk of plaque rupture and thrombus formation (7).

This review aims to draw attention to the main inflammatory mediators involved in vascular remodeling seen in atherosclerosis.

CHEMOKINES

Chemokines are a family of chemoattractant cytokines secreted by various cells, which play a vital role in cell migration from the bloodstream into tissues. They induce cell movement in response to and toward a chemokine gradient also referred to as chemotaxis (8). In addition, chemokines play an important role in various cellular functions including proliferation, survival and differentiation (9). Chemokines can be classified into four structural subfamilies, CC, CXC, CX_3C and C based on the location of the key cysteine residues in the disulfide bonding which are either juxtaposed (CC) or separated by 1 or 3 amino acids (CXC and CX_3C) respectively (10). Chemokines initiate cellular responses through interaction with seven-transmembrane G-protein coupled receptors (GPCRs), more specifically classical chemokine receptors, or with atypical chemokine receptors (ACKRs), which do not signal through G-proteins (11).

CC chemokines have at least 27 distinct members reported in mammals, called CC chemokine ligands (CCL) and are typically responsible for the induction of leukocyte migration (10, 11). Currently, 17 different CXC chemokines have been described in mammals, which can be further subdivided into two subcategories, based on the presence or absence a specific amino acid sequence of glutamic acid-leucine-arginine immediately before the first cysteine of the CXC motif (12). The subgroup of CXC chemokines with this sequence specifically induce the migration of neutrophils, while the subgroup without this sequence typically attract lymphocytes. More unique is CX_3CL1, which possesses three amino acids between the two cysteines and is also termed CX_3C chemokine or fractalkine (12) and the two C chemokines XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β) (12).

The remainder of this chapter will focus on individual chemokines that have been shown to be involved in vascular remodeling during inflammation with a particular focus on atherosclerosis.

CCL2

CCL2 (**Table 1**) also known as monocyte chemoattractant protein-1 is a member of the CC chemokine subfamily and exhibits potent chemotactic activity toward monocytes and T lymphocytes (36). Jones et al. (37) demonstrated that activated VSMCs isolated from mice secreted more CCL2 and CXCL1 and *in vivo* knockout of *Protease-Activated Receptor* 2 (PAR2) in vascular cells reduced their expression of CCL2/CXCL1 and resulted in a reduction of macrophage content in atherosclerotic lesions compared to the control animals. Additional findings demonstrated increased plaques stability, increased smooth *muscle actin alpha* 2 ACTA-2, collagen content and reduced interleukin-1 (IL)-1 and tumor necrosis factor (TNF)-α. This suggests that CCL2 acts on macrophage chemotaxis into the lesion in a paracrine fashion (**Figure 2**). Furthermore, CCL2 may interact on VSMC via an autocrine mechanism to stimulate the phenotypic changes toward a synthetic phenotype. Besides promoting the transmigration of circulating monocytes, CCL2 also promotes cytokine production and adhesion molecule expression on monocytes (38). CCL2 expression is induced by inflammatory cytokines, growth factors, or complement factors in monocytes, ECs, and VSMCs (39, 40). Furthermore, CCL2 seems to be an important chemokine in the development of atherosclerosis, since its expression has been detected in atherosclerotic lesions but not in vessels obtained from healthy individuals (38, 41) and in patients with MI (36, 42). Enhanced CCL2 within the lesion is correlated with histopathologic,

TABLE 1 | Overview of chemokines involved in atherosclerosis remodeling and their physiological effect.

Chemokines	Receptors	Cells affected	Proposed effect in vascular remodeling	References
CCL2	CCR2	Monocytes	Recruitment	(13)
				(14)
				(15)
		VSMCs	Migration via PI3Kγ signaling	(16)
			Activation of NF-kβ and AP-1 leading to cytokine secretion	(17)
			Proliferation	
CCL5 (RANTES)	CCR1	Monocytes	Recruitment, Arrest, infiltration	(18)
	CCR3	Macrophages	Differentiation into foam cells	(19)
	CCR5	VSMCs	Proliferation	(19)
			Phenotypic switch from contractile to synthetic repair cell	
CCL19/CCL21	CCR7	Monocytes	Recruitment	(20)
		Macrophages	Foam cell formation	(21)
				(22)
		VSMCs	Proliferation	(22)
			Increase MMP-1 expression	
CXCL10	CXCR3	CD4+ T lymphocytes	Recrutment of CD4+ T lymphocytes and Tregs	(23)
		Endothelial cells	Reduced wound healing	(24)
CXCL12	CXCR4	Monocytes	Recruitment	(25)
		Macrophages	Differentiation into foam cell	(25)
		VSMCs	Migration	(26)
			Secretion of collagen	(27)
				(28)
CXCL16	CXCR6	Platelets	Deposition on ECs	(29)
		Macrophages	Differentiation into foam cells	(30)
				(31)
		VSMCs	Differentiation into foam cells	(30)
				(31)
CX3CL1 (Fractalkine)	CX3CR1	Monocytes	Recruitment and adhesion	(32)
				(33)
		VSMCs	Proliferation	(34)
				(35)

molecular, and clinical hallmarks of plaque vulnerability, clearly suggesting that this chemokine also plays a role in advanced stages of lesion development (43). Interestingly, circulating CCL2 levels also correlate with subclinical atherosclerosis disease severity in postmenopausal women and may act as a potential early biomarker in this population (44). Further studies have demonstrated that circulating myeloid cells deposit CCL2 on the arterial endothelium to enhance monocyte recruitment and thereby drive atherogenesis (15).

CCL2 is also involved in vascular remodeling as it has been shown to stimulate the binding activity of *nuclear factor kappa-light-chain-enhancer of activated B cells* (NFκB) to *activator protein*-1 (AP-1) in cultured human VSMCs (HVSMCs) grown from unused portions of saphenous veins harvested during coronary artery bypass surgery (17). In addition, CCL2 was reported to induce the proliferation and IL-6 secretion from HVSMCs *in vitro* (17). Furthermore, recombinant CCL2 stimulated HVSMC proliferation *in vitro via* AP-1, which was inhibited by *mitogen-activated protein kinase* (MEK)-1 and MEK2 inhibitor treatment (17). This suggests that CCL2 induces differential activation of NFκB and AP-1 leading to cytokine secretion and proliferation in human VSMCs.

Moreover, pharmacological *phosphatidylinositol* 3-*kinase gamma* (PI3Kγ) inhibition, inactivation of PI3Kγ, as well as genetic deletion of PI3Kγ in mice was used to study the role of CCL2 on the *platelet-derived growth factor* (PDGF) signaling pathway and migration processes in primary aortic VSMCs (16). A wound healing assay illustrated that CCL2 is crucial for VSMC migration via PI3Kγ signaling as blocking the CCL2/CCR2 pathway or the inhibition of PI3Kγ reduced PDGF-stimulated aortic VSMC migration by 50% (16). Furthermore, a *low-density lipoprotein receptor* knock-out ($Ldlr^{-/-}$) mouse model fed a *Western diet* (WD) for 8-weeks treated with a PI3Kγ inhibitor showed decreased atherosclerotic lesion size and increased plaque collagen content (45). Finally, VSMCs isolated from PI3Kγ-deficient mice ($PI3K\gamma^{-/-}$), or mice expressing an inactive PI3Kγ, termed $PI3K\gamma^{KD/KD}$, showed reduced migration when compared to the control cells in response to CCL2 and PDGF (16). Combined these results demonstrate that CCL2 plays a role in lesion formation by promoting VSMC migration in a PI3Kγ dependent manner but can also result in decreased plaque stability by reducing the collagen formation.

Another study demonstrated that a CCL2 competitor (PA508) reduced inflammatory monocyte recruitment, limited neointimal hyperplasia, and attenuated myocardial ischemia/reperfusion injury in an *Apolipoprotein E* knock-out ($Apoe^{-/-}$) mouse model, highlighting the potential of PA508 as novel therapeutic approach to treat MI (13). However, the study did not examine mice on a WD to investigate the therapeutic potential of PA508 on atherosclerosis development. Nevertheless, another study treating $Apoe^{-/-}$ mice, fed with a WD for 6-weeks, daily with a CCR2 antagonist (INCB3344) revealed reduced circulating $CCR2^{+}$ monocytes and diminished atherosclerotic plaques in both the carotid artery and the aortic root (14), proving the therapeutic potential of CCL2/CCR2 targeting for atherogenesis.

Overall, as CCL2 has been shown to be overexpressed in atherosclerotic lesions it is likely that it plays a prominent role in vascular remodeling of VSMCs in atherosclerosis (**Figure 1**). CCL2 may stabilize the plaque by fostering migration of medial VSMCs into the intima and increase their proliferation while in parallel, recruitment of arterial leukocytes into the lesion triggers atherogenesis (15). Furthermore, therapeutic strategies, such as the PI3Kγ inhibition or CCL2/CCR2 pathway inhibition reveal a decrease in lesion size in mice due to the reduced VSMC migration (45) (**Table 2**). Nevertheless, further research is necessary to demonstrate whether the putative beneficial effects of CCL2 on VSMCs, e.g., increased proliferation and subsequent increase of plaque stability, outbalance its pro-atherogenic effects on leukocyte recruitment.

CCL5

CCL5, a chemokine which is also known as RANTES, can bind to a plethora of receptors including CCR1, CCR3 and CCR5 (**Table 1**) (48). Platelet-derived deposition of CCL5 on the activated endothelium results in monocyte arrest (49), which appears to be dependent on P-selectin (50). Wire-injury induced neointima formation in the carotid artery of $Apoe^{-/-}$ mice showed that the systemic inhibition of CCL5 or P-selectin deficiency hindered neointima formation as well as monocyte infiltration (50). Furthermore, it could be demonstrated that *CCL5* mRNA and protein levels were upregulated in the aortic intima of $Ldlr^{-/-}$ mice fed a WD for 3 weeks and a function-blocking antibody to CCR5 significantly reduced monocyte recruitment into the lesions (18). Further investigations using a *bone marrow transplantation* (BMT) experiment demonstrated that hematopoietic CCL5 regulates monocyte recruitment and accumulation of macrophages in the lesions after 3 weeks of cholesterol-rich diet feeding of $Ldlr^{-/-}$ mice. However, after 6 weeks of cholesterol-rich diet, CCL5 only plays a minor role in the recruitment of monocytes into the lesions suggesting that CCL5 only plays a role in early stages of lesion formation and subsequent vascular remodeling (18).

Similar results were seen in a 2-week WD $Ldlr^{-/-}$ mouse model where mice were treated with a CCL5 antagonist (Met-RANTES). Treated animals had reduced atherosclerotic lesion size and a reduction in relative lesional foam cell content compared to the control group (46). Thus, CCL5 mediates vascular remodeling in atherogenesis *via* monocyte arrest and infiltration into the lesion and facilitates the differentiation and development of monocytes into foam cells, although the exact underlying mechanisms behind this CCL5-induced foam cell formation remain to be elucidated. Another study, using $CCL5^{-/-}CCR5^{-/-}$ mice on a 12-week WD, showed significantly reduced expression of the synthetic markers osteopontin and *proliferating cell nuclear antigen* (*PCNA*), while the expression of the contractile VSMC marker SMα was increased in the thoracoabdominal aorta compared to the control group (19). Furthermore, *in vitro* culturing of human aortic SMCs (HASMCs) with palmitic acid resulted in an increased expression of proliferative and synthetic phenotype markers while inhibition of CCR5 using the antagonist maraviroc or RNA interference prevented HASMC proliferation and synthetic phenotype formation (19). Macrophages from stroke patients, exhibit an increased expression of CCL5 which signals through CCR5 on VSMCs driving their proliferation and dedifferentiation, suggesting a paracrine relationship between these macrophages and vascular VSMCs [(19, 51), p. 153]. These data suggest that CCL5 induces VSMC proliferation and phenotypic switching from a contractile to synthetic phenotype via CCR5 (**Figure 2**). The latter would argue for a pro-atherosclerotic role of CCL5 and induction of a synthetic VSMC phenotype promoting vascular remodeling. However, Lin et al. did not backcross the mice on an atherosclerotic background such as $Apoe^{-/-}$ or $Ldlr^{-/-}$, making it impossible to determine whether CCL5 also promotes a synthetic VSMC phenotype during atherogenesis.

CCL5 can also heterodimerize with CXCL4, another platelet-derived chemokine deposited onto vascular endothelium (52). Enhanced CXCL4 presence in human plasma correlates with the severity of human atherosclerotic disease and the platelet specific deletion of CXCL4 decreases atherosclerotic lesions in mice (53, 54). Furthermore, interfering with this heterodimer via the use of the cyclic peptide MKEY resulted in decreased leukocyte recruitment and release of NETs (47). All in all, these studies suggest that CCL5 at least exerts part of its atherogenic effects by heterodimerizing with CXCL4.

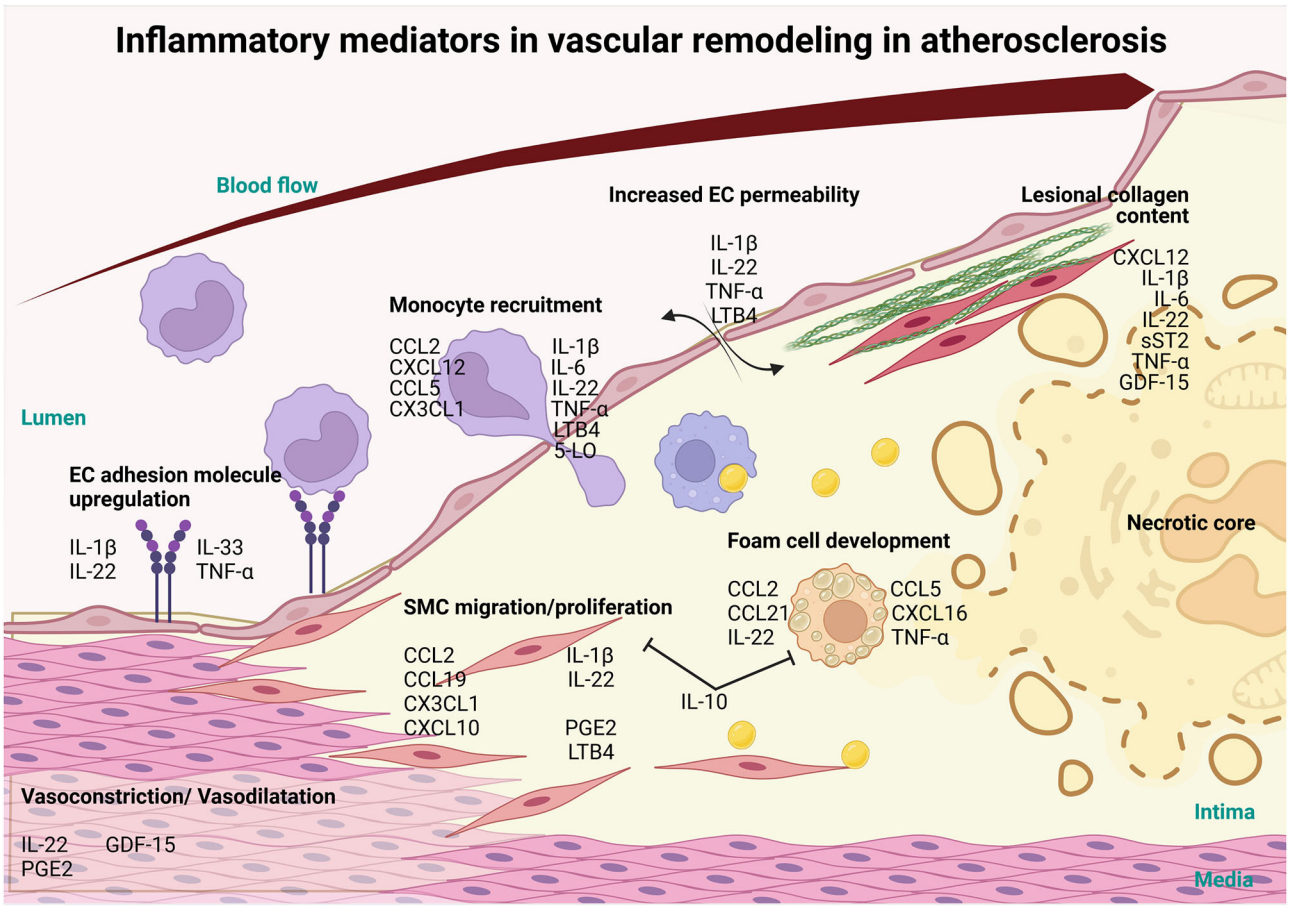

FIGURE 1 | Involvement of inflammatory mediators in vascular remodeling in atherosclerosis. Inflammatory mediators, such as interlukin-1β (IL-1β), IL-22, IL-33 and tumor necrosis factor-α (TNFα) cytokines can influence the progression of atherosclerosis and CVD *via* the activation of the endothelium resulting in the upregulation of adhesion molecules. Furthermore, these mediators increase vascular permeability, through IL-1β, IL-22, TNFα and LTB4 and along with the adhesion molecule upregulation allows for the infiltration of monocytes and other immune cells recruited *via* chemokine ligand 2 (CCL2), C-X-C Motif Chemokine Ligand 12 (CXCL12), CCL5, CX3CL1, IL-1β, IL-6, IL-22, TNF-α Leukotriene B4 (LTB4), 5-LO. Mediators like CCL2, CCL19, CXCL10, CX3CL1, IL-1β, IL-22, Prostaglandin E_2 (PGE2) and LTB4 also induce the migration and proliferation of VSMCs into the intima and affect the production of collagen, which, in turn, modulates plaque stability. Foam cell formation is initiated by CCL2, CCL5, CCL21, CXCL16, IL-22 and TNFα and exhausted foam cells undergoing apoptosis and necrosis to establish the necrotic core of the lesion. On the other hand, IL-10, a potent anti-inflammatory cytokine, prevents the formation of foam cells and SMC migration and proliferation. As the lesion grows, blood vessel lumen is narrowing eventually causing vessel occlusion which may lead to major adverse cardiovascular complications. Inflammatory mediators CXCL12, IL-1β, IL-6, IL-22, soluble suppression of tumorigenesis-2 (sST2), TNFα and Growth/Differentiation Factor-15 (GDF-15) also play a role in the stability of the lesion by controlling collagen in the fibrous cap. In addition, mediators like LL-22, GDF-15 and PGE2 regulate vasoconstriction and vasodilation of the arteries thereby controlling blood pressure and ensuring proper vascular function (this figure was made with Biorender.com).

CD146, a cell adhesion molecule expressed on arterial endothelium was also implicated in CCL5-mediated changes to the vascular wall. $CD146^{-/-}Apoe^{-/-}$ mice fed a WD for 24 weeks displayed lesions with a greater neutrophil and macrophage content correlating with the upregulation of CCL5 secretion when compared to the control group (55). Furthermore, neutrophil recruitment was increased in CD146-deficient mice 12 h after thioglycolate-induced peritonitis, whereby this increased recruitment correlated with the enhanced CCL5 secretion in the peritoneal cavity. The same study showed that mice treated with maraviroc between week 12 to 24 of WD feeding showed smaller atherosclerotic lesions and reduced neutrophilia in $CD146^{-/-}Apoe^{-/-}$ mice to the same level found in $Apoe^{-/-}$ mice (55). These data suggest that CCL5 plays a prominent role in neutrophil recruitment and foam cell formation in atherosclerotic lesions and identifies CD146 agonists as potential therapeutic targets to lower CCL5 levels (**Figure 1**). Hence, increased levels of CCL5 may act as a biomarker for the severity of atherosclerosis and may even be a potent therapeutic target for SMC proliferation and phenotype switching (**Table 2**).

TABLE 2 | Targeting chemokines as therapeutic treatments in vascular remodeling and CVD.

Chemokine	Therapeutic Treatment	Clinical trials	Animal experimentation	Outcomes on atherosclerosis	References
CCL2	Pharmacological phosphatidylinositol 3-kinase gamma (PI3Kγ) inhibitor	–	✓	Reduces PDGF-Stimulates aortic VSMC migration by 50%	(16)
	CCL2 competitor (PA508)	–	✓	Reduces inflammatory monocyte recruitment	(13)
				Limited neointimal hyperplasia and attenuates myocardial ischemia/reperfusion injury	
	CCR2 antagonist (INCB3344)	–	✓	Reduces circulating CCR2+ monocytes, Diminished atherosclerotic plaques	(14)
CCL5	CCL5 antagonist (Met-RANTES)	–	✓	Reduced atherosclerotic lesion size	(46)
				Reduction in foam cells	
	MKEY	–	✓	Decreases leukocyte recruitment into infarcted tissue Decreases release of NETs	(47)
CCL19/CCL21	anti-CCL21 monoclonal antibody	–	✓	Reduction of the infarction size after AMI	(20)
CXCL10	pharmaceutical antagonist specific for CXCR3 (NBI-74330)		✓	Reduced lesion size, CD4+ T lymphocytes content and increased Tregs content	(23)
CXCL12	–	–	–	–	–
CXCL16	–	–	–	–	–
CX3CL1	CX3CL1-Fc fusion protein	–	✓	Reduces atherosclerotic lesions size, independent of the diet	(33)

CCL19/CCL21

Chemokines CCL19 and CCL21 and their receptor CCR7 (**Table 1**) are associated with the modulation of inflammatory responses in lymphoid and nonlymphoid tissues, including atherosclerotic lesions (56–58). Elevated levels of circulating CCL19 and CCL21 can be found in patients with unstable angina pectoris compared to controls (57) and enhanced levels of these chemokines were also observed in patients with carotid atherosclerosis, both systemically (CCL21) and within the lesion (CCL19 and CCL21) (22).

In a mouse model studying MI, the inhibition of CCL21 via intravenous injection of anti-CCL21 monoclonal antibodies led to the reduction of the infarction size after acute MI (20). Anti-CCL21 monoclonal antibody treatment further resulting in reduced MMP-9 and total collagen content in the myocardium. CCL21 was also shown to increase the binding of *acetylated LDL* (ac-LDL) to macrophages, inducing the up-regulation of *LDL receptor*-1 (LOX-1), a scavenger receptor of ox-LDL which has been shown to drive foam cell formation (21, 22). Furthermore, the lipid droplet marker *adipose differentiation-related protein* (ADRP) was upregulated in CCL21-treated macrophages indicating that CCL21 induces foam cell formation (59).

CCL19 has been shown to induce proliferation of VSMCs and results in increased MMP-1 serum levels (22), which positively correlates with total plaque burden (60). Using a BMT experiment of either *CCL*19$^{-/-}$ or *CCL*21$^{-/-}$ bone marrow into *Ldlr*$^{-/-}$ mice it was further demonstrated that CCL21 directs leukocyte homing into atherosclerotic lesions, whereas CCL19 induces the activation of leukocytes, lipid uptake by macrophages and foam-cell formation (61). Thus, CCL19 is involved in vascular remodeling in atherosclerosis, by inducing VSMC proliferation and enhancing protein expression of MMP-1 in VSMCs, which acts to destabilize the plaques, while both CCL19 and CCL21 foster macrophage foam cell formation (**Figure 1**). Blocking CCL19 and/or CCL21 may therefore be a potent therapeutic target to decrease the lipid content in lesions and increase plaque stability (**Table 2**).

CXCL10

EC, VSMCs, and macrophages all express CXCL10 during atherosclerosis while its receptor CXCR3 is mainly expressed on CD4$^+$ T cells (**Table 1**) (62). Evidence from *Apoe*$^{-/-}$ *Cxcl*10$^{-/-}$ mice on a WD for 6 and 12 weeks demonstrated attenuated lesions, reduced CD4$^+$ T cell content and increased regulatory T cells (Tregs) markers within the lesions, suggesting an increase in Tregs within the lesion of *Apoe*$^{-/-}$ *Cxcl*10$^{-/-}$ compared

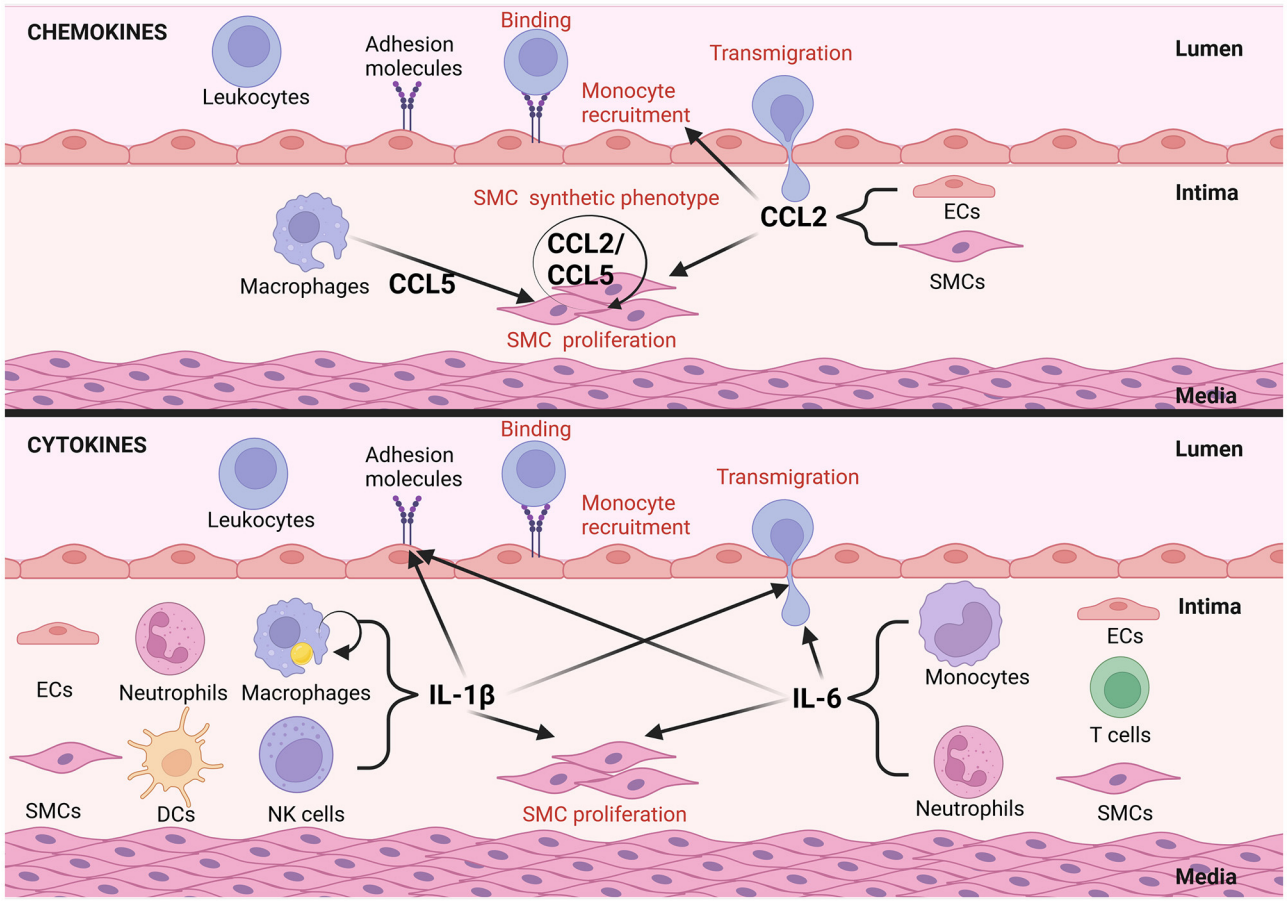

FIGURE 2 | Examples of autocrine/paracrine interactions of inflammatory mediators in vascular remodeling in atherosclerosis. Inflammatory mediators can act in an autocrine manor, CCL2 and CCL5 for example activate VSMCs to undergo phenotypical changes in atherosclerosis. EC and VSMC derived CCL2 can further foster cross-talk between them promoting remodeling. CCL2 from ECs triggers synthetic differentiation of VSMCs and CCL2 from VSMCs promotes monocyte recruitment. Alternatively, mediators like interlukin-1β, expressed by dendritic cells (DCs) and natural killer (NKs) cells promote the upregulation of adhesion molecule expression by ECs, transmigration of circulating leukocytes and VSMC proliferation in a paracrine fashion. IL-1β in addition can act upon macrophages to promote IL-1β secretion in an autocrine manner. IL-6 secreted by ECs, monocytes, T cells, neutrophils and VSMCs promotes adhesion molecule expression by ECs as well as transmigration of circulating immune cells into the atherosclerotic plaque (this figure was made with Biorender.com).

to the control (63). Furthermore, *Ldlr*$^{-/-}$ mice fed a 2-week WD before a collar placement and a further 8 week WD with daily treatments of a pharmaceutical antagonist specific for CXCR3 (NBI-74330) demonstrated a reduced lesion size in the aorta and aortic root compared to the control (23). Further investigation showed reduced CD4^{+} T cells in the lesion and greater expression of genes associated with greater presence of Tregs in the plaques from mice treated with NBI-74330. This suggests that CXCL10 plays a prominent role in vascular remodeling and may be a potential therapeutic target (**Table 2**). Further experiments were conducted in *Apoe*$^{-/-}$ mice fed a WD for 2 weeks followed by induction of an unstable plaque with a flow-altering device around the carotid artery. These animals were further fed a WD for 9 weeks and treated with a bioactivity-neutralizing monoclonal CXCL10 antibody (MAB466) (64). The CXCL10 antibody treatment resulted in a more stable lesion phenotype with increased VSMCs content compared to untreated controls. Hence, CXCL10 may also influence VSMC behavior affecting plaque stability, potentially making CXCL10 an attractive therapeutic target for vascular remodeling (65). Adding to this, in PI3KγKD/KD mice treated with the CXCL10 antibody (MAB466) demonstrated that CXCL10 production by VSMCs inhibited endothelial healing in a wire induced scratch model (24). This suggests that CXCL10 also has a paracrine relationship with endothelial healing and may play a role in maintaining endothelial dysfunction in atherosclerosis. However, this would need to be further investigated in an atherosclerotic mouse model on a WD. Furthermore, studies using an *in vitro* model in which human endothelial cells (SGHEC-7) were co-cultured with the human VSMC cell line SGHVSMC-9 demonstrated that CXCL10 expression contributes to remodeling by altering the motility and differentiation of the VSMCs (66). Future studies could investigate the role of CXCL10 on the phenotypic switching of VSMCs within atherosclerosis.

CXCL12

The chemokine CXCL12 (**Table 1**) is highly expressed in human atherosclerotic lesions (32, 67) and genome-wide association studies noted that the genomic locus 10q11, hosting the *CXCL*12 gene and the intergenic single nucleotide polymorphism (SNP) rs2802492 located near *CXCL*12, are independently associated with CXCL12 plasma levels and *coronary artery disease* (CAD) risk (68, 69). Furthermore, a causative detrimental role of enhanced CXCL12 titers in CAD was described (70) and may also account for CVD in general, rendering CXCL12 a useful biomarker for CVD risk. In line with this, mouse studies could show that EC-specific *CXCL*12 knock out in *Apoe*$^{-/-}$mice fed a WD for 12 weeks reduced the lesion area in the thoracoabdominal aorta and the aortic arch as well as CXCL12 plasma levels compared to control mice (69). Furthermore, a positive correlation between CXCL12 plasma levels and lesion area was seen in the control but not in EC-CXCL12 deficient mice. Combined, these observations suggest that EC-derived CXCL12 promotes atherosclerosis and vascular remodeling (69).

In vitro assays exploring the effects of ox-LDL treatment demonstrated an increase in CXCL12 protein and mRNA expression compared to untreated controls for both THP-1 cells (human monocyte cell line derived from a patient with acute monocytic leukemia) and HASMCs (71). Furthermore, Gao et al. (25) showed that CXCL12-treated THP-1 cells expressed less *ATP Binding Cassette Subfamily A Member* 1 (*ABCA*1) and subsequently resulted in a reduced cholesterol efflux capacity toward apolipoprotein A1 (ApoA-I). The same study also revealed that systemic overexpression of *CXCL*12 in *Apoe*$^{-/-}$ mice, using a lentivirus, resulted in increased lesion formation as well as macrophage accumulation in the plaques. A more detailed analysis demonstrated decreased *ABCA*1 mRNA expression in lesional macrophages in *Apoe*$^{-/-}$ mice which overexpressed CXCL12 supporting the observations made *in vitro* (25). Together, these data suggest that CXCL12 promotes the formation of macrophage-derived foam cells in atherosclerotic lesions via inhibition of *ABCA*1 expression. The potential of CXCL12 to promote foam cell formation by VSMCs or any other analysis of VSMCs was not investigated in the *in vivo* model in this study and should therefore be a subject for future research.

In contrast to the above described observations, CXCL12 is upregulated in the artery via lysophosphatidic acid in response to vessel injury, and the increase in circulating CXCL12 induces the migration of a subtype of Sca-1^{+} Lin^{-} *smooth muscle progenitor cells* (SPCs) (26, 27). Intravenous injection of CXCL12 in *Apoe*$^{-/-}$ mice fed a WD resulted in increased plaque stabilization, characterized by increased collagen content and a thicker fibrous cap, via the accumulation of SPCs (28). However, this study analyzed ligated carotid arteries, which display significant phenotypical differences in their lesion composition compared to ≪native≫ atherosclerotic lesions.Contrary, presence of CXCL12 does also induce recruitment of endothelial cell progenitors and fosters neovascularization in MI (72). While neovascularlarization in the context of MI seems beneficial another recent study has shown that active non-canonical NF-κB signaling in microvessels of carotid atherosclerotic lesions together with enhanced CXCL12 expresssion and neovascularization may induce plaque instability (73).

Overall, CXCL12 plays a critical role in the development of vascular remodeling as demonstrated by EC-derived CXCL12 mediating atheroprogression or the accumulation of VSMCs in the intima in response to increased CXCL12 titers (**Figure 1**) (26, 27, 69). Taken together, CXCL12 may serve as biomarker for CAD risk (70) and seems to drive macrophage foam cell formation and lesion growth. On the other hand, animal studies also suggest that it increases lesion stability by recruitment of SPCs and infiltration of medial VSMCs. Hence, therapeutical targeting of CXCL12 should only be cell specific and considered with great care.

CXCL16

CXCL16 is an atypical chemokine containing a mucin-like stalk, transmembrane and cytoplasmic domains, which are not found in other CXC chemokines (74). CXCL16 has two distinct forms, the membrane-bound form which promotes the firm adhesion of cells expressing the receptor CXCR6 and the soluble form, generated by proteolytic cleavage of membrane-bound CXCL16, which acts as a chemoattractant for CXCR6^{+} cells (74). CXCL16 (**Table 1**) is expressed on stimulated ECs and VSMCs, macrophages, dendritic cells (DC) and platelets, whereas its receptor CXCR6 is expressed on memory and effector T cells, natural killer (NK) cells and NK T cells and is also found on plasma B cells (29, 75). CXCL16 is expressed in human atherosclerotic plaques and lesion severity is correlated with increased CXCL16 levels, as shown in human carotid endarterectomy specimens (76). It could be demonstrated that CXCL16 expression in the plaque enhanced platelet deposition on the endothelium (29). Furthermore, *in vitro* studies using human umbilical vein endothelial cells (HUVECs) established that the immobilization of CXCL16 promoted CXCR6-dependent platelet adhesion to the endothelium during physiologic flow and at low shear rates (77). This data implies that CXCL16 may play a role in vascular inflammation and thrombo-occlusive diseases. However, future investigations need to assess this relationship also on the more pathologically relevant arterial endothelium to understand platelet adhesion in an atherosclerotic model. Zhao et al. demonstrated that during ischemia reperfusion injury, cardiac EC-derived CXCL16 recruits CD11b^{+}Ly6C^{high} inflammatory cells and facilitates the release of *tumor necrosis factor α* (TNFα) (interferon) IFN-γ and interleukin (IL)-17 in the heart. In line with this, silencing of CXCL16 by applying a specific shRNA reduced cardiac apoptosis, inflammation and dysfunction in ischemia reperfusion induced mice (78).

CXCL16 may also play a role in vascular remodeling through alteration of foam cell formation as its expression has been found to be upregulated in lipid-laden intimal macrophages and VSMCs (30). Moreover, CXCL16 may also directly contribute to foam cell formation as it is known to be a scavenger receptor for phosphatidylserine and oxLDL and its expression is upregulated in lipid-laden intimal macrophages and VSMCs via an autocrine mechanism (30). Growing evidence highlights the potential for VSMCs to develop into foam cells (71) and, like

macrophages, VSMCs also express CXCL16 in response to IFN-γ stimulation, which also correlates with an increased uptake of oxLDL by VSMCs (31). Therefore, it can be surmised that CXCL16 facilitates the development of macrophage and VSMC foam cells (**Figure 1**). However, to our best knowledge so far no therapeutic treatment against CXCL16 has been investigated in the context of atherosclerosis and chronic vascular inflammation opening a potential interesting novel avenue of future research.

CX3CL1

CX3CL1 also known as fractalkine is also a chemokine which can be present as a membrane-bound or soluble form (79). Both forms activate the chemokine receptor CX3CR1 (**Table 1**), where the transmembrane form induces integrin-independent leukocyte adhesion and the soluble form is a chemoattractant for leukocytes (80). During atherosclerosis, monocytes expressing CX3CR1 bind and adhere to the endothelium which express the membrane-bound form of CX3CL1 (81). $CD16^{+}CX3CR1^{HIGH}$ monocytes then activate endothelial *signal transducer and activator of transcription* 1 (STAT1), NFκB and p65 phosphorylation to upregulate proliferation and expression of CX3CL1, IL-1β, Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by ECs (82, 83). Hence, the CX3CL1-CX3CR1 axis enhances a pro-atherosclerotic EC phenotype via the upregulation of adhesion molecules and inflammatory mediators. In this context, i*n vitro* studies have shown that CX3CL1 is upregulated on activated VSMCs and triggers monocyte adhesion to VSMCs (32). Further work has demonstrated that $Cx3cr1^{-/-}$ animals—post femoral arterial injury induced *via* an angioplasty guide wire—were protected against intimal hyperplasia due to decreased monocyte trafficking to the lesion compared to the control group (34). In addition, CX3CR1 deficiency resulted in decreased VSMC proliferation and intimal accumulation, which is either directly or indirectly a result of defective monocyte infiltration (34). The relationship between CX3CL1-CX3CR1 and inhibited VSMC proliferation and intimal accumulation in vascular remodeling still needs to be elucidated in detail. One study has demonstrated that a mononuclear $CX3CR1^{+}$ cell population residing in murine bone marrow provides a source of SPCs after wire-induced vascular injury, which differentiate into VSMCs within the neointimal plaque (35). Furthermore, BMT of CX3CR1 deficient bone marrow into C57BL6/J mice demonstrated that Cx_3cr1 expression is essential for VSMC differentiation from SPCs in the vascular wall (35). Thus, CX3CL1 may play a prominent role in intimal hyperplasia promoting atherosclerosis through increased VSMC proliferation and monocyte trafficking. However, it would be worthwhile to perform similar studies using either an $Apoe^{-/-}$ or a $Ldlr^{-/-}$ mouse fed a WD to provide results that truly reflect CVD triggered mechanisms.

Another recent study treated $Ldlr^{-/-}$ mice with a CX_3CL1-Fc fusion protein inhibiting the CX_3CR1-CX3CL1 interaction. This fusion protein treatment significantly reduced atherosclerotic lesion size, independent of WD diet feeding and reduced M1-like macrophage and T cell accumulation in the aortic wall (33). Thus CX3CL1-Fc could be a potent therapeutic option interfering with vascular remodeling and atherosclerosis (**Table 2**). Along this line, another study implemented the use of a DNA vaccine, a vector that contains genes encoding a single-chain antibody specific for the mouse dendritic cell (DC) antigen DEC205 and the receptor CX3CR1 (DEC-CX3CR1) and a non-DC DNA vaccine (Con-CX3CR1). Both vectors were injected into $Apoe^{-/-}$ mice fed a normal chow diet (84). DEC-CX3CR1 vaccinated mice demonstrated a significantly reduced atherosclerotic plaque size compared to both Con-CX3CR1 vaccinated and unvaccinated mice (84). Furthermore, DEC-CX3CR1 mice showed reduced monocyte infiltration and lipid deposition in the lesions compared to unvaccinated mice, although the lesional macrophages still possessed an M1 phenotype. Not only does this further support the role of the CX3CL1-CX3CR1 axis in vascular remodeling (**Figure 1**), but it also emphasizes the need to conduct more studies on DNA vaccination (**Table 2**) as an effective therapeutic strategy against atherosclerosis and vascular remodeling. Overall, CX3CL1 plays an important role in M1 macrophage differentiation and the migration/proliferation of VSMC resulting in vascular changes (**Figure 1**).

CYTOKINES

Cytokines are molecules that are secreted by immune cells and other specific cell types that modulate the inflammatory immune response and mediate cell-cell communication. Cytokines are subdivided into different classes: TNFs, IFNs, ILs, *transforming growth factors* (TGFs) including *growth/differentiation growth factors* (GDF), *colony-stimulating factors* (CSFs) and chemokines as detailed before. In atherosclerosis, pro-inflammatory cytokines play an important role in the initiation and progression of the disease and in the instigation of endothelial dysfunction, upregulation of adhesion molecules and promotion of immune cell migration as well as their infiltration into the lesion. All these different factors lead to arterial remodeling and subsequent changes in vascular function (85). The most important cytokines that are known to contribute to atherosclerosis lesion remodeling are IL-1, IL-6, IL-10, IL-22, IL-33, TNF-α and GDF-15 which will be discussed below in greater detail:

Interleukin 1

IL-1 was one of the first cytokines to be discovered and it is divided into two related but functionally distinct isoforms: IL-1α and IL-1β. Here, the main focus will be on IL-1β as this is a potent driver of the inflammatory response in atherosclerosis and vascular inflammation. IL-1β is synthesized by many cells including neutrophils, NK cells, DCs, ECs macrophages, monocytes and SMCs (**Figure 2**). In atherosclerosis, its synthesis is triggered by the uptake of cholesterol crystals by macrophages activating the *NLR family pyrin domain containing* 3 (NLRP3) inflammasome or by the binding of IL-1 family members to their receptor IL-1R resulting in a positive autocrine inflammatory feedback loop (86). In addition to cholesterol, monocytic inflammasomes and the production of IL-1β can be activated by dying VSMC and in turn promotes VSMC proliferation as shown *in vitro* and *in vivo* in C57BL/6 mice of vein graft injury (87). IL-1β itself induces the upregulation of adhesion molecules such

as ICAM1 and VCAM1 as well as the monocyte chemoattractant chemokine CCL2 (please see section CCL2) on ECs promoting leukocyte recruitment into the atherosclerotic plaque (**Figure 1**; **Table 3**) (88, 115, 116). Pro-inflammatory cytokine IL-1β has also an important role in cross-talk between ECs and the underlying VSMCs. Specific secretion of IL-1β by SMC induces E-selectin expression by ECs (117). In turn, IL-1β secretion by activated ECs promotes VSMC proliferative, synthetic and macrophage-like phenotypes (118). The barrier function of ECs is crucial to maintain vascular wall homeostasis. However, in atherosclerotic conditions, dyslipidemia and proinflammatory cytokines such as IL-1β promote excessive adhesion molecule expression by ECs leading to endothelial dysfunction and increased permeability of the EC barrier due to the disruption of intercellular junctions, resulting in increased leukocyte infiltration and vascular remodeling (**Figures 1**, **2**; **Table 3**) (90). In addition, IL-1β promotes VSMC proliferation by stimulation of autocrine production of PDGF (**Figure 1**) and the production and release of the pro-inflammatory cytokine IL-6 (**Table 3**) (92, 93). IL-1β-induced EC dysfunction is also promoted by plasma *trimethylamine-N-oxide* (TMAO). TMAO is an oxidation product of the liver that is made from compounds synthetized by intestinal bacteria and an elevated concentration of TMAO increases monocyte mobilization and activation leading to low-grade inflammation (119). In line with this, high plasma levels of TMAO are associated with atherosclerosis and increased risk of CVD. In this context Boini et al. (119) showed that TMAO increases the assembly and activation of the NLRP3 inflammasome leading to an increased production of IL-1β in carotid arteries of WT mice with partially ligated carotid artery. In addition, *in vitro* experiments indicate that TMAO treatment induces NLRP3-dependent endothelial hyperpermeability by decreasing *zonula occludens*-1 (ZO-1), a tight junction protein responsible for junction integrity, expression in mouse carotid arterial endothelial cells (CAECs) (119). Therefore, targeting TMAO may help to reduce adverse remodeling in atherosclerosis by preventing EC leakage and infiltration of inflammatory cells as well as reducing IL-1β driven inflammation (119). IL-1β also increases the expression of *dipeptidyl peptidase* 4 (DPP4), which is a transmembrane protein expressed on ECs that is involved in glucose metabolism and cardiometabolic disease (120). Recent studies showed that DPP4 inhibition decreases atherosclerotic plaque burden. For example, Meng et al. investigated the atheroprotective role of the DPP4 inhibitor trelagliptin on HAECs exposed to IL-1β *in vitro*, showing that trelagliptin treatment had a strong inhibitory effect on the expression of adhesion molecules and pro-inflammatory chemokines and cytokines that orchestrate monocyte adhesion on the endothelium (89). Mechanistically, trelagliptin inhibits IL-1β induced NFκB transcription factor activation which subsequently prevents the transcription of the monocyte chemoattractant chemokines CCL2, CXCL1 as well as the pro-inflammatory cytokine IL-6 and the adhesion molecules ICAM1 and VCAM1 (mRNA and protein levels) (**Table 3**) (89).

Results from studies that focus on IL-β antibody blocking or knock out *in vivo* are less straightforward. Earlier work in $Apoe^{-/-}$mice in which the anti-IL-1β antibody XMA052 MG1K was injected twice weekly during 16 weeks of WD showed a decrease of aortic lesion area of 37, 22 and 29% with 0.1, 1.0, 10 mg/kg XMA052 MG1K, respectively, compared to IgG injected control $Apoe^{-/-}$ mice (91). *In vitro* experiments performed in the same study revealed reduced release of other pro-inflammatory cytokines such as IL-6, IL-8, TNF-α and CCL2 from cultured macrophages and reduced release of the proteolytic enzymes MMP-3 and MMP-9 from ECs and VSMCs, after XOMA 052, a human engineered IgG2 anti-IL-1β antibody, treatment (**Tables 3**, **4**) (91). However, MMP-3 and MMP-9 expression were not affected *in vivo,* and no difference was observed in the plaque collagen content suggesting that plaque stability is not modified by this treatment (91). Yet, when anti-IL-1β treatment was tested to investigate its role in established atherosclerotic lesions, it led to adverse remodeling. Cell-specific effects of the IL-1β antibody (mouse monoclonal antibody, Novartis, 10 mg/kg) were tested by Gomez et al. on VSMC lineage tracing $Apoe^{-/-}$ mice where the fate and migration of SMCs can be monitored during the development of atherosclerosis by using an inducible Cre-flox system to label $MYH11^{+}$ SMC specific YFP expression ($Apoe^{-/-}$*Myh*11-$CreER^{T2}$R26R-YFP). The anti-IL-1β monoclonal antibody treatment did not lead to any differences in the aortic plaque size compared to IgG treated control animals after 18 weeks WD. In addition, anti-IL-1β treated mice showed thinner fibrous caps characterized by a 30% decrease of collagen, a 40% decrease of VSMC content, though a surprising 50% increase of lesional macrophages within the fibrous cap (**Table 4**) (121). These results indicate that anti-IL-1β treatment has a detrimental effect on fibrous cap remodeling promoting plaque instability.

Nevertheless, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) was the first big scale clinical trial confirming the potential of anti-inflammatory IL-1β therapy in CVD. In the CANTOS trial, anti-IL-1β (canakinumab) treatment resulted in a decrease of inflammatory markers in the plasma of patients such as high-sensitivity C-reactive protein (hsCRP) (26–41%) and IL-6 (6–25%) (129–131).

The receptor for IL-1β, *IL-1β receptor type* 1 (IL-1R1), was also described to reduce atherosclerotic plaque development (132, 133). However and in sharp contrast, lack of the *IL-1β receptor antagonist* (IL-1ra) promotes atherosclerotic plaque formation (134). More recent studies further support this controversial role of IL-1R1 and IL-1ra in atherosclerosis development and more precisely in atherosclerotic plaque remodeling.

IL-1R1 deletion has a dual role in plaque stability and outward vessel remodeling as shown in a female mouse model lacking IL-1R1 ($IL\text{-}1R1^{-/-}Apoe^{-/-}$) and fed a WD for 30 weeks. These mice showed reduced aortic root lesion size as well as a 50% reduction of the lumen area of the brachiocephalic artery when compared to control $IL\text{-}1R1^{+/+}Apoe^{-/-}$ mice. However, these reductions were accompanied by enhanced features of plaque instability, characterized by decreased VSMCs and collagen content. MMP-3 expression in the brachiocephalic artery was also significantly lower in $IL\text{-}1R1^{-/-}Apoe^{-/-}$ animals which may explain the reduced collagen content in the plaques (135). In line with this, IL-1Ra-deficient mice exhibited considerable inflammation in

TABLE 3 | Overview of cytokines involved in atherosclerosis remodeling and their physiological effect.

Cytokines	Receptors	Cells affected	Proposed effect in vascular remodeling	References
IL-1β	IL-1R	ECs	EC dysfunction	(88)
			Expression of ICAM1, VCAM1, CCL2	(89)
			Leukocyte adhesion and infiltration into the intima	(90)
		Macrophages	Expression of IL-6, IL-8, TNFa, CCL2	(91)
		VSMCs	Proliferation	(92)
			IL-6 expression	(93)
			Collagen expression	
IL-6	Gp130	ECs	ICAM1, VCAM1 expression	(94)
				(95)
		Monocytes	Recruitment, infiltration	(96)
				(97)
		VSMCs	Recruitment and migration	(98)
				(95)
		Neutrophils	Recruitment	(96)
				(97)
		ECM	Collagen deposition	(99)
IL-10	*IL*-10*R*1	Macrophages	Prevent proinflammatory cytokines production	(100)
				(101)
				(102)
			Prevent foam cell formation	
	IL-10*R*2			
			Promotes M2 macrophage polarization	
IL-22	IL-1R1	ECs	ICAM1, VCAM1 expression	(103)
				(104)
				(105)
		Macrophage	Phenotypic change from anti-inflammatory into pro-inflammatory cell type, Reduced cholesterol efflux	(105)
		VSMCs	Migration and proliferation	(106)
			Phenotypic switch	(107)
			from contractile into synthetic repair cell	(105)
IL-33	ST2	ECs	VCAM1, ICAM1, E-selectin, CCL2 expression	(108)
		Macrophage	Inhibits foam cell formation	(109)
		T cells	Differentiation into Th2 cells	(109)
TNFa	TNF1 TNF2	ECs	ICAM1, VCAM1, CCL2 expression	(110)
		Monocytes	Recruitment, Differentiation into macrophages	(111)
				(112)
		Macrophage	Foam cell formation	(110)
		VSMCs	Proliferation	(113)
				(114)

the aorta accompanied by an increased macrophage content in the adventitia and destruction of the elastic lamina, which is normally important to maintain arterial wall stability prevent intima lesion progression (136, 137). Another study also investigated the role of IL-1Ra in atherosclerosis lesion development by making use of *IL*-1$Ra^{+/+}$ and *IL*-1$Ra^{+/-}$ mice (*IL*-1$Ra^{-/-}$ mice were excluded from this study due to reduced weight gain and differential cholesterol metabolism) on $Apoe^{-/-}$ background. Heterozygous and control animals were fed normal chow diet for 16 weeks and *IL*-1$Ra^{+/-}Apoe^{-/-}$ mice showed a 30% increase of atherosclerotic lesion size compared to *IL*-1$Ra^{+/+}Apoe^{-/-}$ mice (134). Interestingly, after 32 weeks of chow diet feeding the lesion size was similar in both groups, but macrophage accumulation in the lesions was reduced in *IL*-1$Ra^{+/-}Apoe^{-/-}$ animals compared to control. VSMCs in the same lesions showed a 15% decrease compared to control *IL*-1$Ra^{+/+}/Apoe^{-/-}$animals (134). These findings suggest that IL1-Ra plays an important role in the development of atherosclerotic lesions and also suggests that it impacts plaque composition, modulation and stability (**Figure 1**).

TABLE 4 | Targeting cytokines as therapeutic treatments in vascular remodeling and CVD.

Cytokines	Therapeutic Treatment	Clinical trials	Animal experimentation	Outcomes on atherosclerosis	References
IL-1 β	Canakinumab	✓	✓	Decreases aortic lesion area, Decreases IL-6, IL-8, TNFα, CCL2, Decreases collagen and VSMC content	(91)
					(121)
IL-6	Raloxifene	✓	✓	Decreases aortic lesion area, Decreases IL-6, Decreases ICAM1, VCAM1 expression, Decrease macrophage and VSMC content	(95)
	Ziltivekimab	✓	–	Decreases of serum CRP	(122)
	Tocilizumab	✓	–	Decreases CRP, Ameliorates FMD, Increase total cholesterol	(123)
IL-10	Nothing yet	–	–	–	–
IL-22	Fezakinumab	✓	–	Not published yet	(124)
IL-33	Nothing yet	–	–	–	–
TNFα	Adalimumab	✓	✓	Decreases VCAM1, E-selectin, CRP, and aortic stiffness; Increases oxLDL	(125)
					(126)
					(127)
	Etanercept	✓	–	Decreases aortic stiffness, Increases cholesterol and triglycerides	(128)

Taken together, IL-1β is detrimental and promotes early-stage atherosclerosis development but on the other hand animal models suggest that it is beneficial in an advanced stage of the disease to maintain plaque stability and avoid major cardiovascular complications. Plaque stability also seems to be affected by interfering with IL1-R1 or IL-1RA. Nevertheless, it is important to keep in mind that research on mice and *in vitro* assays have certain limitations with regards to the translation to human pathologies. Therefore, efficacy and safety of the CANTOS trial and its promising beneficial results in patients with adverse cardiovascular events still hold true even if research on IL-1β on atherosclerosis in mice and *in vitro* findings are sometimes conflictual meaning that the results are not always consistent from one study to another. In addition, one should keep in mind that $Apoe^{-/-}$ mice are severely hypercholesterolemic which does not fully translate with the CANTOS patients who were under statin treatment. Moreover, canakinumab in CANTOS was only administered quarterly and not weekly which may explain why no effects on lesion stability were reported yet. The latter also underlines the importance of careful timing of treatment regimens.

Interleukin 6

IL-6 is secreted by various cells including epithelial cells, VSMCs, vascular ECs, monocytes, and T cells (**Figure 2**) (138) and promotes the synthesis of acute phase proteins such as CRP, amyloid A, β2 protein, hemopexin and haptoglobin. Both CRP and amyloid A are biomarkers of chronic inflammation and their levels predict cardiovascular risk (97, 139). In addition, IL-6 shows chemotactic activity for monocytes and neutrophils and promotes the expression of adhesion molecules (**Table 3**) (94) and chemokines leading to vascular remodeling by the induction of leukocyte adhesion and infiltration into the intima (**Figures 1**, **2**; **Table 3**) (96, 97). More precisely, paracrine secretion of IL-6 by VSMC induces expression of the adhesion molecule E-selectin on ECs (117). In addition, in response to IL-6 signaling ECs and SMC also start to secrete IL-6 (140). TNFα activated ECs also induce SMC production of IL-6 (141).

Circulating IL-6 is a biomarker for CVD such as acute coronary syndrome with atherosclerosis (142). A study on carotid atherosclerosis showed that blood levels of IL-6 and TNFα were higher in patients with carotid atherosclerosis compared to control subjects and the cytokine levels increased with increasing amounts of carotid atherosclerosis stenosis (143). IL-6 was also shown to promote migration and viability of macrophages and VSMCs *in vitro* and regulate ECM deposition and reorganization (**Table 3**) (95, 98). One mechanism of IL-6 driven SMC migration is made via MyD88 and TRIF action and activation of p38 MAPK and ERK1/2 signaling) (144). Schieffer et al. investigated the role of IL-6 deficiency on atherosclerosis development. In this study, IL-6 deficient $Apoe^{-/-}$ mice ($Apoe^{-/-}IL\text{-}6^{-/-}$) were fed with chow diet for 53 ± 4 weeks and showed a significant decrease in aortic transcript and protein levels of MMP-9, tissue inhibitor of metalloproteinase-1, collagen I and V and lysyl oxidase (145), an important protein for the formation and repair of the ECM, compared to $Apoe^{-/-}$ control mice (146). Additionally, $Apoe^{-/-}IL\text{-}6^{-/-}$ mice exhibited a decrease in macrophage and leukocyte infiltration into the lesions, although atherosclerotic lesion formation was enhanced in $Apoe^{-/-}IL\text{-}6^{-/-}$ compared to $Apoe^{-/-}$ animals (28.1 vs. 14.9% respectively). The latter may be explained by the observed increase of total cholesterol, LDL, and very-low-density lipoprotein VLDL in IL-6 deficient animals and a decrease of plasma IL-10, compared to control mice (146).

Interestingly, a recent study showed an atheroprotective effect of raloxifene treatment in male *Apoe*$^{-/-}$ mice fed a WD for 12 weeks. Raloxifene is a drug that is prescribed to post menopause women to prevent osteoporosis and is able to inhibit IL-6 binding to the IL-6 receptor subunit GP130, thereby prohibiting downstream signaling pathways such as STAT3. In this study, it was observed that daily administration of 5 mg/kg raloxifene reduced the area of atherosclerotic lesion size in the aorta and aortic root in *Apoe*$^{-/-}$ mice compared with untreated control *Apoe*$^{-/-}$ mice (95). In addition, raloxifene significantly decreased the expression of IL-6, ICAM1, and VCAM1 in the aortic vascular ECs and reduced the lesional macrophage and VSMC content in these aortic lesions. Mechanistically it could be demonstrated that raloxifene decreases atherosclerosis by preventing IL-6-induced phosphorylation of STAT3 and inhibiting the IL-6/GP130 interaction (**Tables 3**, **4**) (95).

IL-6 secretion by aortic perivascular adipose tissue (PVAT) also promotes aortic stiffness and remodeling in *Ldlr*$^{-/-}$ mice compared to WT C57BL/6 mice fed with chow diet (99). Aortic stiffness, mediated by changes in the ECM protein expression, is caused by an increase in cross-linking of collagen (147). Consistent with other studies, Du et al. (99) also observed an increase in IL-6-ediated collagen type I expression in the aorta of *Ldlr*$^{-/-}$ mice (**Table 3**). These findings suggest that IL-6 may play an important role in inducing changes in the ECM by increasing collagen type I expression and thereby promoting subsequent arterial stiffness and thus vascular remodeling (**Figure 1**).

RESCUE, a trial to evaluate the reduction in inflammation in patients with advanced chronic renal disease utilizing antibody-mediated IL-6 inhibition investigated the effect of ziltivekimab in patients with high cardiovascular risk (122). Patients in this study had elevated serum CRP levels and chronic kidney disease and were randomly allocated into groups with differential monthly subcutaneous administrations of ziltivekimab (total of 7.5 mg, 15 mg or 30 mg) or placebo. After 12 weeks of treatment, CPR levels decreased by 77% in the 7.5 mg group and by 92% in the 30 mg group. Biomarkers of systemic inflammation and thrombosis, relevant to atherosclerosis, were also reduced in a dose-dependent manner, while no severe side effects were detected. Due to the COVID-19 pandemic, the study unfortunately had to be terminated before the planned secondary 24-week endpoint to avoid exogenous causes of increased CPR levels and thereby bias the interpretation of the results. However, it is planned to do a large-scale cardiovascular outcome trial on the same population to investigate the effect of ziltivekimab on the recurrence rate of vascular events (**Table 4**) (122). Another clinical study investigated the efficiency of an anti-IL-6 treatment, tocilizumab, on endothelial function in high-risk CVD patients with rheumatoid arthritis (RA). Tocilizumab is a humanized monoclonal antibody that targets the soluble and membrane-bound IL-6 receptor. The study consisted of three groups, whereby 18 patients received tocilizumab (8 mg/kg IV) every 4 weeks, 24 patients underwent anti-TNFα treatment (methotrexate 15–25 mg/week or leflunomide 20 mg/d) and 18 control patients who were treated with synthetic disease-modifying antirheumatic drugs (123). Endothelial function was evaluated by flow-mediated dilation (FMD) measurements before and after 16 weeks of starting the different treatments. As expected, patients from the tocilizumab treated group showed the most striking reduction in mean CRP levels compared to the other treatments. Furthermore, the mean FMD only significantly improved in the tocilizumab group, increasing from 3.43 to 4.96%. On the other hand, anti-IL-6 treatment greatly enhanced the atherogenic lipid profile and increased total cholesterol levels (**Table 4**) (123). Long-term treatment with tocilizumab was also addressed in a pilot study with 16 female RA patients receiving monthly injections with tocilizumab (8 mg/kg IV) and endothelial function was again measured by FMD. The endothelial function significantly improved after 6 months of treatment compared to 16 non-RA control subjects (148). As underlined by various clinical trials, tocilizumab worsens the lipid profile, total cholesterol burden, LDL, and triglyceride profile. However, it does also improve endothelial function and therefore reduces vascular remodeling. Therefore, to minimize the detrimental effects of elevated lipids, it would be ideal to for example combine tocilizumab with statin treatment.

Interleukin 10

IL- 10 is an anti-inflammatory cytokine produced mainly by macrophages and T cells. IL-10 signaling is mediated through a two-receptor complex named IL-10 receptor 1 (IL-10R1) and IL-10R2. The receptor complex is constitutively expressed on numerous hematopoietic and non-hematopoietic cells such as epithelial cells and fibroblast and is upregulated upon activation (149). In atherosclerosis, IL-10 prevents remodeling by inhibiting macrophage activation and their proinflammatory cytokine production including TNFα, IL-1β, IL-6, IL-8, granulocyte stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), foam cell formation, MMP expression, VSMC proliferation and therefore helps to reduce atherosclerotic plaque formation (**Figure 1**; **Table 3**) (101, 150).

In contrast, IL-10 deficiency in *Apoe*$^{-/-}$ after 16 weeks on chow diet leads to greater atherosclerosis plaque formation, an increase in blood cholesterol (e.g., LDL), an increase in Th1 cell response in the lesion as well as greater tissue factor activities, systemic coagulation and vascular thrombosis compared to control (151). In APOE*3-Leiden mice under cholesterol-enriched high-fat diet with cuff-induced neointima formation in the femoral artery, IL-10 deficiency led to increased neointima formation. On the other hand, in the same mouse model, IL-10 overexpression by means of single intramuscular injection of IL-10 resulted in a 45% decrease of neointima surface (152). However, *Ldlr*$^{-/-}$ mice transplanted with marrow cells from IL-10 transgenic male mice on a C57BL/6J background and fed chow diet for 4 weeks prevents the formation of advanced lesions, shifts Th1 cells toward Th2 phenotype and decreases IFN-γ levels in the lesion (153). Interestingly, IL-10 overexpression leads to increased modified LDL uptake by macrophages, although it also stimulates its efflux and thus prevents foam cell formation (**Figure 1**) (100). Overall, IL-10 diminishes lesion burden by decreasing proinflammatory cytokine levels and promoting an anti-inflammatory environment in the lesion with M2 polarized macrophages as well as Th2 cells and prevents foam cell formation (100, 154, 155). IL-10 also inhibits VSMCs activation

in vitro and *in vivo*. IL-10 treatment of LPS-stimulated rat SMCs resulted in a decreased NF-κB activation, IL-6 secretion as well as reduced SMC migration and proliferation (**Figure 1**). In line with these observations, in a rat model of intimal hyperplasia, IL-10 treatment led to reduced SMC proliferation and intimal growth 14 days after balloon abrasion of the aorta compared with saline-injected control animals (150).

Furthermore, Jung et al. investigated more in detail the protective cellular and molecular mechanisms of IL-10 that prevent adverse MI LV remodeling. The authors show that IL-10 promotes M2 macrophage polarization *in vitro* and in turn the M2 secretome induces cardiac fibroblast activation, proliferation, migration and α-SMA expression. In addition, the same study showed that IL-10 treatment of fibroblasts reduces the ratio of collagen I to III secretion 7 days post-MI leading therefore to decrease fibrosis formation (**Table 3**) (102). These observations could mean that IL-10 levels may be implicated in plaque stability.

Systemic treatment with IL-10 is not ideal as it inhibits inflammation even when it is needed to fight pathogens. Indeed, long-term treatment of IL-10 increases the prevalence of intracellular infection such as Chlamydia and Listeria (156). In this regard, a study has tried cell-specific systems to avoid these off-target side effects. Exosomes loaded with IL-10 mRNA were engineered to target inflamed macrophages in the atherosclerotic lesion. These exosomes were used to treat *Apoe*$^{-/-}$ mice under 8 weeks high fat diet and resulted in decreased atherosclerotic plaque formation compared to PBS receiving control or exosome treatment (157).

Treatments and clinical trials are currently undergoing to explore the anti-inflammatory properties of IL-10 in various chronic diseases including RA, multiple sclerosis, allergies and inflammatory intestinal disease among others. However, IL-10 therapy for atherosclerosis and its effect on vascular remodeling remains to be investigated.

Interleukin 22

IL-22 is a member of the IL-10 cytokine family and is secreted by both innate and adaptive immune cells such as activated T cells especially T helper (Th) 22 cells and Th17 cells, NK cells, neutrophils, fibroblasts, and macrophages. IL-22 is involved in many cellular processes including lipid metabolism regulation, maintenance of bacterial homeostasis in the intestine and tissue regeneration (158).

The function of IL-22 in atherosclerosis is largely unknown, although evidence suggests that IL-22 is involved in vascular remodeling by promoting pro-inflammatory chemokines and antimicrobial peptide secretion as well as increasing VSMC migration and proliferation (**Figure 1**) (159, 160). In addition, IL-22 regulates adhesion molecule expression by ECs such as ICAM1 and VCAM1 as well as the production of chemokine ligands that have been implicated in adhesion, migration and recruitment of monocytes in atherogenesis (**Figure 1**) (103–105) (**Table 3**). The receptor of IL-22, being IL-22R1, is widely expressed on VSMCs, macrophages and ECs and mediates enhanced proliferation and migration through NFκB-, STAT3-, MAPK- and ERK1/2-dependent pathways in VSMCs (**Figure 1**). Furthermore, paracrine IL-22 promotes macrophage differentiation from anti-inflammatory into a pro-inflammatory phenotype and impairs the cholesterol efflux capacity of the cells, thereby promoting foam cell formation (**Figure 1**) (161, 162). Rattik et al. demonstrated that IL-22-deficient *Apoe*$^{-/-}$ mice (*IL-22*$^{-/-}$ *Apoe*$^{-/-}$) fed a WD for 14 weeks had a significant reduction of atherosclerotic plaque size in both the aortic root and aorta, compared to control *Apoe*$^{-/-}$ mice. In addition, *IL-22*$^{-/-}$ *Apoe*$^{-/-}$mice depicted reduced collagen content but increased expression of genes associated with VSMC contraction, namely α-actin, vinculin and caldesmon (**Table 3**) (106). The same study also explored the role of IL-22 in tissue repair mediated by arterial VSMCs in a carotid artery injury model in C57BL/6 mice. Here, an increased expression of IL-22 on VSMCs could be observed in the injured compared with non-injured arteries (106). These results suggest that IL-22 plays a key role in atherosclerotic plaque formation via the stimulation of dedifferentiation of contractile VSMCs toward synthetic repair cells resulting in plaque growth and that IL-22 is involved in plaque stability by thickening the fibrous cap, rendering it more stable by promoting lesional collagen content (**Figure 1**). IL-22 is therefore a double-edged sword. On the one hand, IL-22 worsens atherosclerosis by promoting inflammation, dysregulating macrophage cholesterol metabolism leading to more foam cell formation and promoting VSMCs proliferation and fibrous cap thickening (**Table 3**) (105). While on the other hand, IL-22 also leads to more stable plaques by increasing VSMC proliferation and migration into the intima forming a thick fibrous cap which decreases the risk of rupture and acute cardiovascular events.

Besides the above described protective effect of IL-22 it was also demonstrated that this cytokine can prevent atherosclerosis by promoting the expression of antimicrobial peptides which limit the spread of proatherogenic bacteria such as *Enterobacteriaceae* (*Klebsiella* sp), *Prevotellaceae* (*Prevotella copri*), *Lachnospiraceae, Clostridiaceae* and *Ruminococcaceae*. Additionally, a BMT of *IL-22*$^{-/-}$ BM into *Ldlr*$^{-/-}$ mice fed with WD for 16-weeks revealed larger atherosclerotic lesion sizes in hematopoietic IL-22-deficient *Ldlr*$^{-/-}$ mice compared to the control group (158). In these mice the aortic plaque was characterized by an increase of T cells and myeloid cell content as well as enhanced expression of aortic and intestinal proinflammatory cytokines like IL-1β, TNFα, CCL2 and CCL5. Intestinal gene expression of anti-microbial peptides *C-type regenerating islet derived*-3 (Reg3)-b and Reg3-g were also significantly reduced in hematopoietic IL-22 deficient *Ldlr*$^{-/-}$ mice compared to controls and whole metagenome shotgun sequencing analysis of cecal luminal microbiota revealed an increase of the above mentioned pro-inflammatory and proatherogenic bacterial species in absence of IL-22 (158).

However, in sharp contrast, more recently Shi et al. (107) underlined the pro-atherogenic effects of IL-22 produced by Th22 cells. After 12 weeks of WD feeding while *Apoe*$^{-/-}$ mice were treated with intraperitoneal injections of 2 μg recombinant mouse IL-22 (rIL-22) three times a week, it could be shown that IL-22 treatment resulted in larger atherosclerotic plaque sizes in the aortic root and the aorta as well as an increase in lesional

macrophages. Th22, Th17 cells as well as DCs, collagen and serum IL-6 levels were also enhanced compared to PBS-treated *Apoe*$^{-/-}$ control mice. In addition, SMC α-actin was reduced in mice undergoing rIL-22 treatment. These observations were abolished when mice were treated three times a week with 20 μg anti-IL-22 monoclonal antibody (IL-22 mAb), proving the causal role of IL-22 in the observed effects. The same study also showed that BM-derived DCs from *Apoe*$^{-/-}$ mice treated with 100 ng/mL rIL-22 followed by stimulation with oxLDL displayed enhanced maturation properties and were able to induce differentiation and proliferation of naïve CD4^{+}T cells into Th17 cells (107). The authors concluded that IL-22 secretion by Th22 aggravates atherosclerosis by promoting T cells (Th17), DCs and macrophage infiltration in the plaque and by inducing the dedifferentiation of contractile SMCs into synthetic SMCs (**Table 3**).

Human studies have also observed a correlation between circulating IL-22 levels and atherosclerosis. 45 patients with carotid artery disease were selected and were classified as symptomatic or asymptomatic based on the presence or absence of cerebrovascular symptoms. Immunostaining of plaques revealed a 7.15-fold higher IL-22 occurrence in symptomatic patients compared to the asymptomatic ones (104). Moreover, significant higher plasma levels of IL-22 were measured in patients with acute MI compared to healthy controls (163). Pre-clinical and clinical studies testing fezakinumab, a human anti-IL-22 mAb, and mAbs targeting the IL-22 receptor are still ongoing in patients with severe inflammatory diseases such as psoriasis, atopic dermatitis and rheumatoid arthritis. The limited results that are already published suggest no adverse safety concerns and <50% skin improvement, based on Percentage Change in the Scoring of Atopic Dermatitis (SCORAD) scores in atopic dermatitis patients (**Table 4**) (124, 164). Since atherosclerosis is also an inflammatory disease, interfering with IL-22 or IL-22 receptor may also represent a promising therapeutic target for CVD, although more elaborate research is needed to pinpoint the exact mechanisms of action.

Interleukin 33

IL-33, the most recently discovered member of the IL-1 cytokine family may also play a crucial role in vascular remodeling. This cytokine is expressed by ECs, VSMCs, epithelial cells and immune cells such as macrophages and T cells. For example, IL-33 is released from injured or necrotic ECs and acts as an alarmin leading to pro-inflammatory responses, both from innate and adaptive immune cells (165, 166). IL-33 binds to *IL-1 receptor like protein* (IL-1RL1 or also known as ST2). ST2 has two forms, a transmembrane (ST2L) and a soluble (sST2) one, which compete with each other for IL-33 binding. ST2L is expressed by various immune cells such as macrophages, T cells (predominantly Th2), mast cells, and innate lymphoid cells (167). Elevated levels of IL-33 and its soluble receptor sST2 were observed in patients suffering from pathologies such as diabetes, obesity, CAD, stroke and atherosclerosis (168).

IL-33 promotes the expression and secretion of various pro-inflammatory cytokines, adhesion molecules, proteolytic and coagulation factors. Investigations of IL-33 in atherosclerosis revealed that this cytokine stimulates the expression of the endothelial adhesion molecules VCAM1, ICAM1 and E-selectin as well as the expression of CCL2 in HUVECs in a concentration-dependent manner resulting in increased leukocyte adhesion (**Figure 1**; **Table 3**) (108). On the other hand, by binding to the ST2L, IL-33 can also influence the phenotype and function of macrophages and T cells (167). Furthermore, Miller et al. (109) found that IL-33 reduces atherosclerotic lesion size in the aortic sinus of 6-week-old male *Apoe*$^{-/-}$ mice fed with WD for 12 weeks while treated twice weekly with IL-33 injections (1 μg/injection) compared to the PBS-injected control group. In the same study, it could be demonstrated using *in vitro* serum assays that the atheroprotective effect of IL-33 was due to IL-33-mediated polarization of T cells into Th2 cells, inhibition of foam cell formation and an increase of antibody production targeting oxidized LDL. Nevertheless, plaque stability from the treated and untreated group remained unchanged, characterized by similar VSMC and collagen content, suggesting that IL-33 treatment decreases atherosclerosis plaque size without affecting plaque stability (**Table 3**) (109).

However, also some controversial results regarding the effects of IL-33 were found in human and rat studies. In humans, circulating IL-33 levels were found to correlate with vulnerable and high-risk plaques in 191 patients with carotid artery atherosclerosis. Analysis of carotid endarterectomies (CEA) from these patients also revealed an increased plaque expression of IL-33 and its receptor ST2 as well as enhanced IL-33 serum levels in CEA patients with vulnerable plaques compared with CEA patient with stable plaques (166). In contrast to what was observed in humans, obese rats showed increased sST2 levels in the aorta which correlated with an increase in the production of collagen, fibronectin and profibrotic molecules enhancing ECM formation and vascular fibrosis (**Figure 1**) (169). IL-33 seems to have differential effects on the stability of the atherosclerotic plaque depending on the species, in humans it was associated with vulnerable plaques however in rats, IL-33 seems to increase plaque stability.

In addition, it was found that certain genetic polymorphisms in the IL-33 locus resulted in deferential atherosclerosis development in patients with RA. From 576 RA patients carrying the mutant TT genotype of IL-33 rs3939286 polymorphism had a significantly lower carotid intima-media thickness (cIMT) evaluated by carotid ultrasound, compared to the wild-type CC genotype. The heterozygous CT genotype had an intermediate cIMT value. Combined, these results suggest a potential protective effect of the IL-33 rs3939286 T allele in atherosclerosis development by decreasing IL-33 expression (170).

However, there are also studies showing that atherosclerosis severity was not affected by IL-33/ST2 signaling. For example, there were no significant differences in atherosclerotic lesion area between *IL33*$^{-/-}$*Apoe*$^{-/-}$, *ST2*$^{-/-}$*Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ mice fed with 10-weeks-high cholesterol diet (171).

In conclusion, research on IL-33 and its multiple controversial effects on atherosclerosis are not yet fully elucidated and need to be better understood. A potential explanation for the controversial results are differences in tissue specific expression patterns, the applied model (e.g., deficiency vs. receptor blocking)

or the prescribed medication, which should be investigated and compared more closely in future studies.

Tumor Necrosis Factor α

TNFα is a pro-inflammatory cytokine that is expressed mainly by macrophages, DCs and T cells and activates various pathways including cell survival, apoptosis, necrosis, migration, proliferation, barrier disruption, cell adhesion and actin cytoskeleton modification (172). It has two receptors TNFR1 and TNFR2, although several studies showed that the majority of TNFα signaling is mediated through TNFR1 (173–175). TNFα modulates vascular remodeling by increasing EC permeability, up-regulation of endothelial adhesion molecules leading to monocyte adhesion to the endothelium, matrix degradation and VSMCs proliferation in the intima (**Figure 1**; **Table 3**) (111, 113, 176, 177). TNFα can act both in a paracrine or autocrine manner. For example, TNFα-activated ECs stimulate VSMC expression of the pro-inflammatory cytokine IL-6 but also vascular endothelial growth factor (VEGF) and further TNFα expression (141). Furthermore, TNFα secreted by ECs leads to VSMC proliferation as well as promoting synthetic and macrophage-like phenotype differentiation (118).

Therefore, TNFα is a key atherosclerotic cytokine in atherosclerosis (178) and its genetic deletion in atherogenic *Apoe*$^{-/-}$ mice fed 10 weeks of WD was found to decrease atherosclerotic lesion size by 50% compared to control *Apoe*$^{-/-}$ animals (112). In the same study a BMT of 10-week-old *Apoe*$^{-/-}$ mice with age-matched *Apoe*$^{-/-}$*TNFα*$^{-/-}$ BM resulted in a 83% reduction of lesion size after 25 weeks of WD in mice with hematopoietic TNFα-deficiency (112). Unexpectedly, no differences in lipid burden, VCAM1 expression, macrophage, B cell and T cell numbers in the circulation could be observed comparing TNFα-deficient mice with control animals. These results suggest that hematopoietic TNFα may not be involved in monocyte mobilization, since adhesion molecules as well as macrophage content in the plaques were similar in both groups (**Table 3**) (112). Another study using *Apoe*$^{-/-}$*TNFα*$^{-/-}$ mice found a decreased expression of the adhesion molecules VCAM1 and ICAM1 and the cytokine CCL2 in TNFα-deficient mice on chow diet compared to *Apoe*$^{-/-}$ control mice (**Table 3**) (110). The same study also described an reduced capacity of macrophages to phagocytose LDL particles, thereby promoting foam cell formation as well as an decreased expression level of the scavenger receptor class A in TNFα-deficient *Apoe*$^{-/-}$ mice compared to *Apoe*$^{-/-}$ controls (110) (**Figure 1; Table 3**). Taken together, it seems that somatic TNFα deficiency has a greater impact on atherosclerosis development as it decreases endothelial adhesion molecule expression as well as foam cell formation compared to hematopoietic TNF-α deficiency.

Anti-TNFα therapies in RA patients are also known to decrease the level of serum chemerin, an adipokine that has an important role in CVD, although the relationship of chemerin and TNFα in remodeling remains unknown (179). Nevertheless, vascular remodeling seems to be affected by chemerin levels as shown *in vitro*, where chemerin deficiency decreases VSMCs proliferation and *in vivo* where it decreases neointima hyperplasia after angioplasty (180). Lack of chemerin also leads to the reduction of pro-inflammatory cytokines, like TNFα, in the serum suggesting a feedback loop between the two (179). Another component that modulates TNFα-induced remodeling is the *erythropoietin-producing human hepatocellular receptor* (EphA), which is a receptor tyrosine kinase that mediates cell-adhesion and leukocyte homing in atherosclerosis by promoting ICAM1 and VCAM1 expression on ECs (181). EphA2-deficient *Apoe*$^{-/-}$ mice which were fed a WD for 12 weeks developed smaller innominate artery and carotid sinus plaque size compared to *Apoe*$^{-/-}$control animals (182). Furthermore, *in vitro* knockout of EphA2 in HAECs revealed that TNFα treatment is unable to induce monocyte adhesion to ECs lacking EphA2, suggesting that the TNFα-induced expression of adhesion molecules is inhibited upon EphA2 deficiency (182).

The effect of TNFα on endothelial function may also be modulated by food consumption. For example, aged garlic extract (AGE) and its sulfur-containing constituents improve the endothelial barrier function elicited by TNFα through stimulation of anti-inflammatory, anti-oxidative and anti-hypersensitive pathways in humans thereby also preventing CVD development including atherosclerosis. Active substances in AGE consisting of *S*-1-*prpenylcysteine* (S1PC) particularly interfere with TNFα-induced hyperpermeability of the endothelium (183). Therefore, adverse remodeling in atherosclerosis may also be reduced by food supplements.

Furthermore, tongxinluo (TXL), a traditional Chinese medicine product has anti-inflammatory as well as vasoprotective properties. In C57BL/6 mice subjected to carotid artery ligation, TXL treatment significantly reduced neointima hyperplasia in a dose-dependent manner by inhibiting macrophage infiltration as well as VSMCs proliferation in the intima of the artery, compared to untreated control mice (114). Further analysis revealed that the protective effects of TXL in hyperplasia are due to the inhibition of TNFα-induced miRNA-155 expression, a generally pro-inflammatory acting miRNA (114).

Nevertheless, until recently the therapeutic potential of TNFα blockage in atherosclerosis by pharmacological inhibitors such as monoclonal antibodies remained unknown. Oberoi et al. tested weekly injections of mouse-specific anti-TNFα monoclonal antibody CNTO5048 (12 mg/kg) in 10-week-old *Ldlr*$^{-/-}$ mice fed with high fat, high cholesterol diet for either 6 or 12 weeks. Plasma inflammatory markers such as IL-6, CCL2 and TNFα were significantly decreased in mice receiving CNTO5048 after 12 weeks of treatment compared to control animals injected with IgG antibody (184). However, no differences were observed in the 6 weeks group, although mRNA expression levels of IL-6, CXCL1 and ICAM1 in vascular tissue of the aortic arch were increased after both 6- and 12-weeks treatment. On the other hand, plasma lipid profiles revealed a significant increase in VLDL cholesterol and triglycerides due to CNTO5048 treatment. Moreover, atherosclerotic plaque burden was also augmented in CNTO5048 mice. Detailed examination of the plaque composition revealed a reduction of intimal VSMC infiltration and lower collagen type I deposition within the atherosclerotic plaque in the CNTO5048-treated group which is associated with plaque instability. These counter-intuitive results revealing reduced systemic inflammation though enlarged lesion

growth are most likely due to the enhanced cholesterol levels observed in the treated group. Yet, analysis of genes regulating lipid and cholesterol metabolism, such as *Apob*, *Mttp* or *Apoa*5 in the liver, did not reveal any differences between the two groups (184). In contrast, adalimumab, a human-specific anti-TNFα monoclonal antibody binding and blocking both soluble and membrane bound TNFα, administered to $Ldlr^{-/-}$ mice fed 10-weeks of WD, revealed reduced atherosclerotic lesion size by 52% in the anti-TNFα treated mice (2.2 mg/kg twice weekly *via* intraperitoneal injection) compared to IgG controls, while the cholesterol and triglyceride levels did not change between the groups (185). These differential results may be due to the different antibodies that were applied, since the mouse model and length of WD feeding were comparable between the two studies (184, 185).

Various clinical trials have already been conducted to evaluate the effect of anti-TNFα treatment in patients with CVD. For example, recent findings reveal a decrease in cardiovascular biomarkers, such as soluble VCAM1, in patients after treatment with adalimumab. Psoriasis is well known to increase CVD risk as well as sharing different biomarkers and pathophysiological mechanisms such as systemic inflammation and endothelial dysfunction with CVD, suggesting that this pathology might benefit from similar therapeutic approaches (186, 187). Therefore, Zdanowska et al. conducted a study using 34 patients with psoriasis and 8 healthy volunteers between 30 and 73 years old, which were treated with an initial dose of 80 mg and then 40 mg of adalimumab every 2 weeks. After 12 weeks of treatment, soluble VCAM1 serum levels were significantly decreased comparing psoriasis patients with controls, while E-selectin was not affected (**Table 4**) (125). On the other hand, E-selectin levels were decreased in a bi-yearly study examining the effect of adalimumab in 17 patients with psoriasis compared with 24 healthy age-, gender- and BMI-matched volunteers with the same treatment regimen. Serum levels of E-selectin as well as plasma levels of IL-22 were significantly decreased compared to baseline, both after 12 and 24 weeks of treatment (126). Plasma CPR levels also decreased but only reached a significant difference compared to baseline after 24 weeks of treatment. Notably and in sharp contrast to the previously described beneficial outcomes, circulating oxLDL levels increased during the 2 years follow-up (**Table 4**) (126). The atheroprotective effects of adalimumab may therefore be a cumulative effect from a repression of inflammation and reduction of cholesterol uptake by macrophages leading to reduced foam cell formation. However, since cholesterol uptake by macrophage is decreased, it causes an augmentation of circulating cholesterol, which is a side-effect that could be solved with a combinational treatment with statins.

In addition, adalinumab decreases aortic stiffness as tested in 18 RA patients receiving either a monotherapy of subcutaneous adalinumab (40 mg/ 2 weeks) or a combination with disease modifying antirheumatic drugs in comparison to control patients using methotrexate (MTX). After 3 months of therapy, adalinumab treatment significantly decreased the aortic stiffness indices as measured by carotid-femoral pulse wave velocity (cfPWV) from 8.18 m/s to 7.01 m/s while no difference was observed in patients using MTX. On the other hand, there was no significant difference in the disease augmentation index (AIx), a systemic arterial stiffness parameter (188), or other cardiovascular risk factors after the adalinumab treatment (**Table 4**) (127). Besides adalinumab, also another TNFα inhibitor called etanercept is used as therapy for patients with various chronic inflammatory diseases such as RA. Based on a recent meta-analysis, both treatments significantly decreased vascular remodeling by limiting aortic stiffness and wave reflection in RA patients, independently of the treatment duration (duration varied from 6 to 56 weeks) (**Table 4**) (128). However, in all the studies mentioned above, TNFα treatment led to enhanced cholesterol and triglyceride titers (**Table 4**).

In conclusion, TNFα therapy holds great promise in reducing CVD and benefitting cardiovascular patients by decreasing atherosclerotic plaque size as well as decreasing several pro-inflammatory markers and ameliorating aortic stiffness. However, the long-term effects of anti-TFNα treatment and augmentation of cholesterol levels need to be critically considered when judging on the superiority of the treatment for CVD patients in light of the unfavorable lipid profile (128). However, based on clinical studies, the increase in lipid profiles in patients undergoing TNF-α treatment could be reduced by combining it with statin treatment (189).

OTHER MEDIATORS

In addition to chemokines and cytokines also other factors influence vascular function and composition. Among them are for example growth factors, prostaglandins and leukotrienes which will be summarized with respect to their role in vascular remodeling in the following paragraphs.

Growth Differentiation Factor-15

Growth differentiation factor-15 (GDF-15), also known as macrophage inhibitory cytokine-1, is a member of the TGF-β family which is induced under stress conditions such as oxidative stress, inflammation, ischemia, or mechanical stretch (190). GDF-15 is known to be expressed in cardiomyocytes, especially in response to MI, but also by adipocytes, macrophages, ECs, and VSMCs from both healthy and injured tissues (191). A receptor for GDF-15 has been identified called *glial cell-derived neurotrophic factor* (GDNF) *family receptor α-like* (GFRAL). However, this receptor is mainly expressed on human brain stem cells and based on the various physiological effects of GDF-15 throughout the body it is hypothesized that other receptors should exist which are still to be discovered (192). Inflammatory proteins such as IL-1β, TNFα, IL-2 and *macrophage stimulating factor* (MCSF)-1, all of which are upregulated in atherosclerosis, induce the expression of GDF-15 (192). In line with this, an increase in circulating GDF-15 protein was recently associated with an elevated risk of adverse events in patients suffering from acute coronary syndrome, chronic kidney disease or heart failure (193–197). For example, the study by Gohar et al. (194), demonstrated that high plasma levels of GDF-15 in women with carotid atherosclerotic disease are predictive for secondary cardiovascular events. However, the effect of GDF-15 on cardiac remodeling is still poorly understood. One study from Xu et

al. (198) showed that GDF-15 blocks norepinephrine-induced myocardial hypertrophy through a novel pathway involving the inhibition of epidermal growth factor receptor (EGFR) transactivation. In line with this observation, a recent overview from Wesseling et al. (199) emphasizes the role of GDF-15 in endothelial dysfunction, hypertrophy, and fibrosis (**Table 3**). GDF-15 was also associated with an elevated risk of adverse events in patients suffering from coronary syndrome, chronic kidney disease or heart failure (199). Heart failure involves cardiac remodeling following tissue injury which is caused by inflammation, volume, or pressure overload (199).

Growing evidence indicates that GDF-15 has detrimental effects on endothelial function by causing an increase in adhesion molecule expression and influencing the balance between vasoconstriction and vasodilatation (**Figure 1**). Indeed, GDF-15 leads to endothelial dysfunction by reducing vascular contraction and relaxation, which ultimately leads to an impaired cardiac function (**Table 3**) (190, 200). In addition, GDF-15 is implicated in cardiac hypertrophy which is described as an increased heart size and insufficient cardiac output (199). The exact role of GDF-15 on cardiomyocytes is still not fully understood but it seems that GDF-15 has both pro-hypertrophic and anti-hypertrophic effects depending on environmental cues. For example, GDF-15 promotes cardiac hypertrophy by protecting cardiomyocytes from apoptotic stimuli (201), but in constrast GDF-15 also seems to reduce myocardial hypertrophy by inhibiting the transactivation of EGFR (**Table 3**) (198). Further research also suggests that the anti-hypertrophic effects of GDF-15 on cardiomyocytes may be due to GDF-15-induced activation of *small mother against decapentaplegic* (SMAD) 2/3 proteins (202). GDF-15 also promotes cardiac fibrosis in heart failure and increases collagen turnover in MI as well as collagen deposition in atherosclerotic plaques (**Figure 1**; **Table 3**) (203–205).

In addition, elevated circulating GDF-15 levels are suggested as a potential biomarker for cardiovascular risk and outcome as it is directly linked to atherosclerosis progression. GDF-15 promotes plaque vulnerability through pro-inflammatory and angiogenic effects, especially in the early stages of the disease (205, 206). Furthermore, GDF-15 has been linked to cardiovascular event prediction in general and identification of high-risk patients (205, 206). Therefore, monitoring and targeting GDF-15 in CVD may improve early diagnosis and treatment strategies (206).

Prostaglandins

Prostaglandins are a group of lipids synthesized at sites of tissue damage or infection and play an important role in resolving injury and illness by regulating inflammation, blood flow and the formation of blood clots (207). The production of prostaglandins is initiated by *cyclooxygenase* (COX) which induces the production of *thromboxane A*2 (TXA2) and prostaglandins, such as *prostaglandin* (*PG*)-*D*2, PG-I2, PG-E2 and PG-F2α (208). TNFα induces the expression of the prostaglandin endoperoxide synthase COX2 in a variety of cell types including ECs and VSMCs (186, 209, 210). PGs have also been found to induce diverse effects on VSMCs. For example, PG-D2 dictates the balance of VSMC proliferation and apoptosis and PG-E2 causes VSMCs to contract by inhibiting the potassium current (211, 212). PG-E2 also regulates VSMC tone through the *prostaglandin E* 1 (EP-1) and EP-3 receptors. Furthermore, EP-1 and EP-3 activation mediates intracellular Ca^{2+} pathway activation and the reduction of cAMP induced vasoconstriction (213). In sharp contrast, PG-E2 stimulation of EP-2 and EP-4 increases cAMP promoting vasodilation (214). Moreover, stimulating contractile VSMCs with PG-D2 activates the ERK pathway resulting in PG-D2 dependent VSMC phenotypic switching (215, 216). In CVD animal models, e.g. in *microsomal prostaglandin E*2 *synthase*-$1^{-/-}$ (*mPGES*$1^{-/-}$) *Ldlr*$^{-/-}$mice on a 3- and 6-month WD, PGES1-deficiency, resulting in reduced circulating PG-E2 levels and resulted inreduced plaque burden and foam cell accumulation without affecting the blood pressure (217). PG-D2 is also induced by TNFα and, in turn, propagates the dedifferentiation of contractile VSMCs into a synthetic phenotype via the upregulation of *proliferator-activated receptor* (PPAR) (218). Furthermore, the loss of TNFα or the inhibition of COX2 repressed the induction of intimal thickening in mice (218). The same study also found that TNFα stimulates the activity of COX2 and thereby enhances COX2 expression in contractile VSMCs, while inhibition of COX2 suppressed TNFα-induced contractile VSMC phenotypic switching and neointima formation (218). Taken together, PGs have a diverse role in vascular remodeling (**Figure 1**), from regulating vasoconstriction and vasodilation to VSMC proliferation and apoptosis. Yet, specifically in atherosclerosis PGs like PG-E2 can dictate lesion size (217) and PG-D2 plays an important role in the phenotypic switching of VSMCs to their pro-atherosclerotic synthetic phenotype (218). However, it is difficult to suggest PGs as a potential therapeutic target as COX2 selective inhibitors are associated with increased atherothrombotic risk (219). Therefore, further research is needed to better understand the role of PGs in vascular remodeling before developing specific treatment approaches.

Leukotrienes

Leukotrienes (LTs) are biologically active molecules produced by leukocytes, mastocytoma cells and macrophages in response to immunological and nonimmunological stimuli. LTs are well known as allergic, acute and chronic inflammatory mediators and are involved in several inflammatory conditions such as human arthritis, asthma, allograft rejection and atherosclerosis (220, 221). These inflammatory LTs originate from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism and, together with the 5-*LO-activating protein* (FLAP), catalyze the arachidonic acid metabolism from membrane phospholipids resulting in the formation of LT-A4, which is an unstable precursor leukotriene. 5-LO can further be metabolized into LT-B4 or form cysteinyl-leukotrienes like LT-C4, -D4 and -E4 after conjugation with glutathione (222, 223). Macrophages are the main producers of 5-LO and a correlation has been found between macrophage content of 5-LO and 5-LO localized in DCs, foams cells, mast cells and neutrophils and atherosclerotic plaque size in humans (224). In addition, clinical, population genetic, cell biological and mouse studies have all linked the 5-LO pathway to atherogenesis and arterial wall remodeling (223).

In atherosclerosis, LTs promote the migration and accumulation of inflammatory cells into the intima of the vascular wall resulting in the initiation and progression of the disease (**Figure 1**). In addition, the inflammatory response is enhanced by the activation of leukotriene B_4 receptors 1 (BLT_1) and 2 (BLT_2) as well as the *cysteinyl-leukotrienes receptors* (CysL)-T_1 and $CysLT_2$.These receptors are expressed on immune cells and vascular cells which are associated with atherogenesis such as ECs, VSMCs and monocytes/macrophages and their activation through leukotriene binding lead to structural alterations of the vascular wall and thus vascular remodeling (222, 223).

Direct effects of LTs on blood vessels include left ventricular contractility modifications, blood pressure regulation, coronary artery contraction and leukocyte recruitment into the perivascular space (225–228). *In vitro* studies revealed that LT treatment of ECs enhances the surface expression of P-selectin, secretion of von Willebrand factor and stimulates the synthesis of platelet activation factors and promotes VSMCs proliferation (229–231). LT-B4 is expressed on diverse cell types and for example neutrophils, eosinophils, VSMCs and monocytes all respond to LT-B4-dependent cell migration and recruitment to sites of inflammation (**Figure 1**) (220, 228, 232). In addition, the LT-B4-BLT1 pathway mediates VSMC recruitment and proliferation in the atherosclerotic plaque leading to intima hyperplasia as shown in rats treated with the BLT receptor antagonist BIIL 284 (10 mg/kg, once daily for 14 days) after balloon-induced injury of the carotid artery (233). Overall, it is clear that LT-B4 plays an important role in leukocyte attraction and adhesion to the vascular endothelium and the proliferation and migration of VSMCs (**Figure 1**) (231, 234).

As 5-LO is the regulator of the production of LT-B_4 and cysteinyl leukotrienes, it is an interesting therapeutic target. 5-LO inhibition with intake of 0,1% BHB-TZD (5-(3,5-di-tert-butyl-4-hydroxybenzylidene)thiazolidin-2,4-dione) mixed in food was described to prevent plaque progression in atherogenic $Ldlr^{-/-}$ mice fed a WD for 8 weeks (235). Moreover, 5-LO inhibition with licofelone in rabbits significantly decreased the femoral artery intima/media ratio as well as macrophage infiltration in the neointima, CCL2 expression and the activation of NFκB in the vascular lesion (236). Therefore, licofelone seems to diminish neointima formation following arterial wall injury and reduces inflammatory cell recruitment and adhesion into the arterial wall, thereby reducing atherosclerosis vascular remodeling.

Targeting LT receptors may represent an additional putative therapeutic target for the treatment of atherosclerosis and for preventing intimal hyperplasia after angioplasty. The *Carotid Atherosclerosis Progression Study* (CAPS) investigated 8 genetic polymorphisms associated with the leukotriene pathway and early atherosclerosis and remodeling in 969 patients. However, no significant effect of these polymorphisms was observed on atherosclerosis and remodeling risk based on carotid intima-media thickness (237). This result may be explained by the fact that most patients only showed signs of early atherosclerosis, while there was insufficient plaque advancement and stenosis to demonstrate associations with advanced atherosclerosis. Another randomly sampled cohort of 470 healthy, middle-aged women and men from the *Los Angeles Atherosclerosis Study* (LAAS) investigated the association between 5-LO gene promoter polymorphism, dietary arachidonic acid intake and the effect on atherosclerosis. An increase in intima-media thickness and atherosclerotic plaques could be observed in patients carrying two variant alleles of the 5-lipoxygenase compared with patients with the common allele. In addition, among the persons with the two variant alleles, the ones ingesting more arachidonic acid had significantly elevated intima-media thickness compared to patients with a marine n-3 fatty acid rich diet. These findings suggest a diet-gene interaction effect which impacts the development of atherosclerosis (238).

An ongoing clinical study (started in May 2020, estimated end in December 2023) is examining the role of a cyteinyl leukotriene antagonist in atherosclerosis (NCT04277702). They aim to study its effect on lower limb artery re-occlusion rate in 200 patients with peripheral artery disease after endovascular treatment. Results will further demonstrate whether LTs are a promising therapeutic option.

CONCLUSION

Taken together many mediators exert divers and, in some cases, even opposing functions in atherosclerotic vascular remodeling. Future studies are needed to demonstrate whether the effects of CCL2, CCL5, CCL19, CXCL12, CXCL16 and CX3CL1 on VSMCs like increased proliferation and subsequent increase of plaque stability but also induction of phenotypic switching balance out their more pro-atherogenic effects on leukocyte recruitment and lesional foam cell formation. Hence, while considering the therapeutic targeting potential of chemokines in general one should keep in mind that chemokine blocking could reduce plaque size at costs of reduced lesion stability. Still, some chemokines may be useful as biomarkers since augmented levels of CCL5 or CXCL12 correlate with the severity of atherosclerosis or CAD respectively.

Cytokines like IL1-β, IL-6, IL-22, TNFα and GDF-15 seem to particularly foster EC activation and drive early atherogenesis while the effects of IL-33, PGs and LTs are less clear. In addition, IL1-β and IL-22 are also involved in VSMC proliferation and phenotypic switching, but animal models also suggest that IL-1β is beneficial in an advanced stage of the disease by maintaining plaque stability. Caution is also warranted when blocking IL-6 and TNFα. Blocking of those two cytokines enhances total cholesterol burden, LDL, and triglyceride profiles despite improving endothelial function and reducing vascular remodeling. Therefore, to minimize the detrimental effects of elevated lipids, it is important to combine IL-6 and TNFα blocking with lipid lowering strategies like statin treatment. On the other hand, fostering beneficial effects of IL-10 for example to inhibit phenotypic switching of VSMCs could also be a promising therapeutic approach to delay vascular wall remodeling.

Overall, it is important keep in mind that tissue specific expression patterns, the applied model, the prescribed medication and deficiency vs. receptor blocking all differentially impact on well-orchestrated immune functions and, in addition,

time and spatial resolution significantly contribute to the results summarized above.

AUTHOR CONTRIBUTIONS

BE, AY, and YD performed literature research, drafted the manuscript, and made the figures. IB, SB, EV, and MS wrote the manuscript and provided corrections. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Identifying novel mechanisms of abdominal aortic aneurysm *via* unbiased proteomics and systems biology

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Background: Abdominal aortic aneurysm (AAA), characterized by a continued expansion of the aorta, leads to rupture if not surgically repaired. Mice aid the study of disease progression and its underlying mechanisms since sequential studies of aneurysm development are not feasible in humans. The present study used unbiased proteomics and systems biology to understand the molecular relationship between the mouse models of AAA and the human disease.

Methods and results: Aortic tissues of developing and established aneurysms produced by either angiotensin II (AngII) infusion in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice or intraluminal elastase incubation in wildtype C57BL/6J mice were examined. Aortas were dissected free and separated into eight anatomical segments for proteomics in comparison to their appropriate controls. High-dimensional proteome cluster analyses identified site-specific protein signatures in the suprarenal segment for AngII-infused mice (159 for *Apoe*$^{-/-}$ and 158 for *Ldlr*$^{-/-}$) and the infrarenal segment for elastase-incubated mice (173). Network analysis revealed a predominance of inflammatory and coagulation factors in developing aneurysms, and a predominance of fibrosis-related pathways in established aneurysms for both models. To further substantiate our discovery platform, proteomics was performed on human infrarenal aortic aneurysm tissues as well as aortic tissue collected from age-matched controls. Protein processing and inflammatory pathways, particularly neutrophil-associated inflammation, dominated the proteome of the human aneurysm abdominal tissue. Aneurysmal tissue from both mouse and human

had inflammation, coagulation, and protein processing signatures, but differed in the prevalence of neutrophil-associated pathways, and erythrocyte and oxidative stress-dominated networks in the human aneurysms.

Conclusions: Identifying changes unique to each mouse model will help to contextualize model-specific findings. Focusing on shared proteins between mouse experimental models or between mouse and human tissues may help to better understand the mechanisms for AAA and establish molecular bases for novel therapies.

KEYWORDS

inflammation, thrombosis, network analysis, angiotensin II, elastase, mouse model

Introduction

An aneurysm is characterized by a localized expansion of arterial wall, which may portend rupture (1). Abdominal aortic aneurysms (AAA) occur primarily between the renal arteries and the bifurcation of the iliac arteries (2), affecting up to 8% of men over the age of 65 (3). In addition to male sex, other risks for AAA include smoking, advanced age, previous myocardial infarction, and family history (4). Many AAAs remain asymptomatic and only incidentally undergo detection through abdominal imaging such as ultrasonography, computed tomography angiography, and magnetic resonance imaging (1). Despite many efforts to establish predictive or diagnostic imaging techniques (5), circulating biomarkers (6), or disease features such as intraluminal thrombus (ILT) (7) for indication of rupture-prone aneurysms, none have proven to be sufficiently robust to be implemented in clinical practice. No medical approaches to reducing AAA expansion have been validated, rather, once an aneurysm diameter has reached an established "threshold" [4.5–5.0 cm for women and 5.5 cm for men (8)], the only treatment option involves surgical repair. Better understanding the pathogenesis of AAA may provide a molecular basis for new targeted medical therapies for the prevention of AAA progression and rupture.

Studies of tissues collected from patients undergoing surgical repair have defined the pathological characteristics of AAAs, including loss of extracellular matrix (ECM) integrity, medial degeneration, and inflammation (9). Proteomics-based studies have explored further features of human AAA such as the contribution of the ILT (10), identifying differential markers within the ECM (11), or pursuing potential circulating biomarkers (12). While the reliance on mice to study this disease is convenient, the relevance or extent of overlap between AAA mice and the human condition remains unclear.

Among several types of experimental AAA in mice (13, 14), the most common are provoked by either angiotensin II (AngII) or elastase (15). AngII is infused using a subcutaneously implanted osmotic pump and produces suprarenal AAAs in $\leq$1 weeks (13). AngII–infusion is usually performed in hypercholesteremic *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice fed a high fat and cholesterol enriched "Western" diet (16). In the elastase model, either intraluminal elastase perfusion or topical elastase incubation in/on the infrarenal aorta results in immediate arterial dilation with cell death, elastin fragmentation, and inflammation driving further diameter expansion through 2 weeks after surgery (14). Each model recapitulates selected features of human AAA (e.g., inflammation, ECM destruction, luminal dilation, intact intima, and rupture-induced death), but neither covers the full spectrum of pathology. While the AngII model in *Apoe*$^{-/-}$ mice more closely mirrors the human disease in its inclusion of hypercholesterolemia, a relatively chronic disease development, and the heterogeneity of aneurysms between mice, its mortality rate reaches higher (nearly 40%) than in humans (17) and the thrombus occurs extraluminally due to medial rupture resulting in adventitial dissection (17). In the elastase model, surgical intervention and manipulation drives initial inflammation and aortic dilation producing consistent and uniform aneurysms without requirement of genetic alteration. However, the elastase model relies on experienced microsurgical skills and the administration of a foreign substance (i.e., porcine elastase).

Prior studies have explored mechanisms of aneurysm expansion by examination of temporal changes in tissue pathology (18, 19), or similarities between mouse and human tissues (20). The present study uses a systems approach, involving unbiased mass spectrometry-enabled proteomics, high-dimensional bioinformatics, and systems biology, to complete proteome profiling of the three most commonly used mouse models of AAA: systemic AngII infusion into *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice and localized elastase perfusion into wildtype mice. The initial goal involved identifying both commonality and disparity amongst these three models. To further substantiate such an approach in mice, we performed proteomics in a set of human AAAs which may unify the human condition with each model *via* their common and potentially critical molecular signatures. Ultimately, this study aims to

better understand mechanisms that drive AAA, providing molecular bases for new therapies.

Methods

The Online Data Supplement contains additional detailed methods. Male *Apoe*$^{-/-}$ ($n = 12$) or *Ldlr*$^{-/-}$ ($n = 13$) mice in a C57BL/6J background at 10–12 weeks of age, underwent infusion of AngII *via* a subcutaneously placed osmotic minipump (13). Male wildtype C57BL/6J mice (10–12 weeks of age, $n = 10$) underwent experimental AAA creation with the luminal infusion of porcine pancreatic elastase (14). The aorta was cut into 8 segments (segment 1, aortic arch; segments 2–4, thoracic; segment 5, suprarenal; segment 6 corresponded with renal vessels; segments 7 and 8, infrarenal). Human AAA tissue was obtained from nine patients (4 female and 5 male) aged 65 ± 7.7 years undergoing open aneurysm repair at the University of Rochester Medical Center. The patient criteria for open surgical repair were defined as AAA diameter exceeding 55 mm for males, AAA diameters between 50 and 55 mm for females, rapidly expanding aortic diameters (≥5 mm in 6 months with a minimum diameter of 40 mm) or symptoms attributable to AAA and AAA rupture. Mural thrombi were collected during surgery, along with residual aortic wall fragments, and were immediately flash frozen until protein processing. Control tissue was procured from the infrarenal aortas (5 males and 5 females) of deceased individuals aged 58 ± 6.6 years and rapidly frozen for analysis. Mouse and human tissue was proteolyzed using either a chloroform:methanol extraction strategy or the standard protocol provided by the PreOmics iST Kit. Peptide samples were analyzed with the LTQ-Orbitrap mass spectrometer or the Orbitrap Fusion Lumos mass spectrometer, each coupled to an Easy-nLC1000 HPLC pump (Thermo Fisher Scientific). Proteins were identified and quantified using Proteome Discoverer (v2.1, Thermo Fisher Scientific). Proteomics data were analyzed further using the statistical software suite, Qlucore (version 3.3, http://www.qlucore.com/), XINA (21) and the protein-protein interaction network (Supplementary material).

Results

Mouse study rationale and design

The primary aim of the present study involved identifying and comparing proteins and molecular pathways defining the two most commonly used mouse AAA models: AngII infusion into *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ male mice, and the localized, luminal perfusion with elastase into the infrarenal aorta of male wildtype mice (Figure 1A). To capture a proteome correlated with a "developing" aneurysm as well as one that correlated with an "established" aneurysm, we chose two time-intervals for aortic tissue harvest: 2 and 4 weeks after initiation of AngII infusion, and 4 days and 2 weeks after elastase exposure (Figure 1B). The extent of aortic expansion was determined by ultrasound-based measurements of aortic diameter (Figure 1C; Supplementary Table 1), depicted as percent increase at each time interval (Figure 1D). Aortic diameter dilation of >50% (relative to baseline prior to aneurysm induction) was considered aneurysmal. Compared to elastase, expansions due to AngII infusion demonstrated greater variability (Figure 1D), consistent with previous reports (16, 22).

Histopathology demonstrated adventitial thickening, cellular infiltrate, and elastin layer tears or breaks common to both two models (Figures 1E,F). The difference between diameter of representative aortas from saline-infused from each model can be attributed to the suprarenal or infrarenal locality of the AngII and elastase models, respectively.

Global proteomic profiling of mouse AAAs

To capture the spatiotemporal protein expression patterns within and across experimental AAAs, we performed label-free proteomic profiling of the arch, thoracic, suprarenal, and infrarenal segments of the aorta (Figure 2A). The proteomics data reflected the variation in the aneurysmal expansion between and within the AAA models (Supplementary Figure 1; all mice studied). This variability, particularly notable in AngII-infused mice, could pose problems if the proteomes of mice with an aortic expansion < 50% would dilute the aneurysm-specific variation in the proteome. We therefore selected a subset of mice from each group that would specifically capture the aneurysm-specific proteome, prioritizing the final analyses on data acquired from three mice, per procedure, with the most severe aneurysm pathology (Figure 2B) for subsequent analyses.

Proteome profiling revealed distinct aortic molecular patterns

A total of 2,477 proteins from all three AAA groups and appropriate controls were quantified (Supplementary Table 2). A Venn diagram demonstrated the interconnectivity of these models (1,913 proteins were shared amongst all three groups), as well as the unique features resulting from the specific aneurysm model and/or the genetic background of each group (Figure 2C). Hierarchical clustering of protein abundances revealed three major patterns (Figure 2D). Pattern 1, a baseline pattern, contains predominately proteins associated with AngII model controls, or segments of artery not directly affected by surgery (i.e., artery segments 1 through 6) in the elastase model. Pattern 2, a general pathology pattern, contains proteins whose expression coincides with aneurysm locality within each model

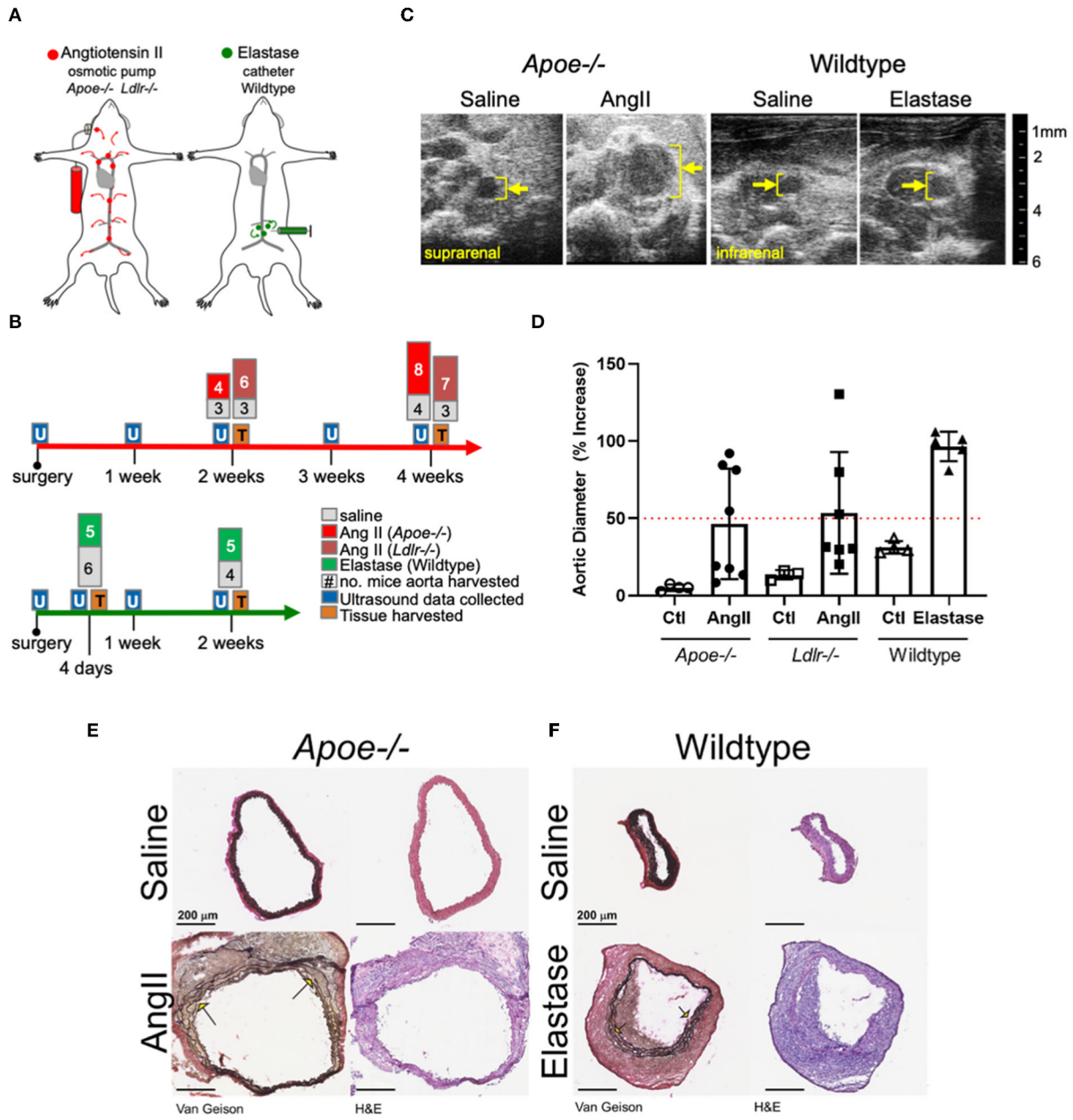

FIGURE 1
Study design and abdominal aortic aneurysm model phenotypes. **(A)** AngII vs. elastase administration in mice. AngII was infused subcutaneously using an osmotic pump; elastase was administered directly into the infrarenal aortic lumen using a catheter. **(B)** Timeline and experimental design detailing collection of ultrasound data and tissues for proteomics. The number of aortas collected are indicated at tissue collection time points. **(C)** Representative cross-sectional ultrasound images for final time point of each model. Yellow bracket indicates luminal area and expansion. **(D)** Graph depicting percent increase in aortic diameter over baseline ultrasound measurements in each model. *Apoe*$^{-/-}$ represented as circles, *Ldlr*$^{-/-}$ by squares, and elastase wildtype by triangles, with saline controls represented by empty shapes respective to group; red dotted line indicates AAA (50% increase over baseline). **(E,F)** Van Gieson (collagen and elastin) and H&E (nuclear and cytosol) staining of the suprarenal and smaller infrarenal segments of *Apoe*$^{-/-}$ and wildtype aortas, respectively. Arrows identify elastin breaks.

(suprarenal segments in the AngII groups, infrarenal segments in the elastase model); and Pattern 3 is populated by proteins with increased expression in the arch segments of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ aneurysmal mice.

The principal component analyses (PCA, Figure 2E) further delineated the intra-group sources of variability. Segments of the suprarenal and arch aorta revealed the greatest variation in the *Apoe*$^{-/-}$ group, likely due to the

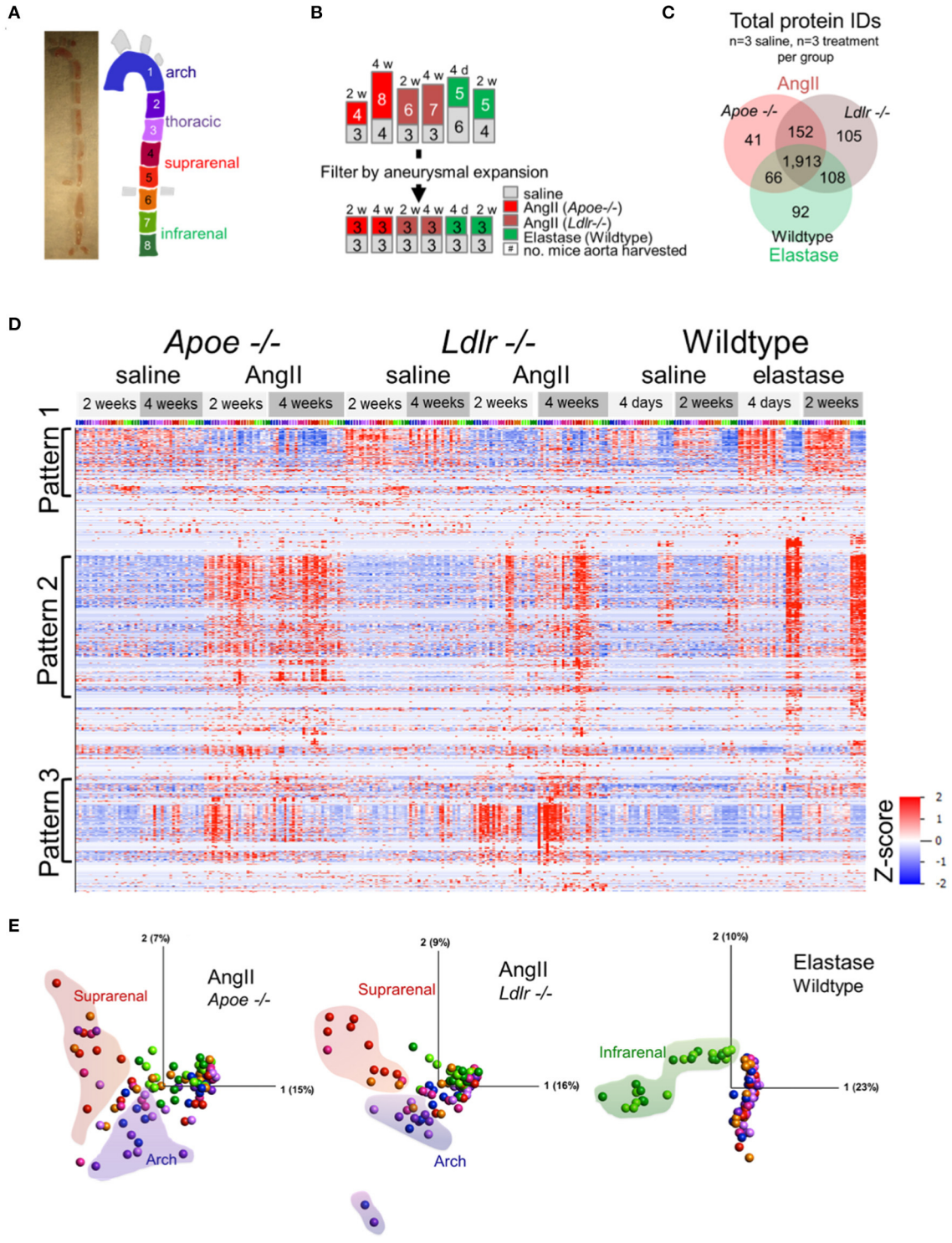

FIGURE 2
Proteomic profiling of three AAA models. **(A)** Gross image of artery and segmentation method, and cartoon depiction of segments with corresponding numbers and colors. **(B)** Representation of the number of mice included in proteomic analysis (top set of numbers) and filtration of this group by aneurysm severity (bottom set). **(C)** Venn diagrams for the number of proteins, increased or decreased, identified across the three animal models. **(D)** Hierarchical cluster analyses (by protein, rows) showed their relative abundances across models (grouped at top) and segments [colored across top correlated to coloration in **(A)**]. **(E)** Principal component analysis representing protein expression for each artery fragment from each individual model, including saline controls. Coloration of the fractions follows cartoon in **(A)** with shaded outlines indicating general grouping of arterial segments on the plot.

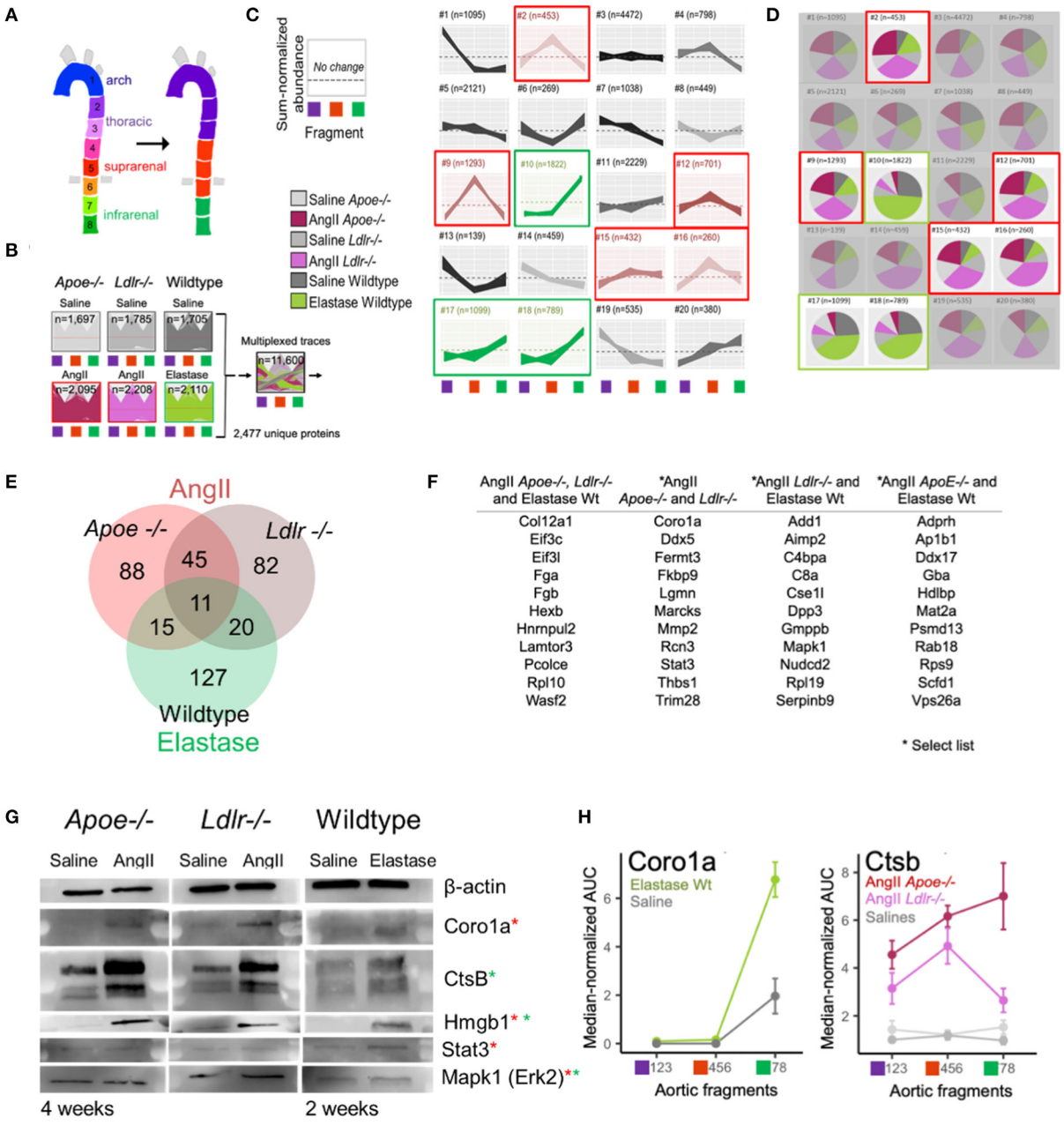

FIGURE 3
High-dimensional cluster analyses identified AAA model proteins. **(A)** Original eight arterial fragments re-colored to represent three major segment groups for subsequent clustering. **(B)** Protein signatures from all three AAA models were combined to generate a single cluster depicting the variance from the combined protein data. **(C)** Protein abundance clusters showing sum-normalized quantified data on the y-axis and arterial segment group [colored according to scheme in panel **(A)**] on the x-axis. Selected expression patterns colored red (AngII) or green (elastase) according to model. **(D)** Composition pie charts depict source (i.e., model) from which each cluster derives its constituent proteins. Gray shades represent saline groups, dark (*Apoe*$^{-/-}$) and light (*Ldlr*$^{-/-}$) pink represent AngII model, and green represents the elastase-perfused mice. Red (AngII) and green (elastase) outlines mirror highlighted clusters in **(B)**. **(E)** Venn diagram shows the number of proteins identified as being correlated significantly with aneurysm development in each model. **(F)** A select set of proteins unique to one model or shared across two or more models are listed. **(G)** Western blots of candidate aneurysmal proteins. Red and green asterisks indicate from which model(s) the proteins were enriched. **(H)** Coro1a and Ctsb proteins were omitted from elastase and AngII dataset filtering, respectively, due to strict filtering criteria, but still appear to reflect aneurysmal signatures.

aneurysmal and atherosclerotic plaque features, respectively. Several points representing the arch and suprarenal segments remain intermingled, possibly demonstrating the interference of atherosclerotic and aneurysmal features in these mice, even in the absence of a "Western" diet (Figure 2E). The *Ldlr*$^{-/-}$ plot, on the other hand, showed the fewer intermingled suprarenal and arch segment points, possibly due to the expected absence of atherosclerotic plaques in these mice in the absence of a "Western" diet (23). In complete contrast, and as expected for the elastase group in wildtype mice, infrarenal fractions cluster closely together and provide the most notable variation (Figure 2E).

High-dimensional model-based clustering for spatial resolution of aneurysmal proteins

The PCA (Figure 2E) revealed that the protein abundance patterns of the eight segments can be simplified into three arterial segments of interest: arch (segments 1–3), suprarenal/thoracic (4–6), and infrarenal (7 and 8) (Figure 3A). Subsequent, in-depth analyses of the data was performed using these data-identified arterial segments. The multi-dimensional nature of our dataset, including model, treatment, time interval, and aortic section, required careful consideration to identify proteins contributing to aneurysm development and progression. Thus, we turned to a high dimensional dataset clustering strategy that combined protein abundance profiles from multiple datasets into a single dataset for clustering (21) (Figure 3B). The single 20-cluster output, comprising a combined 11,600 protein profiles pertaining to 2,477 unique proteins, depicted the variation across all six groups—the three aneurysms and their saline control counterparts (Figures 3B,C). Abundance profiles expected from aneurysm-specific responses for the AngII model (suprarenal, red-highlighted clusters) or the elastase model (infrarenal, green-highlighted clusters) were selected (Figure 3C). The pie charts depict group compositions for each cluster, with red-highlighted, red/pink dominated pie charts demonstrating a dominance of proteins from the AngII groups whereas green-highlighted pie charts show a dominance of proteins from the elastase group (green shades; Figure 3D).

Proteins from the five AngII- and three elastase- dominated clusters were further filtered to identify those with a profile unique to the aneurysm, i.e., the counterpart saline profile did not fall within any of the selected cluster patterns. These filtered protein lists were refined by identifying proteins with a fold change ≥ 2 or ≤ 0.5 as compared to their localized saline controls, p-value ≤ 0.05, and false discovery rate (FDR) ≤ 0.05. This high dimensional clustering and filtering step reduced the proteins of interest to include 159 and 158 proteins in AngII-infused *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, respectively, and 173 proteins in the elastase mouse group (Figure 3E; Supplementary Table 3). The elastase model contains 127 unique proteins and shares 31 and 26 proteins with AngII-infused *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice, respectively; the two groups in the AngII model share 56 common proteins.

The list of eleven proteins shared across all groups includes three proteins of particular interest: regulator complex protein Lamtor 3 (LAMTOR3), procollagen C-endopeptidase enhancer 1 (PCOLCE), and collagen alpha-1 (XII) chain (COL12A) (Figure 3F; Supplementary Table 3). LAMTOR3 participates in mitogen activated kinase 1 (MAPK1/ERK2) signaling (24, 25), a protein studied previously in aneurysm (26, 27) and enriched in AngII *Ldlr*$^{-/-}$ and wildtype elastase aneurysms. PCOLCE interacts with THBS1 (28) and MMPs such as MMP2 (29), two proteins identified in both AngII groups and implicated previously in mouse aneurysm models as well as human aneurysms (26, 27, 30–33). In addition to PCOLCE, the presence of COL12A in all three aneurysm groups indicates a role for fibrosis and/or ECM interactions (34). Other proteins listed in Figure 3F include those with relevance to the networks shown further below. A full list of filtered aneurysmal proteins is presented in Supplementary Table 3.

Western blot analyses and tissue immunostaining validated select proteins (Supplementary Table 4). For the later interval in each model, we readily detected and verified an increase in signal for CORO1A (AngII-filtered protein), CTSB (elastase-filtered) and HMGB1 (AngII and elastase-commonly filtered) across all three groups (Figure 3G). Although the presence of STAT3 and MAPK1 could be confirmed in each model using Western blot analysis, the expression was not increased in aneurysmal tissues as compared to control (Figure 3G). While immunostaining revealed FERMT3/KINDLIN3 to be increased moderately in AngII *Apoe*$^{-/-}$ tissues as compared to control, the difference did not reach significance (Supplementary Figure 2).

Due to the multi-step, stringent filtration process chosen for this proteomics dataset, there are proteins ultimately listed as associated with only one AAA model (e.g., CORO1A in AngII, and CTSB in elastase), which are in actuality increased in the respective aneurysm region for both models. CORO1A increased in elastase-administered tissues but was excluded from the elastase model proteome because corresponding saline-perfused tissues exhibited a similar expression pattern (Figure 3H). AngII-infused *Apoe*$^{-/-}$ mice excluded CTSB due to its elevated expression in both supra- and infra-renal segments (Cluster #11 in Figure 3C). This retrospective examination of the proteomes reflected the pros and cons of any given protein filtering strategy.

Temporal trends in the aneurysmal proteomes differed within and between AAA models

Our study aimed to monitor the proteomes of early (developing) and late (established) aneurysms, providing a

means to address a potential mechanism and investigate factors in disease progression. Considering each model separately, Venn diagrams provided an overview of aneurysm-related proteins in early and late time intervals (Figure 4A). With the aim to visualize potential relationships across different AAA proteomes and identify the molecular mediators between early and late intervals, we built subnetworks for each model by mapping the temporally labeled (early, late, or both) proteins on a fixed coordinate system on the protein-protein interaction (PPI) network (Figures 4B–D). The PPI network was built from human interaction databases, and thus the human orthologs for proteins are indicated in the subnetworks. Inspecting the connections within each model's subnetwork revealed the following trends: (1) the largest PPI subnetwork for AngII mice comprised ribosomal proteins that primarily associate with the early interval for *Ldlr*$^{-/-}$ and the late interval for *Apoe*$^{-/-}$ (Figures 4B,C); and (2) the largest PPI subnetwork for the elastase model comprises a distinct group of ribosomal proteins that remain connected to EGFR and MAPK subnetworks primarily associated with the late interval (Figure 4D). This MAPK node connected to LAMTOR3 and WASF2, two proteins identified in all three aneurysm groups. These proteins were associated with the late stage/established AAA in AngII *Ldlr*$^{-/-}$, but with early/developing, late, or both stages for AngII *Apoe*$^{-/-}$ and elastase-perfused mice. Other proteins that were relatively close to the MAPK node include THBS1, MMP2 in both AngII groups, and PCOLCE in all three groups (Figures 4B–D). Particularly in the context of the integrins found in proximity to this subnetwork in the late stage AngII groups (ITGAM/CD11b, ITGB2/CD18, ITGA2B, ITGB3) (35–37), THBS1 (30, 38) and WASF2 (39) may have contributed to adhesion and transmigration activities. The overall topology of the fixed coordinate PPI confirmed, as expected, that the AngII *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ models share more similarities to each other than to the elastase model; but a closer look at subnetworks demonstrated that peak expression times vary across all three groups.

A representative subset of significantly enriched pathways derived from AAA proteomes at early and late time intervals of each group are presented in a heatmap (Figure 4E; Supplementary Figure 3 for the complete list of pathways). Signaling pathways related to translation and transcription, inflammation and inflammatory cell activities, and coagulation pathways were associated with all three groups, primarily the early and late interval proteomes of both AngII models, and the late interval proteome of the elastase model (Figure 4E). The early interval proteome for the elastase model uniquely associates with apoptosis-related pathways and phagocytosis (Fc gamma R-mediated phagocytosis), while oxidative stress (ROS, RNS production in phagocytes) is identified in the early proteome of the AngII *Ldlr*$^{-/-}$ group. The early and late interval proteome of the AngII *Apoe*$^{-/-}$ group share several features including cell damage and apoptosis (apoptosis-induced DNA fragmentation, phagosome), and features that may indicate endoplasmic reticulum (ER) stress (retrograde and anterograde transport, ER associated degradation, and cargo concentration in the ER) (Supplementary Figure 3; Figure 4E). The late interval proteomes of the AngII model shared pathways related to ECM and collagen formation and leukocyte infiltration, while the late interval of the elastase model included pathways of nuclear factor kappa B (NF-κB)-driven inflammation and shared pathways of vasculogenesis (vascular endothelial growth factor, VEGF), cell proliferation, and mitogen-activated protein kinase (MAPK) signaling with the late interval AngII *Ldlr*$^{-/-}$ proteome (Figure 4E).

Thrombus did not bias aneurysmal proteome data

The proteomic signal from the thrombus could potentially supersede that of the arterial wall at the site of aneurysm expansion. We used laser capture microdissection (LCM) to separate the thrombus from the surrounding arterial wall for proteomics (Supplementary Figure 4; Supplementary Table 5). From six *Apoe*$^{-/-}$ and three *Ldlr*$^{-/-}$ aneurysm LCM samples, we quantified a total of 2,311 proteins. A two-group comparison between the arterial wall and thrombus proteomes was performed for each genotype (Supplementary Figure 4; Supplementary Table 5). Most differentiating proteins within each group were increased in the arterial wall. Since the wall and thrombus proteomes largely overlap, the thrombus itself did not account for the differences between aneurysm and control tissues.

Human aneurysmal tissues provided limited but distinct proteome signatures

To further substantiate the clinical relevance of the mouse models, we performed a proteomics study on human diseased tissues. Nine human infrarenal AAA samples were acquired, and eight were separated into thrombus and AAA tissues, leaving one sample with no distinct thrombus as an intact sample. Non-aneurysmal, infrarenal aortic tissues from 10 autopsy cases served as controls (Figure 5A; Supplementary Table 6). A total 2,485 proteins were quantified, however, initial analyses showed control tissues to contribute 43 unique proteins as compared to 6 from AAA tissues and none from thrombus tissues (Figure 5B).

Hierarchical clustering of the protein abundances yielded three major clusters that showed not only a marked difference between the control and aneurysmal tissues, but also a subdivision within the aneurysmal aortic wall samples (Figure 5C). Specifically, five AAA samples clustered closer to the thrombus samples, hence designated thrombus-like

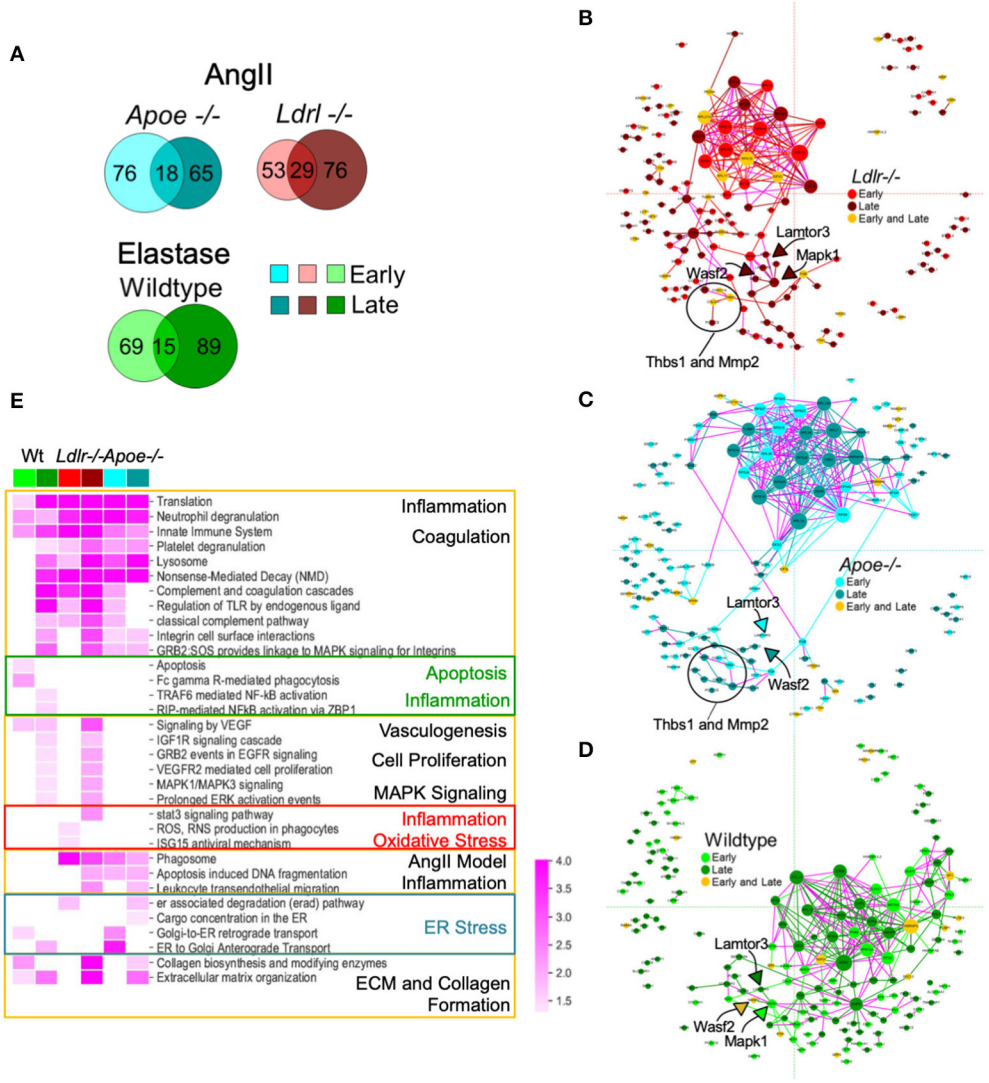

FIGURE 4
Protein-protein interaction (PPI) networks associated with aneurysmal tissue in each AAA group and model. **(A)** Venn diagrams showing aneurysm-associated proteins group and interval. **(B–D)** A fixed coordinate PPI network depicting described interactions among each AAA model's aneurysm-enriched proteins. When superimposed, proteins shared across the networks occupy the same coordinates. Links connecting early and late interval proteins are represented in purple. Node size is proportional to degree (i.e., the number of connections). **(E)** Heatmap showing the enriched pathways (FDR < 0.05) of each mouse model and time interval (early or late). A darker shade indicates higher enrichment while empty cells indicate non-significant (FDR > 0.05) enrichment. Pathways with similar enrichment values are clustered together. The color scheme follows panels **(B–D)**.

AAA (T-AAA), whereas the remaining four aneurysmal aortic wall samples formed a distinct cluster and were designated non-thrombus AAA (nT-AAA, Figure 5D). The heatmap also demonstrated that, when compared to the control tissues, aneurysmal tissues contain a low abundance of most detected proteins, likely reflecting the ECM breakdown, medial degeneration, and acellular composition of these tissues. Only a few proteins comprised the tissue-specific proteome signal, defined primarily by Cluster 1 for nT-AAA samples and Cluster 3 for T-AAA and thrombus samples (Figure 5C). The full list of the protein abundances within each tissue type is represented in Supplementary Table 6.

Since the protein distribution signal varied greatly between control and aneurysmal tissues, we instead compared tissues using a percentile ranking of their averaged protein abundances. Each protein is plotted according to relative abundance within each tissue type, and each plot divided to five percentile ranks (pentiles) in order to identify proteins moving from lower percentile ranks in control tissues to higher percentile ranks in the aneurysmal tissues (Figure 5E). We mapped the locations

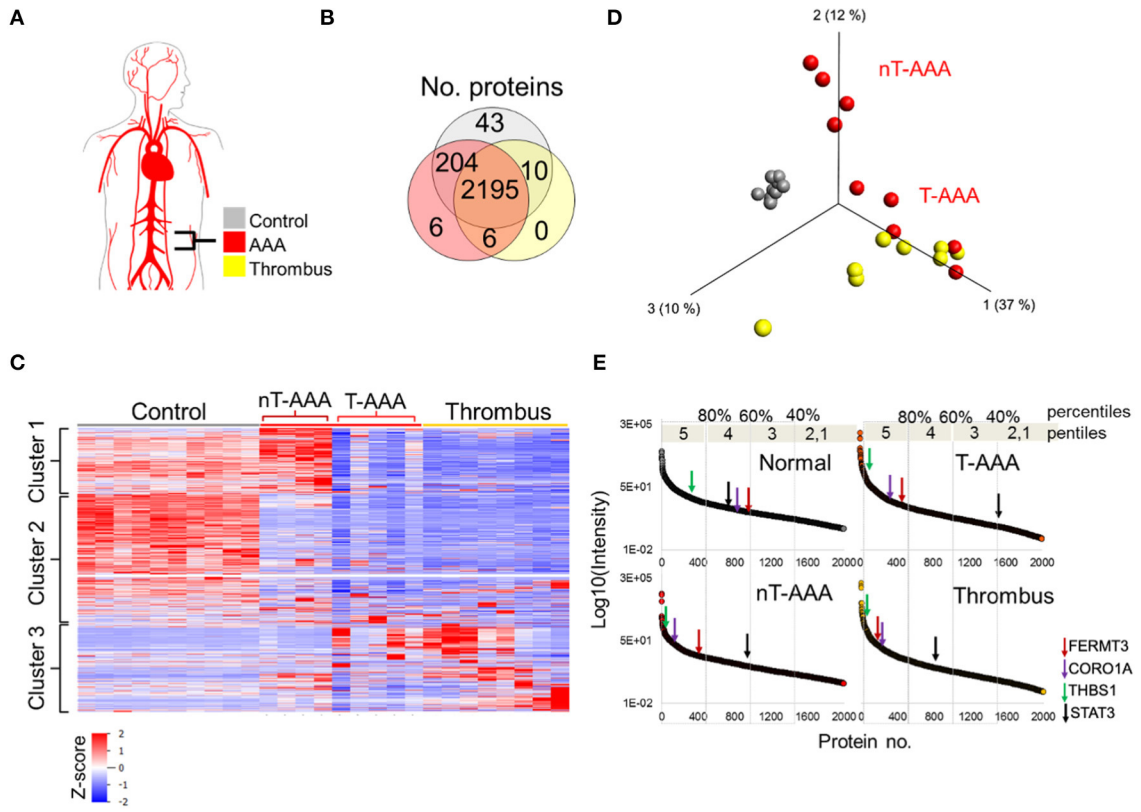

FIGURE 5
Human aortic aneurysmal tissue proteomics. **(A)** Schematic of aneurysm tissue samples and sample color scheme. **(B)** Venn diagram comparing the number of proteins identified across the three tissue types. **(C)** Hierarchical cluster analysis showing the relative abundances of proteins across the human tissue samples. **(D)** PCA depicting that control samples exhibit a unique proteome whereas some aneurysmal tissues cluster closer to the thrombus samples (T-AAA), but others do not (nT-AAA). **(E)** Protein abundances are ranked from high to low for each tissue sample in order to compare the relative change in abundance of proteins across the samples. The changes in abundance for four selected proteins are shown using colored arrows along each abundance curve.

of some proteins of interest as identified from the animal models such as FERMT3/KINDLIN3, CORO1A, THBS1, and STAT3, and observed interesting trends: FERMT3/KINDLIN3, CORO1A, and THBS1 move up in abundance rank between control and each aneurysmal tissue, with FERMT3/KINDLIN3 and CORO1A moving from the 60th to 80th percentiles, and THBS1 remaining in the upper most pencentile range (Figure 5E). STAT3, on the other hand, moved to lower abundance ranks in all three aneurysmal tissues, dropping to as low as the 40th percentile in the T-AAA tissues.

Murine AAA models exhibited overlapping and unique features compared to the human disease network

The increase in abundance rank for FERMT3/KINDLIN3 and CORO1A led to the examination of other proteins exhibiting a similar behavior (Supplementary Table 7). These analyses resulted in 122 (nT-AAA), 160 (T-AAA) and 192 (thrombus) proteins that were mapped to the PPI network (Figure 6). Larger font and arrows are used to highlight several topological features, proteins, and/or subnetworks shared among the three networks. For example, a subnetwork of proteins related to translation and transcription predominates the nT-AAA network (Figure 6A) but remained almost completely absent from the T-AAA and thrombus networks (Figures 6B,C). The latter two networks, while generally comprising a complementary topology, contain notable attributes. Specifically, the thrombus network contained a subnetwork populated by proteasome-related proteins such as proteasome subunit alpha 1 (PSMA1), 4 (PSMA4), 6 (PSMA6), and 7 (PSMA7), which further extends to both an erythrocyte subnetwork and a platelet subnetwork, including scavenger receptor CD36, platelet glycoproteins GP1BB and GP9, von Willebrand factor (VWF) (Figure 6C). The erythrocyte subnetwork, including erythrocyte membrane protein band 4.2

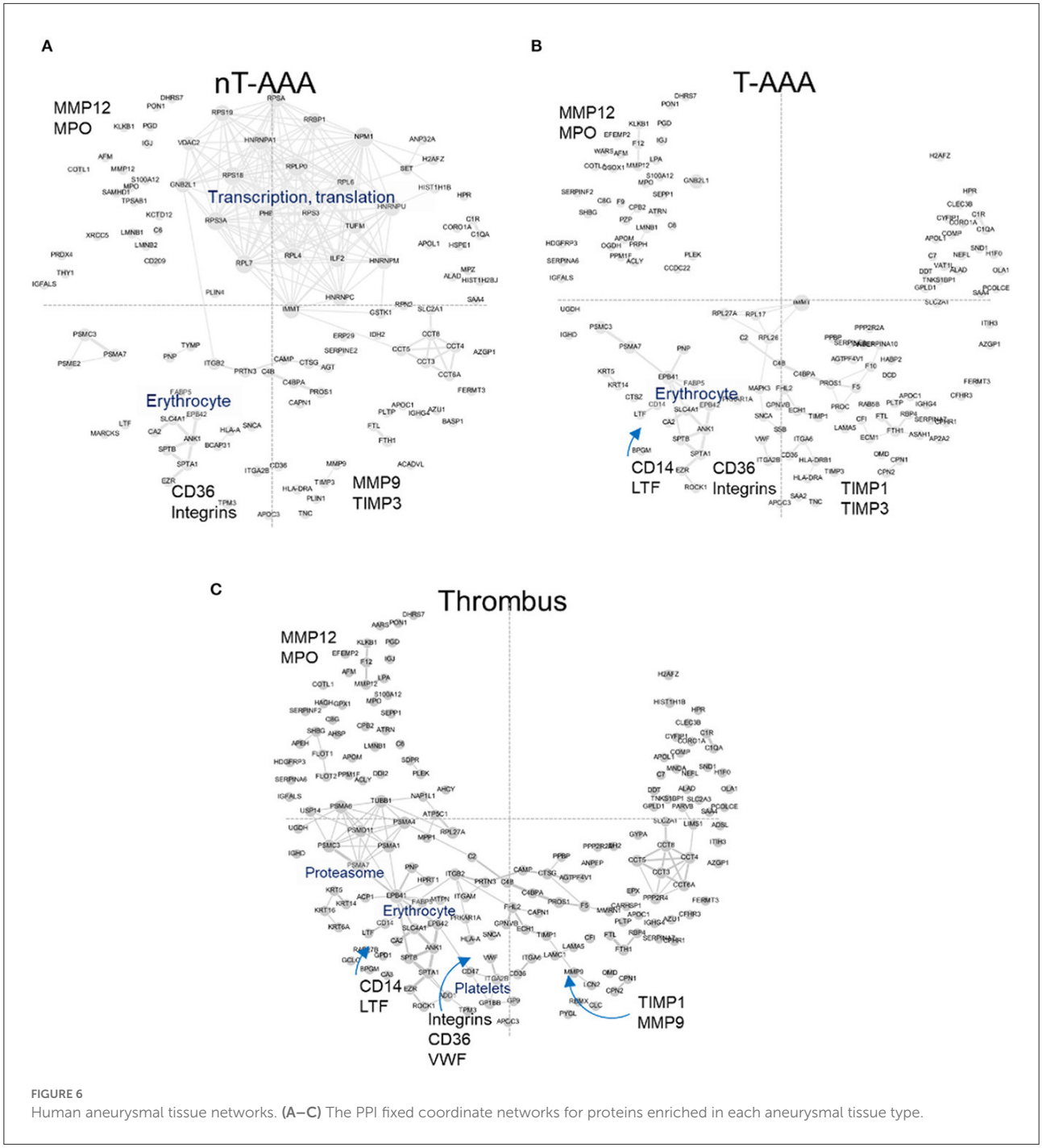

FIGURE 6
Human aneurysmal tissue networks. **(A–C)** The PPI fixed coordinate networks for proteins enriched in each aneurysmal tissue type.

(EPB42) and/or protein 4.1 (EPB41), spectrins beta and alpha 1 (SPTB and SPTA1), and ankyrin-1 (ANK1) is present in each the T-AAA, nT-AAA, and thrombus network (Figures 6A,B). The interactions between this erythrocyte subnetwork and the proteasome and platelet subnetworks specifically in the thrombus PPI network could reflect inflammation and oxidative stress contributed by a thrombus (7, 10, 40, 41). Proteins shared among the three networks include matrix metalloproteinases MMP9 and MMP12, the metalloproteinase inhibitors TIMP1 and TIMP3, and myeloperoxidase (MPO) are identified in at least two the networks, and each protein has been identified previously in at least one proteomics-based aneurysm study (11). In addition, integrins including ITGB2 (CD18), ITGAM (CD11b), and ITGA2B are identified, supporting a role for leukocyte infiltration within aneurysmal tissues (35, 37, 42). The monocyte/macrophage marker CD14 is linked to LTF

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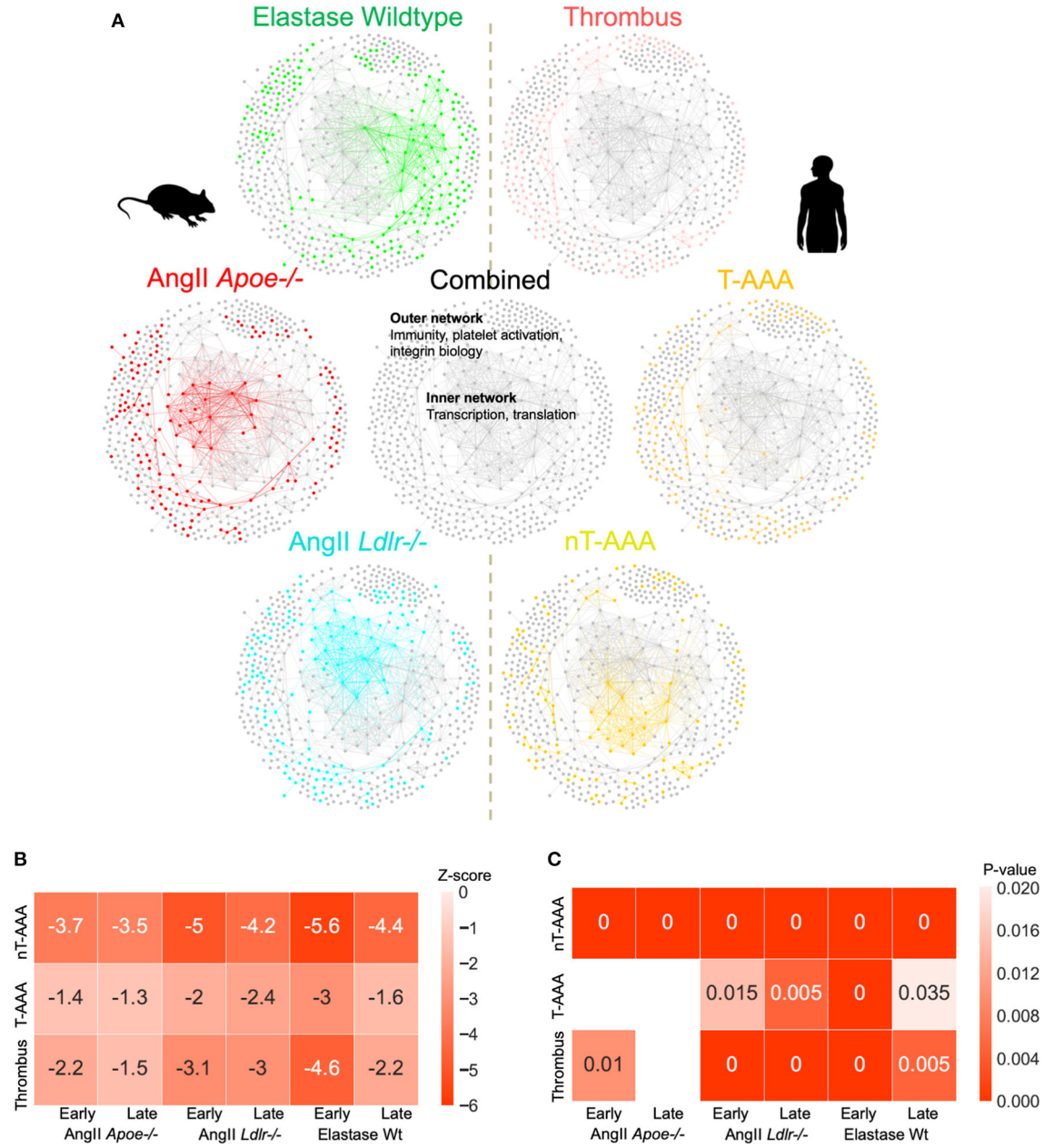

FIGURE 7
AAA animal model and human tissue consensus network. **(A)** The contribution of each proteome to the combined/consensus network. **(B)** Z-scores of the network closeness between human tissue subnetworks and mouse model subnetworks. A lower z-score (higher absolute values) indicates a higher closeness between the respective pair of subnetworks, suggesting a higher molecular association in the interactome. **(C)** Empirical *p*-values based on 200 randomizations. Heatmap cells annotated as "0" indicate a *p*-value < 0.005. Empty cells indicate insignificant ($p > 0.05$) associations. Z-scores and *p*-values are derived from large numbers of randomized subnetwork instances that were constructed using degree-preserving randomization.

(lactoferrin) that has been shown to have inflammatory effects on macrophages (43–45).

We next combined all three sets of mouse and human aneurysmal proteomes, and created a combined consensus network (Figure 7; Supplementary Figure 5; Supplementary Table 8). The combined network contains a highly interconnected, central subnetwork comprised largely of ribosomal proteins and other transcription/translation-related proteins, and an outer subnetwork that comprises an extensive network of immunity, platelet activation, and integrin biology-related proteins (Figure 7). The proteome of the three mouse model datasets and the human nT-AAA contributed

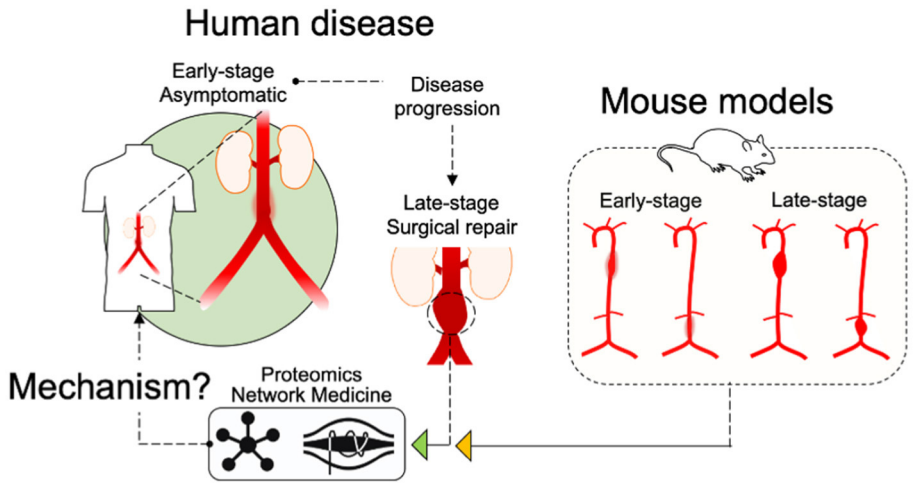

FIGURE 8
Schematic of study design. An illustration of the study design indicates that "Early-stage" and "Late-stage" tissues from the mouse models, as well as "Late-Stage Surgical repair" human tissues were assessed by Proteomics and Network Medicine to search out common mechanisms.

unique and overlapping nodes to the central subnetwork, whereas no contribution by human thrombus and T-AAA proteomes occurs (Figure 7; Supplementary Figures 5E–G). A more general assessment of this combined network leads to the following initial impressions: (1) a prominent deviation between the elastase model and any of the other networks due to its largest subnetwork localized to the right side of the network (Figure 4D); and (2) the consistent presence of inflammation and leukocyte migration-related pathways throughout all six networks.

To assess the degree of association between human tissue and mouse model subnetworks at the molecular level, we performed network closeness analyses (Figures 7B,C). Both analyses depict the highest level of similarity between all three aneurysm model groups and the nT-AAA human tissue type, likely attributed in part to the central transcription/translation subnetwork. In contrast to the human thrombus tissue, which has relatively high overlap with nearly all mouse groups, the disparity between T-AAA and all three mouse groups indicates this tissue type to be relatively unique to the human disease.

Discussion

The use of murine AAA models in research studies has several purposes, including the potential to examine elements of disease development over time. Our study design includes the consideration of temporal features for each group aiming to capture developing and late-stage aneurysmal molecular features. As a consequence, the current study reveals how these models progress and, when considered in context of the human tissue proteome(s), how their progression relates to the human disease (Figure 8). A comparison of "early" and "late" tissue proteomes reveals several consistent elements of diseased tissue in all time intervals and aneurysm groups including translation, inflammation, platelet activity and coagulation, and MAPK signaling. The differences between time intervals provide valuable insight to the progression of each model. Early time interval pathways include indications of apoptosis and phagocytosis (elastase *wildtype* and AngII-infused *Apoe*$^{-/-}$) and oxidative stress (AngII-infused *Ldlr*$^{-/-}$). The late time interval proteome is populated by pro-fibrotic and leukocyte infiltration pathways (AngII model), angiogenesis, cell proliferation, and MAPK signaling (elastase wildtype and AngII *Ldlr*$^{-/-}$), and NF-κB-mediated inflammation (elastase wildtype). Interestingly, both the early and the late proteome of the AngII model include pathways potentially related to ER stress, a feature shown to influence aneurysm (46, 47). The late proteome of each model appears at least partially influenced by injury response(s) to tissue damage at the early stages of aneurysm development. Notably, continued AngII infusion past 28 days can lead to rupture (48), whereas elastase-induced aneurysm expansion ceases by about 14 days without a secondary treatment (49). Continued expansion in this modified elastase model is attributed to chronic inflammation, highlighting a role for leukocyte infiltration (identified in late-interval AngII model proteomes) as a driver of continued expansion in AngII aneurysms (48).

The choice to include only male mice in this study was made primarily to adhere to convention of the field with the hope of producing a dataset applicable to the largest number of studies. Existing evidence suggests that aneurysms produced in female mice are significantly different from those produced in male mice (50). Future studies may further identify the

precise differences between the sexes in the context of these mouse models.

Comparison between the human and mouse aneurysmal proteomes in the next stage of this study permitted us to explore the extent of molecular conservation between the human and mouse aneurysmal tissues. The PPI networks of the human aneurysmal tissues indicated many platelet- and acute inflammatory phase-related proteins for the T-AAA tissues; whereas the nT-AAA tissue network was defined by a prevalent subnetwork related primarily to transcription, translation, and protein processing. This heavily interconnected subnetwork comprised the inner network of the combined murine-human AAA PPI network (Figure 7), and the shared nature of this inner network may suggest its relationship to the vascular cells present in these tissue types as compared to the relatively decellularized nature of the thrombus-related tissues in the human samples (51). PCOLCE, CORO1A, and FERMT3/KINDLIN3 in the combined networks suggest a role for collagen turnover and angiogenesis (28, 52, 53), platelet function (54), and integrin biology (54–57) pathways also identified in the murine aneurysm proteomes. The prominence of neutrophil-related proteins and the presence of both MMP9 and MMP12 in the human networks supports the role for sustained influx of damaging inflammatory cell types in human tissues (58), a feature mirrored in the late interval AngII infusion models. A similar conclusion was reached in a study examining the AngII *Apoe*$^{-/-}$ model, the elastase model, and human tissues through the use of genomics (59). Findings in that study led those authors to conclude, in part, that the AngII model likely provides a more "chronic" disease state with continued inflammatory cell infiltrate, while the elastase model eventually reaches a "healing" stage in which fibrosis occurs. Interestingly, despite several study design differences, several of our pathways of interest converge with these previous studies including leukocyte motility, integrin signaling, and THBS1 activity.

The previously described platelet and erythrocyte subnetwork within the thrombus-related human tissues may contribute to the continued inflammatory response, specifically through accumulation of leukocytes, platelets, and erythrocytes, oxidative stress, and the activity of proteolytic pathways (40, 51). The laser capture microdissection component of our study demonstrated that the extramural thrombus found in the AngII model has little or no proteomic difference to the surrounding arterial wall, whereas our proteomic assessment of the human tissue demonstrates differences between the thrombotic and non-thrombotic AAA tissues. The isolation of the AngII-induced thrombus in the extravascular space, as compared to the constant exposure to blood experienced by the human ILT, likely drives this difference. While this evidence does not necessarily determine the thrombus present in murine models to be biologically inert, it lends support to the idea that the thrombus of human AAA holds a significant role in driving the disease (10, 40, 47, 51, 60).

An in-depth exploration of each pathway of interest and its constituent proteins is beyond the scope of this study, however, the study does provide a valuable road map for further analyses. The study provides insight to the ways in which the experimental mouse models represent and/or deviate from the human disease they represent. While we demonstrate that the most common models share several specific proteins and protein pathways with the human disease, the data also reveal interesting details: (1) in contrast to the human ILT, AngII aneurysm-associated, extraluminal thrombus does not appear to contribute to aneurysmal expansion; (2) although the proteomes of the two mouse aneurysm models indicate similar signaling pathways, differences emerging at the late interval proteome point toward events including continued leukocyte infiltration and ER stress setting the stage for continued aneurysmal expansion in the AngII model. Identifying the specific proteins or signaling pathways underlying aneurysm expansion may provide key targets for therapeutic strategies.

Data availability statement

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a article that does not adhere to our open data policies. Data are available *via* ProteomeXchange with identifier PXD032293.

Ethics statement

The studies involving human participants were reviewed and approved by University of Rochester Cardiovascular Tissue Bank Internal Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center.

Author contributions

SM contributed to the study design, performed most *in vitro* and *in vivo* experiments and data analysis, and drafted and finalized the manuscript. LL and AH conducted bioinformatics and network analyses. JN assisted in *in vitro* experiments and histological assays. HH performed mass spectrometry. AHH and JW performed histological analysis. AD and PL provided advice and critically read the manuscript. SC and DM provided human AAA samples. EA supervised histological assays, provided advice, and critically read the manuscript. AO contributed to scientific discussions, provided human AAA samples, and critically read the manuscript. SS contributed to the

study design, supervised mass spectrometry-assisted proteomics and its data analysis, and critically read and finalized the manuscript. MA directed the project, contributed to the study design and data interpretation, critically read and finalized the manuscript, and was responsible for funding and overall administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author HH is an employee of Kowa and was visiting scientist at Brigham and Women's Hospital when the study was conducted. Author MA has received research grants from Pfizer and Sanofi. Author PL is an unpaid consultant to or involved in clinical trials for Amgen, AstraZeneca, Esperion Therapeutics, Ionis Pharmaceuticals, Kowa, Novartis, Pfizer, Sanofi-Regeneron, and XBiotech, is a member of scientific advisory board for Amgen, Corvidia Therapeutics, DalCor Pharmaceuticals, IFM Therapeutics, Kowa, Olatec Therapeutics, Medimmune, Novartis, and XBiotech, and serves on the Board of XBiotech. The laboratory of PL has received research funding in the last 2 years from Novartis. Author PL has a financial interest in Xbiotech. His interests were reviewed and are managed by Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.889994/full#supplementary-material

SUPPLEMENTARY TABLE 1
Raw ultrasound measurements.

SUPPLEMENTARY TABLE 2
Median normalized, label-free based quantification of the two AAA mouse models and their saline counterparts: AngII (*ApoE-/-* and *Ldlr-/-*) and elastase (wildtype *C57BL/6J*).

SUPPLEMENTARY TABLE 3
AAA-induced suprarenal and infrarenal filtered proteins.

SUPPLEMENTARY TABLE 4
Antibodies for Western blot and immunostaining.

SUPPLEMENTARY TABLE 5
Laser capture microdissection of thrombus and arterial wall tissues in AngII-infused $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice.

SUPPLEMENTARY TABLE 6
Label-free based quantification of human aneurysm and control group artery samples.

SUPPLEMENTARY TABLE 7
Control and aneurysmal protein abundance rank comparisons.

SUPPLEMENTARY TABLE 8
Mouse and Human Uniprot and MGI IDs.

SUPPLEMENTARY FIGURE 1
Proteomic profiling of three aneurysm models with all mice included.

SUPPLEMENTARY FIGURE 2
Fermt3/Kindlin3 immunostaining. **(A)** Representative images of Fermt3/Kindlin3 immunostaining in abdominal aortic cross-sections from saline- and AngII-infused $Apoe^{-/-}$ mice. Scale = 300 μm. **(B)** Quantification of Fermt3/Kindlin3 immunostaining in tissue sections from saline ($n = 3$) and AngII ($n = 3$)-infused $Apoe^{-/-}$ mice. **(C)** Representative image of an aneurysmal cross-section from an $Apoe^{-/-}$ mouse; Adventitia (A), media (M) and intima (I). Scale = 100 μm. **(D)** Representative tissue section of a thrombotic region from an $Apoe^{-/-}$ mouse. Scale = 100 μm.

SUPPLEMENTARY FIGURE 3
Extended heatmap of enriched pathways.

SUPPLEMENTARY FIGURE 4
Proteome profiling of LCM aneurysmal tissues from AngII-infused mice. **(A)** LCM was performed on aortic segments of 4 and 5 $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice, respectively. **(B)** Example LCM samples prepared from proteomics. **(C,D)** Heatmap of a two-group comparison between thrombus and arterial wall samples. Protein lists are provided in Supplementary Table 5.

SUPPLEMENTARY FIGURE 5
High-resolution, node-readable consensus networks with animal and human tissue superimpositions. **(A)** Consensus; **(B)** Elastase wildtype; **(C)** AngII $Apoe^{-/-}$; **(D)** AngII $Ldlr^{-/-}$; **(E)** Human nT-AAA $Apoe^{-/-}$; **(F)** Human T-AAA; **(G)** Human thrombus.

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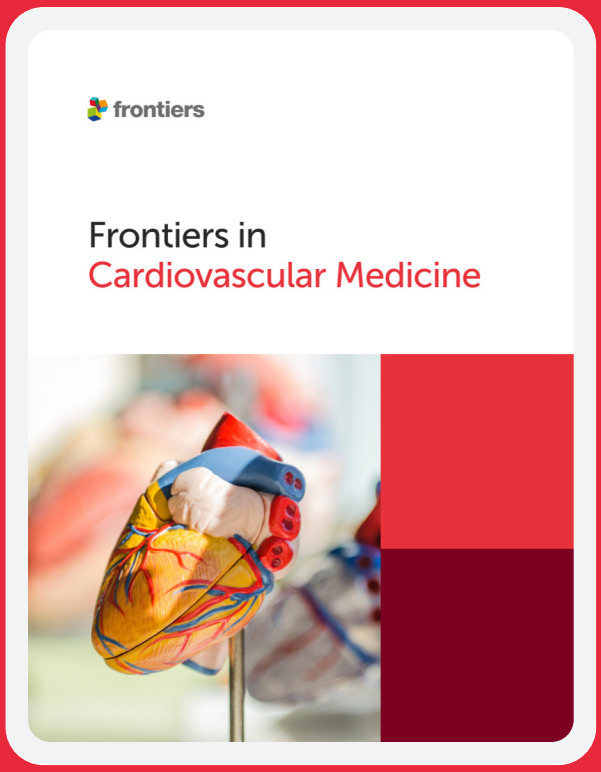

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