IMMUNOMODULATORY APPROACHES IN CARDIOVASCULAR DISEASES

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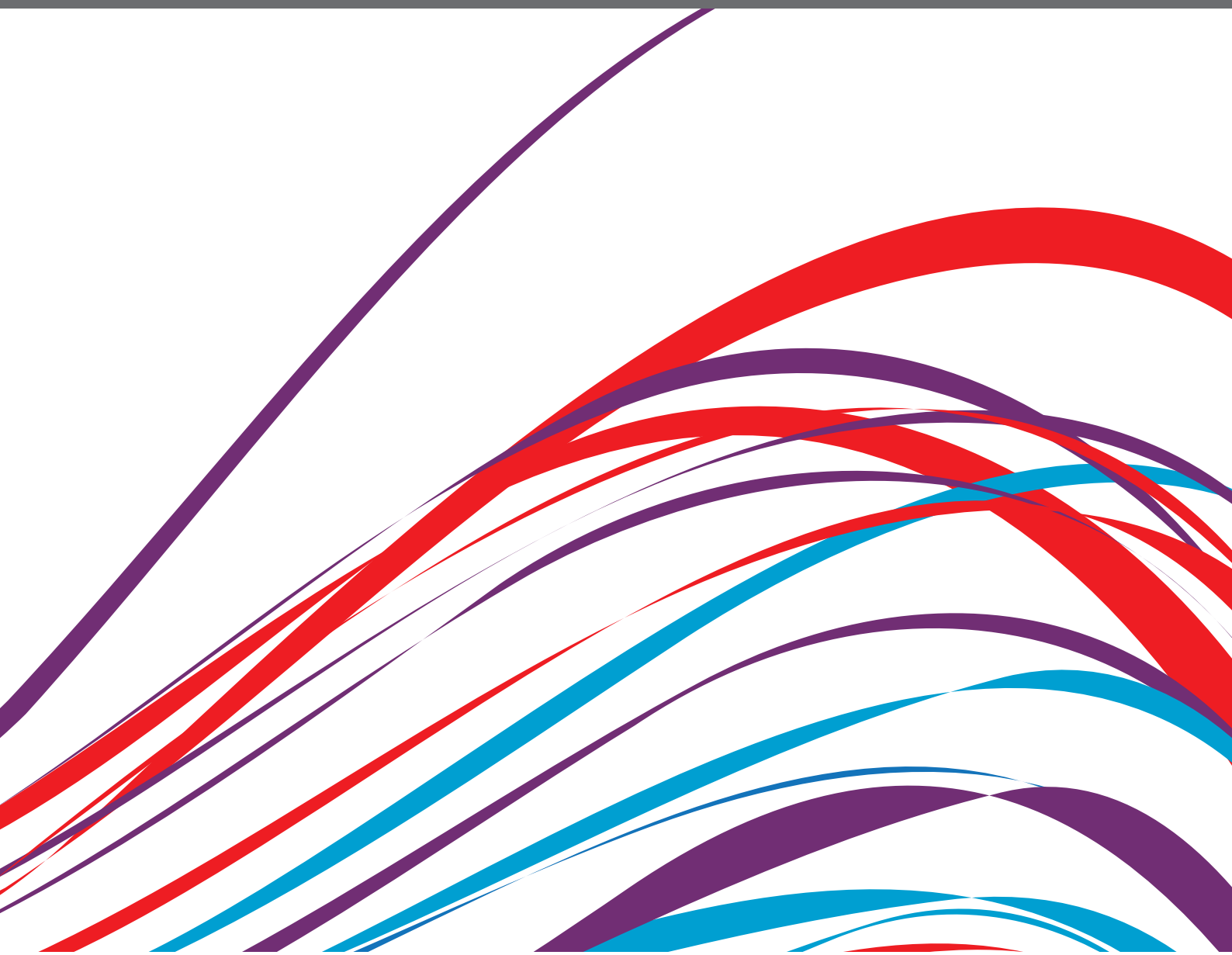

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IMMUNOMODULATORY APPROACHES IN CARDIOVASCULAR DISEASES

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EDITORIAL
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Editorial: Immunomodulatory Approaches in Cardiovascular Diseases

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Editorial on Research Topic

Immunomodulatory Approaches in Cardiovascular Diseases

A revolution has unfolded in our midst. Over the last two decades or so, a substantial body of evidence has developed showing that the immune and cardiovascular systems are intertwined. There are two aspects to this revolution. First, it is by now firmly established that the immune system contributes to the progression of cardiovascular disease (CVD), atherosclerosis in particular, although the intricacies are not fully understood. Nor do we understand how the immune system affects the initiation and progression of various cardiac conditions or myopathies. The second aspect is that the immune system can be engaged to target CVD. For instance, proof of concept evidence was recently presented that cardiac fibrosis can be attenuated by immunotherapy, involving adoptive transfer of CAR T cells expressing a cognate T cell receptor against fibroblast activation protein (FAP) (1). A subsequent study provides further evidence to support the therapeutic feasibility of this approach to counter maladaptive cardiac remodeling (2). In this study, T cell–targeted lipid nanoparticles were used to produce transient antifibrotic CAR T cells *in vivo*, which in a mouse model of heart injury, reduced fibrosis and restored cardiac function.

To celebrate this revolution, Frontiers in Cardiovascular Medicine presents the series entitled *Immunomodulatory Approaches in Cardiovascular Diseases*. Immunomodulatory approaches can be defined as "all interventions that modulate and curb the immune response of the host rather than targeting the disease itself" (Ammar et al.). This collection of 11 articles covers a broad swath, including reviews and original research articles, preclinical studies, a clinical trial, and case reports. They cover such topics as cardiovascular and metabolic diseases, cardiorenal syndromes, atrial fibrillation, Graves' disease, cardiac sarcoidosis, COVID-19, stress-induced myocardial remodeling, and dilated cardiomyopathy (**Figure 1**).

A comprehensive review by AlZaim et al. tackles the topic of adipose tissue immunomodulation and its impact on CVDs. This impact varies according to the type of adipose tissue depot. Significant crosstalk occurs among adipocytes, adipokines, and both resident and infiltrating cells of the innate and adaptive immune systems. As discussed, immune cell and adipokine profile dysfunction underpins adipose tissue inflammation, which in turn influences the heart and vasculature. The authors provide an overview of immunomodulatory approaches targeted toward adipose tissue for treating metabolic disorders and CVDs, including exercise and lifestyle modifications and anti-diabetic drugs, as well as several novel approaches, such as those that alter the gut microbiota. Another review article, deals with

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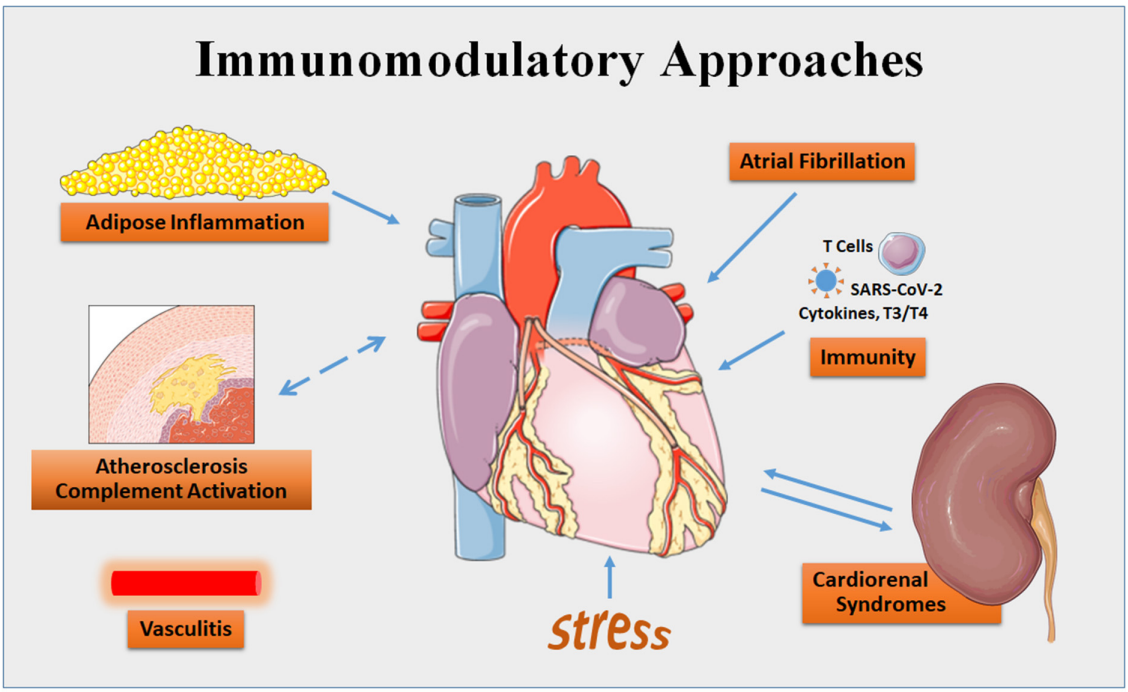

FIGURE 1 | Areas of cardiovascular disease targeted by immunomodulatory approaches that are covered by the series in Frontiers in Cardiovascular Medicine. Some content is adapted from Servier Medical Art (https://smart.servier.com/).

immunomodulatory approaches in diabetes-induced cardiorenal syndromes (Ammar et al.). After a brief introduction to immunomodulatory approaches in diabetic cardiomyopathy and nephropathy, this comprehensive article reviews the epidemiology and classifications of cardiorenal syndrome, which denotes the confluence of heart-kidney relationships such that dysfunction of one may initiate disease in the other via common neurohormonal, hemodynamic, biochemical, and/or immunological feedback pathways. The authors focus in on therapeutic approaches that target the immunomodulatory pathways implicated in diabetes-induced cardiorenal syndrome, namely the renin-angiotensin system, JAK/STAT signaling, and oxidative stress.

Two case-based reviews highlight the impact of the immune system on the heart and/or vascular system and the therapeutic importance of targeting it in the real world. One article deals with treatment of autoimmune myocarditis in Graves' disease, which is an immune system disorder that results in overproduction of thyroid hormones (hyperthyroidism) (Wu et al.). A second article presents a case series reporting the successful treatment of cardiac sarcoidosis with tumor necrosis factor alpha (TNFα) antagonists (Stievenart et al.). This life-threatening disease, which lacks clear recommendations for treatment, is a rare inflammatory condition in which clusters of white blood cells form granulomas in different areas of the heart, which may disrupt heart rhythm, blood flow, and normal heart function. Apropos of the present COVID-19 pandemic, an article by Zareef et al. addresses the course of SARS-CoV-2–induced illness in pediatric patients, both otherwise healthy children and those with congenital heart disease. Consequences include myocarditis, cardiogenic shock, arrhythmias, and multisystem inflammatory syndrome. Another original research study by Itani et al. presents data supporting the hypothesis that the inflammatory immune response is exacerbated in patients with both atrial fibrillation and cardiometabolic syndrome compared to either condition alone. They examined inflammatory cytokines and fibrotic markers as well as cytokine genetic profiles. Their approach has importance for the development of novel immunomodulatory therapeutic strategies for treating atrial fibrillation.

Two articles in the series deal with atherosclerosis. One gene expression-based bioinformatics study provides a comprehensive analysis of the immune cell infiltrates and aberrant pathway activation in atherosclerotic plaque (Han et al.). The findings support the idea that patients with coronary artery disease have an inflammatory immune microenvironment, which may be responsive to anti-inflammatory therapies under investigation. A preclinical study in Apoe$^{-/-}$ mice examined the therapeutic possibility of targeting complement activation in atherosclerosis (Dai et al.). Complement activation has been implicated in the development of atherosclerosis, but how that occurs is not known. The present study was based upon the observation that in ischemia-reperfusion injury, ischemia induces exposure of neoepitopes recognized by natural self-reactive IgM antibodies, which in turn activate complement. The authors used a novel construct (C2scFv-Crry), consisting of a single chain antibody (scFv) linked

to a complement inhibitor (Crry) that functions at C3 activation. The scFv moiety was derived from C2 IgM mAb, which recognizes phospholipid neoepitopes known to be expressed after ischemia. In Apoe$^{-/-}$ mice fed a high-fat diet, C2scFv-Crry administration decreased atherosclerotic plaque in the aorta and aortic root, reduced deposition of endogenous total IgM in the plaque, decreased lipid content in the lesion, and reduced serum oxLDL levels. Thus, neoepitope targeted complement inhibitors may be a novel therapeutic strategy to combat atherosclerosis. Somewhat related, leukocytoclastic vasculitis is a systemic autoimmune disease that is characterized by inflammation of the vascular endothelium and includes cutaneous small vessel vasculitis (CSVV) and anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV). Risk haplotypes, genetic variants, susceptibility loci and pathways associated with vasculitis immunopathogenesis have been identified and have laid the foundation for personalized medicine with targeted therapies. A review article by Yap et al. discusses pathways involved in disease pathogenesis and the underlying genetic associations in different populations worldwide. As noted, specific genetic variants predisposing individuals to CSVV and their pathogenic mechanisms are incompletely defined. Determining the immunopathogenic pathways in vasculitis and associated genetic variations will enable development of targeted personalized therapies.

Stress, including the psychological pressures of daily life, can adversely affect the structure and function of the heart, for example takotsubo cardiomyopathy. A study in the series examined maladaptive cardiac remodeling in an isoproterenol-induced cardiomyopathy mouse model (Adzika et al.). The authors found that amlexanox, an anti-inflammatory and immunomodulatory drug, attenuated the myocardial hypertrophy, fibrosis, and inflammation seen with isoproterenol. These actions were enhanced by the adenylyl cyclase activator, forskolin. In isolated macrophages, amlexanox acted by inhibiting a GRK5-mediated proinflammatory effect and, along with forskolin, facilitating cAMP-mediated immunoregulation. Finally, dilated cardiomyopathy, characterized by dilation and systolic dysfunction of one or both ventricles, may have a genetic basis or occur due to various etiologies that cause myocardium inflammation. New insights provide a better understanding of the pathogenesis of dilated cardiomyopathy by linking the genetic and inflammatory causes together. A review by Kadhi et al. summarizes the genetic and inflammatory causes underlying dilated cardiomyopathy and the pathways amenable to immunomodulatory strategies to salvage and prevent heart failure linked to the disease.

In recent years, we have made much progress in understanding the interplay between the immune system and CVDs. We stand on the cusp of another revolution that implements this knowledge into novel therapeutic approaches, possibly in the context of personalized medicine. The exciting articles in *Immunomodulatory Approaches in Cardiovascular Diseases* should attract much attention and foster further investigations to advance medical knowledge.

AUTHOR CONTRIBUTIONS

GB and FZ helped edit the text. All authors contributed to the inception and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Adipose Tissue Immunomodulation: A Novel Therapeutic Approach in Cardiovascular and Metabolic Diseases

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Adipose tissue is a critical regulator of systemic metabolism and bodily homeostasis as it secretes a myriad of adipokines, including inflammatory and anti-inflammatory cytokines. As the main storage pool of lipids, subcutaneous and visceral adipose tissues undergo marked hypertrophy and hyperplasia in response to nutritional excess leading to hypoxia, adipokine dysregulation, and subsequent low-grade inflammation that is characterized by increased infiltration and activation of innate and adaptive immune cells. The specific localization, physiology, susceptibility to inflammation and the heterogeneity of the inflammatory cell population of each adipose depot are unique and thus dictate the possible complications of adipose tissue chronic inflammation. Several lines of evidence link visceral and particularly perivascular, pericardial, and perirenal adipose tissue inflammation to the development of metabolic syndrome, insulin resistance, type 2 diabetes and cardiovascular diseases. In addition to the implication of the immune system in the regulation of adipose tissue function, adipose tissue immune components are pivotal in detrimental or otherwise favorable adipose tissue remodeling and thermogenesis. Adipose tissue resident and infiltrating immune cells undergo metabolic and morphological adaptation based on the systemic energy status and thus a better comprehension of the metabolic regulation of immune cells in adipose tissues is pivotal to address complications of chronic adipose tissue inflammation. In this review, we discuss the role of adipose innate and adaptive immune cells across various physiological and pathophysiological states that pertain to the development or progression of cardiovascular diseases associated with metabolic disorders. Understanding such mechanisms allows for the exploitation of the adipose tissue-immune system crosstalk, exploring how the adipose immune system might be targeted as a strategy to treat cardiovascular derangements associated with metabolic dysfunctions.

Keywords: adipose tissue, adipose tissue inflammation-definition of metabolic syndrome-insulin resistance-myokines-systemic inflammation, immunometabolism, adipose tissue immunology, adipose tissue browning

INTRODUCTION

Over the past two decades, the traditional view of adipose tissue (AT) as a passive store of excess calories evolved to implicate an endocrine role that is particularly pertinent to glucose and lipid homeostasis (1). This endocrine function is the result of a complex interaction between adipocytes and cells of the stromal vascular fraction of AT, which modulate the type of mediators produced in different conditions of health and disease. Importantly, this endocrine role is ascribed to the white adipose tissue (WAT); one of the two major types of AT. While, WAT comprises unilocular adipocytes that specialize in the storage of energy and the regulation of metabolic homeostasis by the production of adipokines, brown AT (BAT) is formed of mitochondria-rich multilocular adipocytes whose main function is energy dissipation through thermogenesis (2). Interestingly, accumulating evidence shows that both endocrine and thermogenic functions are modulated by resident and infiltrating immune cells. In fact, AT harbors a plethora of immune cells belonging to both the innate and adaptive immune systems, which either exert a pro- or an anti-inflammatory role depending on the microenvironmental stimulation and metabolic rewiring. Obese AT represents a state of chronic inflammation due to increased adipocyte hypertrophy, hyperplasia and apoptosis accompanied by an alteration in the production of adipokines and inflammatory mediators. This has been linked to the development of insulin resistance (IR), metabolic syndrome (MetS) and type 2 diabetes (T2D) (3). The manifestations of AT inflammation are proposed to alter the phenotype and gene expression profile of adipose immune cells, which was proposed to underlie major comorbidities of obesity including cardiovascular diseases (CVDs) (4).

In this review, we elaborate on the metabolic rewiring of AT-resident and infiltrating immune cells in health and disease and their participation in the inflammatory phenotype of AT relevant to the development of metabolic and cardiovascular disorders. We also touch upon recent evidence implicating AT-resident and infiltrating immune cells in the induction or suppression of AT thermogenesis and its possible outcomes. Finally, we discuss how several interventions immuno-modulate AT function and the exciting future perspectives in the field of AT immunometabolism.

OBESITY, AT INFLAMMATION AND THE METABOLIC SYNDROME

AT Inflammation and Remodeling

The incidence of obesity is increasing globally at an alarming rate with a parallel increase in the associated conditions including IR, CVD, and T2D (5, 6). Obesity is considered a chronic inflammatory disease that is linked to metabolic disorders (7). In this context, AT chronic low-grade inflammation and the progressive infiltration of immune cells into the AT contribute to IR (5, 8). The precise triggers of obesity-correlated inflammation are not fully understood. However, it is widely accepted that overnutrition drives a state of hyperinsulinemia, which participates in AT inflammation by inducing adipocyte hypertrophy followed by hypoxia, adipocyte death, lipotoxicity, and altered extracellular matrix (ECM).

WAT is a poorly vascularized tissue that exhibits a further decreased blood supply during AT expansion resulting in hypoxia. This hypoxic atmosphere is stimulated by increased adipocyte dimensions beyond the oxygen diffusing-ability, increased oxygen demand and lack of proper compensatory vascularization (9, 10). Infiltrating immune cells and ECM alterations also contribute to this hypoxic phenotype (11). Indeed, hypoxia induces the release of pro-inflammatory cytokines, chemokines, and angiogenic as well as fibrotic factors from adipocytes, which favor AT inflammation, vasculature remodeling, and AT dysfunction (9, 12). Hypoxia-induced AT dysfunction is characterized by an extensive lipolytic activity and free fatty acids (FAs) release leading to lipotoxicity, which was shown to exacerbate AT inflammation and participate in the pathogenesis of IR by promoting endoplasmic reticulum (ER) stress, adipocyte apoptosis, and inflammation (8, 13, 14). Hypoxia also causes necrosis-like adipocyte death, which initiates inflammation via interacting with macrophages (15). Nevertheless, AT reacts to adipocyte death by initiating a self-limiting wound healing response, which is characterized by intensive infiltration of immune cells, especially macrophages, that encircle dead fat cells, creating histological crown-like structures (CLS) (15). These macrophages generate toxic reactive oxygen species (ROS) and nitric oxide (NO), which further damage neighboring cells and support fibrosis (16). On the other hand, as the injury signal sustains in obesity, the chronic stimulation of myofibroblasts and immune cells causes additional damage, fibrosis, ECM remodeling and eventually AT dysfunction as well as IR (17, 18).

AT low-grade inflammation is driven by the excessive production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, which activate and recruit immune cells to AT, promoting its remodeling and causing an imbalance between homeostatic AT-resident immune cells and infiltrating inflammatory immune cells (19). The latter cells consist of macrophages, neutrophils, mast cells and T and B lymphocytes among others that secrete cytokines promoting the recruitment and polarization of other inflammatory cells in the AT. Moreover, dendritic cells (DCs), macrophages, and B cells induce the expansion of CD4 and CD8 T cells in the AT (20–22). In case of obese AT, macrophages exceed 50% of the immune cell population compared to lean AT (23, 24), and the production of CXCL12, CCR5, and MCP-1 by the AT tends to recruit and activate macrophages, making macrophages the major producers of cytokines in the AT (25–27).

Differences in Inflammation Susceptibility of Different AT Depots

Various molecular, physiological, and metabolic differences exist among adipose depots (28). Differences found in the microenvironment of WAT depots lead to unequal AT expansion and inflammation susceptibility under metabolic stress. Indeed, BAT is less prone to inflammation in comparison to WAT (29–31). Another good example is the difference in inflammation

susceptibility between PVAT and other VAT depots. We have shown that PVAT localized inflammation, which was associated with uncoupling protein 1 (UCP1)-mediated hypoxic preconditioning, occurs in isolation of systemic inflammation in a prediabetic rat model (32). Moreover, EpiCAT has small adipocyte size, high protein content and high rate of FA synthesis compared to other adipose depots making it susceptible to a metabolic profile shift (33). Additionally, during EpiCAT expansion, a quick proinflammatory microenvironment is generated due to the extensive inflammatory immune cell infiltration (34).

Adipokine Profile Dysregulation

Adipokines, which encompass endocrine and other biologically-active proteins, are released by WAT and function as hormones that regulate insulin sensitivity, energy balance, immune system functions and whole-body homeostasis (35). Metabolically healthy individuals possess a balance between proinflammatory and anti-inflammatory adipokines. This balance shifts in favor of proinflammatory mediators as the AT expands in the course of metabolic syndrome and obesity. This adipokine profile dysregulation has been associated with an increased risk of metabolic dysfunction, T2D and CVDs. Importantly, these adipokines profoundly influence the activation state, differentiation, and proliferation of AT-resident and infiltrating immune cells. Anti-inflammatory adipokines include adiponectin, C1q/TNF-related proteins (CTRPs), omentin, and secreted frizzled-related protein 5 (SFRP5) (36–38). Proinflammatory adipokines include leptin, resistin, chemerin, visfatin, retinol binding protein 4 (RBP4), and lipocalin 2 (LCN2) (35).

Adiponectin

Adiponectin is the best-known and most abundant adipokine found in human serum with insulin-sensitizing and cardioprotective actions (39, 40). Adiponectin serum levels decrease in obesity, T2D, and in states of high oxidative stress (41, 42). Total plasma adiponectin levels are also inversely correlated with MI risk (43, 44). Adiponectin-deficient mice exhibit an exacerbated myocardial ischemic injury, and adiponectin supplementation protects the heart against ischemia/reperfusion injury (45, 46). In circulation, adiponectin forms low, intermediate, and high molecular weight complexes where the high molecular weight complex was shown to block NF-κB activation and the production of proinflammatory cytokines (47, 48). Adiponectin exerts its effects by binding to its tissue-specific receptors, AdipoR1 and AdipoR2, which results in the downstream activation of AMPK, Akt-eNOS phosphorylation, and NO production (49–51). Moreover, adiponectin exerts an antioxidant (oxidative and nitrative stress) activity that is AMPK-independent and that is largely mediated via PKA-dependent NF-κB inhibition (52). Importantly, adiponectin modulates the activity of several immune cells in the AT including macrophages (53, 54), eosinophils (55), and mast cells (56). Indeed, profound mechanistic frameworks for this modulation are still lacking and require further investigation.

CTRPs

CTRPs are structurally similar, paralogs of adiponectin, with at least 15 isoforms being described to date where they exhibit broadly diverse effects (57, 58). For example, CTRP1 plays an important role in regulating body energy homeostasis and insulin sensitivity (59). Plasma CTRP1 was higher and negatively correlated with insulin resistance in diabetic subjects (60, 61). A recent study highlighted a significant association between increased CTRP1 levels and metabolic syndrome, obesity, T2D and non-alcoholic fatty liver disease (62). It was suggested that CTRP1 improves insulin resistance by reducing the phosphorylation of IRS-1 Ser1101 (61). In line with that, it was shown that elevated concentrations of CTRP1 reduce weight gain and diet-induced insulin resistance (59). Moreover, CTRP1 was shown to enhance glucose uptake through an increased GLUT4 translocation to the plasma membrane and enhanced glycolysis in HFD-fed CTRP1 transgenic mice (63). Moreover, CTRP1 promoted fatty acid oxidation and therefore, CTRP1 seems to perform a defensive catabolic effect in response to nutritional challenges. Interestingly, CTRP1-deficient mice fed a low-fat diet developed insulin resistance and hepatic steatosis (64). At the level of the cardiovascular system, CTRP1 was shown to regulate blood pressure through the induction of vasoconstriction (65). As such, mice overexpressing CTRP1 are hypertensive and hypertensive patients display a higher CTRP1 levels in comparison to healthy individuals (65). Moreover, CTRP1 was demonstrated to limit the extent of ischemia-reperfusion injury in acute myocardial infarction (59). The level of CTRP1 was also significantly increased in CAD patients and was suggested as a superior biomarker for the diagnosis of severity of vessel-lesion in CAD patients (66, 67). Interestingly, CRTP1 levels positively correlated with concentrations of IL-6 and TNF-α in CAD patients (66). In congestive heart failure patients, the levels of CTRP1 in serum and EpiCAT were higher than in controls, which was associated with a worse prognosis (68). Nevertheless, the implication of CTRP1 serum levels alteration on the activity of immune cells in models of metabolic and cardiovascular diseases has not yet been assessed.

CTRP3 (also known as cartducin) regulates adiponectin secretion from adipocytes (69, 70). CTRP3 was also shown to regulate glucose homeostasis (71), to stimulate *in vitro* endothelial cell proliferation and migration (58), and to inhibit TLR4 signaling and cytokine production in LPS- and FFA-stimulated adipocytes and monocytes (58). Importantly, CTRP3 serum level decrease following myocardial infarction and its restoration post-MI attenuates post-ischemic pathological remodeling (72).

Plasma CTRP9 levels are decreased in rodent models of obesity and diabetes (73, 74). Importantly, CTRP9 heterodimerizes with adiponectin and shares AdipoR1 stimulation in cultured cardiomyocytes and endothelial cells (73, 75, 76). CTRP9 promotes eNOS activity and NO production via AdipoR1-mediated activation of AMPK, resulting in endothelium-dependent vasorelaxation of aortic rings (76).

Moreover, CTRP9 attenuates inflammation in TNF-α-stimulated endothelial cells via AMPK activation and inhibits inflammatory responses in ox-LDL-stimulated macrophages (77, 78). Indeed, CTRP9-deficient mice are obese and insulin resistant (79). Importantly, several studies demonstrated a cardioprotective effect of CTRP9 (73, 74, 80, 81).

Adipolin (CTRP12) is an insulin-sensitizing adipokine that is abundantly produced by AT and whose expression levels decrease in rodent models of obesity (82, 83). The systemic administration of adipolin ameliorated glucose intolerance and insulin resistance in HFD-fed obese mice (82). Adipolin administration also attenuated macrophage infiltration and proinflammatory genes expression in AT of obese mice (82). Importantly, it was demonstrated that adipolin levels increase in response to hyperinsulinemia induction in healthy lean human subjects or following PPARγ agonism (84). This indicates that adipolin, as a novel anti-inflammatory adipokine, increases in the early stages of the metabolic insult to curb metabolic derangements and these levels are not sustained following prolonged metabolic disease induction. Importantly, adipolin levels were found to be lower in CAD patients compared to healthy controls (85). Moreover, adipolin levels were inversely correlated with HOMA-IR and TNF-α and positively correlated with adiponectin expression levels (85). Another study highlighted that adipolin levels decrease in acute myocardial infarction patients and that these levels are negatively associated with epicardial fat thickness (86). Indeed, adipolin-deficient mice exhibited an exacerbated neointimal thickening following vascular injury which was accompanied by enhanced inflammation and vascular cell proliferation (87). Adipolin-treated LPS-stimulated macrophages *in vitro* exhibited a reduced expression of IL-6 and TNF-α. Moreover, adipolin-deficient MI mice had increased myocardial apoptosis, cardiomyocyte hypertrophy, and perivascular fibrosis at the remote zone of infarct heart through an Akt-dependent mechanism (88). This indicates that adipolin exerts a protective effect against pathological processes of vascular and cardiac remodeling.

The adipokine CTRP6 regulates metabolism and inflammation (89, 90). CTRP6 improves cardiac function and ameliorates ventricular remodeling post-MI (91). CTRP13 was also shown to improve insulin sensitivity and inhibit the inflammation of lipid-loaded hepatocytes (92).

Omentin

Omentin is a novel adipokine whose levels decrease in obese subjects and negatively correlate with carotid intima media thickness (93–95). Moreover, omentin expression is negatively associated with the prevalence and the angiographic severity of coronary artery disease (96). Omentin inhibits TNF-α-induced endothelial COX2 expression and induces the activity of eNOS (97). Moreover, omentin enhances isolated aortic rings dilation in mice in an eNOS-dependent manner (98). Omentin systemic delivery also attenuated neointimal thickening and vascular smooth muscle proliferation in an AMPK-dependent mechanism (99). Therefore, omentin functions as an anti-atherogenic and anti-inflammatory adipokine similar to adiponectin and the CTRPs.

SFRP5

SFRP5 has anti-inflammatory effects in AT and in macrophages where it was shown to suppress the noncanonical Wnt5a/JNK signaling which inhibits the synthesis of macrophage TNF-α, IL-1β, and CCL2-MCP1 (100).

Leptin

First described as a satiety hormone, leptin was shown to bind to long form of leptin receptor expressed in nearly all immune cells to initiate innate immune responses (101). Leptin enhances the production of proinflammatory cytokines in peripheral blood monocytes and tissue-resident macrophages in mice and humans (102–105). Leptin also induces ROS production in macrophages, neutrophils, and endothelial cells and potentiate the expression of INFγ-induced nitric oxide synthase (106–108). Leptin also enhances Th1 and Th17 immune responses and prevents T cell apoptosis (109).

Resistin

Resistin was first characterized as a mediator of insulin resistance, metabolic syndrome, and T2D in mice (110). Although WAT represents the primary source of resistin in mice, monocytes and macrophages are the most important source of resistin in humans (111). The proinflammatory actions of resistin are mediated by CAP-1, a resistin receptor, with downstream activation of NF-κB in human monocytes (112). Resistin binds to TLR4 and regulates the production of TNF-α and IL-6 in macrophages through the activation of NF-κB signaling (113). Importantly, resistin levels are elevated in obese humans and are associated with an increased risk of CVDs (114).

Visfatin

Visfatin, also known as pre-B cell colony-enhancing factor (PBEF), is a novel, highly conserved adipokine that is abundantly expressed in visceral fat (115, 116). Visfatin plays a determinant role in the pathophysiology of metabolic and cardiovascular diseases (117). Visfatin elicits insulomimetic effects in adipocytes and an increased blood glucose level prompts an increase of serum visfatin (115, 118). Nevertheless, it was suggested that the effects of visfatin do not involve the classical insulin signaling pathways in skeletal muscles (115, 119). Indeed, several studies demonstrated an association between increased plasma visfatin level and diabetes (120, 121). In contrast, other studies reported opposite or no association between visfatin plasma levels and diabetes (122, 123). Similar controversy was also documented when correlating visfatin plasma levels with obesity (124–126). Despite the role of visfatin in metabolic disorders remaining debatable (127), it does not rule out visfatin implication in these disorders and its participation in metabolic dysfunction-associated cardiovascular diseases. Several studies suggested a pro-inflammatory role of visfatin in both VAT and scWAT (128). In fact, visfatin was shown to enhance monocyte-mediated recruitment of T cells and B cells by increasing the expression of CD80, CD40, and ICAM-1 (129). Moreover, visfatin-stimulated human leukocytes exhibit a dose-dependent induction in the expression of IL-1β, IL-1Ra, IL-10, and IL-6 (129).

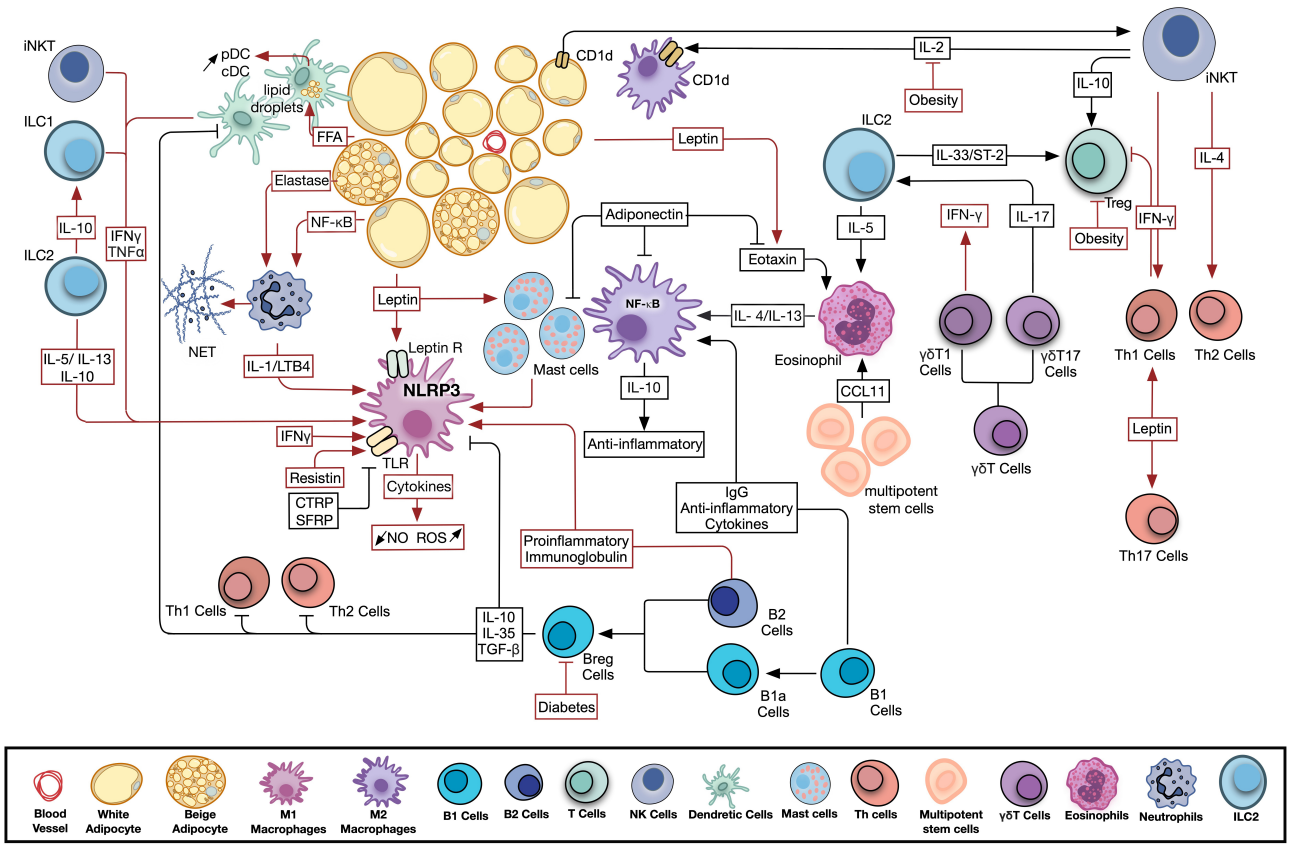

FIGURE 1 | Adipose tissue resident and infiltrating immune cells activity in health and disease. Significant crosstalk exists among adipocytes, adipokines, and resident and infiltrating, innate and adaptive immune cells. Metabolic disease conditions modulate the adipokine profile and immune cell activity leading to the observed chronic low-grade inflammation. Pathways involved in AT homeostasis are depicted in black arrows, while those activated by metabolic dysfunction are shown in red. B Cell, B Lymphocyte; Breg Cells, Regulatory B Lymphocyte; CCL11, C-C motif chemokine 11; CD1d, Cluster of Differentiation 1d; cDC, Conventional Dendritic Cell; FFA, Free Fatty Acids; IFNγ, Interferon Gamma; IgG, Immunoglobulin G; IL, Interleukin; ILC, Innate Lymphoid Cell; iNKT Cell, Invariant Natural Killer T Cell; LTB4, Leukotriene B4; NET, Neutrophil Extracellular Trap; NF-κB, Nuclear Factor Kapp-light-chain-enhancer of Activated B cells; NLRP3, NLR Family Pyrin Domain Containing 3; NO, Nitric Oxide; pDC, Plasmacytoid Dendritic Cell; ROS, Reactive Oxygen Species; T Cell, T Lymphocyte; TGF-β, Transforming Growth Factor Beta; Th Cell, Helper T Lymphocyte; TLR, Toll Like Receptor; TNFα, Tumor Necrosis Factor Alpha; Treg, Regulatory T Lymphocyte.

LCN2 and RBP4

LCN2, also known as neutrophil gelatinase-associated lipocalin (NGAL) is upregulated in the presence of IFN-γ and TNF-α in obese individuals (130, 131). Similarly, RBP4, which is mostly complexed with retinol in circulation, was shown to promote IR and increases the risk of T2D (132, 133). RBP4 activates antigen presenting cells and is suggested as a cardiometabolic marker in MetS (134).

Alongside the above-mentioned changes observed in the adipokine profile with the induction and progression of metabolic disease, a bi-directional interaction proceeds within the AT microenvironment among adipocytes and different types of resident and infiltrating immune cells. Details and outcomes of this interaction will be discussed for each of the cell types below. A summary of the different pathways and mediators involved is provided in **Figure 1**.

METABOLIC REGULATION AND ADAPTATION OF TISSUE RESIDENT AND INFILTRATING MYELOID CELLS

Macrophages

Tissue-resident macrophages are highly heterogeneous with distinct, localization-dependent transcriptomes (135). Classically-activated M1 macrophages, which drive CLS formation, can be induced by LPS, toll-like receptor (TLR) ligands or interferon (IFN)-γ, secrete pro-inflammatory cytokines and upregulate the production of ROS and NO following activation (136). Conversely, alternatively-activated M2 macrophages, which contribute to AT homeostasis, are induced by IL-4 and IL-13, are implicated in the resolution of inflammation, and produce anti-inflammatory cytokines such as IL-10 (137). Although being useful to highlight the inflammatory state of tissues in health and disease, the M1/M2 macrophage classification paradigm is now

TABLE 1 | Metabolic pathways of classically activated M1 macrophages and alternatively activated M2 macrophages.

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
M1 macrophages	Glycolysis	⇑	Inflammatory cytokines production	Myeloid-specific HIF-1α overexpressing mice	(139)
				HFD-fed mice and myeloid-specific HIF-1$\alpha^{-/-}$ mice	(140)
			Vascular remodeling	ATMs deletion in HFD-fed mice LPS and High Glucose stimulated macrophages	(141)
	Oxidative phosphorylation	⇓			(138)
	Pentose phosphate pathway	⇑	Production of inflammatory cytokines, ROS and NO	*Trypanosoma cruzi*-infected macrophages	(142)
			Inflammatory cytokines production	Myeloid-specific HIF-1α overexpressing mice	(139)
	TCA Cycle	Truncated	Production of prostaglandins, NO and ROS		(143)
	Lipogenesis	⇑	Required for Inflammasome activation and production of inflammatory mediators	Cecal ligation puncture-induced endotoxic shock in SREBP-1a-deficient mice LPS stimulated macrophages from mutant mice	(144)
			Required for Phagocytosis	SREBP-1a-deficient macrophages	(145)
			Required for inflammasome activation	Polymicrobial sepsis UCP2$^{-/-}$ mouse model	(146)
	Glutamine metabolism	⇓	Required for polarization and T cell recruitment	Glutamine synthase (GS)-inhibited macrophages and GS$^{-/-}$ macrophages	(147)
	Fatty acid oxidation	⇑	Inflammasome activation	NOX4-deficient mice	(148)
M2 macrophages	Glycolysis	⇑	Not essential for polarization unless OXPHOS is affected	BMDMs and Raw264.7 cells	(149)
	Oxidative phosphorylation	⇑	Required for polarization	BMDMs and Raw264.7 cells	(149)
	Glutamine metabolism	⇑	Required for polarization		(150)
	Fatty acid oxidation	⇑	Required for polarization and activation of the anti-inflammatory program	STAT6–/– BMDMs, embryonic-derived myeloid progenitors and PGC-1β transgenic mice	(151)
			Essentiality for polarization ?		(152–154)

⇑, *high metabolic rate;* ⇓, *low metabolic rate;* ?, *requires further investigation.*

considered an oversimplification as it does not consider microenvironmental factors.

Macrophages exhibit differential metabolic profiles based on their specific polarization and microenvironmental factors (138). These metabolic alterations are summarized in **Table 1**. Indeed, the dynamic inflammatory milieu of obese AT drives ATMs metabolic profile modifications. ATMs in obese mice exhibit an increased activation of OXPHOS and glycolysis (140). In addition, the activation of the NLRP3 inflammasome in macrophages by the increasing exogenous FAs in obesity contributes to the emergence of M1 macrophages (155–157). Moreover, monocytes and macrophages express the leptin receptor, which induces the proliferation of macrophages and the production of pro-inflammatory cytokines in response to leptin (158). In contrast to leptin, adiponectin suppresses the NF-κB-dependent expression of pro-inflammatory cytokines and promotes M2 polarization (53, 54). Nevertheless, another study argued that adiponectin induced the production of pro-inflammatory cytokines in M2 macrophages without interfering with their polarization (159).

Dendritic Cells

Dendritic cells (DCs) are professional antigen-presenting cells that either instigate or suppress immune responses based on their maturation state. DCs are divided into two categories, plasmacytoid DC (pDC) and myeloid or conventional DC (cDC). Accumulating evidence implicates DCs and particularly cDCs in the regulation of AT inflammation. The DC population expands, promotes macrophage recruitment and induce a Th17-driven inflammatory response in HFD-fed mice (160–162). Indeed, HFD-fed mice exhibited an increased number of CD11c^{+} DCs in the AT whose ablation attenuated visceral AT inflammation (160, 161, 163). The accumulation of cDC during obesity was also attenuated in CCR7-deficient mice, which was associated with decreased AT inflammation (164, 165). Conversely, cDCs in AT were shown to acquire a tolerogenic phenotype through

TABLE 2 | Metabolic pathways utilized by activated dendritic cells.

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
Activated DCs	Glycolysis	⇑	Required for activation	PAMPs-stimulated human monocyte-derived DCs	(173)
				TLR agonist-stimulated DCs	(174)
			Required for migration	*In vitro* stimulated bone marrow-derived DCs and splenic DCs	(175)
	Oxidative phosphorylation	-		PAMPs-stimulated human monocyte-derived DCs	(173)
		⇓			(176)
	Pentose phosphate pathway	⇑	Required for the enhanced synthesis of fatty acids		(177)
	TCA cycle	Truncated	Production of NO and ROS		(176)
	Lipogenesis	⇑	Required for activation	TLR agonist-stimulated DCs	(174)

⇑, *high metabolic rate;* ⇓, *low metabolic rate;* -, *no change in metabolic rate.*

the activation of β-catenin and PPARγ without affecting weight gain (166). pDCs were also shown to accumulate in AT and to have detrimental effects in mice and humans (167, 168). DCs also influence the normal expansion of lean AT, where increased adiposity was accompanied by a reduction of $CD11c^+$ AT DCs (169). Moreover, the uptake and accumulation of FAs in DCs and the formation of lipid droplets (LDs) were associated with increased DC immunogenicity (170, 171). Due to the lipid-rich environment of WAT especially in obesity, AT DCs are expected to acquire more LDs. Nevertheless, the functional impacts of this remain to be investigated. Different DC subsets exhibit distinct metabolic programs (172). In fact, resting and stimulated DCs have different metabolic requirements and thus, employ differential metabolic pathways. These pathways are highlighted in **Table 2**.

Neutrophils

Neutrophils are relatively rare in WAT of lean mice, where they are suggested to maintain tissue homeostasis (178). Neutrophils are among the first immune cells to be recruited to the AT of HFD-fed mice with a sustained infiltration. Neutrophils drive AT inflammation and IR through the production of inflammatory mediators and the formation of neutrophil extracellular traps (NETs) (179–181). Neutrophils accumulation in AT is dependent on the production of elastase, whose activity is enhanced in the AT of HFD-fed mice (179). WAT-infiltrating neutrophils exhibit an upregulation of IL-1β expression via NF-κB activation in an adipocyte contact-dependent manner (182). Adipocyte lipolysis and LTB4 production in WAT also accumulates neutrophils prior to macrophages and increases the production of IL-1β, which enhances macrophage recruitment into the AT (182). Nevertheless, it was proposed that neutrophils, similar to macrophages, exhibit phenotypic heterogeneity by which N1 neutrophils are pro-inflammatory and N2 neutrophils are anti-inflammatory (183, 184).

Neutrophils were believed not to require extensive metabolic networks and to solely depend on glycolysis as they exhibit a relatively low transcriptional and translational activity where (185). Nevertheless, novel evidence suggests the implication of the TCA cycle, OXPHOS, PPP, FAO, and glutaminolysis in neutrophil metabolism, demonstrating a broad metabolic plasticity (185) (**Table 3**).

Eosinophils

Eosinophils are multifunctional phagocytic granulocytes that are typically associated with helminth infection and allergic disorders (206). A growing body of evidence suggests a homeostatic role for AT-resident eosinophils (178). AT-resident eosinophils are sustained by AT multipotent stromal cells-derived CCL11 and ILC2-derived IL-5 (207, 208). Indeed, AT-resident eosinophils produce IL-4 and IL-13 that drive macrophage M2 polarization, trigger Th2 differentiation, enhance B cell activation and promote metabolic homeostasis (209, 210). Eosinophil-deficient HFD-fed mice showed pronounced IR (209, 211). Moreover, it was shown that HFD-induced adiposity can be inhibited by increasing the number of eosinophils in mice (207, 209). Conversely, another study demonstrated an increase in gonadal AT eosinophils in HFD-fed mice, which was supposed to be regulated by increased CCL11 expression (211). Indeed, HFD-fed ΔdblGATA and IL-5-KO mice lacking eosinophils or almost having no gonadal AT eosinophils, exhibited impaired insulin sensitivity (207, 212). The forced increase of AT eosinophils in different models demonstrated an enhanced metabolic homeostasis (209, 213, 214). Indeed, IL-4-stimulated eosinophils induced M2 macrophage polarization, while oxidized LDL-mediated induction promoted M1 macrophage polarization (209, 215, 216). HFD-fed transgenic mice overexpressing eotaxin2 specifically in AT exhibited an increased eosinophil migration into AT that was accompanied by enhanced glucose tolerance (217). It is also worth mentioning that leptin promotes while adiponectin attenuates eotaxin-induced human eosinophil adhesion and chemotaxis (55, 218, 219). Nevertheless, enhancing AT eosinophil abundance is debated since several studies demonstrated no beneficial or even negative outcomes of this approach (220).

TABLE 3 | Metabolic pathways of neutrophils, eosinophils and mast cells.

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
Neutrophils	Glycolysis	⇑	Required for phagocytosis	Stimulated human neutrophils *in vitro*	(186)
			Required for NETosis	Phorbol myristate acetate-stimulated human neutrophils *in vitro*	(187)
	Oxidative phosphorylation	⇑	Required for differentiation	shRNA induced knockdown of adenylate kinase 2 in neutrophil progenitor cells	(188)
			Required for chemotaxis and respiratory burst	Oligomycin and FCCP-treated human neutrophils	(189)
			Required for the production of ROS	LPS-treated mouse bone marrow-derived neutrophils treated with Antimycin A or myxothiazol	(190)
			Required for migration	Polg CRISPR/Cas9-mediated neutrophil-specific knockout in Zebra fish	(191)
	Pentose phosphate pathway	⇑	Required for NETosis	Amyloid fibril- and phorbol myristate acetate-stimulated human neutrophils	(192)
			Required for ROS generation and NETosis	G6PD-deficient patients G6PD-deficient mice	(193, 194)
	TCA cycle	⇑	Required for chemotaxis	Isocitrate dehydrogenase 1 mutant mice	(195)
			Required for differentiation	Mouse Atg5-deficient neutrophils and an *in vitro* model of differentiating neutrophils	(196)
	Lipogenesis	⇑	Required for differentiation	Atg7-deficient neutrophil precursors	(197)
			Required for neutrophil maintanence	FASlox/lox-Rosa26-CreER mice	(198)
	Glutamine metabolism	⇑	Not required for NETosis	Phorbol myristate acetate-stimulated human neutrophils *in vitro*	(187)
	Fatty acid oxidation	⇑	Required for NOX-2-dependent respiratory burst and ROS production	NOX deficient $p47^{-/-}$ mice, bone marrow c-$Kit^{+/-}$ neutrophils and human neutrophils	(199)
			Required for NETosis		(185)
			Required for differentiation	Atg7-deficient neutrophil precursors	(197)
Eosinophils	Glycolysis	⇑		Peripheral blood-derived human eosinophils	(200)
				IL-3, IL-5, or GM-CSF-stimulated human eosinophils	(201)
	Oxidative phosphorylation	⇑		Peripheral blood-derived human eosinophils	(200)
				IL-3, IL-5, or GM-CSF-stimulated human eosinophils	(201)
	TCA cycle	⇑		IL-3, IL-5, or GM-CSF-stimulated human eosinophils	(201)
	Glutamine metabolism	⇑		IL-3, IL-5, or GM-CSF-stimulated human eosinophils	(201)
Mast Cells	Glycolysis	⇑	Required for histamine release	2-DG-treated rat mast cells	(202)
			Required for IgE-mediated degranulation	High glucose-treated bone marrow-derived mouse mast cells	(203)
	Oxidative phosphorylation	⇑			(204)
	Pentose Phosphate Pathway	⇑			(204)
	TCA Cycle	Truncated	Accumulation of upstream intermediates that channel through the PPP Mast cell degranulation	Basophilic leukemia (RBL-2H3) cells and a mouse model of allergen-induced airway hyper-responsiveness	(204, 205)
	Lipogenesis	⇑			(204)

⇑, *high metabolic rate;* ⇓, *low metabolic rate.*

It was suggested that circulating eosinophils display a greater metabolic flexibility in comparison to neutrophils (200, 201). Further investigation into the metabolic rewiring of eosinophils (shown in **Table 3**) is required as emerging roles of eosinophils suggest a central modulatory function in AT homeostasis.

Mast Cells

Mast cells (MCs) are innate immune cells that originate from multipotent hematopoietic stem cells, then migrate to peripheral organs, where they undergo maturation giving rise to heterogeneous populations of mature MCs (21). MCs are enriched in visceral AT of mice and humans and are increased in settings of obesity and T2D, where they drive AT inflammation partly by enhancing macrophage infiltration (21, 221, 222). Indeed, the increased abundance of MCs in sub-cutaneous WAT of MetS subjects positively correlated with IR and markers of fibrosis and angiogenesis linking them to AT fibrosis and remodeling (223, 224). It was also suggested that MCs infiltration precedes the development of overt obesity (225). Indeed, the genetic ablation of MCs or manipulations impairing their function in HFD-fed mice resulted in decreased weight gain and reduced IR (221, 226, 227). Nevertheless, other studies utilizing different mouse models could not find a correlation between MC deficiency and the amelioration of AT inflammation (228, 229).

The activation of MCs is accompanied by a major metabolic reprogramming (**Table 3**). Indeed, these metabolic processes regulate MCs inflammatory cytokines and ROS production and IGE-mediated degranulation (203, 204, 230). A role for adipokines in the regulation of MC function has been recently revealed (56). Leptin and adiponectin were shown to exert opposite effects on MC polarization to promote a pro-inflammatory or an anti-inflammatory cytokine profile, respectively.

METABOLIC REGULATION AND ADAPTATION OF TISSUE RESIDENT AND INFILTRATING LYMPHOID CELLS

T Cells

T lymphocytes play major immunoregulatory and immunometabolic roles in AT homeostasis and dysfunction. Indeed, T cells were increased in VAT of obese mice and humans (231). Different effector T cells including helper T (Th) cells (T-bet-regulated Th1, GATA3-regulated Th2 and ROR-γt-regulated Th17) and cytotoxic T lymphocytes (CTLs) were shown to actively participate in obesity-associated WAT inflammation (178). Conversely, anti-inflammatory T cells such as regulatory T (Treg) cells and invariant natural killer T (iNKT) cells that reside in the AT under physiological conditions were reduced in obesity (232, 233). Based on the composition of the T-cell antigen receptors (TCR), T cells are categorized into two populations, αβ T and γδ T cells. αβ T cells are further classified, based on their surface markers, into $CD4^+$ T cells and $CD8^+$ T cells that upon activation, differentiate into Th cells and CTLs, respectively. Tregs emerge as a subset of $CD4^+$ T cells that negatively regulate immune responses with a characteristic signature $CD4^+$ $CD25^+$ $Foxp3^+$.

αβT Cells

αβ T cells represent the second largest immune population in WAT (178). In obesity, T cells are enriched in visceral AT of mice and humans, and are possibly recruited through a CCR5-CCL5-mediated interaction (231, 234). It was proposed that T cells infiltration precedes that of macrophages (235). However, this is debated as other studies did not arrive at a similar result (236, 237). $CD4^+$ T cells represent the more abundant subtype in visceral AT and are further enriched in obesity (234, 238). In addition to their recruitment from the general circulation, both $CD4^+$ and $CD8^+$ T cells undergo clonal expansion in epicardial WAT (239, 240). MHCII expression was increased post-HFD feeding and mice deficient in MHCII exhibited greater insulin sensitivity (241). Nevertheless, inhibiting MHCII in HFD-fed mice did not improve glucose tolerance, an improvement seen with conventional T cells deficiency (242–244). The depletion of $CD8^+$ cells in HFD-fed mice decreased the expression of TNF-α and IL-6 in epicardial WAT, which was accompanied by an enhanced glucose and insulin tolerance (235). Similarly, $CD4^+$ Th1 cells were shown to drive AT inflammation and glucose intolerance (245). In fact, Th1 cells are similar in proportion to Tregs in lean conditions, while they occur at a higher frequency in comparison to other $CD4^+$ cell subtypes in obesity (242, 246). Similarly, Th17 cells accumulated in sub-cutaneous WAT of insulin resistant individuals (247). IL-17-deficient mice displayed a better insulin and glucose tolerance, this was however abrogated by HFD feeding (248). Th2 cells were shown to have a beneficial effect on AT inflammation. Rag-deficient HFD-fed mice showed marked obesity and IR in comparison to their WT counterparts, a phenotype that was abrogated by the adoptive transfer of $CD4^+$, but not $CD8^+$ T cells (242). Indeed, most of $CD4^+$ cells that homed to epicardial WAT expressed GATA3 (242).

Increasing evidence suggests a pivotal role for metabolic pathways in naïve and activated T cells maintenance and function (249). On activation, naïve T cells undergo major metabolic reprogramming, which highly depends on the duration and strength of TCR stimulation, including glucose metabolism, glutamine metabolism, and biosynthetic pathways (249). These metabolic alterations are summarized in **Table 4**.

Regulatory T Cells

Phenotypically-distinct AT-resident Tregs were reported to be enriched in visceral AT of lean mice, where they originate from enhanced proliferation rather than circulating Tregs infiltration (178). Visceral AT Tregs are markedly reduced in obese mice and humans, which promotes inflammation (232, 260, 261). Conversely, expanding Tregs in HFD-fed mice improved metabolic parameters (262). Indeed, PPARγ is essential for the accumulation and function of Tregs in AT of lean mice, where it collaborates with Foxp3 to induce their distinct phenotype, a phenotype abrogated by obesity through phosphorylating PPARγ at position Ser273 (263–265). Moreover, it was shown that IL-33/ST-2 axis amplified Tregs in visceral AT, which was accompanied by an attenuation of inflammation in obese mice (266, 267). Indeed, Tregs are highly enriched in visceral AT and to a lesser extent in sub-cutaneous WAT, where IL-33 is expressed (268–270). Other immune cells including γδ T cells, ILC2s and iNKT cells were also shown to regulate AT Tregs accumulation promoting insulin sensitivity (178).

TABLE 4 | Metabolic pathways required for T lymphocyte proliferation, differentiation, function, and activity.

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
αβ T cells	Glycolysis	⇑	Required for cell growth and clonal proliferation		(249)
				Glut1 transgenic mice Murine model of asthma	(250)
	Oxidative phosphorylation	⇑	Required for the survival, proliferation, generation and function		(249)
	Pentose phosphate pathway	⇑	Nucleotide and ribosome biosynthesis		(249)
	TCA Cycle	⇑			(249)
	Lipogenesis	⇑	Required for Th17 development, production of membrane phospholipids and inflammatory function	Pharmacological and genetic inhibition of ACC1 in mice Human T cell cultures Murine model of experimental autoimmune encephalomyelitis	(251)
			Required for growth and proliferation		(249)
	Glutamine Metabolism	⇑	Regulates T cell activation		(249)
	Fatty Acid Oxidation	⇑		*Ex vivo* human $CD4^+CD25^-Foxp3^-$	(252)
		⇓		Glut1 transgenic mice Murine model of asthma	(250)
Regulatory T Cells	Glycolysis	⇑	Required for cellular migration	Treg-specific HIF-$1\alpha^{-/-}$ mice	(253)
			Required for cellular migration but not immunosuppressive function	Foxp3-GFP and $Cd28^{Y170F}$ genetically targeted mice on C57BL/6 background $Ctla4^{-/-}$ mice Murine lung microvascular endothelial cells, bone marrow-derived dendritic cells H2-d allospecific Treg cells Loss-of-function GCK mutation human blood samples	(254)
			Required for proliferation	*Ex vivo* $CD4^+CD25^{hi}Foxp3^+CD127^-$ Treg cells	(252)
	Lipogenesis	⇓		Pharmacological and genetic inhibition of ACC1 in mice Human T cell cultures Murine model of experimental autoimmune encephalomyelitis	(251)
	Fatty Acid Oxidation	⇑	Required for proliferation	*Ex vivo* $CD4^+CD25^{hi}Foxp3^+CD127^-$ Treg cells	(252)
			Required for immunosuppressive activity	Treg-specific HIF-$1\alpha^{-/-}$ mice	(253)
				Glut1 transgenic mice Murine model of asthma	(250)
γδ T cells	Glycolysis	⇑	In γδ T1 cells and is required for differentiation and cytokine production	CD2-cre;Raptor-f/f mice	(255)
	Oxidative phosphorylation	⇑	In γδ T17 cells and is required for the production of IL-17	$MyD88^{-/-}$, $Il1r1^{-/-}$ and IL-23R KO and conditional (CD2-cre; $Raptor^{fl/fl}$, CD-2-cre;$Rictor^{fl/fl}$ and CD-2-cre;$Stat3^{fl/fl}$) KO mice Human subjects with psoriasis vulgaris Psoriasis-like mouse model	(256)
	TCA cycle	⇑	Required for the production of IL-17	$MyD88^{-/-}$, $Il1r1^{-/-}$, and IL-23R KO and conditional (CD2-cre; $Raptor^{fl/fl}$, CD-2-cre;$Rictor^{fl/fl}$ and CD-2-cre;$Stat3^{fl/fl}$) KO mice Human subjects with psoriasis vulgaris Psoriasis-like mouse model	(256)

(Continued)

TABLE 4 | Continued

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
iNKT cells	Glycolysis	⇑	Required for the production of IFN-γ and TCR recycling and accumulation in the immune synapse	Murine spleen and liver Vα14 Tg.cxcr6$^{gfp/+}$ iNKT cells	(257)
	Oxidative PHOSPHORYLATION	⇓		Murine spleen and liver Vα14 Tg.cxcr6$^{gfp/+}$ iNKT cells	(257)
		⇑	Essential for survival, proliferation and selective cytokine production	PLZF$^{+/-}$ and PLFZ^{Tg} mice spleen NKT cells	(258)
	Pentose phosphate pathway	⇑	Required for effector functions	PLZF$^{+/-}$ and PLFZ^{Tg} mice spleen NKT cells	(258)
	Lipogenesis	⇑	Required for the production of IFN-γ	Murine spleen Vα14 Tg.cxcr6$^{gfp/+}$ iNKT cells Clinical tumor biospecimens from HCC patients	(259)

⇑, *high metabolic rate;* ⇓, *low metabolic rate.*

Conversely, IFN-γ producing Th1 cells inhibited AT Tregs and thus promoted IR (271).

The development, function, and phenotype stabilization of Tregs is metabolically regulated by several pathways highlighted in **Table 4** (272). Moreover, leptin metabolism was shown to partially induce Tregs *in vitro* and mice deficient in leptin exhibited an increased proliferative ability of Tregs (273). Several lines of evidence suggest that Treg deficiency or insufficiency can lead to both T1D and T2D (274). Although studies demonstrated no difference in Treg frequency in diabetes, Treg phenotype and suppressive function were altered (275).

γδT Cells

γδ T cells can be classified into two major functional groups, IFN-γ-producing γδ T1 cells and IL-17-producing γδ T17 cells (178). In comparison to αβ T cells, γδ T cells harbor a restricted TCR repertoire, and the antigens recognized by these cells remain largely unknown. γδ T cells are as abundant as MCs, neutrophils, and CD8$^+$ T cells in lean WAT, and are increased in response to HFD consumption (268, 276). Earlier investigations into the role of γδ T cells in AT demonstrated a pro-inflammatory function in HFD-fed mice (276). Nevertheless, circulating γδ T cells were decreased in obese subjects and were negatively correlated with BMI (277). More recently, γδ T cells of epicardial WAT were shown to comprise two distinct populations; PLZF$^-$, CD3ε^{low}, CD27$^+$, RORγT$^-$, T-bet$^+$ γδ T cells that produce IFN-γ and PLZF$^+$, CD3ε^{high}, CD27$^-$, RORγT$^+$, T-bet$^-$ cells that produce TNF-α and IL-17A (268). Indeed, mice deficient in PLZF$^+$ γδ T cells or IL-17A KO mice exhibited reduced IL-33 levels and failed to accumulate ILC2s and Tregs in AT, suggesting that PLZF$^+$ γδ T cell-produced cytokines modulate the number of IL-33-producing stromal cells. Moreover, PPARβ overexpressing mice, which exhibited lower αβ T cells and higher γδ T cells, were protected from HFD-induced AT inflammation and IR (278). Intriguingly, it was demonstrated that γδ T cells were initially increased in AT of ketogenic diet-fed mice but then decreased following the development of obesity (279). **Table 4** highlights the fact that different γδ T cell subtypes exhibit a differential metabolic profile depending on their polarization (256).

iNKT Cells

iNKT cells represent a subset of the innate-like T lymphocytes, NKT cells, that recognize glycolipids presented on MHC-I-like family protein CD1d and express a conserved semi-invariant TCR that recognizes the prototypic ligand α-galactosylceramide (178). Indeed, adipose iNKT cells exhibit a distinct transcriptome from that of iNKT cells residing in other tissues with both anti-inflammatory and pro-inflammatory characteristics, secreting Th1-recruiting IFN-γ and Th2-recruiting IL-4 (280, 281). iNKT cells are suggested to modulate WAT immunity in setting of leanness and obesity (282, 283). iNKT cells were enriched in visceral AT of humans and mice and in mouse sub-cutaneous WAT, where CD1d-expressing M2 macrophages and adipocytes promptly activate iNKT cells (283–285). WAT iNKT cells contribute to metabolic homeostasis through the secretion of IL-2 and IL-10, which regulate M2 macrophages and Tregs function, respectively (283). In obesity, the number of AT iNKT cells decline with WAT inflammation (284–288). Also, iNKT cells were shown to be dysfunctional in patients suffering from obesity or T2D exhibited by a diminished capacity to secrete IL-2 (289). Alternatively, HFD-fed mice deficient in AT iNKT cells were prone to obesity and IR which were reversed upon the adoptive transfer of iNKT cells (290, 291). Importantly, the hypoxic condition of expanding AT favors the upregulation of HIF-1α (10, 292). iNKT cells respond to hypoxia by upregulating the CD1d-mediated cytokine response (293). Furthermore, leptin activates iNKT cells resulting in their anergy and PD-1 upregulation (294, 295). Moreover, the inhibition of the synthesis of glucosylceramide, which can be presented on CD1d, in adipocytes was shown to impair iNKT cell activity and cytokine production (296). Several lines of evidence suggest that iNKT cell metabolism contributes to their development and functioning (shown in **Table 4**).

Innate Lymphoid Cells

Innate lymphoid cells have been previously regarded as enigmatic lymphocyte-like cells that possess the morphological features of a lymphocyte in the immature state but lack its surface markers, and are thus described as "lineage negative." ILCs

TABLE 5 | Metabolic pathways of innate lymphoid cells.

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
ILC1s and NK Cells	Glycolysis	⇑	Required for cytotoxicity and IFN-γ production Not required for NK cell degranulation	IL-2 or IL-12/15-stimulated peripheral blood NK cells	(308)
			NK cell proliferation and cytotoxicity	IL-15-activated NK cells *in vitro* and MCMV infection in mice	(309)
	Oxidative Phosphorylation	⇑		IL-2 or IL-12/15-stimulated peripheral blood NK cells	(308)
				Primary murine NK cells	(310)
			Not required for IFN-γ production	IL-12 and IL-18-stimulated primary murine NK cells	
			Required for NK cell activation	NK receptor-activating stimulation of primary murine NK cells	
ILC2s	Glycolysis	⇓	Required for proliferation and cytokines production	Arg1-deficient ILCs in mice	(311, 312)
			?	Arg1-deficient ILC2s in a mouse model of helminth infection	(56)
			Required for ILC2 development	Conditional deletion of E3 ubiquitin ligase VHL in innate lymphoid progenitors	(313)
			Required for ILC2 homeostasis and cytokine production	$Atg5^{-/-}$ mice	(314)
	Fatty Acid Oxidation	⇑	Required for accumulation and production of IL-13 and IL-5	$Rag1^{-/-}$ mouse model of helminth infection and malnutrition	(315)
			Required for ILC2 homeostasis and cytokine production	$Atg5^{-/-}$ mice	(314)

⇑, *high metabolic rate;* ⇓, *low metabolic rate;* ?, *require further investigation.*

include three transcriptionally-defined groups; Tbet-dependent ILC1s (which include NK cells) that secrete IFN-γ and TNF-α, GATA3-dependent ILC2s that secrete IL-5/IL-13 and IL-10, ROR-γt-dependent ILC3s that secrete IL-17A/IL-22 and finally Id3-dependent ILCregs that produce IL-10 and require autocrine TGF-β1 (297). Importantly, recent evidence demonstrated the presence of all ILC subsets in different AT depots, where they are implicated in AT immune responses (298).

Adipose Tissue ILC1s and NK Cells

AT-resident ILC1s and NK cells are highly enriched in WAT in both humans and mice, and further increase at the setting of obesity and T2D, where they positively correlated with IR (299, 300). ILC1s and NK cells drive AT inflammation in obesity by secreting IFN-γ and promoting M1 macrophage polarization (299, 301). However, the enrichment of ILC1s and NK cells in WAT at steady state suggests homeostatic roles (302). Indeed, ILC1s and NK cells were shown to regulate the survival of ATMs by killing AT M2 macrophages (302). Nevertheless, the physiological relevance of this regulation is questioned since mice and human deficient in ILC1s do not display major metabolic derangements (303, 304). ILC1s and NK cells exhibit a distinct metabolic program following activation. These alterations drive cellular functions, cytotoxicity, and inflammatory cytokines production (305–307). **Table 5** highlights metabolic pathways implicated in ILC1s and NK cells activity and function.

Adipose Tissue ILC2s

ILC2 are key regulators of lean AT homeostasis (298). ILC2s are enriched in visceral AT, where they represent the predominant producers of IL-5 and IL-13 which are essential for the recruitment of eosinophils (207). Indeed, the recruitment and proliferation of AT ILC2s is driven by IL-33 whose origin is still debated (297, 316). Moreover, AT ILC2s express ICOSL, which signals to Tregs through ICOS and drive their accumulation in visceral AT at steady state, a process abrogated in obesity by IFN-γ (317). Moreover, ILC2s upregulate OX40L following their stimulation by IL-33 which is essential for the recruitment of Treg cells into the AT (318). The exact mechanisms leading to the reduction of AT ILC2s number in obesity is not well-understood. Nevertheless, one possible mechanism includes the expansion of ILC1s where ILC1-derived IFN-γ antagonizes ILC2s (317). Another mechanism implicates IL-12 in driving the conversion of ILC2s to ILC1s in the context of diet-induced obesity (DIO) (319). Metabolic pathways in ILC2s govern their proliferation and function. Shifting the balance between OXPHOS and glycolysis toward glycolysis impairs the development and function of ILC2s (311, 312). Metabolic pathways implicated in ILC2s metabolism are summarized in **Table 5**.

B Cells

B lymphocytes are further subdivided into 2 major classes; B-1 and B-2 depending on their developmental origin,

microenvironmental niches and the requirement of Th cells to produce antibodies (320). B-1 cells are further stratified to B1-a and B1-b cells, which differ by their surface expression of CD5. B cell-secreted IL-10, IL-35 and TGF-β are characteristics of the functionally-distinct Breg cells that can derive from both B-1 and B-2 cells. Breg cells suppress Th1 and Th2 polarization, and inhibit macrophage and dendritic cell activation and cytokine production. B-1 and B-2 cells coexist in perivascular AT, epicardial WAT and BAT, where the B1:B2 ratio is higher than that in sub-cutaneous WAT but yet varies greatly in a depot-specific manner (321, 322). B cells are among the first immune cells to infiltrate AT following the consumption of a HFD consistent with an increased IR, where AT B-2 cells are thought to promote inflammation (323–327). B cell abundance also increases in BAT following the consumption of a HFD, where their role is poorly understood (328). Indeed, B cells global deficiency in mice attenuated HFD-induced AT inflammation and reduced IR (324, 325). Consistently, circulating B cells from obese, diabetic and obese diabetic individuals produced higher amounts of pro-inflammatory cytokines in comparison to healthy individuals (329, 330). In addition, B cell-produced pro-inflammatory immunoglobulins were elevated in visceral AT of obese mice activating macrophages to secrete inflammatory cytokines (324, 331). Contrary to B-2 cells, B-1 cell-derived natural IgG and anti-inflammatory cytokines block AT inflammation and improve glucose tolerance through inducing M2 macrophage polarization and increasing their production of IL-10, while reducing their production of IL-6 and TNF-α (321, 322, 332–335). The number of Breg cells, which are present in AT, is reduced in diabetic patients in comparison to healthy donors (336, 337). Moreover, Breg cells from diabetic patients secrete less IL-10 (329, 330). Indeed, the adoptive transfer of Breg cells ameliorated AT inflammation and IR in DIO mice (336). Different B cell subsets exhibit distinctive metabolic profiles depending on their particular microenvironments and thus, careful interpretation of B cell metabolic data is pivotal (338). The metabolic pathways implicated in B cell metabolism are summarized in **Table 6**. Metabolic rewiring of different subsets of B cells and plasma cells have also been discussed in details elsewhere (338).

IMMUNE CELL CONTRIBUTION TO DEPOT-SPECIFIC ADIPOSE TISSUE INFLAMMATION

While AT is broadly classified in WAT and BAT, WAT is further divided into several distinct depots that differ in their properties and microenvironments including subcutaneous (scWAT) and visceral WAT (VAT) depots. The latter includes epicardial (EpiCAT), perivascular (PVAT), epididymal (EpiWAT), mesenteric (MAT) and perirenal (PRAT) AT. VAT has been extensively studied due to the association between visceral obesity and the emergence of CVD risks (345, 346). It was demonstrated that scWAT exhibits a greater potential than VAT to undergo beiging, a process by which white adipocytes become brown-like and participate in energy dissipation (2, 347). Indeed, the induction of VAT beiging has been largely regarded as an approach to curb obesity and its accompanying metabolic and cardiovascular derangements (348, 349). Nevertheless, several visceral adipose depots including PVAT and EpiCAT intrinsically possess a beige phenotype and the implications of thermogenic induction in these particular tissues on cardiovascular functioning is not yet well-characterized, especially that the immune landscape of these tissues is less known (33). In the below sections, an overview of changes in immune cell function, population, and activity, as well as the alterations in adipokine and cytokine profile across different depots will be provided with particular emphasis on PVAT and EpiCAT due to their relevance to CVD in metabolic impairment.

Subcutaneous Adipose Tissue

The mass of scWAT is positively correlated with BMI in obese subjects (350). A dysregulated scWAT in patients with MetS exhibits higher macrophage infiltration and CLS formation, which is accompanied by a dysregulated adipokine profile (351). Indeed, scWAT inflammation is linked to the development of IR (214, 352). Interestingly, a sustained scWAT low-grade inflammation extending beyond weight loss was reported (353, 354). This sustained inflammation has been attributed to the accumulation of effector memory T cells (354). This contradicts with recent reports that demonstrated VAT but not scWAT inflammation as a manifestation of obesity (355, 356). Such discrepancies may arise from differences in diet composition and the duration of HFD-feeding. The general consensus however is that scWAT inflammation participates in driving IR and the MetS in obese subjects with the implication of various immune cells.

It was shown that an increased abundance of eosinophils in scWAT of MetS patients, is accompanied with IR, tissue fibrosis, and adipokine dysregulation (357). Macrophage infiltration and CLS formation are also elevated in scWAT of obese and diabetic patients (351, 358). Interestingly, an accumulation of M2 macrophages in scWAT but not in VAT of obese patients is associated with inflammation limitation (359). Moreover, the abundance of MCs is increased in scWAT of MetS subjects and is significantly correlated with IR, leptin, IL-1β, IL-6, and the activities of MAPK and NF-κB in circulating monocytes (224). Similar to eosinophils, scWAT MCs are correlated with markers of AT fibrosis and angiogenesis (224). The number of total dendritic cells is reduced but that of pDC increases in the scWAT of subjects with T2D implicating pDCs in scWAT low-grade inflammation (360).

Epididymal Adipose Tissue

EpiWAT is a metabolically active visceral fat pad, which is anatomically attached to the testis and epididymis, then stretches out toward the diaphragm (361). EpiWAT low-grade inflammation is thought to contribute to the initiation of IR, MetS and its related cardiovascular derangements (362). Indeed, macrophage infiltration and accumulation in EpiWAT is at the core of this inflammation (222, 362). Interestingly, Chronic DIO eventually leads to decreased EpiWAT mass, which correlated negatively with body weight and was associated with a widespread of CLSs and MCs, together with an impaired

TABLE 6 | Metabolic pathways implicated in B lymphocyte proliferation, differentiation, activation, and function.

Immune cell	Metabolic pathway	Metabolic reprogramming	Relevance of metabolic pathway to cellular function	Model	References
B cells	Glycolysis	⇑	Required for proliferation and antibody secretion	Rag1$^{-/-}$ mice LPS, anti-IgM or CpG oligodeoxynucleotide-stimulated mouse B cells B cell-specific Glut1 deletion Chronic BAFF stimulation of B cells	(339)
			B cell antigen receptor (BCR)-mediated growth	p85α-deficient mice	(340)
			Limited time frame BCR-mediated metabolic activation	Anti-IgM and CpG-stimulated mouse B cells B cells of MD4 mice treated with oligomycin, 2-DG and FCCP	(341)
		⇓	In germinal center B cells	*Ex vivo* germinal Center B cells Cpt2-knockdown B cells	(342)
	Oxidative phosphorylation	⇑	Required for B cell growth and differentiation	IL-4-stimulated and oligomycin-treated primary mouse B cells	(343)
			Limited time frame BCR-mediated metabolic activation	Anti-IgM and CpG-stimulated mouse B cells B cells of MD4 mice treated with oligomycin, 2-DG and FCCP	(341)
	Pentose phosphate pathway	⇑		p85α-deficient mice	(340)
	tca cycle	⇑		IL-4-stimulated and oligomycin-treated primary mouse B cells	(343)
		⇓	In Germinal Center B cells	*Ex vivo* germinal Center B cells Cpt2-knockdown B cells	(342)
	Lipogenesis	⇑	Proliferation and expansion of endomembrane network in response to LPS	LPS-stimulated murine splenic B lymphocytes CH12 B lymphoma cells	(344)
	Glutamine metabolism	⇑	Required for B cell growth and differentiation	IL-4-stimulated and oligomycin-treated primary mouse B cells	(343)

⇑, *high metabolic rate;* ⇓, *low metabolic rate.*

adipokines gene expression, which could be attributed to the increased abundance of dysfunctional or dead fat cells (361).

EpiWAT neutrophils of HFD-fed mice exhibited an increased IL-6 expression in EpiWAT due to an adipocyte-contact-dependent activation of NF-κB (182). Moreover, it was suggested that HFD-induced EpiWAT fibrosis was attributed to macrophages and MCs (222). MCs were also shown to have a role in HFD-stimulated adipocytes senescence in EpiWAT (222). It has also been shown that the consumption of a HFD induces an elevation of NK count and the production of pro-inflammatory cytokines in EpiWAT at an early phase of obesity induction, which was linked to increased fasting glucose, insulin levels and ATMs count (301). Interestingly, NK-mediated EpiWAT derangements were IFN-γ-dependent at early stages and then became TNFα-dependent (363). Moreover, studies revealed an increase in B cells in EpiWAT of HFD-fed mice, which was associated with IR (320). These B cells were shown to drive VAT inflammation by regulating the activity of VAT T cells and macrophages through the production of pro-inflammatory cytokines including IL-6 and INF-γ (320). HFD induces an increase of the immature DCs count in EpiWAT, which has a role in adipose tissue inflammation through triggering a parallel increased production of Th17 cells via promoting an excessive production of IL-6, TGF-β, and IL-23 (162).

Perivascular Adipose Tissue

PVAT environs most of the blood vessels including the aorta and coronary and subcutaneous small arteries. PVAT play a crucial role in supporting blood vessels by maintaining vasomotor tone and insulating them from surrounding environment (364). It communicates with neighboring VSMCs and ECs in a paracrine manner through the production of various adipokines influencing the vascular tone (365). Indeed, PVAT-derived adipokines infiltrate into vasculature and serve as either vasodilators or vasoconstrictors. As such, an adipokine profile imbalance in PVAT toward proinflammatory adipokines is suggested to drive vascular derangements in metabolic disorders through the disruption of PVAT anticontractile activity. Furthermore, PVAT inflammation has been reported in states of nutritional excess through the recruitment of pro-inflammatory cells. This interaction provides the framework by which PVAT inflammation impairs vascular function (366–369).

PVAT infiltrating immune cells include macrophages, T cells, NK cells, and DCs that produce both inflammatory and anti-inflammatory cytokines, depending on the adipokine profile shifts (363, 370–373). Interestingly, both B cell subtypes occur in PVAT, where B-2s promote the development of diet-induced atherosclerosis and B-1s inhibit it by reducing MCP-1 and TNF-α production (334, 374, 375). However, PVAT possesses a higher

B1:B2 ratio and thus, B cells have an anti-inflammatory role in PVAT (322). Recent studies done on rat PVAT inflammation demonstrated that the increased production of IL-1β and TGF-β1 was correlated with a reduced AMPK activity and endothelial relaxation impairment (32). On the other hand, there was an increase in AT hypertrophy, oxidative stress, and Rho-associated kinase (ROCK)-mediated Ca^{2+} sensitivity (32, 376). Other factors associated with PVAT inflammation include adipocyte derived MCP-1 and low-density lipoprotein receptor related protein-1 (377, 378).

On the other hand, several PVAT adipokines were reported to have an ameliorative effect on vascular function. Indeed, the treatment with the adipokine irisin normalizes the reduced anti-contractile properties of aortic PVAT in obese mice (379). Adiponectin was also shown to attenuate vascular inflammation and atherosclerosis possibly through blocking NF-κB signaling and downregulating the expression of VCAM-1 and ICAM-1 (380–383). Moreover, PVAT-derived adiponectin normalizes endothelial function partly through enhancing endothelial eNOS phosphorylation (384). Similarly, omentin was suggested to have an anti-atherogenic potential in concert with circulating adiponectin (385). In fact, omentin was demonstrated to restore endothelium-dependent relaxation by inhibiting ROS and enhancing NO production in high glucose-treated endothelial cells (386, 387).

Other PVAT-derived adipokines include vaspin, which modulates ER stress by upregulating the phosphorylation of Akt and AMPK (388), and apelin, which maintains vascular structure by upregulating endothelial NO (389, 390). Despite the positive correlation between serum leptin levels and vascular calcification (391), PVAT-derived leptin was reported to exert an anticontractile effect when in synergy with other vasorelaxing factors (392, 393). Nevertheless, PVAT-derived leptin also promotes VSMC phenotypic switch by increasing the phosphorylation of p38 MAPK (394–396). Chemerin was demonstrated to promote aortic atherosclerosis by promoting NF-κB signaling and p38 MAPK phosphorylation (397, 398). Additionally, and via the activation of NLRP3 signaling, the adipokine visfatin was shown to induce vascular endothelial dysfunction and tissue inflammation (399, 400). Finally, resistin was demonstrated to activate the renin-angiotensin system inducing hypertension (401, 402). Changes in PVAT adipokine environment and immune cell activity brought about by metabolic dysfunction is depicted in **Figure 2**.

Epicardial Adipose Tissue

EpiCAT is located in the atrioventricular and interventricular heart grooves and plays a role in providing FA to the myocardium. Indeed, EpiCAT represents 15% of the cardiac mass, and as the epicardial fat increases, the ventricles and the epicardial surfaces get covered by EpiCAT. Moreover, EpiCAT surrounds the adventitia of coronary arteries and plays a cardioprotective role during metabolic and mechanical insults (33). The endocrine function of EpiCAT has witnessed extensive investigation as EpiCAT dysfunction was implicated in cardiovascular diseases. It regulates FA homeostasis to prevent lipotoxicity, while secreting anti-inflammatory and anti-atherogenic adipokines under healthy conditions (403, 404). However, EpiCAT alters its adipokines to release FA and pro-inflammatory cytokines under metabolic insults (404, 405). Several studies reported the expression of numerous adipokines in EpiCAT including adiponectin, omentin, adipsin, leptin, resistin, visfatin, chemerin and adrenomedullin (406). While the EpiCAT expression of resistin, leptin and TNF-α increase in obesity, the expression of adiponectin is markedly reduced (407, 408). In addition, EpiCAT adiponectin expression is also decreased while that of leptin increased in CAD patients (409, 410). Importantly, the administration of recombinant adiponectin can reverse the harmful effects of dysfunctional EpiCAT-derived factors (409). Another study demonstrated a differential expression of adiponectin, visfatin, leptin, chemerin and vaspin in periaortic, pericoronary and apical EpiCAT, where these adipokines were correlated with either aortic or coronary atherosclerosis (411). Furthermore, the expression level of omentin was decreased in CAD patients (412). Importantly, it was suggested that exogenous omentin supplementation might support a cardioprotective role through its anti-inflammatory effect on EpiCAT (413). Increased levels of resistin in EpiCAT were also reported in patients with advanced coronary atherosclerosis and patients with acute coronary syndrome (414, 415). Similarly, higher chemerin levels were observed in CAD patients which was correlated with an increased EpiCAT volume (416, 417). Interestingly, the level of chemerin was positively correlated with the severity of coronary atherosclerosis in CAD patients (416).

Immunohistochemistry done on EpiCAT confirmed the presence of infiltrating $CD3^+$ T cells, $tryptase^+$ mast cells, and $CD68^+$ macrophages. These immune cells have been shown to be unique to EpiCAT when compared to scWAT (418). A study showed that patients with coronary artery disease (CAD) had a significant increase in macrophage infiltration into EpiCAT compared to individuals without CAD (419). Furthermore, the levels of IL-6, IL-1β, MCP-1, and TNF-α were higher in EpiCAT compared to scWAT (418). Changes in EpiCAT adipokine environment and immune cell activity brought about by metabolic dysfunction is depicted in **Figure 2**.

Mesenteric Adipose Tissue

MAT is located between the gut and the liver. Several lines of evidence associate MAT expansion to an elevated risk for the development of peripheral and central IR as well as CVDs (327, 420). In response to HFD consumption, MAT adipocytes secrete high amounts of MCP-1, which intensifies the inflammatory response by modulating macrophage infiltration driving IR and atherosclerosis (421). Similar to MCP-1, GM-CSF is highly expressed in MAT of obese animals. It has been shown that both GM-CSF and B cells play an important role in the activation and accumulation of macrophages besides the production of pro-inflammatory cytokines in MAT of HFD-fed animals (327, 422). It is worth noting that B cells are among the earliest immune cells infiltrating MAT in HFD-induced AT inflammation models (327). Likewise, an increased accumulation of mast cells was shown in MAT of HFD-fed mice, which was associated with

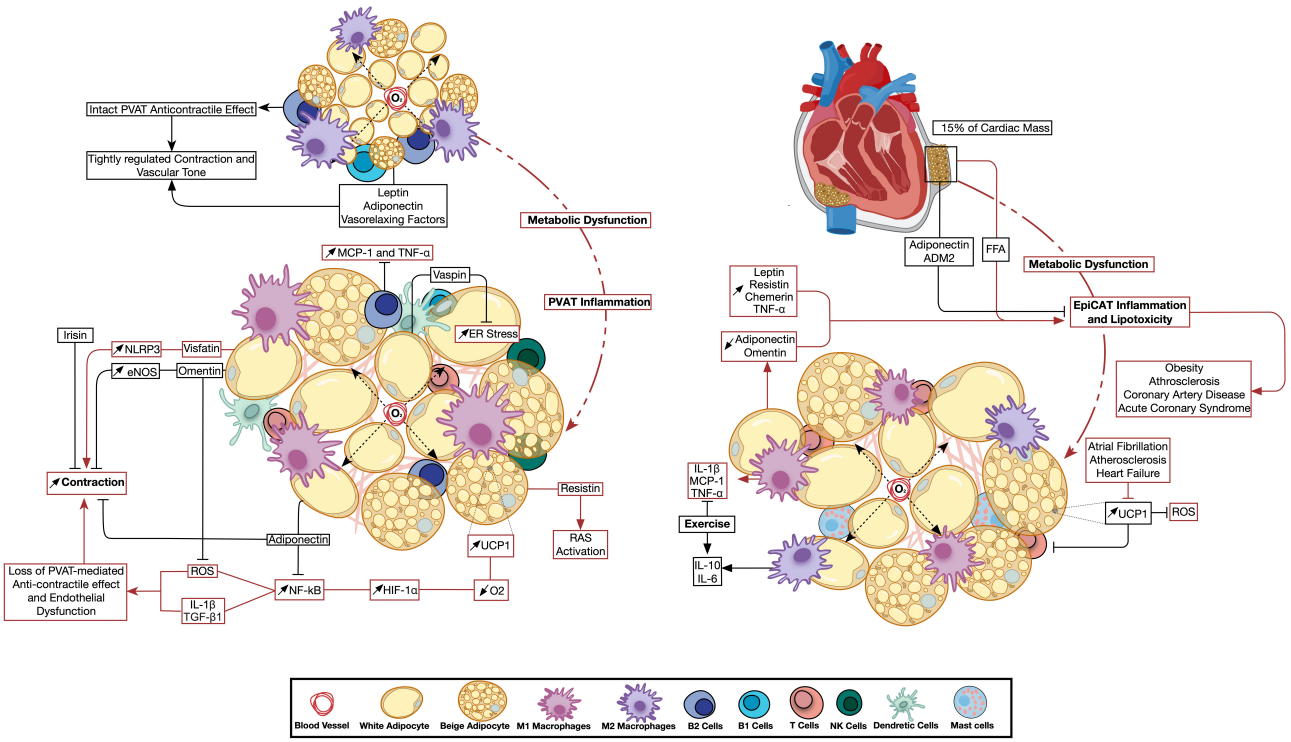

FIGURE 2 | Perivascular and epicardiac adipose tissue dysfunction: the emerging role of immune cell and adipokine profile dysregulation. Metabolic impairment triggers changes in PVAT (left) and EpiCAT (right) adipokine environment and immune cell activity. Inflammation has a direct effect on the neighboring vascular and cardiac tissue. Changes in UCP1 expression were reported to have opposite effects in either depot. Pathways active in basal PVAT and EpiCAT homeostasis are depicted in black arrows, while those activated during inflammation are shown in red. ADM2, Adrenomedullin-2; eNOS, Endothelial Nitric Oxide Synthase; EpiCAT, Epicardial Adipose Tissue; ER Stress, Endoplasmic Reticulum Stress; HIF-1α, Hypoxia-induced Factor 1 Alpha; IL, Interleukin; MCP-1, Monocyte Chemoattractant Protein 1; NF-κB, Nuclear Factor Kapp-light-chain-enhancer of Activated B cells; NLRP3, NLR Family Pyrin Domain Containing 3; O2, Oxygen; PVAT, Perivascular Adipose Tissue; RAS, Renin Angiotensin System; ROS, Reactive Oxygen Species; TGF-β, Transforming Growth Factor Beta; TNFα, Tumor Necrosis Factor Alpha; UCP1, Uncoupling Protein 1.

tissue fibrosis and IR. These alterations occurred coincidently with the progression of obesity and diabetes (225).

Perirenal Adipose Tissue

PRAT, the AT surrounding the kidney, was previously assumed to merely mechanically support the kidneys. However, several studies postulated that not only PRAT has a pronounced role in regulating kidney function but is also associated with cardiometabolic risk factors. Clinical studies suggest that excess PRAT is associated with higher risk of CVDs (423, 424). The weight of PRAT has the highest partial correlation coefficient with CVDs among other AT (425). Indeed, excess PRAT is believed to contribute to the decrease in kidney function, regardless of obesity, in hypertensive patients (426). A recent study reviewed the possible mechanisms of PRAT in regulating CVDs including neural, humoral, and direct kidney related regulation (425). PRAT is shown to synthesize and secrete adipokines and several pro-inflammatory cytokines (425, 427). PRAT in pigs with obesity-related metabolic dysfunction showed elevated levels of pro-inflammatory macrophage infiltration and TNF-α expression (428). Moreover, excess PRAT secrets leptin which in turn activates MAPK pathway and further exacerbates renal vascular and endothelial damage (429). An interesting study in rats have shown that injecting Leptin directly into PRAT activated the adipose afferent reflex without changing the systemic sympathetic activity, indicating a direct regulation of cardiovascular function by PRAT (430). Interestingly a study on diabetic fatty rats found that the inhibition of PRAT inflammation, mainly inhibiting IL-6, IL-1b, and TNF-α, reduced renal inflammation and alleviated diabetic nephropathy (431). As such PRAT inflammation is assorted with adverse cardiometabolic risk factors and is a main predictor of CVD.

THE ADIPOSE IMMUNE SYSTEM AS O REGULATOR OF ADAPTIVE THERMOGENESIS

UCP1-Dependent and Independent Thermogenesis

Uncoupling protein 1 (UCP1) is an inner mitochondrial membrane protein that uncouples oxidative phosphorylation from the production of ATP through a FA/H^+ symport mechanism (2). UCP1 expression is mainly driven through

β3-adrenoreceptors (β3-AR) stimulation by sympathetically and non-sympathetically produced norepinephrine in thermogenically active adipocytes. Although being the most efficient and qualitatively significant thermogenic effector, it was demonstrated that UCP1 is dispensable for cold-induced and diet-induced thermogenesis. Therefore, it was proposed that less-efficient thermogenic pathways downstream of β3-ARs also contribute to adaptive thermogenesis (432).

Creatine cycling, that is the phosphorylation of creatine by creatine kinase and its subsequent hydrolysis, participates in energy transfer from ATP-rich to ATP-poor cellular regions (2). Creatine futile cycling appears to occur in all fat depots and blocking creatine cycling promotes obesity in HFD-fed mice (2). Lipolysis/re-esterification cycling has also been proposed to mediate adaptive thermogenesis based on the ATP demand of triacylglycerol synthesis (2). This pathway proposes that adipocytes break down fat and subsequently re-esterifies FAs by way of glycerol 3-phosphate. Importantly, It was also shown that triglyceride/FA cycling is induced in WAT upon HFD feeding (433).

A role for calcium transport in non-shivering thermogenesis has also been proposed (2). Calcium sequestration in the sarcoplasmic (SR) and endoplasmic (ER) reticulum is mediated by the SR/ER calcium ATPase (SERCA) pump. SERCA activity in the AT is regulated by phospholamban (PLB) (434). Interestingly, it was shown that PLB is upregulated in UCP1-deficient beige fat with no difference in the expression of SERCA suggesting compensatory thermogenesis (435).

Finally, the UCP1-independent proton leak by the ubiquitously expressed inner membrane protein, mitochondrial ADP/ATP carrier (ACC), that is initiated at high membrane potential, contributes to adaptive thermogenesis (2).

Adaptive Thermogenesis Across Adipose Depots

Brown Adipose Tissue

The first insights into the implication of BAT in thermogenesis and its contribution to energy expenditure started with the demonstration of a reduced GDP binding to BAT mitochondria of cold-exposed obese ob/ob mice relative to lean siblings (436). Then, Rothwell and Stock observed an increased sympathetic activity in BAT following overnutrition in rats (437). The identification of human BAT and the subsequent observations that a reduced BAT level induces obesity ignited investigation into BAT-mediated non-shivering thermogenesis. In comparison to WAT, which is more prone to inflammation than BAT (31), relatively little is known about the processes driving BAT chronic inflammation. However, increasing evidence suggests that BAT inflammation alters its thermogenic activity through the induction of IR (438, 439). Although mainly composed of brown adipocytes and their precursors, BAT also contains a variety of immune cells such as neutrophils, macrophages and lymphocytes (440, 441). Chronic inflammation of BAT was associated with a shift of BAT immune cells where M1 macrophages drive BAT whitening (442, 443).

Subcutaneous Adipose Tissue

Cold exposure and β_3-AR stimulation induced the expression of UCP1 in scWAT of humans (444, 445). Nevertheless, despite the increased UCP1 expression in scWAT, cold acclimation was shown to reduce mitochondrial uncoupling-mediated fat oxidation in inguinal scWAT, while increasing the capacity to export FAs (446). Indeed, the consumption of HFD induced scWAT inflammatory and immune responses (447). These derangements were reversed by intermittent fasting, which increased the expression of UCP1, β_3-ARs and adiponectin, while it attenuated the expression of pro-inflammatory and pro-apoptotic markers in scWAT (448). In an AMPK gain of function mutant mice, scWAT exhibited a morphological similarity to brown adipocytes with no detectable UCP1 expression but increased energy expenditure suggesting the activation of UCP1-independent thermogenesis (449). It was demonstrated that PPARγ agonism induced scWAT browning, while PPARγ deletion in inguinal scWAT inhibited thermogenesis and was associated with IR (450, 451).

Perivascular Adipose Tissue

The peculiarity of PVAT, being a hybrid AT and especially the resemblance of aortic PVAT to classical BAT in morphology and UCP1 expression, suggests that PVAT possesses a similar thermogenic potential (369). Indeed, it was shown that PVAT deletion resulted in a reduction of whole body temperature (452). The proximity of PVAT to the vascular wall suggests a possible implication of PVAT thermogenic processes on the pathophysiology of vascular diseases (33). We recently identified an increased expression of UCP1 in PVAT of HFD-fed rats, which was associated with localized PVAT inflammation contributing to MetS-associated vascular dysfunction (32). The targeting of PVAT UCP1 was also put forward as means to limit its detrimental effect on PVAT hypoxic predisposition (33). Such a proposition was made based on an assumed exaggerated oxygen consumption triggered by increased UCP1 expression and further complicated by the observed adipocyte hypertrophy in a combination of events less likely to occur in other adipose depots. However, increased UCP1 expression is typically viewed as beneficial where it serves as a route of energy assimilation that might be of value in diabetes and obesity. Yet, many of the tools shown to increase adipocyte glucose consumption and increased UCP1 expression *in vitro* failed to produce any effect when used *in vivo*, and even resulted in an opposite effect of decreased UCP1 expression (453, 454).

Epicardial Adipose Tissue

EpiCAT adipocytes express genes and secrete adipokines that are involved in thermogenesis (455). Adult human EpiCAT was shown to possess molecular features characteristic of beige adipocytes with relatively abundant expression of UCP1 (456). Opposite to findings in PVAT, an increased expression of UCP1 in EpiCAT was associated with a downregulation of ROS production and immune response (457, 458). Indeed, EpiCAT thermogenic activity was impaired in patients suffering from atrial fibrillation and heart failure with reduced ejection fraction (459, 460). Moreover, during the progression of atherosclerosis,

EpiCAT was shown to undergo a phenotypic conversion from BAT to WAT, which further promoted the development of atherosclerosis (461). Nevertheless, exploiting EpiCAT browning for the treatment of CVDs remains controversial (33). As such, detailed examination of the role of thermogenesis modulation in PVAT and EpiCAT is required since both depots are particularly pertinent to the development of CVD in metabolic dysfunction.

Perirenal Adipose Tissue

Human PRAT has been shown to possess unilocular and multilocular UCP1$^+$ adipocytes (462, 463). Indeed, several studies associated PRAT browning with aging and the female sex (464, 465). Also, bigger unilocular adipocytes with reduced UCP1 expression were detected in the PRAT of hypertensive patients (466).

Epididymal Adipose Tissue

EpiWAT expresses UCP1 in rats age-dependently (467). The chronic agonism of PPARγ in EpiWAT promoted UCP1 expression and WAT browning (347). Indeed, the ectopic expression of very low levels of UCP1 in EpiWAT was shown to reverse IR in obese mice and epididymal beige adipocytes were shown to employ prominent creatine cycling (468, 469). Cold exposure improved metabolic dysfunction in obese mice through activating BAT thermogenesis and inducing EpiWAT browning (470). Moreover, cold-induced browning of VAT and improvement of insulin sensitivity were blunted following the knockdown of UCP1 in EpiWAT (471). Additionally, housing mice at room temperature induced EpiWAT thermogenesis, which was associated with a decreased M1 macrophage infiltration and improved insulin sensitivity (472). It was also shown that infused M2 macrophages in obese rats homed to EpiWAT reversing the M1 macrophage-dominant phenotype, enhancing UCP1 expression and ameliorating IR (473).

Mesenteric Adipose Tissue

It was demonstrated that β3-AR agonism in HFD-fed rats not only decreased the mass of WAT but also induced the appearance of multilocular, UCP1$^+$ adipocytes in MAT (474, 475). These early observations indicated that MAT can be thermogenically induced. Indeed, cold exposure induced a sympathetic response in MAT of rats, evidenced by an increased level of tyrosine hydroxylase (476). Importantly, chronic cold exposure induces non-sympathetic catecholamine production leading to an increased level of NE in addition to the stimulation of M2 macrophage infiltration, pro-inflammatory cytokines reduction, and UCP1 induction (476, 477).

Adipose Immune System and Adaptive Thermogenesis

Adipose Immune Cells and β_3-AR Stimulation

β-AR stimulation is pivotal to the induction of thermogenesis. Sympathetically-released NE stimulates the release of adipokines and FGF21 from adipocytes, promoting PGC1α and UCP1 expression, oxidative metabolism, and mitochondrial biogenesis (478, 479). FGF21 also induces the release of CCL11 in murine scWAT, which promotes the recruitment of IL-4-secreting eosinophils and the proliferation of PDGFRα$^+$ beige adipocytes in an IL-4Rα-dependent manner (480, 481). Moreover, eosinophils and ILC2s were shown to induce β_3-AR signaling through IL-4/IL-13-dependent induction of tyrosine hydroxylase expression in ATMs, promoting the release of NE (215, 482). Also, the selective deletion of Mecp2 in BAT macrophages reduces UCP1 expression as a result of impaired innervation (441). Nevertheless, recent evidence suggests that ATMs are not likely to contribute to the induction of adaptive thermogenesis by directly producing NE (483). Sympathetic neuron-associated macrophages increased in HFD-fed mice AT and were recently shown to express the NE transporter, SLC6A2 and the NE degrader, monoamine oxidase (MAO), where the inhibition of SLC6A2 increased AT thermogenesis (484). Conversely, CLS-associated ATMs were shown to phagocytose white adipocytes and secrete chemokines that drive the recruitment of beige adipocyte precursors (263). Tregs were also shown to enhance β_3-AR signaling in scWAT but not in VAT of female and to a lesser extent in male mice by suppressing M1 and inducing M2 macrophages (485). γδT cells were also shown to promote AT innervation by driving the expression of TGFβ1 in parenchymal cells via the IL-17 receptor C (IL-17RC), where the ablation of IL-17RC signaling pathway or γδT cells impaired sympathetic innervation and thermogenesis (486). The interaction among immune cells, adipocytes, and sympathetic nerve terminals is summarized in **Figure 3**.

Macrophages

M1 macrophages suppress the induction of thermogenic adipocytes in obese AT of mice (487). Conversely, adiponectin-induced M2 macrophages drive scWAT thermogenesis in cold-exposed mice and the depletion of either the macrophages or adiponectin reduces scWAT browning (488). The browning effect of adrenomedullin 2 (ADM2), a white adipocyte-produced factor that increases UCP1 expression, is also mediated by M2 macrophages (489).

The activation of pattern recognition receptors in AT-infiltrating macrophages was shown to suppress thermogenesis. LPS-activated TLR4 receptors of macrophages repressed β3-AR-induced adipocyte browning, caused mitochondrial dysfunction, and increased ROS production (490). Moreover, the activation of NLRP3 inflammasome in macrophages attenuated UCP1 induction in cultured adipocytes in an IL-1β-dependent manner (490). Furthermore, adipocyte-specific deletion of transforming growth factor-activated kinase 1 (TAK1) but not TNF receptor associated factor 6 (TRAF6), increased the expression of beige markers in WAT. TAK1 deletion in WAT increases AMPK phosphorylation, PGC-1α abundance, non-canonical NF-κB signaling, and markers of M2 macrophages while inhibiting canonical NF-κB signaling (491). Conversely, the deletion of TRAF1, an inhibitory adapter of TNFα, IL-1β, and TLRs enhanced leukocyte accumulation and potentiated the proinflammatory signaling of macrophages in HFD-fed mice (492). Nevertheless, TRAF1-deficient mice were protected from metabolic derangements and exhibited an improved IR partially by β3-AR-mediated induction of UCP1-dependent thermogenesis (492).

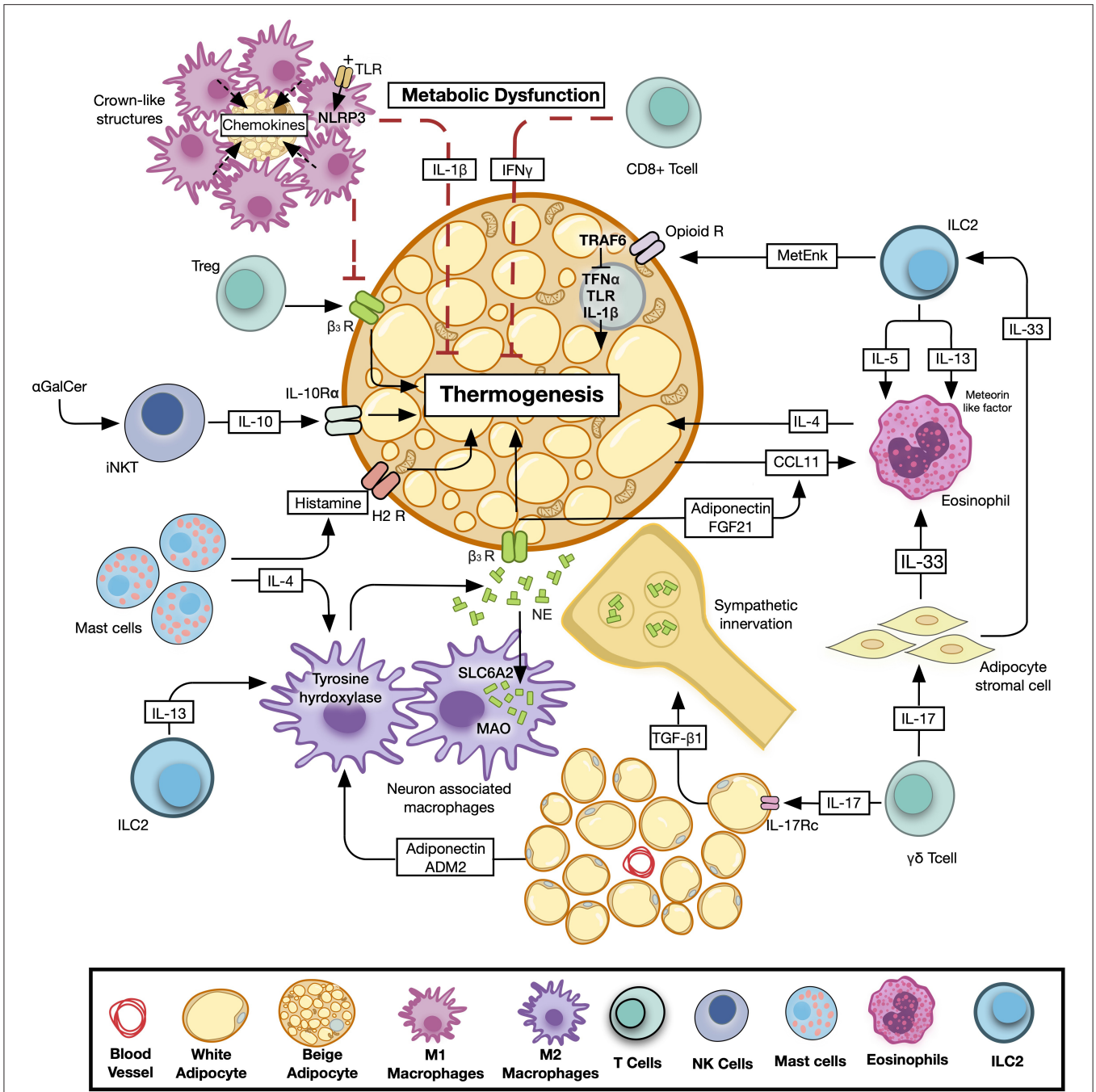

FIGURE 3 | Immune cells-mediated regulation of adaptive thermogenesis. Different types of immune cells exert various modes of control on thermogenesis by either directly modulating the adipocyte function or affecting sympathetic nerve activity and norepinephrine turn-over. Pathways promoting thermogenesis are depicted in black, while inhibitory pathways are shown in red. ADM2, Adrenomedullin-1; β3-AR, Beta 3-adrenergic Receptor; CCL11, C-C motif chemokine 11; FGF21, Fibroblast Growth Factor 21; H2R, Histamine 2 Receptor; γGalCer, Alpha-galactosylceramide; IL, Interleukin; ILC, Innate Lymphoid Cell; MAO, Monoamine Oxidase; NE, Norepinephrine; Opioid R, Opioid Receptor; SCL6A2, Solute Carrier Family 6 Member 2; TGF-β, Transforming Growth Factor Beta; Treg, Regulatory T Lymphocyte.

ILC2s

Activation of murine ILC2s with IL-33 induced the proliferation of beige adipocyte progenitors and increased WAT browning through an IL-4/IL-13-dependent pathway involving eosinophils (480). The recruitment of IL-4^{+} eosinophils was driven by ILC2-secreted IL-5 and IL-13. ILC2-produced BMP7 was also demonstrated to induced the differentiation of adipocyte progenitors into brown adipocytes (493). ILC2s also induce thermogenesis through the production of the opioid-like peptide methionine enkephalin (MetEnk) (316, 494). The stimulation

of ILC2s with IL-33 induced the production of MetEnk that signaled through opioid receptors in scWAT and BAT to promote thermogenesis (316). Mice either treated with MetEnk or adoptive transfer of IL-33-activated ILC2s increased the expression of UCP1 in scWAT even in mice deficient in eosinophils or IL-4Rα demonstrating a direct activity of ILC2s on opioid receptors to induce thermogenesis (316).

γδT Cells

AT-resident γδT cells were recently shown to regulate body temperature through the production of IL-17A upon cold exposure, which regulated IL-33 production by adipose stromal cells (268). Mice deficient in γδT cells or IL-17A exhibited decreases in both $ST2^+$ Tregs and IL-33 abundance in VAT and dysregulated core body temperature at thermoneutrality and upon cold exposure (268). Given the critical role of IL-33 in regulating insulin homeostasis and thermogenesis (495), IL-17-deficient mice were cold-intolerant (268). γδT cell-deficient mice also exhibited a reduced UCP1 expression and energy expenditure upon cold exposure (268).

iNKT Cells

The selective loss of IL-10Rα in adipocytes as well as the global depletion of IL-10 enhanced thermogenesis (496). Early reports demonstrated that the activation of adipose iNKT cells with αGalCer induced potent weight loss in obese mice (282, 284, 286, 497, 498). It was recently shown that iNKT cell-induced weight loss occur through the induction of FGF21-dependent adaptive thermogenesis. The intraperitoneal administration of αGalCer into obese mice induced a significant reduction of AT mass under thermoneutral conditions, which was accompanied by an increased WAT browning and energy expenditure (499). FGF21-deficient mice exhibited a blunted, but not fully ablated response toward αGalCer suggesting that iNKT cells drive thermogenesis through an FGF21-independent mechanism (499).

Mast Cells

BAT MC-released histamine is thought to play a β3-AR-independent role in thermogenesis through its interaction with H2 receptors (500). Upon cold exposure, MCs were recruited to WAT and exhibited an enhanced histamine degranulation in both lean and obese subjects, which was positively correlated with UCP1 expression and thermogenesis (501). Indeed, in response to cold, MCs also release IL-4 along with other factors driving UCP1 expression and WAT browning (502). Nevertheless, it was also proposed that MC deficiency in mice increases WAT browning by promoting adipocyte differentiation as MC-derived serotonin inhibited WAT browning (503). Nevertheless, these findings were based on a murine model in which c-kit tyrosine kinase is mutated and thus, a careful interpretation of the results is required. Furthermore, several other genetic models of MC depletion showed no association between MC function and obesity (228, 229).

T and B Lymphocytes

Several studies revealed a potential function of Tregs of scWAT and BAT in regulating thermogenic homeostasis. Systemic deletion of Tregs impaired oxygen consumption upon cold exposure (504). Additionally, the T cell-specific STAT6/PTEN axis is thought to mediate the link between β_3-AR stimulation and Treg cell induction in both BAT and scWAT (505, 506). Indeed, UCP1-deficient mice exhibited reduced Tregs in BAT and scWAT (506). B and T lymphocytes were also shown to play a role in thermoregulation. Rag1-deficient HFD-fed mice, lacking both T and B lymphocytes, exhibited decreased UCP1 expression (507). Conversely, deleting Rag1 in lean mice housed at room temperature resulted in an increased UCP1 expression and energy expenditure (508). Moreover, a decreased $CD8^+$ but not $CD4^+$ T cells is believed to contribute to adipocyte browning mainly due to a decreased IFN-γ secretion (508).

Eosinophils

Eosinophil-derived IL-4 drives M2 macrophage polarization, promoting the secretion of catecholamines that drive WAT browning (215). As mentioned before, the role of M2 macrophages in local catecholamine production has been questioned. Nevertheless, this does not preclude the implication of eosinophil-derived catecholamines in WAT browning. Indeed, PVAT eosinophils were shown to promote PVAT browning by locally producing catecholamines (509). Moreover, mice lacking eosinophils exhibited an impaired thermogenic capacity of scWAT following cold exposure (215). Meteorin-like is another factor linking eosinophils to WAT browning, where it was shown to stimulate IL-4 secretion from eosinophils and macrophage M2 polarization in AT following cold exposure (510). In addition to ILC2-derived IL-33-dependent eosinophils recruitment to AT, IL-33 was also shown to recruit eosinophils in the absence of ILC2s (178). It was recently demonstrated that the transcriptional repressor krüppel-like factor 3 (KLF3)-deficient mice exhibited profound WAT beiging, which was accompanied by an accumulation of AT eosinophils (511).

IMMUNOMODULATING ADIPOSE TISSUE INFLAMMATION IN METABOLIC DISORDERS AND CARDIOVASCULAR DISEASES

Strategies to modulate AT inflammation are multi-faceted, they include physical exercise, lifestyle modifications, in addition to several pharmacological and non-pharmacological interventions. Likewise, treatment of CVDs focuses on similar strategies that impact AT. In this section, we will tackle the contribution of different modalities on AT inflammation in CVDs.

Exercise and Lifestyle Modifications

One of the first strategies to decrease the severity and complications of CVDs is to limit food intake and increase energy expenditure, this is mainly due to the fact that most patients with CVDs are overweight or obese. Exercise and lifestyle modifications lower the mortality risk, improve quality of life and have been extensively studied. Physical activity improves insulin sensitivity and alters AT adipokine expression, which affect whole-body metabolic health in human subjects (512, 513). Recent studies highlighted the mechanistic pathways, linking

those two interventions with decreasing AT inflammation. The protective effects of regular physical activity is accompanied with reduction of visceral fat mass along with an anti-inflammatory pathway (514). Physical exercise exerts a direct anti-inflammatory effect by inducing an acute elevation in IL-6 and IL-10 and an inhibition of TNF-α (515). The anti-inflammatory effect of exercise also comprises the inhibition of macrophage infiltration and the induction of ATMs phenotypic switch toward the M2 phenotype in obese mice (515, 516). Zielger et al. demonstrated that exercise enhances the anti-inflammatory phenotype in VATs of old mice (517). Endurance training regardless of weight loss induced an increase in M2 macrophages in scWAT (513, 518). The role of physical activity in thermogenesis and WAT browning is debated as some reported scWAT browning with bicycle training programs while another study failed to find a correlation between aerobic exercises and recruitment of beige adipocytes (519, 520). Nevertheless, more studies should target the exact role of AT remodeling following physical activity. Indeed, long-term anti-inflammatory effects of chronic physical activity could be required for a pronounced AT remodeling required to decrease CVD risks.

Diet Modification and Weight Loss

Fasting

Several fasting regimens were introduced as an alternative or a complementary intervention to restricted caloric diets in improving cardiometabolic endpoints in related diseases (521, 522). Indeed, several studies on experimental animals and recent human investigations highlighted the importance of fasting in metabolic activity regulation, blood pressure, and atherosclerosis reduction, as well as health optimization (522–524). The most common types of fasting regimens include intermittent fasting (IF), periodic fasting (PF), short term fasting, and religious fasting. IF has a crucial role in adaptive cellular responses being able to reduce inflammation, oxidative stress, optimize energy metabolism, and cellular bioenergetics (524). Fasting is an effective strategy for improving cardiometabolic profile in cases of IR, stroke, prediabetes, and diabetes (525, 526). Moreover, IF modulates the susceptibility of inflammatory diseases by decreasing peripheral monocyte pools and modifying their metabolic activity through AMPK and PPARα pathways (527). On the AT level, fasting enhances mitochondrial biogenesis in visceral adipocytes. Short term fasting suppressed thermogenesis in inguinal WAT and iBAT in a mouse model (528). Another fasting regimen, every other day fasting, was shown to induce beiging of WAT thus reversing HFD-induced obesity and associated metabolic disorders in mice (529). The metabolic effects of IF are largely mediated by adipose thermogenesis; fasting-induced adipose VEGF, which is thought to act on eosinophils, Th2, and ILC2 to promote M2 polarization was linked to WAT browning (530). Moreover, fasting induces a reduction of IL-1 and IL-6 in VAT and scWAT and IL-6 in intraperitoneal WAT (531). Moreover, biomarkers of inflammation in EpiWAT and BAT were reduced in mice following IF. The latter study presented IF as a preventive and therapeutic intervention to protect mice against MetS and obesity (532). On the other hand, fasting reduces leptin levels triggering a profound metabolic state as well as regulating T lymphocytes and cytokine production in obese animal models and human trials (533–535). In addition to that, a new randomized control trial has also linked fasting to reduction of leptin levels (536).

Dietary Modifications

Dietary modification is the cornerstone in preventing cardiometabolic diseases. Weight loss approaches as well as initiating certain diet regimens lower CVD events significantly and reduce mortality (537). Reports correlating dietary manipulation, such as in high protein diets, phosphate diet, and ketogenic diet, to AT inflammation suggest that certain diet regimens can play a critical role in modulating cardiometabolic diseases.

Long term intake of high protein diet in obesity-prone rats reduced food intake and WAT mass while improving basal blood sugar, insulin levels, leptin, and triglyceride levels in addition to glucose tolerance (538). On the other hand, a clinical study suggested that phosphorus supplementation is involved in modulating glucose and insulin serum levels (539). Another study reported that high dietary intake of phosphate in rats can influence lipid and glucose metabolism by upregulating lipolytic gene expression and reducing WAT accumulation (540).

Ketogenic diet (KD), which consists mainly of ingesting healthy fats, improved long-term blood glucose control and subsequently decreased the use of anti-diabetic agents in human studies (541, 542). KD also improved the CVD biomarkers in T2DM patients (543). Moreover, short term feeding of KD was shown to modulate AT immune cells, where it reduced macrophage infiltration and the expansion of γδ T cells in VAT (279).

Mediterranean Diet (MD) is composed of a balanced combination of fruits, vegetables, fibers, fish, poly saturated fats as well as low intake of meat and dairy products in addition to moderate intake of red wine (544). The adherence to MD is known to protect humans against CVDs, MetS, onset of various types of cancer, and aging (545, 546). Several studies documented that certain typical food of the MD including olive oil, tomato, and red wine induce anti-inflammatory properties and could be even insulin-sensitizing (547, 548). For example, tomato juice mitigates AT inflammation; a 20-days duration of consumption could decrease TNF-α, IL-6, and IL-8 (549, 550). Moreover, tomato juice supplementation could reduce body weight, blood cholesterol levels as well MCP-1 (551).

Anti-diabetic Drugs

Metformin

Besides being widely used for DM2 treatment, Metformin reduces CVD risk, induces weight loss, and improves insulin sensitivity (552). Metformin has been proposed to reduce adipocyte stores and initiate a metabolically healthy adipocytes distribution (553). The beneficial effects of metformin also include reducing visceral AT, a mechanism that is thought to be related to FA oxidation and an upregulation of adaptive thermogenesis (554). Emerging body of evidence, including work done by our team, documented that metformin can exhibit immunomodulatory features, an anti-inflammatory

effect that is shown to be independent on glycemic control (32, 555, 556). Metformin activates the anti-inflammatory macrophage polarization; it lowers the pro-inflammatory cytokine production through elevating M2 macrophage and lowering M1 macrophages (557).

Metformin also decreases MCP-1 in isolated human AT cultures, suggesting an improved low-grade inflammation (558). Moreover, metformin has been shown to alter CRP, NF-κB expression in addition to reducing advanced glycation end products (559–561). All in all, the anti-inflammatory effects of metformin and its ability to reduce several inflammatory related illnesses is becoming more apparent (552).

Thiazolidinediones (TZDs)

Besides the use of TZDs in T2DM, a beneficial role of Pioglitazone lies in reducing cardiovascular events (562). In fact, TZDs activation of PPARγ not only enhances adipogenesis but also reduces fat deposition in tissues, and attenuates the inflammatory cytokines release in obesity (563, 564). Moreover, TZDs repress NF-kB thus restore M2 macrophage phenotype, and prompt the recruitment of regulatory T cells in AT (263, 565, 566). Therefore, this reveals a possible involvement of TZDs in an immunomodulatory mechanism in AT that may benefit patients with CVDs, yet the exact definitive pathway has not been established. Expanding on the beneficial effects of PPARγ agonism in limiting AT chronic low-grade inflammation in metabolic disorders, glitazones-like, multi-targeting drug ligands (MTDLs) were rationally designed (567). Importantly, these drugs were partial PPARγ agonists, potent COX-2 antagonists and moderate 15-LOX inhibitors. This balanced modulation of the three inflammatory targets allows for a more effective targeting of AT inflammation and possibly limit its cardiovascular complications (568).

Glucagon-Like Peptide-1 (GLP-1) Receptor Agonists

Several GLP-1 receptor agonists, as Liraglutide, have been developed to mimic the glucose-lowering and anorexic effects of Glucagon-like peptide-1 (GLP-1) to treat obesity and T2DM. As AT express GLP-1, Liraglutide has been effective in controlling glucose levels, promoting weight loss, and reducing total adiposity (569–571). In addition, in a clinical trial, Liraglutide has been shown to decrease the risk of myocardial infarction in patients with T2DM and high CVD risk (572). More studies on the effect of GLP-1 agonists documented their protective roles against endothelial cell dysfunction, and therefore atherosclerosis by reducing CRP and plasma lipids (573, 574). As such, GLP-1 agonists could provide protection against CVDs through AT mass reduction and inflammation.

Sodium-Glucose Cotransporter (SGLT2) Inhibitors

Similar to GLP-1 receptor agonists, SGLT-2 inhibitors are shown to reduce blood glucose levels and CVDs risk and mortality (575, 576). Treatment with Empagliflozin, an SGLT-2 inhibitor, has been shown to induce weight loss when given in combination with other anti-diabetic medication (577, 578). In obese mice, Empagliflozin was shown to promote utilization and browning of AT as well as reduction of IR and inflammation, a pathway linked to M2 macrophage polarization (579). As such, beside the anti-diabetic effects of SGLT-2 inhibitors, strong evidence appears in their effect on AT remodeling and anti-inflammatory pathway; yet the exact mechanism is to be elucidated.

Surgical Interventions

Bariatric surgery is the most effective treatment option in obese patients for weight loss whether due to food restriction, malabsorption, or both (580). Following the surgery and independent on weight loss; IR, CVDs, and mortality rates are all reduced (537, 581, 582). Importantly, it is expected that a late phase reduction of AT inflammation could be in favor of all the metabolic consequences of the surgery (583). However, when looking back to literature, contradictory results are revealed. Some have documented a decrease in inflammatory mediators of AT after the surgery-induced weight loss, and others have reported no further change (584–587). Reports generally assess subcutaneous AT depot, as it is easier in sampling. However, more studies should be warned to confirm or elucidate the effects of bariatric surgery on AT inflammation. Sampling from visceral AT and other sites should be done as it is more prone to inflammatory changes.

NEW AVENUES FOR ADIPOSE TISSUE IMMUNOMODULATION IN METABOLIC DISORDERS AND CARDIOVASCULAR DISEASES

AT inflammation is associated with an increased production of pro-inflammatory cytokines including IL-1β, TNF-α, IL-6, and IFN-γ. Anti-inflammatory treatments were proposed to contribute to the treatment of diabetes and its vascular complications (588). The antagonism of IL-1R improved IR in T1D patients and DIO mice (589, 590), and in patients with impaired glucose tolerance (591). One study however highlighted the importance of combining IL-1R antagonism treatment with proper dieting for the treatment of obesity (592). Moreover, inhibiting TNF-α in psoriasis patients with MetS decreased macrophage infiltration and pro-inflammatory cytokines levels in umbilical fat (593). Interestingly, a combined inhibition of IL-1β and TNF-α was more effective in improving IR in T2D rats (594). Similarly targeting IL-6 improved IR and normalized adipokine levels in MetS and fructose-fed rats (595, 596). Nevertheless, this mechanistic link was not evident in clinical trials (597). Indeed, IL-6 was shown to drive exercise-induced weight loss in subjects with visceral obesity (598). The complexity of the AT immune landscape driving AT inflammation in the MetS and the response of these cells to the various pro-inflammatory cytokines dictate the efficacy of these approaches. However, simply targeting pro-inflammatory cytokines with either receptor inhibitors or monoclonal antibodies for the treatment of metabolic and cardiovascular diseases is not yet a valid therapeutic strategy and requires further investigation.

The metabolic reprogramming in response to nutritional excess and scarcity of the various immune cells is not universal. Indeed, metabolic modulation emerged as a novel concept in

cancer immunotherapy (599). Th2 immunity in AT supports metabolic health and thus, targeting Th2 immunometabolism represents a valuable therapeutic strategy in metabolic disorders. Several reports on immunometabolism-targeted treatments in cancer and autoimmunity can be repurposed for metabolic diseases. For example, in a model of allograft rejection, blocking glycolysis and glutamine metabolism inhibited $CD4^+$ and $CD8^+$ T cell induction and promoted the generation of allospecific Treg cells (600). Indeed, expanding AT Treg cells hold much promise for the treatment of metabolic disorders and their cardiovascular complications. Additionally, nanoparticles, liposomes, and glucan-shells carrying siRNA or specific drugs can be engineered to tissue-specifically target specific immune cells as exemplified by ATMs (601). Nevertheless, a profound knowledge of metabolic profile shifts of immune cells within the AT is still lacking and thus, direct interpretations of such shifts in other tissues cannot be extrapolated, particularly in the complex, dynamically-evolving AT. Emerging technologies such as RNA sequencing, metabolomics, proteomics and phage display will surely allow for the identification of novel peptide targets (602). Moreover, adaptive immunity and the acquisition of memory T cells in HFD-fed mice suggests an effect on subsequent episodes of weight gain following weight loss (603, 604). Modulating T cell memory has been achieved by targeting antigen presentation in conventional and non-conventional APCs (271) and checkpoint co-inhibitory interactions (605). Finally, emerging evidence indicates that immunometabolism is controlled epigenetically and through miRNAs, which affects cellular differentiation and polarization (606, 607). Nevertheless, further research is required to determine how metabolic dysfunction drives alterations in epigenetic histone modifications and how miRNAs affects the AT immune profile.

Accumulating evidence suggests a role for the gut microbiota in modulating metabolic homeostasis. Indeed, it was shown that the adoptive transfer of Th 17 cells contributed to metabolic homeostasis through an IL-17-dependent microbiota development (608). Moreover, it was demonstrated that the M2 macrophage-mediated helminth-associated Th2/Treg responses induce alterations in microbiota composition which was accompanied by protection against obesity (609, 610).

We have highlighted a key role for physical activity, different fasting regimens, and dietary modifications in limiting AT inflammation and IR. Nevertheless, their implications on different adipose depots, especially those of cardiovascular interest are not well-characterized. In fact, a depot-specific metabolic profiling is pivotal to delineate their differential effects. Moreover, anti-diabetic drugs and surgical procedures showed a favorable outcome on metabolic parameters and CVDs. In fact, these approaches reduced AT inflammation through mechanisms being revealed only recently. Therefore, it is pivotal that AT immune profiles in different depots be characterized following the administration of anti-diabetic drugs.

Finally, the induction of BAT activity and WAT browning has been proposed as a mean to curb obesity and combat CVDs. Indeed, the induction of different AT depots browning resulted in either favorable or detrimental outcomes. Targeting UCP1 was even proposed as the induction of browning in PVAT and EpiCAT was supposed to deteriorate vascular and cardiac functionality (33). Indeed, this strategy is still debated as clinical trials have not shown a significant improvement of metabolic parameters following the induction of thermogenesis (33). In addition to the non-selective impact on all adipose depots, the available UCP1 inhibitors possess a fairly high IC_{50} value ($\sim 20\,\mu M$) (611) precluding systemic administration without significant off-target and adverse effects. As such, immune modulation of thermogenesis might constitute a lucrative target for depot-specific intervention. As different depots possess variable intrinsic brown-like character, it is pivotal to further characterize this phenotype, the manner by which it is affected by the immune system in states of health and disease, and how increased energy expenditure leads to clinical significance. Significantly, the relative impact of the activation of different thermogenic pathways on AT inflammation in various adipose depots requires systematic examination. Whether the selection of one pathway over the other modulates activity and/or recruitment of disparate immune cells remains unknown. As well, the ability of a specific immune cell product/function to favor one pathway over the other has not been investigated.

CONCLUSION

Metabolic and Cardiovascular diseases are multifactorial disorders to which contributes the inflammation of the AT. Several pharmacological and non-pharmacological interventions have been shown to exert their positive effects in these diseases, at least in part by modulating AT inflammation. Accumulating evidence implicates different immune cells in the regulation of AT inflammation and its consequences including IR. The metabolic reprogramming of AT immune cells and the alteration of the AT immune landscape are believed to drive AT inflammation, BAT thermogenesis and WAT browning. Further investigation is required to delineate the exact role of different immune cells and the consequences of their metabolic profile alteration in different adipose depots inflammation. A better comprehension of the mechanisms driving AT inflammation allows for the emergence of novel therapeutic strategies aimed at immunomodulating the AT.

AUTHOR CONTRIBUTIONS

IA, SH, HA-K, and AG participated in literature review and screening and contributed to manuscript writing. IA wrote the first draft of the manuscript. AE helped in overseeing and coordinating the work and participated in manuscript draft review. AE-Y developed the idea, supervised the work, reviewed and modified manuscript draft, and provided research funding support. All authors contributed to the article and approved the submitted version.

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REVIEW
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COVID-19 in Pediatric Patients: A Focus on CHD Patients

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Coronavirus disease 2019 (COVID-19) is a global pandemic caused by SARS-CoV-2 virus. As of the 30th of September 2020, around 34,000,000 cases have been reported globally. Pediatrics with underlying congenital heart disease represent a small yet a critical proportion of these patients. In general, the majority of infected children experience mild to moderate disease with significant interindividual variability in laboratory and radiographic findings. Nevertheless, in healthy children with COVID-19, cardiac involvement has been documented and is attributed to various causes. Myocarditis, arrhythmias, cardiogenic shock, and serious multisystem inflammatory syndrome in children are all encountered. Since COVID-19 is a recent novel disease and based on previous experience with respiratory infections, children with underlying congenital heart disease should be given special attention. To date, little data is available about COVID-19 presentation, complications, and appropriate treatment in this population. However, variable and inconsistent disease presentation and severity have been observed. This paper discusses COVID-19 course of illness in pediatric population with a special emphasis on the cardiac manifestations of the disease in healthy population and also on the disease course in congenital heart disease patients in particular.

Keywords: children, congenital heart disease, CHD, COVID-19, coronavirus, pediatric cardiology

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INTRODUCTION

Coronaviruses (CoVs) are enveloped single stranded RNA viruses that belong to the Coronaviridae family (1). They are implicated in a wide spectrum of diseases ranging from mild illness such as common cold to more serious life-threatening syndromes such as the Middle East Respiratory Syndrome (MERS) and the Severe Acute Respiratory Syndrome (SARS) (2). Toward the end of 2019, a novel coronavirus, called SARS-CoV-2, led to the unprecedented pandemic of Coronavirus Disease 2019 (COVID-19) (3). At the time of writing this manuscript, the total COVID-19 cases reported are around 34 million, with over 1 million deaths reported.

SARS-CoV-2 infection is not only associated with respiratory symptoms but also with multi-organ manifestations that include cardiac, gastrointestinal, hematologic, renal, and neurologic ones (4–7). Despite the rapid global spread of the pandemic, the disease characteristics in pediatrics with congenital heart disease (CHD) remains largely unclear. This article discusses the clinical course of COVID-19 in pediatric patients along with laboratory and radiologic findings with emphasis on cardiovascular complications and on pediatric patients with CHD.

CONGENITAL HEART DISEASE

CHD is the most commonly encountered congenital anomaly accounting for around 30% of all congenital defects (8). Its incidence varies greatly among populations with average of 8 per 1,000 annual live births (9). In recent decades, the incidence of CHD increased due to development in screening and detection methods (8, 10). CHD is classified into two main categories: cyanotic and acyanotic heart diseases [**Figure 1**; (11)]. The acyanotic heart diseases represent the milder types of CHD. They include ventricular septal defects, atrial septal defects, pulmonary stenosis, aortic stenosis among others. On the other hand, the group of cyanotic CHDs usually presents with severe illness early in life. They include Tetralogy of Fallot, hypoplastic right heart, hypoplastic left heart, total anomalous pulmonary venous return among others (11, 12).

With the rapid development in detection methods and surgical techniques, the survival rate of CHD patients significantly improved. It greatly hinges on the type and severity of CHD, where in the milder diseases survival to adulthood reached almost 98% (13). However, these children remain at remarkable risk for increased morbidity and mortality from lower respiratory tract infections (14). For instance, respiratory syncytial virus infection is associated with increased risk of hospitalization, intensive care unit (ICU) admission, and mechanical ventilation requirement in these patients (3). Consequently, it is critical to assess the burden of COVID-19 pandemic on CHD patients. Unfortunately, this remains challenging ought to the low number of reported cases. This article summarizes the available evidence that describes the characteristics of COVID-19 in CHD patients.

SARS-CoV-2 IN PEDIATRIC PATIENTS

In the early phase of the global pandemic, the reported number of infected children was low. As the disease progressed, more cases were reported. Later on, It has been noted that children are less susceptible to COVID-19 and if infected the majority display mild to no symptoms (15). Eventually, attention has drifted toward the pediatric population to explore the disease features and discover their role in spreading the virus. In fact, out of the first 11,791 cases diagnosed in China, only 74 cases were children below 18 years old (16). The first reported pediatric case was on January 20, 2020 (16, 17). Besides, it is estimated that pediatric cases account for around 1–5% of the total reported cases worldwide (18). In the below section we review the clinical features, laboratory and radiologic findings, and treatment of COVID-19 in the pediatric population.

Clinical Features

Reported disease severity of infected children range from asymptomatic to mild, moderate, severe and critically ill cases (**Table 1**) depicts the clinical features of cases classified with different disease severity. Interestingly, gastrointestinal manifestations could be the initial and even the only symptoms of SARS-CoV-2 infection in children (24). Indeed, China's first critically ill pediatric case displayed only gastrointestinal symptoms in early disease state and then progressed rapidly to acute respiratory distress syndrome, septic shock, and renal failure (24). Most of the infected pediatric patients are asymptomatic, or exhibit mild disease, usually recovering in 2 weeks from the onset of symptoms (17, 19, 23). In concordance, out of 171 cases in a Chinese study around 15% were clinically asymptomatic with negative CT chest findings (25).

Studies from different countries reporting pediatric cases highlight the mild to moderate disease presentation in this population. Pediatric cases from all ages were reported. In a report by Dong et al., out of 2,135 Chinese children diagnosed with COVID-19 more than 90% were classified in the asymptomatic, mild or moderate disease categories (15). In Turkey, more than 50% of infected children had mild disease (26). Moreover, compared to adults, pediatric cases are less likely to report the common COVID-19 symptoms (25, 27, 28). Among 291 confirmed pediatric cases from the United States, only 56, 54, and 13% reported fever, cough and shortness of breath, respectively (28). Similarly, out of the 171 confirmed pediatric cases in a Chinese study, only 41.5% reported fever (25).

While as mentioned above most cases are mild, severe and critically ill cases were also reported. Of the 171 cases in the Chinese study, 3 patients required pediatric intensive care unit (PICU) admission, and all had underlying medical illness (leukemia on chemotherapy, hydronephrosis and intussusception). Eventually one death was reported for the 10 months old patient with intussusception, at week 4 post-admission, due to multi-organ failure (25). Congruently, among 745 reported cases in the United States, 147 required hospitalization and of which 15 patients required ICU admission (28). Dong el al., reported that of the 2,143 COVID-positive children, around 5% had severe disease while 0.6% were critically ill (21).

Remarkably, the highest hospitalization rate among the pediatrics population was reported in patients aged <12 months, accounting for up to 62% of the total hospitalized COVID-19 positive children (28). Similarly, 32 and 10.6% of the severely ill children were <1 year old (21, 29). In a Turkish study, 80% of PICU admissions were <1 year old (26). The percentage of severely and critically ill patients decreased with age, it is estimated to be 7.3, 4.2, 4.1, and 3% in age groups 1–5 years, 6–10 years, 11–15 years, and 16–17 years, respectively (21).

Fortunately, no evidence of vertical transmission has been detected. All tested neonates ($n = 10$) born to COVID-19 mothers had negative PCR results, although adverse effects were reported (30). Furthermore, infected neonates tend to show mild to moderate clinical symptoms (31). Only 1 out of 3 neonates had complicated hospital course associated with disseminated intravascular coagulation (DIC) and required non-invasive ventilation, but gradually improved (31). However, this neonate was preterm born at 31 weeks of gestation with neonatal respiratory distress syndrome (31).

Children susceptible to develop severe illness are those with underlying cardiac, respiratory or immunologic diseases such as CHD, asthma or immunodeficiency. 77% (28/37) of hospitalized patients and 100% of the ICU admitted with known information

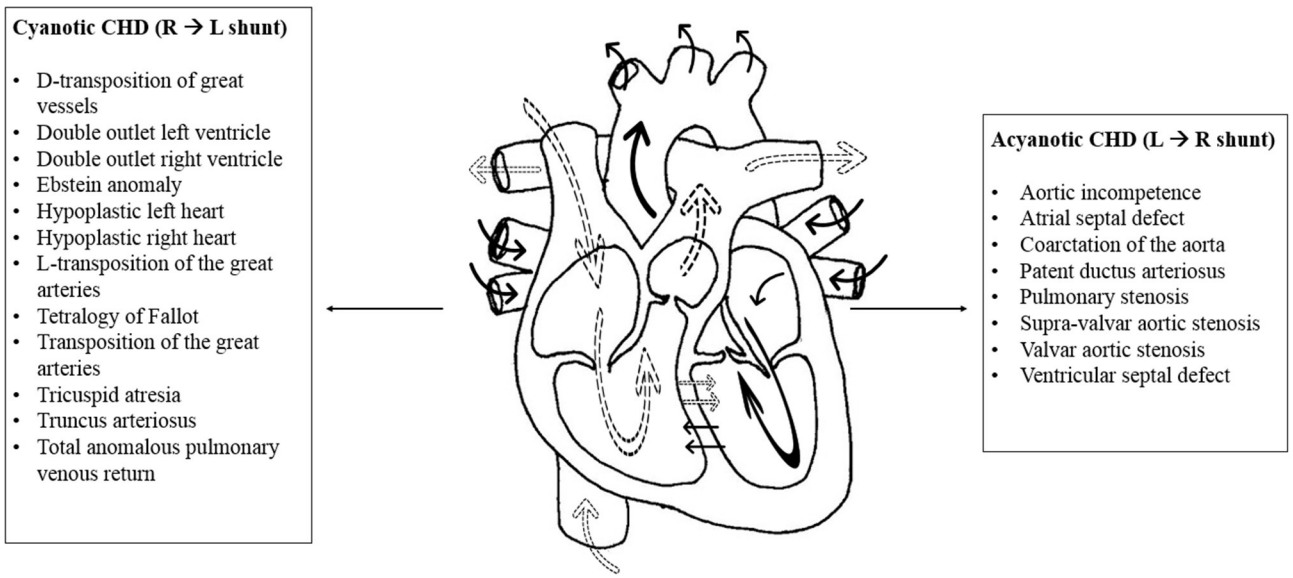

FIGURE 1 | Cyanotic and acyanotic congenital heart diseases. The normal physiology of the heart is represented in the above illustration with the effect of shunts. The dotted arrows represent deoxygenated blood while solid arrows represent oxygenated blood. Shunt is an abnormal communication between the right and left sides of the heart or between the pulmonary and systemic circulations. The cyanotic congenital heart diseases are listed to the left of the diagram. In general, cyanotic lesions cause shunting of deoxygenated blood from the right to the left side of the heart, leading to decreased systemic oxygen saturation and cyanosis. Hence, they present early in life. Acyanotic congenital heart diseases are listed to the right of the diagram. Acyanotic lesions cause left to right shunting of oxygenated blood. They increase blood flow to the pulmonary circulation without affecting oxygen saturation in the systemic blood (11, 12).

TABLE 1 | This table summarizes the clinical features encountered in pediatric COVID-19 patients according to disease severity and as described by various studies (15, 19–23).

Severity	Clinical features
Asymptomatic	Positive PCR test without clinical manifestations or radiologic findings.
Mild	Low- or high-grade fever, fatigue, myalgia, sore throat, headache, sneezing, dry cough, runny nose, nasal congestion, and gastrointestinal symptoms such as nausea, vomiting, abdominal pain, diarrhea, and non-severe pneumoniae on chest imaging.
Moderate	Lower respiratory tract infection symptoms, fever, dry or productive cough, wheezing, and positive findings on chest imaging.
Severe	Rapid progression of the disease with dyspnea, central cyanosis, increased respiratory rate above 70/min for infants and above 50/min for children greater above the age of 1 year, coma, convulsions, somnolence, severe dehydration, and oxygen saturation below 92%.
Critically ill	Rapidly progressing to acute respiratory symptoms, respiratory failure and end organ damage such as heart failure, shock, acute kidney injury, liver injury, encephalopathy, coagulopathy, or myocardial damage.

about hospitalization and medical status had underlying medical condition (28).

In summary, children with COVID-19 usually exhibit mild disease with minority requiring hospitalization and PICU admission. Infants have the highest risk of developing severe and critically ill disease. In addition, children with underlying medical condition are more likely to experience complications requiring hospital admission. Remarkably, vertical transmission has not been documented. Infected neonates have mild illness unless complicated by underlying medical problems or by prematurity.

Laboratory Findings

Strangely, the majority of infected children tend to have normal laboratory markers including CRP and liver function enzymes (32, 33). In one study, procalcitonin and CK-MB elevation was reported in 16/20 and 15/20 of infected children, respectively (32). Variability in lymphocyte counts was reported. The majority (around 70%) had normal lymphocyte count (34); however lymphocytopenia was reported in 2 studies accounting for 3 and 3.5% of the population (25, 34). Besides, increase in WBCs counts and leucopenia were both reported (23, 25, 35). Some laboratory markers found to be associated with severe disease including: decreased lymphocyte count and increased procalcitonin, d-dimer, and CK-MB (19, 23). Coinfection with other pathogens was documented. Such pathogens include influenza viruses A and B, respiratory syncytial virus (RSV), cytomegalovirus (CMV) among others (32).

Radiologic Findings

Published data describing the radiographic findings in pediatric patients with COVID-19 is scarce. During routine practice, chest x-ray (CXR) is the preferred modality in children; however, it has low specificity and sensitivity in evaluating lung involvement in confirmed or suspected COVID-19 children (36). In general, children have lower incidence and limited lung involvement

compared to adults, as evident on chest imaging (37, 38). Ground glass opacities (GGO) with peripheral distribution in the lower lung fields is the most common reported finding (37, 39–41). Besides, the extent of pulmonary changes on imaging is related to disease severity. Patients with mild disease presentation often exhibit no radiographic changes; however, in one study, GGO were detected in 100% of patients with moderate COVID-19 (23).

More serious changes were seen in patients admitted to the PICU (42). In a study describing eight PICU patients, seven had multiple patch-like shadows, six had GGO, and one had pleural effusion and white lung-like change (42). These changes persisted even after resolution of clinical symptoms. Lesions may also persist in the absence of viral detection on PCR testing (32).

Mixture of findings with heterogenous pattern were reported on chest CT. Among the reported changes are bilateral or unilateral ground glass opacities, patchy ground glass opacities, local and bilateral patchy shadowing, interstitial abnormalities, halo signs, small nodular ground glass opacities, and speckled ground glass opacities, and bronchial pneumonia-like changes (25, 39–41, 43, 44). Rarely, pleural effusion, crazy-paving sign, and lymphadenopathy were reported but were witnessed mainly in severe cases (37, 39, 41). Due to the high radiation associated with CT scan and to the fact that most children experience milder lung involvement than adults, it is suggested that chest CT should not be routinely used unless necessary (45).

Treatment

Most studies and trials targeting COVID-19 treatment were performed on adults. In general, in the pediatric population, treatment is symptomatic and supportive. It aims to provide rest, sufficient caloric intake, and maintaining water and electrolyte balance (19). It includes antipyretics for fever, sedatives in case of seizure, oxygen therapy including mask, nasal catheter, high flow nasal cannula or invasive ventilation, and antibiotics for bacterial superinfection (19, 46). In some cases, antiviral therapies are used as well (47).

Ultimately, vaccination remains the optimal preventive measure that can attenuate the global propagation of COVID-19. Researchers worldwide are working with unpreceded efforts to develop an effective vaccine. Currently, 42 vaccine candidates are being tested in pre-clinical or clinical trials (phases 1–3). Nevertheless, on the 11th of August 2020, the first COVID-19 vaccine has been granted approval. This vaccine stands for the Sputnik V vaccine developed by the Gamaleya Research Institute in Moscow. However, the use of this vaccine has raised a lot of arguments since it has not been tested in phase 3 clinical trials yet (48, 49). Hence, further trials are definitely needed to evaluate the role of any vaccine targeted against SARS-CoV-2.

CARDIAC MANIFESTATIONS OF COVID-19 IN PEDIATRIC PATIENTS

In previously healthy COVID-19 pediatric patients, Kawasaki-like disease and myocarditis have been the main cardiovascular manifestations of SARS-CoV-2. These manifestations are triggered primarily by the massive immune response mounted against the viral infection (50–55). In fact, elevated levels of inflammatory markers have been noted in patients with COVID-19 associated Kawasaki-like disease or myocarditis (52, 54, 56). Il-6 was found to be inappropriately elevated in two distinct cases of pediatric myocarditis (52, 54). Similarly, CRP, pro-calcitonin and ferritin were elevated in most cases of Kawasaki-like disease and myocarditis (54, 56–58). Kawasaki-like disease may occur following a prior infection of COVID-19 documented by the presence of SARS-CoV-2 IgG antibodies or a known contact with a confirmed case of COVID-19 (58). This suggests the presence of a post-infectious state of immune dysregulation in children previously infected with or exposed to COVID-19 (51, 57).

Besides, compromised lung function may result in compromised cardiac function; this is attributed to numerous COVID-19-induced pulmonary defects denoted by oxidative stress, tissue injury, respiratory failure and ventilation perfusion mismatch (50, 51). Cardiac dysfunction may be possibly caused by direct myocardial damage. SARS-CoV-2 can gain entry to the cardiomyocytes through the ACE2 receptor (59). Subsequently, myocardial infiltration by SARS-CoV-2 and inflammatory cells may result in lethal complications that include fulminant myocarditis and cardiogenic shock (59).

After several cases were reported, the Kawasaki-like disease induced by COVID-19 was defined by the Centers for Disease Control and Prevention (CDC) as the multisystem inflammatory syndrome in children (MIS-C) associated with COVID-19 (60). As of the 15th of July, 342 cases of MIS-C and 6 deaths were declared in 37 American states (60). Eighty-one percent of the cases were aged between 1 and 14 years. Nevertheless, the disease may develop 2–4 weeks after a COVID-19 infection in any patient aged <21 years (60). SARS-CoV-2 infections complicated by MIS-C are often associated with fatal conditions denoted by heart failure, hepatic injury, renal dysfunction and coagulopathies (56).

Furthermore, in a cohort of 58 pediatric patients with MIS-C, 50% of the patients were admitted to the PICU (58). Acute kidney injury was noted in 13 patients, and cardiogenic shock necessitating inotropic agents was experienced by 27 patients (58). Of these 58 patients, 25 were bound to mechanical ventilators (58). Similarly, in a cross-sectional study of 48 patients with severe COVID-19 disease requiring PICU admission, death was witnessed only in two patients (61). Multi-organ dysfunction was the cause of death in both of them. Congruently, this endorses in turn the presence of a state of hyperinflammation in critically ill patients with multi-organ involvement (61). Cases of MIS-C have been reported in further studies as depicted in **Table 2**. To date, Kawasaki-like manifestations and myocarditis have constituted the key clinical presentations of this syndrome as revealed by most studies.

Finally, despite being less common, arrhythmias were also encountered in children with COVID-19. As compared to adult patients, milder forms of arrhythmias were reported in pediatric patients. In one pediatric study, ventricular tachycardia and ventricular fibrillation were not reported (32). Yet, sinus tachycardia, atrial tachycardias, atrial and ventricular premature beats, bundle branch blocks, and first-degree AV block were the main arrhythmic manifestations of COVID-19 among the studied patients (32).

TABLE 2 | This table summarizes the clinical characteristics of children with multisystem inflammatory syndrome reported in a few additional clinical studies.

Study	Date of publication	Study type	Country	Number of patients	Age/Age range	Highlights/Findings
Cardiac Dysfunction and Shock in Pediatric Patients With COVID-19 (62)	15 July 2020	Case Series	USA	3	6–13 years	• A picture of multisystem inflammatory syndrome was seen in the three patients • Ventricular dysfunction and cardiac shock were among the encountered complications • The three patients were admitted to PICU and discharged few days after admission
Kawasaki-like multisystem inflammatory syndrome in children during the covid-19 pandemic in Paris, France: prospective observational study (63)	3 June 2020	Prospective observational study	France	21	3.7–16.6 years	• A picture of multisystem inflammatory syndrome was seen in all patients • Myocarditis was noted in 76% of the patients • SARS-CoV-2 infection was detected in 19 patients • Gastrointestinal symptoms were reported by all patients • IVIG was given to all patients, and corticosteroids to 10 of them
COVID-19–Associated Pediatric Multisystem Inflammatory Syndrome (64)	22 May 2020	Letter to editor	USA	1	6 years	• A 6-year-old female patient presented with symptoms of fever, sore throat and decreased oral intake. She was initially diagnosed as group A streptococcus pharyngitis • Her condition was complicated by respiratory distress, cardiac failure, and electrolytic abnormalities (i.e., Hyponatremia, hyperkalemia, and azotemia) • In short, she had findings suggestive of Kawasaki-like disease and myocarditis, and was admitted to PICU • After a week of illness, COVID-19 PCR was found to be positive
Multisystem Inflammatory Syndrome in Children in New York State (65)	23 July 2020	Retrospective observational study	USA	191	0–20 years	• Of the 191 patients, 95 had confirmed MIS-C • Fever was noted in all patients • CRP was elevated in all patients. D-dimer and troponin were found elevated in 91 and 71% of the patients • Vasopressors were given to 62% of the patients • 80% of the patients were transferred to ICU • 2 deaths were reported
Multisystem Inflammatory Syndrome in U.S. Children and Adolescents (66)	23 July 2020	Observational study	USA	186	0–20 years	• A picture of multisystem inflammatory syndrome was seen in all patients • Gastrointestinal symptoms followed by cardiovascular symptoms were reported in most patients • 80% of the patients were admitted to ICU Mechanical ventilation and vasopressors were provided to 20 and 48% of the patients, respectively • Inflammatory markers were elevated in almost all patients • 4 deaths were reported
Outbreak of Kawasaki disease in children during COVID-19 pandemic: a prospective observational study in Paris, France (67)	14 May 2020	Prospective observational study	France	17	3.7–16.6 years	• A picture of Kawasaki disease was seen in all patients • Gastrointestinal symptoms were initially reported by all patients • Inflammatory markers were elevated in all patients • 11 patients were admitted to ICU • 12 patients had myocarditis • No deaths were reported
Multisystem Inflammatory Syndrome in Children (MIS-C) Related to COVID-19: A New York City Experience (68)	25 June 2020	Retrospective observational study	USA	15	3–20 years	• A picture of MIS-C was seen in 15% of patients • Not all patients had positive PCR tests, but all had positive serology suggesting post-infection inflammatory response rather than direct viral injury • Initially, lymphopenia (in 13 patients), thrombocytopenia (6), hypoalbuminemia (8), and elevated fibrinogen (14) were noted • Inflammatory markers were elevated: CRP and D-dimer (100% of patients), ferritin (87%), and ESR (93%) • Interleukin-6 and interleukin-8 were elevated in all patients • 13 patients had gastrointestinal symptoms while only 5 patients reported respiratory symptoms • 13 patients showed features of severe cardiac involvement • 9 patients required vasopressors or inotropes, and 1 patient required intra-aortic balloon pump • 20% required intubation, 33% non-invasive ventilation • 1 death in a child who required extra-corporeal membrane oxygenation for 9 days

(Continued)

TABLE 2 | Continued

Study	Date of publication	Study type	Country	Number of patients	Age/Age range	Highlights/Findings
Acute heart failure in multisystem inflammatory syndrome in children (MIS-C) in the context of global SARS-CoV-2 pandemic (69)	17 May 2020	Retrospective observational study	France and Switzerland	35	2–16 years	• 31/35 had positive PCR tests • 28% had comorbidities • Gastrointestinal symptoms were reported in 80% • 33% had left ventricular ejection fraction below 30%, 72% below 50%, 80% had cardiogenic shock, 3% ventricular arrhythmia, 17% coronary artery dilation • 100% required ICU admission • 80% needed inotropic agents and 28% needed ECMO • Elevated BNP, troponin, CRP, and D-dimer was documented • IL-6 elevated in 13 patients • Left ventricular function returned to normal in 25 patients in 7 days • No deaths were reported
Systemic Inflammation With Cardiac Involvement in Pediatric Patients With Evidence of COVID-19 in a Community Hospital in the Bronx, New York (70)	20 July 2020	Case Series	USA	4	3–20 years	• All patients had initial negative PCR tests but positive serology • 3 patients had gastrointestinal symptoms, all had fever, and none had respiratory symptoms • Elevated CRP, ferritin, troponin and pro-BNP noted in all patients, D-dimer in 3 patients and fibrinogen in 2 patients • 3 patients had mild and 1 severe depression of left ventricular function • 1 patient required intubation • 1 patient developed vasogenic shock required ECMO and died due intracranial hemorrhage associated with herniation
COVID-19 and Kawasaki Disease: Novel Virus and Novel Case (71)	7 April 2020	Case report	USA	1	6 months	• A 6-month-old baby girl presented with a picture of Kawasaki disease • COVID-19 PCR was found to be positive • Inflammatory markers (i.e., CRP and ESR) were elevated • No cardiac abnormalities were noted • Patient was treated with IVIG and aspirin
SARS-CoV-2-related pediatric inflammatory multisystem syndrome, an epidemiological study, France, 1 March to 17 May 2020 (72)	4 June 2020	Case series	France	156	5–11 years	• Cases were classified into: confirmed/proven SARS-Cov-2 related pediatric inflammatory multisystem syndrome (CoV-PIMS) (79 patients), probable CoV-PIMS (16), possible CoV-PIMS (13), and non—CoV-PIMS (48) • Myocarditis and Kawasaki-like disease were noted in 70 and 61% of the CoV-PIMS, respectively • Macrophage activation syndrome and seritis documented in 25 and 24% of the CoV-PIMS, respectively • 67% of the CoV-PIMS required ICU admission, of these 43% required intubation and mechanical ventilation, and 73% needed vasopressors or inotropes • 1 death was reported

COVID-19 IN PEDIATRIC CHD PATIENTS

As previously mentioned, children are less prone to acquiring COVID-19. Additionally, as compared to adults, they often exhibit mild disease ranging from flu-like to no symptoms. Nonetheless, children, particularly those with CHD, may develop serious COVID-19 related cardiovascular complications (51, 73). CHD patients are more likely to require ICU admission and artificial respiratory support, especially those with cyanotic defects. In these patients, COVID-19 may lead to worsened hypoxemia and compromised tissue perfusion (51, 73). Additionally, patients with complex CHD complicated by depressed myocardial contractility, pulmonary hypertension, immunodeficiencies (i.e., DiGeorge syndrome) among other comorbid conditions may likely develop severe and critical COVID-19 disease (73, 74). Indeed, as per the British Congenital Cardiac Association (BCCA), CHD patients and particularly those with complex disease (**Figure 2**) are considered at-risk patients prone to develop severe COVID-19 (75).

Despite the scarcity of available evidence in this area, few studies have reported the complications encountered in SARS-COV-2 positive pediatric cardiac patients. In this context, *Lu et al.* declared that severe disease was mainly witnessed in children with preexisting life-threatening conditions. Out of 230 patients, two had severe symptoms. One child had a past medical history of surgically treated CHD. The other had complicated kidney disease (22).

Furthermore, in a former Chinese study published on February 2020, Chinese experts have discussed the characteristics of the COVID-19 pandemic along with its diagnosis, treatment and prevention in children aged up to 17 years. They defined CHD as a risk factor for critical SARS-CoV-2 infection (76). This has been likely endorsed by a retrospective study of 25 Chinese children aged between 3 months and 14 years. In this study, serious and complicated COVID-19 was observed only in two patients having a past medical history of surgically treated CHD (77).

Additionally, in a cross-sectional study of 48 participants, Shekerdemian et al. assessed the clinical presentation, characteristics, and outcomes of COVID-19 pediatric patients admitted to ICU in North America. In this study, underlying comorbid conditions were noted in 83% of the patients. A History of CHD was observed in 3 patients (61). Similarly, in a multi-center observation study, data concerning COVID-19 in CHD patients was collected from eight Italian CHD centers (74). Throughout a period of 6 weeks extending from the 21st of February till the 4th of April, a total of 76 SARS-CoV-2 positive CHD patients were reported. Four were children and the remaining 72 were adults aged more than 18 years. The reported cases were likely subdivided into confirmed (9 patients) and suspected COVID-19 cases (67 patients) (74). Cardiovascular complications, such as heart failure, arrhythmias, stroke, myocardial injury, pericardial effusion and pulmonary hypertension were mainly observed in the confirmed cases. Nonetheless, a mild disease course was witnessed in most patients and zero deaths were reported (74).

As deduced from above and just like in healthy individuals, COVID-19 may exhibit distinct clinical courses in CHD patients ranging from no symptoms to critical disease. Nonetheless, COVID-19 manifestations such as chest pain, cyanosis, dyspnea, and palpitation may imitate symptoms of cardiovascular deterioration in these patients (73). Hence, comprehensive evaluation and meticulous care should be offered to any CHD patient presenting with these symptoms that may indicate worsening CHD or new onset SARS-CoV-2 infection. Additionally, the diagnosis of COVID-19 in CHD patients is made primarily through RT-PCR testing of nasopharyngeal samples (51, 59). The detection of suggestive chest CT scan findings plays likely an imperative role in confirming the diagnosis of COVID-19 in suspected cases (51, 59).

In most cases, patients with CHD are managed with supportive measures geared toward fever reduction, symptoms control and oxygen correction. The use of repurposed medicines such as azithromycin, hydroxychloroquine, dexamethasone, remdesivir, and immunotherapies is not part of the standard management (51, 78). Yet, the addition of these medications to the management of CHD patients necessitates careful dosing and thorough monitoring particularly when dealing with cardiotoxic medications (51, 78). Besides, none of the medications that are often used in treating CHD manifestations and complications, and also in maintaining cardiac functions in these patients are shown to affect or exacerbate the clinical course of COVID-19 in this population (73). For instance, the discontinuation of angiotensin converting enzyme inhibitors (ACE-i) and angiotensin II receptor blockers (ARB) in cardiac patients with confirmed or suspected COVID-19 was prohibited by the Heart failure Society of America (HFSA), American College of Cardiology (ACC), American Heart Association (AHA), and the European Society of Cardiology (ESC) (79, 80).

Finally, numerous studies have emphasized the importance of COVID-19 prevention in this population (51, 59, 73, 78, 81). Indeed, effective screening and early detection of SARS-CoV-2 infection in patients with CHD are key for avoiding severe life-threatening manifestations of the disease. Congruently, CHD patients should be educated about the signs and symptoms of COVID-19, and also about the importance of adopting protective measures such as social distancing, repeated hand washing, and effective wearing of face masks and goggles (51, 59, 73, 78, 81).

LIMITATIONS

The studies reported in our manuscript have several limitations. First, they are restricted in terms of population size. Most reported studies were from single-centers and had a small number of pateints. Second, no randomized controlled trials were found; the studies were mostly observational, case

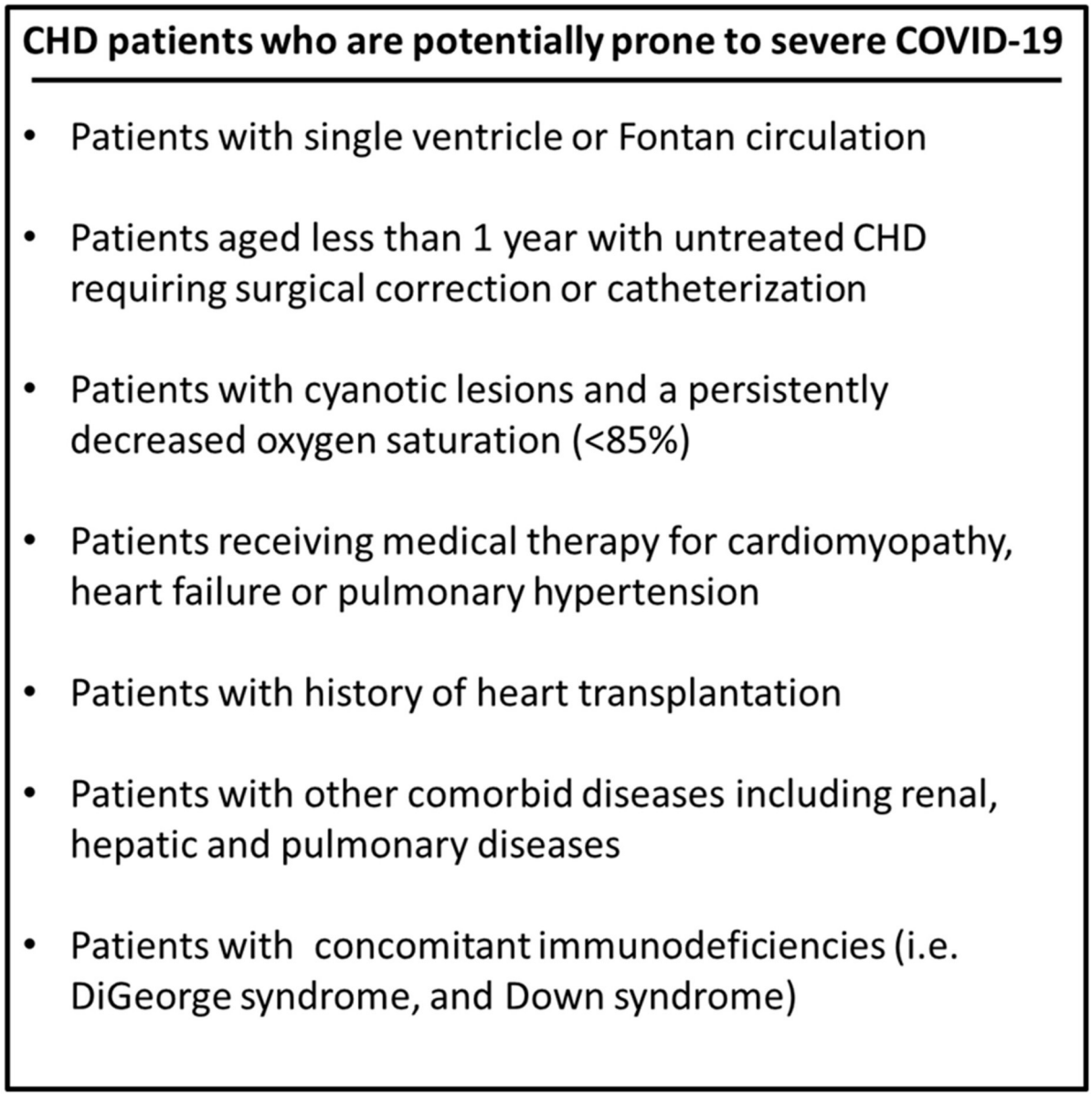

FIGURE 2 | Patients with complex CHD who are considered at high-risk of developing severe COVID-19 as per the British Congenital Cardiac Association (BCCA).

series and retrospective chart reviews. Hence, conclusive evidence concerning infection rate among various pediatric age groups, genders and those with comorbidities cannot be ascertained.

Similarly, the literaure lacks studies comparing COVID-19 infection in heathy children to those with CHD. In addition, the published data regarding children with multisystem inflammatory syndrome is limited by the fact that patients were only from 3 countries. Seven out of the eleven included studies were from centers across the United States, three were from France and one had patients from both France and Switzerland. Besides, the studies were heterogenous in terms of the assessed laboratory markers. Finally, data describing laboratory values and imagings are scarce and highly variable among infected children with CHD. Despite these limitations, this manuscript was able to describe the spectrum of disease presentation and prognosis in CHD patients with superimposed COVID-19 infection.

CONCLUSION

Little is known about the SARS-CoV-2 infection in CHD patients. The majority of the published data consist of

documentaries, narrative reviews, and letters. Yet, as depicted above, it is now considered that serious clinical symptoms and end-organ complications of COVID-19 may develop in children with CHD or previous history of surgically treated CHD. Ultimately, as the COVID-19 pandemic continues to spread globally at enormous rates, further higher-quality clinical trials with enrolled pediatric CHD patients are required to assess the clinical burdens of COVID-19 in these patients. Similarly, internists, pediatricians, and cardiologists should understand the influence of this pandemic on CHD patients and should provide meticulous care to this at-risk population.

AUTHOR CONTRIBUTIONS

MA, FB, and AE developed the idea and the review framework. RZ and NY wrote the first draft of the manuscript. AE did the final editing. All authors contributed to corrections and adjustment of subsequent iterations of the manuscript. All authors approve and agree with the content.

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Genomic and Proteomic Study of the Inflammatory Pathway in Patients With Atrial Fibrillation and Cardiometabolic Syndrome

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Atrial fibrillation (AF) and cardiometabolic syndrome (CMS) have been linked to inflammation and fibrosis. However, it is still unknown which inflammatory cytokines contribute to the pathogenesis of AF. Furthermore, cardiometabolic syndrome (CMS) risk factors such as obesity, hypertension, insulin resistance/glucose intolerance are also associated with inflammation and increased level of cytokines and adipokines. We hypothesized that the inflammatory immune response is exacerbated in patients with both AF and CMS compared to either AF or CMS alone. We investigated inflammatory cytokines and fibrotic markers as well as cytokine genetic profiles in patients with lone AF and CMS. CMS, lone AF patients, patients with both lone AF and CMS, and control patients were recruited. Genetic polymorphisms in inflammatory and fibrotic markers were assessed. Serum levels of connective tissue growth factor (CTGF) were tested along with other inflammatory markers including platelet-to-lymphocyte ratio (PLR), monocyte-to-HDL ratio (MHR) in three groups of AF+CMS, AF, and CMS patients. There was a trend in the CTGF levels for statistical significance between the AF and AF+CMS group ($P = 0.084$). Genotyping showed high percentages of patients in all groups with high secretor genotypes of Interleukin-6 (IL-6) ($P = 0.037$). Genotyping of IFN-γ and IL-10 at high level showed an increase in expression in the AF + CMS group compared to AF and CMS alone suggesting an imbalance between the inflammatory and anti-inflammatory cytokines which is exacerbated by AF. Serum cytokine inflammatory cytokine levels showed that IL-4, IL-5, IL-10, IL-17F, and IL-22 were significant between the AF, AF+CMS, and CMS patients. Combination of both CMS and AF may be

associated with a higher degree of inflammation than what is seen in either CMS or AF alone. Thus, the identification of a biomarker capable of identifying metabolic syndrome associated with disease will help in identification of a therapeutic target in treating this devastating disease.

Keywords: cardiac arrhythmia, cardiovascular diseases, heart diseases, atrial fibrillation, inflammatory markers, metabolic syndrome, gene expression

INTRODUCTION

Cardiometabolic syndrome (CMS) is a substantial cause of worldwide morbidity and mortality and represents a cluster of metabolic abnormalities and significant cardiovascular disease risk factors that can lead to non-communicable diseases (NCD) (1, 2). It has been recently reported that 25% of adults in the US have metabolic syndrome which is attributed to a higher risk for developing atherosclerotic CVD and premature cardiovascular mortality (2–4). Metabolic disorders in metabolic syndrome are associated with insulin resistance, visceral adiposity, obesity, dyslipidemia, endothelial dysfunction, and hypertension with prominent end-organ damages in the cardiovascular system, pancreas, and liver (4, 5). Mitochondrial dysfunction, chronic inflammation, gut microbiome, genetic variation, and environmental contaminants are factors that contribute to the pathogenesis of metabolic syndrome and its transition to CVD (2, 4). Genome-wide association studies (GWAS) studies performed on metabolic syndrome identified genetic variants that are involved in glucose and lipid metabolism (6). Recent studies revealed the importance of the pro-inflammatory state in metabolic syndrome through mediating vascular dysfunction (5). In addition, high levels of serum TNF-α and IL-6 have shown to be linked to obesity and insulin resistance which are key players in metabolic syndrome (2). Thus, far, there is no single biomarker capable of identifying metabolic syndrome, yet a promising panel of biomarkers were shown to be associated with disease (7). Elevated levels of pro-inflammatory cytokines (IL-6, TNF-α), adipokines (Leptin, adiponectin, ghrelin), LAR, prothrombotic factors (PAI-1), uric acid, and pro-oxidant (oxidized LDL) coupled with lower levels of anti-inflammatory cytokines (IL-10) and antioxidant factors (PON-1) are noted in metabolic syndrome (7).

Atrial fibrillation (AF), on the other hand, is a major arrhythmia defined as fast and disorganized electrical excitation of the atria affecting cardiac function (8, 9). AF is considered as a risk factor for stroke, as more than 15% of strokes in the US are due to AF (8). AF together with hypertension could lead to thromboembolic complications and congestive heart failure (10). As a consequence, AF is correlated with increased morbidity and mortality (8). Current treatments of AF target stroke prevention, rate and rhythm control (8, 9). Many research studies proposed gene therapy as a method to reduce the expression of abnormal genes related to the pathogenesis of AF such as ion channels, gap junctions, parasympathetic nervous system, and fibrosis (11). In addition, the impact of gender differences on AF is found to be prominent. Significant differences between women and men with AF are related to AF mechanisms, therapy response stroke risk reduction strategies, as well as other outcomes such as quality of life (8).

Type II diabetes, a major component of CMS, is considered a strong risk factor for AF, and metabolic syndrome has been associated with AF recently (12). Most metabolic syndrome components are found to have an additive effect on the risk of AF (13, 14). Oxidative stress and inflammation are common components related to the pathogenesis of both metabolic syndrome and AF (13). Other components such as hypertension, dyslipidemia, and abdominal adiposity increase the risk of new-onset AF (14, 15). Given the circumstances, studies determined the strong association of low HDL cholesterol, elevated fasting glucose levels in increasing the risk of AF (14, 15). Furthermore, elevated levels of inflammatory mediators were encountered in atrial biopsies from patients with AF (16). The synergistic effect of CMS components is capable of exacerbating the outcome of AF and coronary heart diseases leading to cardiovascular mortality (17). Regardless, the exact mechanism behind the association between CMS and AF is still insufficient and further studies are required to understand the etiology and relation between CMS in AF (15). Increasing evidence supports the role of inflammation-associated cytokines and chemokines in the pathogenesis of atrial fibrillation (AF). Several pharmacological interventions with established anti-inflammatory effects and corticosteroids are associated with AF. Many inflammatory pathways lead to electrical and structural remodeling of the atria that predispose to AF. The Infiltration of immune cells including macrophages and T lymphocytes releasing inflammatory cytokines and inflammatory mediators such as C-reactive protein (CRP) enhance the inflammatory response in cardiac tissue are major players in the development of AF. In the current study, we investigated the inflammatory profile of fibrotic markers as well as cytokine genetic profiles in patients with lone AF, CMS, and combination of both AF and CMS. We further compared the plasma levels of inflammatory cytokines in those patients. The understanding of the inflammatory pathophysiology in AF and CMS patients will help us to identify specific and potential therapeutic strategies for the prevention of AF.

MATERIALS AND METHODS

Study Population

This prospective study included four study groups: patients with CMS and lone AF, patients with CMS without lone AF, patients with lone AF without CMS, and a control group

with neither CMS nor lone AF (previously healthy). According to the modified NCEP ATP III criteria, the following are cardiometabolic risk factors and the presence of any three of these factors is required for a diagnosis of CMS: Abdominal Obesity [>40"/102 cm in men and >35"/88 cm in women], hypertriglyceridemia (≥150 mg/dL or ≥1.7 mmol/L), reduced HDL-C [<40 mg/dL (1.03 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women], elevated blood pressure [≥130/85 mm Hg or use of medication for hypertension], impaired Fasting Glucose [≥100 mg/dL (5.6 mmol/L) or use of medication for hyperglycemia]. Subjects were excluded from this study if they have a history of moderate or severe mitral regurgitation or stenosis, severe aortic stenosis, hyperthyroidism, acute infection, chronic inflammatory diseases, active malignancy, chronic liver disease, cardiomyopathy, or heart failure (EF < 40%) or history of cardiac surgery including CABG or valve replacements.

CMS patients ($n = 11$), lone AF patients ($n = 27$), and patients with both lone AF with CMS ($n = 33$) and Controls ($n = 4$) were recruited for this study from the American University of Beirut Medical Center (AUBMC). All patients provided an informed consent form approved by the Institutional Review Board (IRB) at the AUBMC. Patients were asked to complete a standardized questionnaires to collect baseline information.

Assessing the Cytokine Genotyping and Expression Levels

The cytokine genotyping is based on PCR-SSP methodology which provides sequence-specific oligonucleotide primers for amplification of selected TNF-α, TGF-β1, IFN-γ, IL-6, and IL-10 alleles and the human β-globin gene by the polymerase chain reaction (PCR). These alleles are known to be associated with the expression level of these factors. The primer pairs are designed to have perfect matches only with a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly matched primer pairs result in the amplification of target sequences (i.e., a positive result), while mismatched primer pairs do not result in amplification (i.e., a negative result).

Pre-optimized primers are presented (dried) in different wells of a 96-well 0.2 ml thin-walled tube tray for PCR and are ready for the addition of DNA samples, recombinant Taq polymerase, and specially formulated dNTP-buffer mix (D-mix). Each tray includes a negative control reaction tube that detects the presence of the internal control product generated by the tray. After the PCR process, the amplified DNA fragments are separated by agarose gel electrophoresis and are visualized by staining with ethidium bromide and exposure to ultraviolet light. Interpretation of PCR-SSP results is based on the presence or absence of a specific amplified DNA fragment. Since amplification during the PCR reaction may be adversely affected by various factors (pipetting errors, poor DNA quality, presence of inhibitors, etc.) an internal control primer pair is included in every PCR reaction. The control primer pair amplifies a conserved region of the human β-globin gene, which is present in all DNA samples and is used to verify the integrity of the PCR reaction. In the presence of a positive typing band (specific amplification of a cytokine allele), the product of the internal control primer may be weak or absent due to the difference in concentration and melting temperatures between the specific primer pairs and the internal control primer pair. The amplified DNA fragments of the specific cytokine primer pairs are smaller than the product of the internal control primer pair, but larger than the diffuse, unincorporated primer band. Thus, a positive reaction for a specific cytokine allele or allele group is visualized on the gel as an amplified DNA fragment between the internal control product band and the unincorporated primer band.

Measurement of Human Cytokine Levels and Fibrotic Markers

Serum samples were tested for 13 human inflammatory cytokines using the LEGENDplex™ Human Th cytokine panel for interleukins (ILs, pg/mL) IL-2, 4, 5, 6, 9, 10, 13, 17A, 17F, 21, 22, IFN-γ and TNF-α, which are collectively secreted by Th1, Th2, Th9, Th17, Th22, and T follicular cells (Biolegend). Samples were treated following the manufacturer's instructions and measured with a BD FACS Aria™SORP cell sorter (BD Biosciences). Analysis was done using Data Analysis V8.0 software. Serum levels of IL-18, connective tissue growth factor (CTGF) were tested along with other inflammatory markers including platelet-to-lymphocyte ratio (PLR) and monocyte-to-HDL ratio (MHR).

Statistical Analysis

Our analysis was initiated by carrying out descriptive analysis to provide a summary statistics to all parameters. Continuous parameters were summarized using mean and standard deviation, and categorical parameters were presented using count and percent. To assess the crude unadjusted associations between the IL levels and the groups (AF, AF+CMS, and CMS), we employed the conservative non-parametric tests Mann-Whitney *U* test for the comparisons between the two groups, and Kruskal Wallis for comparisons between multiple groups. Our adjusted analysis was carried out to model the ILs as a function of the groups (AF, AF+CMS, and CMS), and selected clinical and demographic characteristics. In this respect, multiple linear regression was carried out using the logarithmic scale of the ILs employed to achieve symmetry in the outcomes. The AF group was chosen as the reference group. Significance level was chosen to be 0.05, and ILs found to be significantly associated with the groups (AF, AF+CMS, and CMS) at the level of unadjusted analysis were further considered in the multivariable linear regression. To assess the association between categorical variables as in the groups (AF, AF+CMS, CMS) and the stratified genetic polymorphisms (High, Intermediate, Low), we employed the Fisher's Exact test to be on the conservative side when reporting the p-values, given that some of the expected cells were <5 (**Table 2**). In **Table 3**, the mean of ILs are presented across the different groups along with the corresponding P-values obtained using the Kruskal-Wallis test, and in **Table 4** results of the multivariable adjusted analysis for the clinical and demographic characteristics are displayed In Appendix 1-Table a, a detailed summary statistics of the ILs are presented across the different groups. In Appendix 1-Table b, the P-values corresponding to the Mann-Whitney *U* tests unadjusted and adjusted for multiple comparisons are presented

between the groups as well as the P-values for the Kruskal Wallis tests for multiple group comparisons. Box-plots pertaining to the ILs that were significantly associated with the groups (AF, AF+CMS, and CMS) were included in Appendix 2 – Figure 1(a–e). Our data analysis was conducted using SPSS 23, and STATA 14.

RESULTS

Characteristics of the Study Population

Table 1 shows the baseline characteristics or parameters for the study population (AF, AF+CMS, CMS) and are described in terms of mean for continuous data, and percentages for categorical data. These parameters included HDL-C, LDL-C, total cholesterol, systolic blood pressure (SBP), diastolic Blood pressure (DBP), age, gender and smoking status. At baseline, the study population included relatively equal distribution of women (58.3, 54.8, and 36.36%) and men (41.67, 45.16, and 63.64%) among the AF, AF+CMS, and CMS groups.

Gene Expression and Fibrotic Markers Tested in Lone AF, CMS, and AF + CMS Patients

To assess genetic polymorphisms in inflammatory markers such as tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 (TGF-β1), interleukin 10 (IL-10), interleukin 6 (IL-6), interferon gamma (IFN-γ), DNA extraction was performed from lone AF, CMS, AF + CMS, and control patients whose clinical data is described in **Table 1**. The levels of these genetic polymorphisms were stratified to high, intermediate and low (**Table 2**) and the associations between these classes and the groups (AF, AF+CMS, and CMS) were determined using Fisher's Exact tests to be on the conservative side when reporting significant associations especially in the presence of small cell count and expected cells that are <5. Genotyping showed high percentages of patients in the AF, AF+CMS, CMS groups with high secretor genotypes of TGF-β1 (81.48, 70, 72.73%, Fisher's exact test $P = 0.19$) but it did not reach statistical significance. Genotyping showed high percentages of patients in the AF, AF+CMS, CMS groups with high secretor genotypes

TABLE 1 | Baseline characteristics of the study population described in terms of mean (standard deviation) for continuous data, and percentages for categorical data.

Characteristic	AF	CMS	AF + CMS
Age			
Male	58.21 (22.66)	67.25 (7.97)	69.65 (12.65)
Female	67.5 (16.87)	57.57 (12.27)	76.43 (7.21)
All	62.08 (20.58)	61.09 (11.55)	72.71 (10.49)
Gender			
Male	58.33%	36.36%	54.84%
Female	41.67%	63.64%	45.16%
BMI			
Normal weight = 18.5–24.9	38.89%	11.11%	10.71%
Overweight = 25–29.9	33.33%	33.33%	39.29%
Obesity = BMI of 30 or greater	27.78%	55.56%	50%
Average	27.11 (5.75)	31.19 (4.7)	30.61 (6.26)
Blood pressure (mmHg)			
SBP	128.35 (12.92)	143.40 (13.37)	127.52 (19.34)
DBP	77.04 (15.51)	77.70 (12.14)	69.29 (11.56)
Mean	94 (11.75)	99.6 (8.31)	88.74 (13.28)
HDL	56.17 (16.23)	41.1 (11.91)	42.97 (13.17)
LDL	110.07 (31.05)	87.9 (29.99)	92.5 (33.03)
Total cholesterol	180.83 (37.25)	174.8 (51.48)	157.57 (36.95)
Smoking			
Yes	17.39%	20.00%	16.13%
Former	21.74%	0%	9.68%
No	60.87%	80.00%	74.19%
Platelet-lymphocyte ratio (PLR)	135.66 (66.37)	145.06 (63.62)	127.63 (53.92)
Monocyte-HDL ratio (MHR)	9.78 (4.94)	16.71 (10.8)	17.63 (12.4)
CTGF level (pg/ml)	2598.25 (1918.02)	2314.07 (1781.41)	1638.48 (1634.13)

TABLE 2 | Genetic polymorphisms in inflammatory (TNFα, TGF-β, IL-10, IL-6, and IFN-γ) stratified to high, intermediate and low levels in patients with AF, CMS, and AF +CMS.

Gene expression	Marker	Level	AF%	CMS%	AF + CMS%	*P*¥
	Tumor necrosis factor alpha (TNF-α)	High	18.52	10	15.15	0.999
		Intermediate	0	0	3.03	
		Low	81.48	90	81.82	
	Transforming growth factor beta 1 (TGF-β1)	High	81.48	70	72.73	0.19
		Intermediate	18.52	10	24.24	
		Low	0	20	3.03	
	Interleukin 10 (IL-10)	High	7.41	0	18.18	0.05*
		Intermediate	51.85	20	54.55	
		Low	40.74	80	27.27	
	Interleukin 6 (IL-6)	High	95.65	60	86.67	0.037*
		Intermediate	4.35	40	13.33	
	Interferon gamma (IFN-γ)	High	22.22	30	33.33	0.226
		Intermediate	55.56	20	48.48	
		Low	22.22	50	18.18	

¥ *P-value from Fisher's Exact test.*

**Statistical significance with P <= 0.05 was reached.*

of IL-6 (95.65, 60, 86.67%, Fisher's exact test $P = 0.037$), respectively as shown in **Table 2**. IL-10 stratified levels were associated with the AF, AF+CMS, CMS groups with Fisher's exact test $P = 0.05$ (**Table 2**). Genotyping of IFN-γ and IL-10 at high level showed an increase in expression in the AF + CMS group compared to AF and CMS alone suggesting an imbalance between the inflammatory and anti-inflammatory cytokines which is exacerbated by AF.

Evidence for Inflammation in Atrial Fibrillation and Cardiometabolic Syndrome

Serum levels of connective tissue growth factor were tested along with other inflammatory fibrotic markers including connective tissue growth factor (CTGF), platelet-to-lymphocyte ratio (PLR) and monocyte-to-HDL ratio (MHR). CTGF levels were not statistically significant among the groups (Kruskal-Wallis test $P = 0.227$); there was a trend in the CTGF levels between the AF and AF+CMS group (Mann-Whitney test $P = 0.084$). PLR was highest in groups with CMS and lowest in the AF group but was not statistically significant (Kruskal-Wallis test $P = 0.75$, and Mann-Whitney test $P > 0.05$) for all group comparisons. The monocyte-to-HDL ratio (MHR) was significantly different between groups (Krukal-Wallis test $P = 0.005$). MHR was significantly different in particular between AF and AF+CMS groups (unadjusted Mann-Whitney test $P = 0.002$ and adjusted $P = 0.006$ for multiple comparisons). Significant difference in MHR was also detected between AF and CMS groups with Mann-Whitney test $P = 0.029$ unadjusted for multiple comparisons; however, the Mann-Whitney test adjusted for multiple comparisons was not significant with $P = 0.087$. No significant difference in MHR was detected between the AF+CMS and the CMS group (unadjusted Mann-Whitney test, $P = 0.816$).

To assess the level of inflammatory cytokines in the above cohort, human serum cytokine levels were quantified in patients with lone AF, CMS, and AF + CMS. Serum interleukin levels of IL-4, IL-5, IL-10, and IL-17F and IL-22 were significantly different in all groups (**Table 3** and Appendix 1-Tables a,b, Appendix 2 Figures 1a–e). The remaining ILs did not exhibit a significant difference across the groups.

IL-4:

The summary statistics for IL-4 across the three groups: AF, AF+CMS, and CMS depicted in Appendix1-Table A, showed that IL-4 was highest in AF group compared to AF+CMS and CMS group (4.290±2. vs. 2.100±1.859 and 0.874± 0.465 pg/mL, respectively). Kruskal Wallis test was carried out in order to determine if there was an overall group effect on IL-4 levels between the three different groups (AF, AF+CMS, CMS). Two of the groups had significant difference in IL-4 ($P < 0.0001$, Kruskal Wallis; **Table 3** and Appendix 1-Table b). To compare the level of IL-4 between all the groups in a pairwise manner (Appendix 1-Table b), we performed Mann-Whitney *U* tests and presented unadjusted and adjusted P-values for multiple comparisons. Our results indicated that there were unadjusted significance in two pairwise comparisons (AF and AF+CMS; AF and CMS ($P < 0.05$), and one insignificant pairwise comparison between AF+CMS and CMS ($P > 0.05$). This indicated that there was a difference in IL-4 between AF and the remaining two groups which are AF+CMS and CMS. IL-4 was higher in AF compared to that of AF+CMS group (4.290 vs. 2.100, unadjusted $P = 0.001$; adjusted $P = 0.006$) as shown in Appendix 1 Tables a,b. In addition, IL-4 was higher in AF compared to the CMS

TABLE 3 | Inflammatory cytokine mean levels in AF, CMS, and AF + CMS patients along with corresponding standard deviations (SD).

Cytokines*	AF Mean (SD)	AF + CMS Mean (SD)	CMS Mean (SD)	*P*
IL-17A, pg/mL	0.579 (0.824)	0.785 (1.466)	0.089 (0.076)	0.272
IL-17F, pg/mL	2.052 (1.935)	1.370 (1.802)	0.332 (0.196)	0.005*
IFN-γ, pg/mL	4.032 (3.073)	6.392 (7.786)	3.152 (0.962)	0.841
TNF-α, pg/mL	3.074 (1.880)	4.813 (3.594)	3.405 (2.634)	0.250
IL-9, pg/mL	2.426 (1.910)	3.166 (3.017)	1.574 (0.848)	0.273
IL-6, pg/mL	5.166 (2.751)	8.656 (9.107)	4.889 (3.764)	0.566
IL-2, pg/mL	0.463 (0.544)	0.404 (0.587)	0.385 (0.584)	0.750
IL-4, pg/mL	4.290 (2.884)	2.100 (1.859)	0.874 (0.465)	0.000*
IL-5, pg/mL	4.497 (2.509)	4.750 (4.098)	2.033 (0.968)	0.037*
IL-10, pg/mL	2.167 (0.815)	1.833 (1.117)	1.166 (0.150)	0.003*
IL-13, pg/mL	14.367 (13.899)	4.718 (4.438)	-	0.095
IL-21, pg/mL	12.616	9.165	5.304	0.228
IL-22, pg/mL	4.028	3.662	1.078	0.084

**TNF-α, Tumor Necrosis Factor alpha; IFN-γ, interferon-γ; IL-17A, interleukin 17A; IL-17F, interleukin 17F; IL-21, interleukin 21; IL-22, interleukin 22; IL-10, interleukin 10; IL-9, interleukin 9; IL-6, interleukin 6; IL-4, interleukin 4; Il-13, interleukin 13; IL-2, interleukin 2. Kruskal Wallis P-value.*

**Statistical significance was reached, P< 0.05.*

group (4.290 vs. 0.874 pg/mL, unadjusted $P = 0.0001$; adjusted $P = 0.0001$). The remaining comparison, between AF+CMS and CMS were insignificant with Mann-Whitney test unadjusted $P = 0.06$ and adjusted $P = 0.256$, respectively.

IL-5:

The summary statistics for IL-5 across the three groups: AF, AF+CMS, and CMS displayed in Appendix1-Table a showed that IL_5 was highest in AF+CMS group compared to lone AF and CMS group (4.497±2.509 vs. 4.750±4.098 and 2.033± 0.968 pg/mL, respectively). To determine if there was an overall group effect on IL-5 between the three different groups (AF, AF+CMS, CMS), we performed a Kruskal Wallis test which indicated that at least two groups had significant difference in IL-5 ($P = 0.037$, **Table 3** and Appendix 1-Table b). To compare the IL-5 between all the groups in pairwise manner (Appendix 1-Table b), we performed Mann-Whitney *U* tests and presented unadjusted and adjusted P-values for multiple comparisons. Our results showed unadjusted Significance ($P < 0.05$) in one pairwise comparison among AF and CMS groups, and two insignificant in two pairwise comparisons between AF and AF+CMS and between AF+CMS and CMS group ($P > 0.05$). This indicated that IL-5 was higher in AF compared to CMS group (4.497 vs. 2.033 pg/mL, unadjusted $P = 0.004$). However, after we adjusted for the multiple comparisons, AF and CMS groups continued to show significant difference in IL-5 values with Adjusted $P = 0.034$.

IL-10:

The summary statistics for IL-10 across the three groups AF, AF+CMS, and CMS were depicted in Appendix 1-Table a showed that IL-10 was highest in AF group compared to AF+CMS and the CMS group(2.167±0.815 vs. 1.833±1.117 and 1.166± 0.150 pg/mL, respectively). To determine if there was an overall group effect on IL-10, Kruskal Wallis test (**Table 3** and Appendix 1-Table b) was performed that indicating that at least two groups had significant difference in IL-10 ($P = 0.003$). Mann-Whitney *U* test was carried out to compare the IL-10 between all the groups (Appendix 1-Table b) and unadjusted and adjusted P for multiple comparisons were reported. Our results indicate significance in all three pairwise comparisons (unadjusted $P < 0.05$). This indicates that IL-10 was highest in the AF groups compared to the AF+CMS group (2.167 vs. 1.833, unadjusted $P = 0.035$) and highest in AF compared to the CMS group (2.167 vs. 1.166 pg/mL, unadjusted $P = 0.002$). In addition, our results indicated that IL-10 was highest in AF+CMS compared to the CMS group (1.833 vs. 1.166 pg/mL, unadjusted $P = 0.039$). However, after we adjusted for the multiple comparison, only AF and CMS groups had significant difference in the IL-10 (adjusted $P = 0.003$). The remaining comparisons between AF and AF+CMS, and AF+CMS and CMS lost the significance difference that was caught before the adjustment for multiple comparisons. These groups exhibited non-significant difference in the IL-10 after adjustment for multiple comparisons (AF and AF+CMS; AF+CMS and CMS, adjusted $P = 0.16$) (Appendix 1-Table b).

IL-17F:

The summary statistics for IL-17F across the three groups AF, AF+CMS, and CMS (Appendix 1-Table a) showed that IL_17F was highest in AF group compared to AF+CMS and the CMS group (2.052±1.935 vs. 1.370±1.802 and 0.332± 0.196 pg/mL, respectively). To determine if there was an overall group effect on *IL-17F*, Kruskal Wallis test (**Table 3** and Appendix 1-Table b) was carried out between the three different groups AF, AF+CMS, and CMS indicating that at least two groups had significant difference in IL-17F ($P = 0.005$). Mann- Whitney U test was carried out to compare the IL-17F values between all the groups (Appendix 1-Table b), and unadjusted and adjusted P for multiple comparisons were presented. Our results indicate significance in two pairwise comparisons (AF and AF+CMS; AF and CMS, Unadjusted $P < 0.05$) and insignificant pairwise comparison (AF+CMS and CMS, unadjusted $P = 0.05$). This indicated that IL-17F was higher in AF compared to the AF+CMS group (2.052 vs. 1.370 pg/mL, unadjusted $P = 0.039$); and higher in AF compared to the CMS group (2.052 vs. 0.332, unadjusted $P = 0.001$). However, after we adjusted for multiple comparisons, only AF and CMS groups had significant difference in the IL-17F (adjusted $P = 0.005$). The remaining comparisons between AF and AF+CMS groups, and the CMS and AF+CMS groups lost the significant differences that were caught before the adjustment for multiple comparisons and showed non-significant difference in the IL-17F after adjustment for multiple comparisons (multiple comparison, adjusted $P = 0.102$ for the comparisons between AF and AF+CMS, and $P = 0.326$ for the comparisons between AF+CMS and CMS).

IL-18

IL-18 level was assessed by ELISA and was not significant across the groups AF (262 pg/mL), AF+CMS (234 pg/mL), CMS (191 pg/mL) and control (224 pg/mL).

IL-22:

The summary statistics for IL-22 across the three groups AF, AF+CMS, and CMS displayed in Appendix 1-Table a, showed that IL_22 was highest in AF group compared to AF+CMS and the CMS group (4.028±4.508 vs. 3.662±4.639 and 1.078± 1.475 pg/mL, respectively). Kruskal Wallis test carried out in order to determine if there was an overall group effect on the IL-22 between the three different groups (**Table 3** and Appendix 1-Table b). Our results indicated that none of the groups had significant difference in IL-22 after adjusting for multiple comparisons ($P = 0.084$). IL-22 levels were insignificant between all the groups in pairwise manner (Appendix 1-Table b) $P > 0.05$ in two comparisons (AF and AF+CMS, unadjusted $P = 0.992$ for multiple comparisons; AF and CMS, $P = 0.076$). However, IL-22 was significantly higher in the AF+CMS compared to the CMS group (3.662 vs. 1.078 pg/mL, unadjusted $P = 0.021$ for multiple comparisons). However, this significant difference in IL-22 between the AF+CMS and CMS group was no longer present after adjusting for multiple comparisons.

In line with gene expression data, the cytokine serum levels revealed a trend of high levels of IL-6, IFNγ, TNFα, and IL-17A in blood samples drawn from patients with AF CMS compared to patients with CMS or AF alone (**Table 3**). These findings suggest that CMS promotes further the inflammatory process in AF patients. To assess the imbalance of inflammation in those patients, we measured cytokines contributing to the anti-inflammatory responses and protective pathways involved in tissue repair. Furthermore, we found that IL-4, IL-10, IL-13, IL-17F were significantly higher in patients with AF compared to AF CMS patients (**Table 3**). There was a trend in higher levels of IL-21, IL-22 in AF patients alone but didn't reach significance as shown in **Table 3**. In summary our results showed that when a group effect was present on any IL, this effect was mainly triggered by AF since it was the group that exhibited the difference in ILs when compared to AF+CMS and the CMS groups.

Multivariable Adjusted Associations Between Cytokine Levels and Patient Clinical Data

To determine whether serum levels of these cytokines correlated with atrial remodeling, we studied correlations between cytokine levels and parameters in the selected cohort of patients. The inflammatory markers that were significantly associated with the groups (AF, AF+CMS, and CMS) were considered in our multivariable analyses that were adjusted for clinical and demographic characteristics. These parameters included HDL, LDL, total cholesterol, SBP, DBP, age, gender and ever smoked. AF was taken as our reference group. The IL were all transformed to logarithmic scale to achieve symmetry and multivariable linear regressions were carried out whereby the ILs were modelled as functions of the aforementioned clinical and demographic patients' characteristics. Significant ILs at the univariable level of analysis which were considered in the multivariable analyses included IL-4, IL-5, IL-10, and IL-17-F. Results of the multivariable analysis were displayed in **Table 4**.

Our results showed that when adjusting for age, gender, ever smoked, HDL, LDL, total Cholesterol, SBP and DBP, a significant difference in IL-4 level was detected between AF group and AF+CMS, and between AF and CMS group, the AF+CMS had reduced levels of IL4 by 2.19 units compared to AF group ($P = 0.022$) as shown in **Table 4**. Moreover, the CMS group also exhibited a decrease in IL-4 by 9.97 pg/mL compared to AF ($P = 0.001$). The clinical and demographic characteristics were not significantly associated with IL-4. With respect to IL-5 the adjusted analysis showed that IL-5 exhibited a significant difference between AF and CMS groups. The CMS group had a decrease in the mean IL-5 by 3.15 pg/mL ($P = 0.031$) compared to AF. Gender was shown to be significantly associated with IL-5 with females having a decrease in IL-5 by 1.99 units compared to males ($P = 0.044$). HDL was shown to have an incremental association with IL-5 whereby IL-5 increases by 3% when HDL increases by 1 pg/mL ($P = 0.021$). IL-10 was not significantly different between the different groups. Only HDL showed a significant incremental association with IL-10 whereby IL-10 increased by 1% when HDL increased by 1 mg/dL ($P = 0.038$). Age had a borderline significant inverse association with IL-10 ($P = 0.059$) whereby IL-10 was shown to decrease by about 1% when age increased by 1 year. IL-17F was shown to be significantly different between the AF and CMS group ($P = 0.027$). CMS exhibited a decrease in the mean IL-17F by 5.98 pg/mL compared to AF. SBP, HDL and LDL were also significantly associated with IL-17F (P-values were, respectively 0.047, 0.003, and 0.048) as shown in **Table 3**.

DISCUSSION

Recently, many studies emphasize a pathogenic link of inflammation to the development of AF and CMS. Here, we show that activated immune mediators play a key role in the pathogenesis of AF that is aggravated in combination with CMS due to an imbalance in pro-inflammatory profile and anti-inflammatory cytokine response. Th-17 cells, a unique subset of CD4+ T cells, are associated with an increase in IL-17A, IL-17F, IL-21, IL-22 and IFNγ production, and that these cytokines play important roles inflammation, autoimmunity, host defense, and tissue repair (**Figure 1**). IL-21 is a pleiotropic cytokine with effects on innate and adaptive immune cells and has been shown to promote Th-17 and Th-1 cells and inhibit Treg cells and has synergistic effects with IL-17A or IL-17F. IL-17A a pleiotropic pro-inflammatory cytokine, has been implicated in promoting a pro-inflammatory response and fibrosis and among signature Th-17 derived effector cytokines. Pro-inflammatory cytokines produced by immune cells such as IL-6 not only induce platelet activation but are also associated with adverse outcomes in AF patients. CRP has been considered a downstream marker of the inflammatory cascade, specific

TABLE 4 | Multivariable analysis with IL being the outcome of interest and group (AF, AF +CMS, and CMS) as the main predictor, and clinical and demographic characteristics as covariates to factor for in the analysis.

IL	Predictor	Coefficient estimate	Standard Error	*P*	95% CI for the coefficient estimate
IL4 (LOGARITHMIC SCALE)					
	AF (Ref)	—	—	—	—
	AF+CMS	−0.788	0.315	0.022*	−1.449; −0128
	CMS	−2.305	0.553	0.001*	−3.464; −1.147
	Gender	−0.427	0.385	0.281	−1.233; 0.379
	Age	0.001	0.107	0.995	−0.0223; 0.022
	Ever smoked	0.268	0.355	0.459	−0.475; 1.012
	HDL	0.021	0.014	0.149	−0.008; 0.051
	LDL	0.006	0.008	0.477	−0.011; 0.022
	Total cholesterol	−0.006	0.007	0.407	−0.021; 0.009
	SBP	0.028	0.015	0.077	−0.003; 0.061
	DBP	−0.019	0.012	0.156	−0.046; 0.007
IL5 (LOGARITHMIC SCALE)					
	AF (Ref)	—	—	—	—
	AF+CMS	−0.047	0.259	0.856	−0.591; 0.496
	CMS	−1.153	0.494	0.031*	−2.189; −0.118
	Gender	−0.690	0.319	0.044*	−1.358; −0.021
	Age	0.004	0.008	0.610	−0.013; 0.022
	Ever smoked	−0.015	0.292	0.958	−0.628; 0.597
	HDL	0.029	0.011	0.021*	0.004; 0.053
	LDL	0.007	0.006	0.236	−0.005; 0.021
	Total cholesterol	−0.007	0.006	0.254	−0.019; 0.005
	SBP	0.021	0.012	0.107	−0.005; 0.047
	DBP	−0.012	0.011	0.263	−0.034; 0.010
IL10 (LOGARITHMIC SCALE)					
	AF (Ref)	–	-	-	-
	AF+CMS	−0.127	0.133	0.345	−0.396; 0.141
	CMS	−0.374	0.197	0.064	−0.772; 0.023
	Gender	−0.038	0.138	0.783	−0.317; 0.240
	Age	−0.008	0.004	0.059	−0.016; 0.000
	Ever smoked	−0.212	0.126	0.100	−0.467; 0.041
	HDL	0.011	0.005	0.038*	0.001; 0.021
	LDL	0.003	0.003	0.321	−0.003; 0.009
	Total cholesterol	−0.002	0.003	0.361	−0.008; 0.003
	SBP	−0.001	0.004	0.865	−0.009; 0.008
	DBP	−0.004	0.005	0.382	−0.015; 0.006
IL17_F (LOGARITHMIC SCALE)					
	AF (Ref)	-	-	-	-
	AF+CMS	−0.409	0.432	0.355	−1.312; 0.492
	CMS	−1.795	0.751	0.027*	−3.363; −0.227
	Gender	−0.959	0.529	0.085	−2.063; 0.143
	Age	−0.002	0.014	0.864	−0.032; 0.027
	Ever smoked	0.132	0.487	0.789	−0.885; 1.149
	HDL	0.064	0.019	0.003*	0.023; 0.104
	LDL	0.021	0.010	0.048*	0.001; 0.042
	Total cholesterol	−0.017	0.009	0.097	−0.037; 0.003
	SBP	0.044	0.020	0.047*	0.001; 0.088
	DBP	−0.016	0.017	0.375	−0.053; 0.021

The AF was taken as the reference group. ILs that were significant at the univariable analysis were further considered in the multivariable regression and logarithmic transformation was employed on these ILs to achieve symmetry in their respective distributions.

**Significant results with $P < 0.05$.*

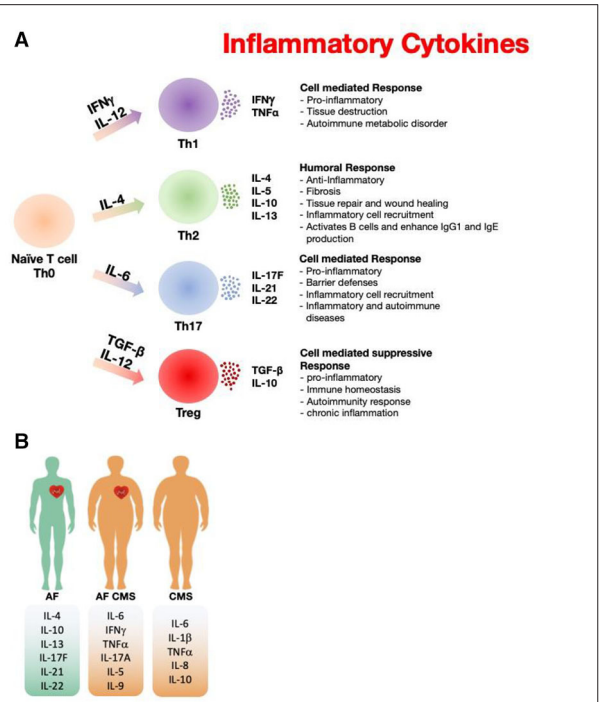

FIGURE 1 | Paradigm illustrating the role of inflammatory markers in atrial fibrillation and cardiometabolic syndrome. **(A)** The inflammatory cytokine released from different types of T cells that have been reported in AF and CMS. **(B)** Differences of the inflammatory cytokine profile among AF, CMS, and AF+CMS recruited patients.

cytokines such as IL-6 and TNF-α have also been linked to AF. IL-6 is a pleotropic cytokine that mediates a variety of biological activities including pro-inflammatory responses and stimulates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. IL-6 is produced by immune cells such as leukocytes and fibroblasts as well as non-immune cells such as endothelial cells, vascular smooth muscle cells and ischemic cardiomyocytes. Previous studies are consistent with our results showing an increase in IL-6 plasma levels in AF. TNF-α secreted by macrophages including those in fat and leukocytes induces activation of the transcription factor nuclear factor (NF)-kB. Thus, both cytokines exhibit a pleiotropic pro-inflammatory response, including myocyte and fibroblast differentiation, proliferation, and migration. Among other "adipokines," IL-6 and TNF-α are also shown to promote CMS, thus, dysregulation of adipokine synthesis and release plays a critical role in insulin resistance. On the other hand, IL-4 is known to suppress the production of some inflammatory cytokines from immune cells and has been shown to promote tissue repair. The elevated levels of IL-4 in blood samples from AF patients compared to combined CMS AF patients highlights IL-4 role in anti-inflammatory responses; thus, less contribution to the inflammation in CMS AF patients. The balance between anti-inflammatory and inflammatory cytokines, such as IL-10 and TNF-α (tumor necrosis factor α), is also associated with AF recurrence. Inducing inhibitory markers or activating pathways that suppress inflammation including IL-10 may be protective and a potential therapeutic strategy for AF that could attenuate adverse cardiac effects.

Despite that both the electrophysiology and structural properties of the atria are critically affected by inflammatory processes, up till today, the anti-inflammatory drugs remain unsatisfactory. Various inflammatory cascades underlying AF may also differ between patients due to genetic polymorphisms such as IL-1, IL-6, and IL-10. For instance, AF patients with high levels of IL-6 owing to its gene polymorphism altering its gene expressions levels as we have shown int his study, did contributes to the pathogenesis of AF. Thus, novel therapies targeting IL-6 in addition to modification of other factors may prove to be beneficial for this disease.

In parallel, an accumulating body of evidence indicates that inflammatory pathways not only interfere with ion channel function of myocytes, but also regulate extracellular homogeneity of atrial tissue and fibrosis. Inflammation is a crucial indicator of fibrosis because of inflammatory signals such as NADPH oxidase, ROS production, cytokines, growth factors, angiotensin II as well as mechanical stretch provoke fibroblast proliferation, migration and differentiation into myofibroblasts. The latter are the principle subtypes in the diseased atrial myocardium to produce cytokines, TGF and MMPs. In this study, genotyping showed high percentages of patients with high secretor genotypes of IL-6 and TGF-β1.

The platelet-to-lymphocyte ratio (PLR) is considered as a new biomarker for predicting inflammation (18). Elevated platelets trigger the infiltration of neutrophils, monocytes, and lymphocytes to the vasculature and hence it is correlated with bad prognosis in CVD (19, 20). On the other hand, monocyte to high-density lipoprotein (HDL) ratio (MHR) is also an inflammatory biomarker predictive for many CVD (21). Monocyte-to-high density lipoprotein ratio (MHR) has been proposed as a novel prognostic indicator of cardiovascular diseases based on the pro-inflammatory effect of monocyte and anti-inflammatory effect of HDL. It has been reported to be related to cardiovascular outcomes in patients with chronic kidney disease and the recurrence of atrial fibrillation. In our present study, interestingly CMS in addition to AF (CMS +AF) displayed a higher MHR ratio suggesting a higher inflammatory response compared to CMS and AF alone. Red blood cell distribution width (RDW) measures the volume range of variation of red blood cells (RBC) (22). The pro-inflammatory state is associated with high RDW (23). As a consequence, RDW was found to be a significant predictor for the development of atrial fibrillation (24).

CONCLUSION

A combination of both CMS and AF may be associated with a higher degree of inflammation in patients than what is seen in either CMS or AF alone. The discovery of a single inflammatory marker or inflammatory pathway contributing to AF may be a promising in the management of these diseases.

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/s.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

BA-S, MK, STA, SA, and MR helped in the patients' recruitment for this study. JE, MS, PZ, JB, AB, RI, MA, and MG helped in the human samples and data collection. HI, MA, AJ, and MR analyzed serum inflammatory cytokine and connective tissue growth factor levels. RM supervised the gene polymorphisms analysis of the inflammatory markers. MR, MAJ, and HI conceptualized the manuscript and the different sections included. MAJ carried out the statistical analysis of the data including the descriptive, non-parametric and multivariable analyses, prepared the tables that displayed the results of these analyses and the boxplots figures, and wrote and interpreted the statistical methods and results in the manuscript. MR supervised the study.

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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.2020.613271/full#supplementary-material

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REVIEW
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Immunomodulatory Approaches in Diabetes-Induced Cardiorenal Syndromes

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Immunomodulatory approaches are defined as all interventions that modulate and curb the immune response of the host rather than targeting the disease itself with the aim of disease prevention or treatment. A better understanding of the immune system continues to offer innovative drug targets and methods for immunomodulatory interventions. Cardiorenal syndrome is a clinical condition that defines disorders of the heart and kidneys, both of which communicate with one another through multiple pathways in an interdependent relationship. Cardiorenal syndrome denotes the confluence of heart-kidney relationships across numerous interfaces. As such, a dysfunctional heart or kidney has the capacity to initiate disease in the other organ via common hemodynamic, neurohormonal, immunological, and/or biochemical feedback pathways. Understanding how immunomodulatory approaches are implemented in diabetes-induced cardiovascular and renal diseases is important for a promising regenerative medicine, which is the process of replacing cells, tissues or organs to establish normal function. In this article, after a brief introduction on the immunomodulatory approaches in diseases, we will be reviewing the epidemiology and classifications of cardiorenal syndrome. We will be emphasizing on the hemodynamic factors and non-hemodynamic factors linking the heart and the kidneys. In addition, we will be elaborating on the immunomodulatory pathways involved in diabetes-induced cardiorenal syndrome namely, RAS, JAK/STAT, and oxidative stress. Moreover, we will be addressing possible therapeutic approaches that target the former pathways in an attempt to modulate the immune system.

Keywords: cardiorenal syndromes, diabetes mellitus, ras pathway, JAK/STAT pathway, oxidative stress, immunomodulatory approaches

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IMMUNOMODULATORY APPROACHES IN DISEASES

Immunomodulatory approaches are defined as the all interventions that modulate and curb the immune response of the host rather than targeting the disease itself (1). With the ongoing attempts of treating infectious diseases amidst the increased pathogen resistance to traditional infectious disease control approaches, immunomodulatory interventions are being closely reviewed (1). Immunomodulatory medicines alter the response of the immune system by increasing or decreasing the production of serum antibodies, using immunostimulators and

immunosuppressives, respectively (2). Immunostimulators are administered in order to enhance the immune response against infectious diseases, tumors, primary or secondary immunodeficiency, and alterations in antibody transfer. However, immunosuppressive drugs are used to reduce the immune response against transplanted organs and to treat autoimmune diseases (2). The ability of immunomodulatory approaches to modulate the immune system's disease response is evidently credited to the disruption of the proinflammatory cascade through various mechanisms involving the antioxidants effects, disruption of bacterial flora, monoclonal antibodies, cytokines, and related extracellular immune mediators and alterations in cell signaling (3). Hence, selectively either inhibiting or intensifying the specific populations and subpopulations of immune responsive cells (2).

Immunomodulatory approaches are heavily used in the medical field. In oncology, particularly for cancers unresponsive to known agents, antitumor immunotherapy is evidently decreasing fatalities through immune-cell-targeted monoclonal antibody (mAb) therapy and adoptive cellular therapy (ACT) (4). Immunomodulatory approaches have also gained attention in cardiovascular diseases by inducing inhibition to early inflammation initiators including reactive oxygen species, inhibition of mast cell degranulation and leukocyte infiltration and blocking the inflammatory cytokines and inhibiting the adaptive B and T-lymphocytes (5). That in addition to nephrology, where manipulation of the patients innate immune system, leads to the enhancement of renal repair and recovery of renal tissue hence, diminishing acute kidney injury without the progression to chronic renal diseases and consequently renal failure (6).

In this regard, understanding how immunomodulatory approaches are implemented in diabetes-induced cardiovascular and renal diseases is important for a promising regenerative medicine. Heart failure, particularly with preserved ejection fraction (HFpEF) is highly prevalent in diabetic patients (7). Several factors increase the risk of heart failure in diabetic patients. These include abnormal cardiac handling of glucose and free fatty acids (FFAs), the effect of the metabolic derangements of diabetes on the cardiovascular system and the effect of most anti-diabetic agents with glucose-lowering molecules that have direct downregulations on the cardiovascular system (8). Consequently, careful assessment should be done on how to treat patients suffering from cardiac and renal disease concurrently. This is where immunomodulatory approaches take effect given that Diabetes Mellitus (DM) is an autoimmune disease with progressive status of chronic, low-grade inflammation (LGI) (9).

Mesenchymal stem cells (MSC) are gaining large interest as being a potential approach for both heart diseases and diabetes (10). MSC are multipotent stromal cells, non-hematopoietic progenitor cells that have shown to have wide immunomodulatory capabilities by altering the adaptive and innate immunity. These capabilities are due to the natural differentiating capacity of MSCs into various different cell lineages (11). When an autoimmune disease is the target, MSC modulate the immune system of the host by inhibiting the proliferation of T cells stimulated with either polyclonal mitogens (12), allogeneic cells or specific antigens (13) through inducing an arrest to the lymphocytes at the G0/G1 phase of the cell cycle (14). For instance, Diabetic Cardiomyopathy (DCM), defined as the cardiac dysfunction that's characterized with structural, functional and metabolic changes in the myocardium that results in impaired cardiac functions (15). DCM is a distinct entity, first proposed by Lundbaek in 1954, as diabetic heart disease independent of hypertension and coronary artery disease (CAD) that are usually highly prevalent in Diabetes Mellitus (Type-2 Diabetes Mellitus) (16). In this context, chronic low-grade inflammation poses great significance in obesity and T2DM which later showed evidence in contributing to the pathogenesis of DCM (17). Mesenchymal stromal cells have been shown to have anti-diabetic as well as cardioprotective features as mentioned earlier. Studies on the mechanisms of action of mesenchymal cells showed several MSCs functions in DCM (18). One of which is the Anti-Inflammatory feature of MSCs. MSCs influence the infiltration of T cells in the pancreas along with reducing the cardiac inflammation (18). Moreover, MSCs also have the capacity to decrease cardiac tumor necrosis factor α (TNF-α) and interleukin-1 expression which both are involved in the initiation and progression of diabetic cardiomyopathy. Hence this anti-inflammatory effect of MSCs protects against myocardial inflammation under Diabetes mellitus (18).

Also, MSCs have an anti-oxidative capacity where MSCs secrete the superoxide dismutase which has the potential to treat diabetic cardiomyopathy (19). This is due to the findings showing that the overexpression of extracellular superoxide dismutase decreases macrophage infiltration and fibrosis thus leading to improved left ventricular function in the diabetic heart (18). This in addition to the anti-fibrotic features of MSCs through reducing cardiac fibrosis via weakening the survival, differentiation, proliferation, and collagen synthesis of cardiac fibroblasts (20). These features are not the only capacities of MSCs on immunomodulatory approaches to Diabetic Cardiomyopathy,

Abbreviations: CRS, Cardiorenal Syndrome; RAS, Renin Angiotensin System; JAK, Janus kinase; STAT, Signal Transducer And Activator of Transcription; DM, Diabetes Mellitus; MSC, Mesenchymal stem cells; DCM, Diabetic Cardiomyopathy; DN, Diabetic Nephropathy; mAb, Monoclonal antibody; ACT, Adoptive cellular therapy; HFpEF, Heart failure with preserved ejection fraction; FFAs, Free fatty acids; LGI, Low-grade inflammation; TNF-α, Tumor necrosis factor-alpha; NF- κB, Nuclear Factor Kappa B; TGF-β, Transforming Growth Factor-Beta; IFN-γ, Interferon-gamma; IL, Interleukin; Treg, T-regulatory cells; Th17, T-helper 17; CAD, Coronary artery disease; GFR, Glomerular filtration rate; GBM, Glomerular basement membrane; LXA4, Lipoxin 4; ADQI, Acute Dialysis Quality Initiative; HF, Heart failure; RBF, Renal blood flow; SNS, Sympathetic nervous system; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; NOX, Nicotinamide Adenine Dinucleotide Phosphate oxidase; NOS, Nitric Oxide Synthase; ACE, Angiotensin Converting Enzyme; AT-1, Angiotensin II type 1 receptor; AT-2, Angiotensin II type 2 receptor; PKC, Protein Kinase C; AP-1, Activator Protein 1; Cx43, Connexin 43; VCAM-1, Vascular Cell Adhesion Molecules-1; ICAM-1, Intracellular Adhesion Molecule-1; ET-1, Endothelin 1; TLR-47, Toll-Like Receptor 4; ERK, Extracellular Receptor Kinase; MAPK, Mitogen Activated Protein Kinase; COX-2, Cyclooxygenase 2; ROS, Reactive Oxygen Species; SOCS3, Cytokine Signaling 3; LPS, Lipopolysaccharide; CD14, Cluster of Differentiation 14; ADMA, Asymmetric Dimethyl Arginine; TxA2, Thromboxane A2; MMP, Matrix Metalloproteinase; XO, Xanthine Oxidase; EMT, Epithelial-Mesenchymal Transition; MAPK, Mitogen-Activated Protein kinase; CoQ10, Coenzyme Q10; STZ, Streptozotocin-induced; DCs, Dendritic cells; PD-1, Programmed death 1; QLQX, Qiliqiangxin.

others include anti-apoptotic features, pro-angiogenic and endothelial-protective features, cardiac progenitor cell-protective features and $Ca2^{+}$ Modulating features (18).

To this end, mesenchymal stem cells "MSC" have been intensively studied as an immunomodulatory approach for the treatment of diabetes and improving the cardiovascular activity. In the context of diabetes research, Bone marrow derived MSCs have been used to form insulin-producing cells and enhance islet engraftment and survival and to treat diabetic ulcers and limb ischemia (21). In experimental models of type 2 diabetes (T2D), the mesenchymal stem cells inoculum improved metabolic control and reduced insulin requirements and of A1C with no significant opposing results after the intra-arterial injection by selective cannulation of the pancreas vasculature (21).

In a study done by Si et al., rat models with induced T2D were used to investigate the effects of autologous MSC inoculum. The autologous MSCs were injected shortly "1 or 3 weeks" after the streptozotocin treatment. streptozotocin "STZ" is used to induce a hyperglycaemic state to induce diabetes' (22). The results showed improved metabolic control through enriched insulin secretion, amelioration of insulin insensitivity and increased islet numbers in the pancreas. These results are in consistency with research evidence on the potential therapeutic properties of MSCs to treat diabetes (22). The potential capacity of bone marrow derived MSCs in enhancing the cardiovascular activity in diabetic cardiomyopathy was also investigated and has been shown to lie in its direct differentiation to cardiomyocytes and the ability to secrete potent trophic and paracrine mediators which induces cardiac regeneration and cardio protection (23). Studies on rats with type 1 DM, intravenous administration of bone-marrow derived MSCs has shown to improve cardiac function by increasing angiogenesis and attenuating cardiac remodeling. This is attributed to the differentiation of the transplanted MSCs into cardiomyocytes and improved angiogenesis and myogenesis therefore increasing matrix metalloproteinases MMP-2 activity, decreasing that of MMP9 and reducing collagen load in the diabetic myocardium (24).

After this overview of various immunomodulatory approaches related to Cardiovascular Diseases and especially Diabetic Cardiomyopathy, we will look at the immunomodulatory approaches to a Renal Disease: Diabetic Nephropathy (DN). The latter is a syndrome characterized by the presence of pathological quantities of urine albumin excretion, diabetic glomerular lesions, and loss of glomerular filtration rate (GFR), thickening of the glomerular basement membrane (GBM) in diabetic patients (25). Many immunomodulatory approaches are proposed to deal with DN. One of which is also MSCs. In a study done at the Kunming Medical University showed that MSCs ameliorate the renal function and extend survival in diabetic rats, regulate the production of lipoxin 4 (LXA4) and ALX/FPR2 (the receptor of LXA4) in kidney tissue of DN, protect renal function and inhibit fibrosis (26). Other approaches include the novel bifunctional cytokine Interleukin-233 (IL-233) that bears IL-2 and IL-33 activities that reverses inflammation and protects against Type 2 Diabetic Nephropathy by promoting T-regulatory cells (Treg) and type 2 immune response. IL-233 also attenuates hyperglycaemia and proteinuria, preserves renal structure and function for long-term and restores glucose clearance and inhibits visceral adiposity (27).

The high prevalence of this syndrome entails the discovery of new treatments to curb the progression of the disease. Because of a better understanding of the disease, there have been many advances in immunomodulatory approaches used for treating diabetes-based cardiorenal diseases. As such, currently there are improvements in the applications of this knowledge to clinical settings, which have led to treatments that are more effective. Since treatments with single agents did not achieve stable metabolic remission as such, future immunomodulatory approaches would focus on dosing, timing, and recognition of the differences between different species. As a result, the next step would be to focus on combined therapy to improve the efficacy of the treatment by promoting additive effects.

In this regard, in what follows we tackle the cardiorenal syndrome; epidemiology & classification, the relation between the cardiac and renal systems and the pathways involved in cardiorenal syndrome in diabetes namely, RAS, JAK/STAT and oxidative stress.

CARDIORENAL SYNDROME

The homeostasis in the human body is maintained by the coordinated work of several organs and systems. The most important key players in the homeostasis are the heart and the kidneys. The heart is the pump of the body, which is responsible for circulating blood within the body. On the other hand, the kidneys are responsible for filtering the blood and for the electrolyte homeostasis. These two organs are interlinked; where a dysfunction in one organ affects the other. From this tight relation emerges the cardiorenal syndrome. According to Ronco et al. (28) the cardiorenal syndrome is "the disorders of the heart and kidneys where one organ affects the other." Another more holistic definition was stated by Bock and Gottlieb (29) in their article in which they have mentioned that the cardiorenal syndrome is when "each dysfunctional organ (heart and kidneys) has the ability to initiate and perpetuate disease in the other organ through common hemodynamic, neurohormonal, immunological, and/or biochemical feedback pathways." Although these two definitions have been established around 10 years ago, to this day a clear mechanistic understanding of the cardiorenal syndrome is not yet agreed upon (30).

Epidemiology of Cardiorenal Syndrome

It is estimated that 25 to 60% of patients with heart failure have some type of cardiorenal syndrome which is associated with high morbidity and mortality (31). In the United States, the prevalence of chronic heart disease is estimated at 2% of people over 45 of age. In comparison, in end stage renal disease, 30% of the patients have chronic heart disease upon the initiation of dialysis (32) and cardiovascular disease is the most common cause of death in those patients (33).

Classifications of Cardiorenal Syndrome

In 2008, a consensus conference on cardio-renal syndromes was held in Venice Italy under the umbrella of the Acute

Dialysis Quality Initiative (ADQI) (28). In this conference the classification of the types of the cardiorenal disease were agreed upon. These classifications are considered clinical which lack structural and functional analysis of the disease mechanisms and therapeutic options (34). However, these classifications are useful as a first step to reach a functional classification (35). The classification system is divided into five types of cardiorenal syndrome (28).

Acute Cardio-Renal Syndrome (Type 1)

This type is characterized by the acute worsening of heart function leading to kidney injury and/or dysfunction. Acute worsening of heart function might include pulmonary oedema, cardiogenic shock, acute heart failure (HF). Approximately 27 to 40% of patients admitted with acute heart failure develop acute kidney failure (36).

Chronic Cardio-Renal Syndrome (Type 2)

This type is similar to the first type, except that the heart failure or dysfunction here is chronic which leads to kidney failure. This is the most common type of cardiorenal syndrome. It has been reported that 63% of patients admitted with congestive heart failure have type 2 cardiorenal syndrome (37).

Acute Cardio-Renal Syndrome (Type 3)

This type is the opposite of type one, wherein the acute renal impairment causes acute cardiac dysfunction or failure. The epidemiology of this subtype has proven to be a challenge to define due to the wide definition of acute kidney injury and limited reporting on it (28).

Chronic Cardio-Renal Syndrome (Type 4)

This type is characterized by chronic kidney injury leading to cardiac injury, disease, and/or dysfunction, such as left ventricular failure or diastolic heart failure. There is a strong correlation between the severity of the kidney injury and the unfavorable heart outcomes (38).

Secondary Cardiorenal Syndrome (Type 5)

This type is characterized by the simultaneous cardiac and renal dysfunction that is caused by a systemic condition that may be chronic or acute. These systemic conditions include sepsis, diabetes mellitus, amyloidosis, and other chronic inflammatory conditions (28).

CARDIORENAL SYNDROME IN DIABETES

The relation between the heart and the kidneys is a pretty complex one. Many factors, including pathways, molecules, and dysfunctions, come at play to make this tight link. It is hard to study each factor on its own because of their interlinked nature; however, some articles were able to classify these factors into hemodynamic factors and non-hemodynamic factors, as shown in **Figure 1** (34). It is important to keep in mind that although this classification is in place, these factors are not independent of each other. It is also evident that these two factors have a strong relation with diabetes. Most of the diabetic complications are due to the macro and microvascular injury, that in turn affect the hemodynamic factors (39). On the other hand, in diabetes the production of mitochondrial ROS plays a huge role in the pathogenesis of diabetes and the development of its complications (40). This lies under the umbrella of non-hemodynamic factors. Moreover, fibrosis is a unifying mechanism linking cardiorenal syndromes. Fibrosis is a result of many metabolic derangements, whether in the heart or in the kidney, which eventually lead to cardiorenal syndromes.

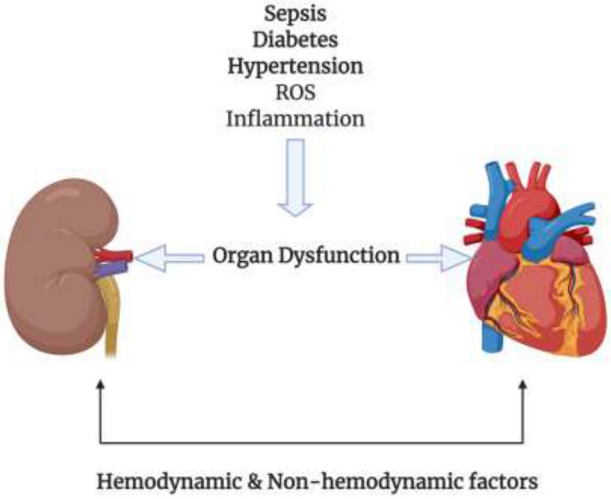

FIGURE 1 | Different conditions such as Sepsis, diabetes, hypertension, ROS, and inflammation cause damage to the kidney or heart where one organ has the ability to injure the other organ by the means of hemodynamic and non-hemodynamic factors linking the heart and the kidneys.

Hemodynamic Factors and Non-hemodynamic Factors

Hemodynamic Factors

Hemodynamics, or in other words dynamics of the blood flow, are tightly controlled by homeostatic mechanisms. Dysfunctions in this well-maintained system have adverse effects on numerous organs and tissues especially the kidneys. The renal blood flow is the primary driver of the glomerular filtration rate (GFR). According to the well-known equation, the GFR equals the renal plasma flow times the filtration fraction. Moreover, many articles that date as back as the mid twentieth century have established that reduced renal blood flow and increased central venous pressure are primary effector mechanisms for renal impairment (38, 41). Later studies suggested the renal autoregulation phenomenon, where the reduction in renal blood flow (RBF) was out of proportion to the reduction in cardiac index, while GFR was relatively maintained (42). However, when the renal blood flow drops further, GFR declines as autoregulatory capacity is exhausted (43). In the last few years, the research focus has shifted to the venous congestion as another important factor in the drop of theGFR, independent of the renal blood flow (44).

Non-hemodynamic Factors

As stated before, hemodynamic and non-hemodynamic factors are not independent of each other. It is very hard to study the effect of one factor in isolation of the other. Especially that the non-hemodynamic factors, also called cardiorenal connectors, act on the glomerular filtration rate by changes on the hemodynamics. Thus, these cardiorenal connectors are more mediators than direct effectors. The non-hemodynamic factors include a wide range of factors that include the renin angiotensin system (RAS), sympathetic nervous system (SNS) activation, inflammation, endothelial dysfunction.

The RAS is considered a prototypical cardiorenal connector since it is activated bidirectionally by the heart and the kidneys upon failure. Renin is released when renal artery pressure is decreased (45), renal venous pressure is increased (46) and when the delivery of sodium to the distal nephron is decreased which all occur in heart failure and chronic kidney disease. Moreover, angiotensin II has an important effect on renal perfusion and it promotes renal fibrosis, which directly affects GFR, induces hypo-responsiveness to natriuretic peptide and mediates SNS activation (47). The SNS is responsible for altering the ultrafiltration coefficient and is associated with tubular injury and the formation of reactive oxygen species (ROS) (40). Angiotensin II is also responsible for modulating oxidative stress and endothelial dysfunction. Through Nicotinamide Adenine Dinucleotide Phosphate [NADP(H)] activation, angiotensin II promotes the formation of reactive oxygen species, which can cause intrarenal (proximal tubular) damage (33).

Fibrosis as a Unifying Pathophysiology of the Cardiorenal Syndromes

Fibrosis is a complex cascade of cellular and molecular processes caused by disease related injury. Over a short period of time, fibrosis serves as an adaptive process that helps the organ. However, over an extended period of time, fibrosis will cause parenchymal scarring and ultimately cellular dysfunction and organ failure (48). The main cause of fibrosis in the heart and kidney is inflammation- and oxidative stress–related endothelial dysfunction in aging, hypertension, diabetes mellitus, and obesity (49).

As much as the details described above shows how the heart and kidney are affected, it is of interest to determine the possible pathways that could be implicated in the cardiorenal syndromes.

IMMUNOMODULATORY PATHWAYS INVOLVED IN CARDIORENAL SYNDROME IN DIABETES

RAS Pathway

RAS is one of the most important cardiorenal connectors. Improper activation of RAS can lead to both heart and renal failure. In heart failure, RAS alongside the SNS are overactivated (50). Our focus in this section will be Angiotensin II which is the most important effector molecule in the RAS pathway, shown in **Figure 2** (51). In response to a drop in blood pressure and/or sodium chloride (NaCl) level, Renin which is also known as Angiotensinogenase, is secreted by the Juxtaglomerular apparatus in the kidneys (52). It is also secreted in response to SNS activity via the β-1-adrenoceptor activation by norepinephrine secretion which induces inflammation via LPS-induced IL-6 production (52–54). Renin acts on Angiotensinogen that is secreted by the liver transforming it to Angiotensin I via hydrolysis; which will then be transformed to Angiotensin II by the action of Angiotensin Converting Enzyme (ACE) that will have multiple effector sites (52). Angiotensin II, upon binding to its AT-1 (Angiotensin II type 1 receptor) and AT-2 receptor (Angiotensin II type 2 receptor) (53, 55), induces the production of Interleukin-6 (IL-6) and of the TNF-α via the Protein Kinase C (PKC) pathway, followed by the activation of two transcription factors: first of which is the Nuclear Factor Kappa B (NF-κB) through phosphorylation of p65 and then the Activator Protein 1 (AP-1) (53). These two transcription factors are important in the pathway for expression of TNF-α and thus for inducing an inflammatory response. This might be an important future perspective in the crosstalk between inflammatory cytokines and RAS in the heart. Another important cytokine induced by Angiotensin II binding to the AT-1 receptor is IL-1β. IL-1β plays a role in heart failure via systolic dysfunction and ventricular remodeling by upregulating Transforming Growth Factor-Beta (TGF-β) (56). Il-1β impairs systolic function by decreasing the expression of genes important in the regulation of calcium homeostasis (57). It also increases Nitric Oxide Synthase (NOS) expression in cardiac myocytes which leads to an increase in Nitric Oxide (NO) activity, a decrease in energy production and a lower myocardial contractility (57). In addition to that, according to a study performed on diabetic mice, IL-1β leads to cardiac arrhythmia by causing a prolongation of action potential duration, a decrease in potassium current and an increase in Calcium sparks in cardiomyocytes (58). IL-1β also affects the synchronized contraction of the heart by decreasing the expression of Connexin 43 (Cx43), a major protein in the cardiac gap junctions (57). Additionally, Angiotensin II induces the production of yet another cytokine, IL-17 via AT-1 receptor binding (59). IL-17, which is produced by T-helper 17 (Th17) cells, triggers the production of other proinflammatory cytokines such as IL-6 and TNF-α, and contributes to the pathogenesis of hypertension and atherosclerosis; as well as, insulin resistance (59).

To add to that, Angiotensin II upregulates the expression of Selectins (P-, E-, and L-selectins), as well as, Vascular Cell Adhesion Molecules-1 (VCAM-1) and Intracellular Adhesion Molecule-1 (ICAM-1), via TNF-α (55, 60). These markers are elevated in Chronic Kidney Disease and Chronic Heart Failure (60). Angiotensin II also increases the kidney expression of Endothelin 1 (ET-1) (61), which stimulates vasoconstriction, inflammation, and fibrosis (cardiac remodeling) (62). Thus, this expression is increased in hypertension, heart failure, and kidney disease. Another role of Angiotensin II in oxidative stress, apoptosis, and inflammation is via Toll-Like Receptor 4 (TLR-4) expression via binding to AT-1 receptor (55). The TLR4 signaling inflammatory cascade mediates renal dysfunction via phosphorylation of Extracellular Receptor Kinase (ERK) and

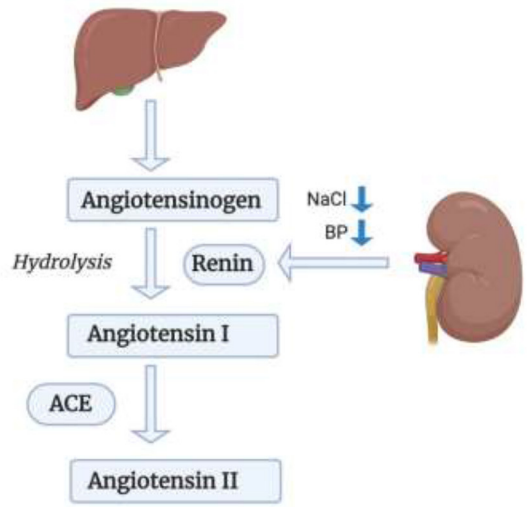

FIGURE 2 | RAS pathway in leading kidney failure. Renin acts on Angiotensinogen secreted by the liver, transforming it to Angiotensin I, which will then be transformed into Angiotensin II via ACE.

Mitogen Activated Protein Kinase (MAPK) (63). The MAPK signaling is also activated by Angiotensin II. Furthermore, Angiotensin II plays a role in oxidative stress via Cyclooxygenase 2 (COX-2) activation to generate vasoactive prostaglandins and ROS which will play a role in endothelial dysfunction (55). It also generates ROS upon binding to its AT-1 receptor via NADPH oxidase (NOX) (55). A summary of some signaling pathways of Angiotensin II is shown in **Figure 3**.

JAK/STAT Pathway

IL-6, which is also activated by Angiotensin II, is secreted as a result of ischemia, and binds to plasma membrane receptors which leads to a signal transduction pathway activating JAK and STAT proteins (64). This signaling pathway has an important role in diabetic nephropathy via an Angiotensin II-dependent mechanism, and is negatively regulated by Suppressor of Cytokine Signaling 3 (SOCS3) by either inhibiting the JAK tyrosine kinase activity or by competing with STATs on cytokine receptors (65). This means SOCS3 negatively regulates IL-6. Another protein mediated by STAT3 is Ephrin-B2, which is increased in diabetes (66). Ephrin-B2 stimulates cardiac fibrosis by the activation and interaction of STAT3 and TGF-β/SMAD3 signaling pathways (67). IL-6 and TNF-α are also regulated by the interaction between Lipopolysaccharide (LPS) and Cluster of Differentiation 14 (CD14) via activation of Nuclear Factor Kappa B (NF-κB) signaling (68).

ROS INDUCED INFLAMMATORY AND CARDIORENAL SYNDROMES IN DIABETES

Oxidative Stress is described as an imbalance between oxidants (like ROS) and antioxidants (like NO), which results in an accumulation of the oxidants (69). So, it is when the production of oxidants (ROS) is greater than the body's antioxidative metabolic ability. ROS play a major role in hypertension, cardiovascular disease, and renal damage, which emphasizes their contribution to cardiorenal syndrome (as shown in **Figure 4**) (55). ROS are small molecules derived from oxygen; they are generated in several cellular processes. One major way is Angiotensin II-induced activation of NADPH oxidase, as we mentioned before.

Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase, also known as NOX, plays a major role in ROS formation via utilizing NADPH as an electron donor to reduce oxygen and produce superoxide anions (O_2^-) and Hydrogen Peroxide (H_2O_2) (70). The high levels of oxygen radicals lead to mitochondrial dysfunction by inactivating mitochondrial enzymes and cause DNA damage at the cellular level (71). At the organ level, our focus will be on the cardiorenal axis and markers implicated. The imbalance between ROS and NO is one of the most reliable markers of oxidative stress. The decrease in NO can be due mainly to two things: Reaction of NO with oxygen radicals and high concentrations of Asymmetric Dimethyl Arginine (ADMA) (33). Superoxide anion (O_2^-) reacts with NO to form Peroxynitrite ($ONOO^-$), the accumulation of which results in vasoconstriction, inflammation, and impaired vascular and renal functions (70). This decrease in NO inhibits P450 enzymes and leads to the production of vasoconstriction molecules (72) via enhancing Cyclooxygenase activity, which promotes the production of Thromboxane A2 (TxA2) (vasoconstrictor) (72). Also, Peroxynitrite increases Thromboxane synthase activity and thus TxA2, so it increases the vasoconstrictor; and inhibits Prostacyclin synthase and thus decreases Prostacyclin-2 production (vasodilator); which leads to an imbalance between the vasoconstrictors and vasodilators which contributes to the pathogenesis of both the heart and the kidney (72). Another factor contributing to the decrease of NO is the high concentration of circulating ADMA, which is an endogenous inhibitor of Nitric Oxide Synthase (NOS) and is highly observed in renal failure (73).

On the other hand, in heart failure, mitochondrial dysfunction plays a role via Angiotensin II by upregulating NOX2; we also have a Mitochondrial-ROS independent pathway by Angiotensin II that results in the upregulation of NOX4 (71). In addition to that, ROS activates Matrix Metalloproteinase (MMP) in cardiac fibroblasts, which leads to structural changes in the myocardium (74). This leads to cardiac remodeling, decrease in contractility, dysfunctional Calcium handling and eventually heart failure (74). Moreover, Hyperuricemia, which is the accumulation of uric acid mainly due to malfunctioning Xanthine Oxidase (XO), also leads to oxidative stress via a dysfunction in the release of ROS and NO. This is associated with kidney disease, heart disease and diabetes (75). ROS also has proinflammatory effect by releasing cytokines; in addition to profibrotic effect by inducing Epithelial-Mesenchymal Transition (EMT) via the Mitogen-Activated Protein kinase (MAPK) activation or SMAD signaling and this will lead to renal fibrosis, in addition to cellular hypertrophy via Extracellular Receptor Kinase (ERK1/ERK2) pathways (70). These most important proinflammatory cytokines are TL-6 and TNF-alpha, and the main transcription factor responsible for

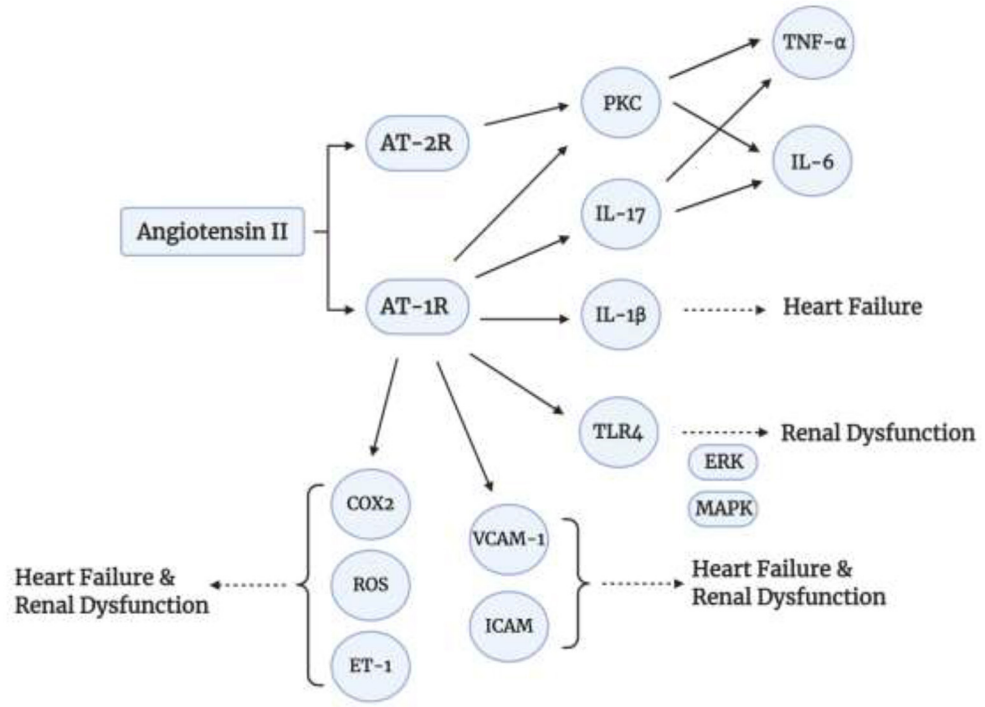

FIGURE 3 | Angiotensin II signaling pathways. Angiotensin II induces inflammatory cytokine production via binding to AT-1 and AT-2.

initiating the proinflammatory response is NF-kB, the expression of which is increased in diabetic experimental models (76). So, the downstream effects of NF-kB are proinflammatory via MCP-1, TNF-alpha, and IL-6 regulation. Furthermore, at the level of the mitochondria, excess-glucose leads to increased glucose-derived pyruvate oxidation which increases the number of electron donors in the electron transport chain resulting in over production of superoxide (76).

ANTIOXIDANTS AND ANTI-INFLAMMATORY APPROACHES TO TREAT DIABETES-INDUCED CARDIORENAL SYNDROME

As discussed previously and evidenced in the literature, oxidative stress has a huge role in injury and pathogenesis of cardiorenal syndrome (77). Therefore, it logically entails that antioxidants have a possible protective ability that would prevent cardiorenal syndrome. The role of oxidative stress and antioxidants appears clearly in diabetes mellitus related cardiorenal syndrome (type 5) (78). Therefore, we will focus in this part on evidence that has explained and proven the protective role of antioxidants in diabetes mellitus and type 5 cardiorenal syndrome. Since the 1960s, the role of antioxidants in improving health and well-being was discovered by biologists (79). In the next decade, Cameron and Pauling (80) were able to find that ascorbic acid (vitamin C) is a potential human cancer protective agent. Since then, antioxidants have become a hot topic in medical research and scientists are looking deeper into the mechanisms, molecular targets, and molecular interactions of antioxidants (81). The antioxidant defense mechanisms are divided into 2 categories: enzymatic and non-enzymatic strategies. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. While non-enzymatic antioxidants include the vitamins A, C, and E, glutathione, α-lipoic acid, mixed carotenoids, coenzyme Q10 (CoQ10), several bioflavonoids, antioxidant minerals (copper, zinc, manganese and selenium), and cofactors like folic acid, uric acid, albumin (82).

In his experiment, Kunisaki et al. (83) was able to show that when he administered vitamin E to diabetic rats, the retinal blood flow and PKC activity in the vascular tissue were normalized. Also, another two short-term experimental studies proved that high doses of vitamin C and lipoic acid can improve some aspects of endothelial dysfunction in diabetes (84, 85). Furthermore, it has been recently reported that vitamin E has the ability to reduce the oxidative stress that builds up in the macrophages in diabetic mice (86). Finally, other studies were able to find prophylactic effects of vitamin E on heart failure patients that have type 1 diabetes. They were able to show that the "supplementation of streptozotocin-induced (STZ)-diabetic rats with 2000 IU of vitamin E/kg of feed beginning immediately after induction of DM and continuing for 8 weeks provided significant protection against cardiac dysfunction induced by T1DM" (87).

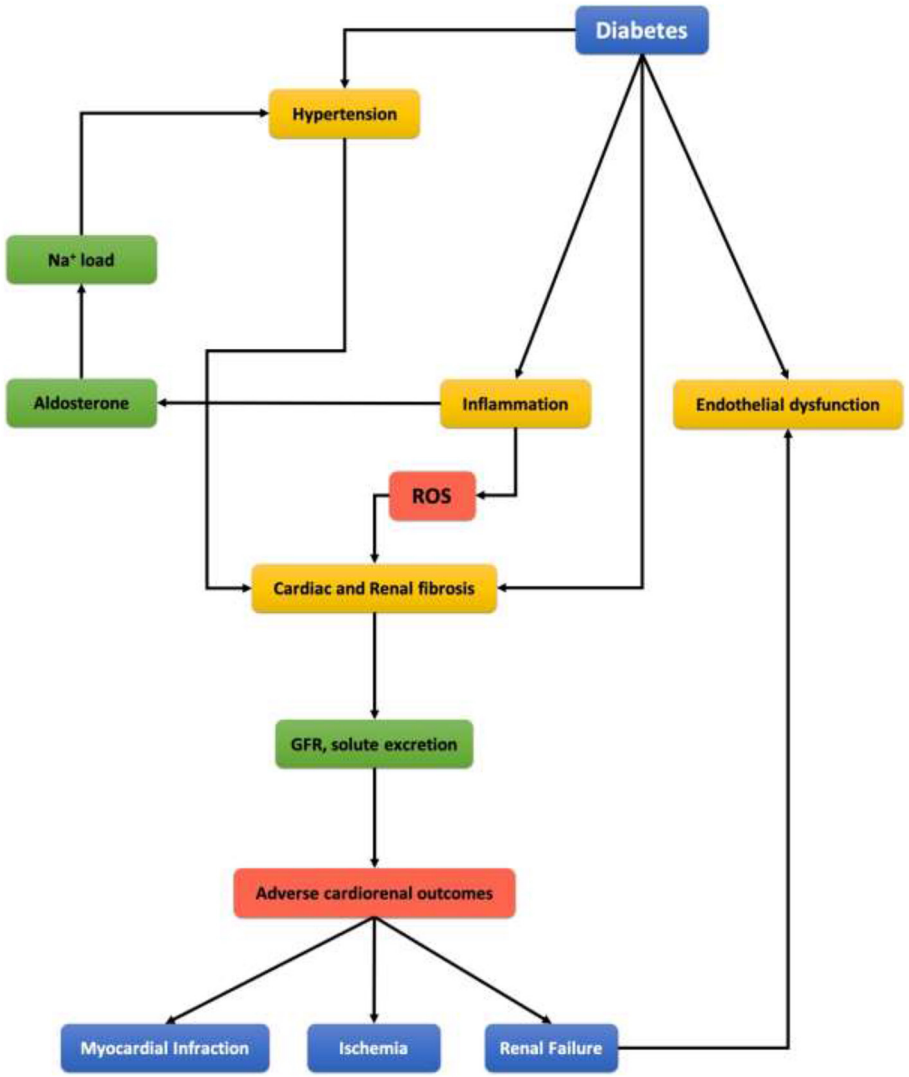

FIGURE 4 | Representing scheme of clinical link between diabetes mellitus, cardiovascular disease, and chronic kidney disease.

As previously revisited mesenchymal stem cells "MSCs"; these cells have the capacity to self-renew rendering them an important immunomodulatory approach to autoimmune diseases (88). MSCs produce soluble factors that can increase the production of anti–inflammatory cytokine interleukin (IL)−10 and decreased production of interferon–gamma (IFN–γ) and IL−12 through altering the secretion of dendritic cells (DCs) (89, 90) MSCs engage the inhibitory molecule programmed death 1 (PD−1) to its ligands PD–L1 and PD–L2 thus, suppressing T–cell proliferation (90, 91). This control over T-cells including $CD4^+CD25^+FoxP3^+$ (90), has influence on the susceptibility to diabetes induction (92) Moreover MSCs, through the production of soluble factors can also inhibit the proliferation and secretion of B cells (90). This release of trophic and immunomodulatory factors by MSCs seems to hold the therapeutic capacity of these cells (90).

Another therapeutic approach for the cardiorenal syndrome is via the Chinese herbal medication Qiliqiangxin (QLQX). QLQX is composed of 11 different herbs that are alismatis rhizome, carthami flos, cinnamomi ramulus, ginseng radix et rhizome, astragali radix, citri reticulatae pericarpium, salvia miltiorrhiza radix et rhizome, aconiti lateralis radix preparata, semen descurainiae lepidii, periploca cortex, and polygonati odorati rhizome (93). It has been shown that QLQX can be used for regulating the immune response and improving circulation via the Astragali Radix component and acts by reducing the production of TNF-α (93). In addition to that, QLQX has a similar effect as Olmesartan by inhibiting the AT-1 receptor; and thus, inhibits the Ang II-induced cardiac fibroblasts' transdifferentiation via reducing IL-6 transcription and regulating nuclear activity of Nuclear Factor of Activated T-cells (NFAT3) (94). By that, QLQX attenuates cardiac

inflammatory reactions and protects myocardial structure and function in HF (94). On the other hand, QLQX can also protect against renal injury in cardiorenal syndrome (CRS) by regulating the oxidative stress and inflammation signaling (95). QLQX significantly reduced inflammatory cytokines and AT receptors in the kidney reducing the inflammatory response; in addition to, reducing the ROS content and thereby regulating the oxidative stress response (95).

Additionally, a study has shown that QLQX improves autophagy via TRPV-1 dependent mechanism in the diabetic heart (9). It also showed that QLQX treatment improves cardiac function and myocardial phenotype in the diabetic mouse model (96). To add to that, QLQX improves endothelial aortic function in diabetic rats via RAS and NO pathways; by inhibiting the expression of ACE and AT-1, and by regulating the NO balance (97).

CONCLUSION

CRS defines the different clinical conditions in which heart dysfunction and kidney dysfunction overlap, it describes the negative effects of an impaired renal function on the heart and circulation. In this review, we have thoroughly explained CRS beginning from epidemiological data and classifications aiming to clarify the historical lack of a clear definition due to the complexity of this disease. Immunomodulation encompasses all therapeutic interventions aimed at modifying the immune response as such offering innovative drug targets and methods for immunomodulatory interventions. A better understanding of CRS holds the promise of regenerative medicine, which points toward repairing damaged tissues, stimulating the healing mechanism of organs and implanting laboratory grown tissues when the body is unable to heal itself.

AUTHOR CONTRIBUTIONS

AE and FH conceived and designed the review article and approved the final version to be submitted. LA, MN, and NS performed the literature review and wrote the first draft of the review. HG discussed, assembled the data, and revised the manuscript for intellectual content. NA worked on the figures of this review. All authors contributed to the article and approved the submitted version.

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Comprehensive Analysis of the Immune Infiltrates and Aberrant Pathways Activation in Atherosclerotic Plaque

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Atherosclerosis is the pathological basis of many cardiovascular and cerebrovascular diseases. The development of gene chip and high-throughput sequencing technologies revealed that the immune microenvironment of coronary artery disease (CAD) in high-risk populations played an important role in the formation and development of atherosclerotic plaques. Three gene expression datasets related to CAD were assessed using high-throughput profiling. CIBERSORT analysis revealed significant differences in five types of immune cells: activated dendritic cells (DCs), T follicular helper cells (Tfhs), resting CD4+ T cells, regulatory T cells (Tregs), and γδ T cells. Immune transcriptome analysis indicated higher levels of inflammatory markers (cytolytic activity, antigen presentation, chemokines, and cytokines) in the cases than in the controls. The level of activated DCs and the lipid clearance signaling score were negatively correlated. We observed a positive correlation between the fraction of Tfhs and lipid biosynthesis. Resting CD4+ T cells and the activity of pathways related to ossification in bone remodeling and glutathione synthesis showed a negative correlation. Gamma delta T cells negatively correlated with IL-23 signaling activity. GSEA revealed a close association with the inflammatory immune microenvironment. The present study revealed that CAD patients may have an inflammatory immune microenvironment and provides a timely update on anti-inflammatory therapies under current investigation.

Keywords: immune infiltrates, atherosclerotic plaque, coronary artery disease, pathways, immune microenvironment

INTRODUCTION

Coronary artery disease (CAD) leads to myocardial infarction (MI), ischemic cardiomyopathy, and arrhythmia, and it is the main source of cardiovascular morbidity, mortality, and the economic health burden worldwide (1). The past decade of research has provided a deeper understanding of the pathogenesis and treatment of CAD (2). Despite this success, little progress has been made on elucidating the function of the immune microenvironment and its therapeutic Implications (3). Growing evidence suggests that that many aforementioned risk factors, such as smoking habits, obesity, hypertension, insulin resistance, diabetes, stress, and hyperlipidemia are primarily

responsible for CAD (4). A significant link exists between the pathophysiology of CAD and inflammatory mediators, immune cells, oxidative stress, lipid infiltration, extracellular matrix, and hormone metabolism (4–8).

High-throughput sequencing technologies examine the role of the immune microenvironment of CAD in high-risk populations in the formation and development of atherosclerotic plaques (APs) (4, 9–12). A growing number of studies indicate that cardiovascular events are determined by multiple biological processes that require different tailored therapeutic approaches, and future therapies for CAD should tackle the residual inflammatory process that statin therapy only partially addresses (13). Inhibitors of specific components of the immune microenvironment in atherosclerosis were developed for considerable treatment (13–15). For example, trials involving anti-cytokine therapy for atherosclerotic CAD targeted interleukin (IL)-1β, which plays numerous roles in atherogenesis, plaque growth, and subsequent rupture (16). Pre-clinical studies also revealed that the inhibition of IL-6 or its receptor achieved atheroprotective effects (17, 18).

Converging lines of evidence support the importance of the immune microenvironment in the initiation, progression, and vulnerability of atherosclerotic plaques. Therefore, we analyzed the immune microenvironment based on three CAD datasets. Several immune infiltration analyses were used to reveal the molecular mechanisms of various factors, such as immune cells, cytokines, chemokines, and abnormally activated pathways, in the occurrence and development of CAD and provide a theoretical basis for anti-atherosclerotic treatment strategies and the prevention of this disease.

MATERIALS AND METHODS

Data Processing

We collected three datasets related to CAD from the Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo/) (19), for analysis using the GEOquery R package (20). The GSE40231 dataset based on the GPL570 platform included samples from 40 CAD patients (40 atherosclerotic aortic walls vs. internal mammary arteries) and was used to examine differences in immune filtration in APs. The GSE20681 dataset, based on GPL4133, included 99 whole blood samples from CAD patients and 99 whole blood samples from healthy controls. The GSE20681 samples were paired based on case/control status, age, and gender. GSE20680 included whole blood samples from 143 CAD patients and 52 healthy individuals and were both based on GPL4133. We used normalizeBetweenArrays of the limma R package to the normalize expression data in GSE40231, GSE20681, and GSE20680 (21). **Figure 1A** shows the workflow of our study.

Abbreviations: CAD, coronary artery disease; DCs, dendritic cells; Tfhs, T follicular helper cells; Tregs, regulatory T cells; AS, Atherosclerosis; APs, atherosclerotic plaques; TNF, tumor necrosis factor; ROS, reactive oxygen species; GEO, Gene Expression Omnibus database; CYT, cytolytic activity; ssGSEA, single-sample gene set enrichment analysis; GSEA, gene set enrichment analysis; GEP, gene expression profile; γδ T cells, gamma delta T cells; NKs, natural killer cells; LDL, low density lipoprotein.

Immune Profiling Based on the CIBERSORT Algorithm

The CIBERSORT algorithm uses the LM22 gene sets to estimate the proportion of 22 infiltrating immune cells in samples based on the expression data (22). We used this algorithm based on the default parameters to calculate the relative proportion of 22 infiltrating immune cells in our samples.

We also used this LM22 gene set to compare differences in the detailed gene expression of the significantly altered immune cells. The genes related to cytolytic activity (CYT), antigen presentation, chemokines, and cytokines in CAD identified in previous studies were used to evaluate differences in immune function between the CAD and control groups (23, 24).

Evaluation of the Abnormal Signaling Signature Based on Single-Sample Gene Set Enrichment Analysis (ssGSEA) and Gene Set Enrichment Analysis (GSEA)

The abnormal signaling score was calculated from expression data (GSE40231, GSE20681, and GSE20680) using the ssGSEA algorithm and the GSVA R package (25–27). Gene sets of pathways were collected from the MsigDB database (28). The limma R package was used to calculate the difference in gene expression (logFC) between the CAD and control groups in this study (21). GSEA was performed using the "clusterProfiler" R package and the Molecular Signatures Database (MSigDB) to annotate the dataset, and Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome terms were considered significant at $P < 0.05$ (29).

Statistical Analysis

The Wilcoxon signed-rank test (paired) was used to compare differences in the relative proportion of infiltrating immune cells and differences in the immune-related gene expression profile (GEP) between the CAD and control groups. $P < 0.05$ was considered statistically significant, and all statistical tests were two-sided. R software (version 3.6) was used for statistical analyses. The R package ComplexHeatmap was used to visualize the heatmap (30). The R package ggplot2 was used to create the violin plot (31). The corrplot R package was used to visualize the correlation heatmap (32).

RESULTS

Immune Cells in CAD Cases

To identify a robust immune infiltrating pattern, we normalized three gene expression datasets (GSE40231, GSE20681, and GSE20680) based on the normalizeBetweenArrays method of the limma R package (**Supplementary Figure 1**). The CIBERSORT algorithm was used to calculate the proportion of 22 immune cells based on the expression data of the discovery dataset (GSE40231; **Figure 1B**). Notably, the fractions of activated DCs and Tfhs were significantly higher in the CAD than in the control groups (**Figure 1C**). In contrast, the CAD group had fewer Tregs, γδ T cells, and resting CD4+ T cells (**Figure 1C**) than the control

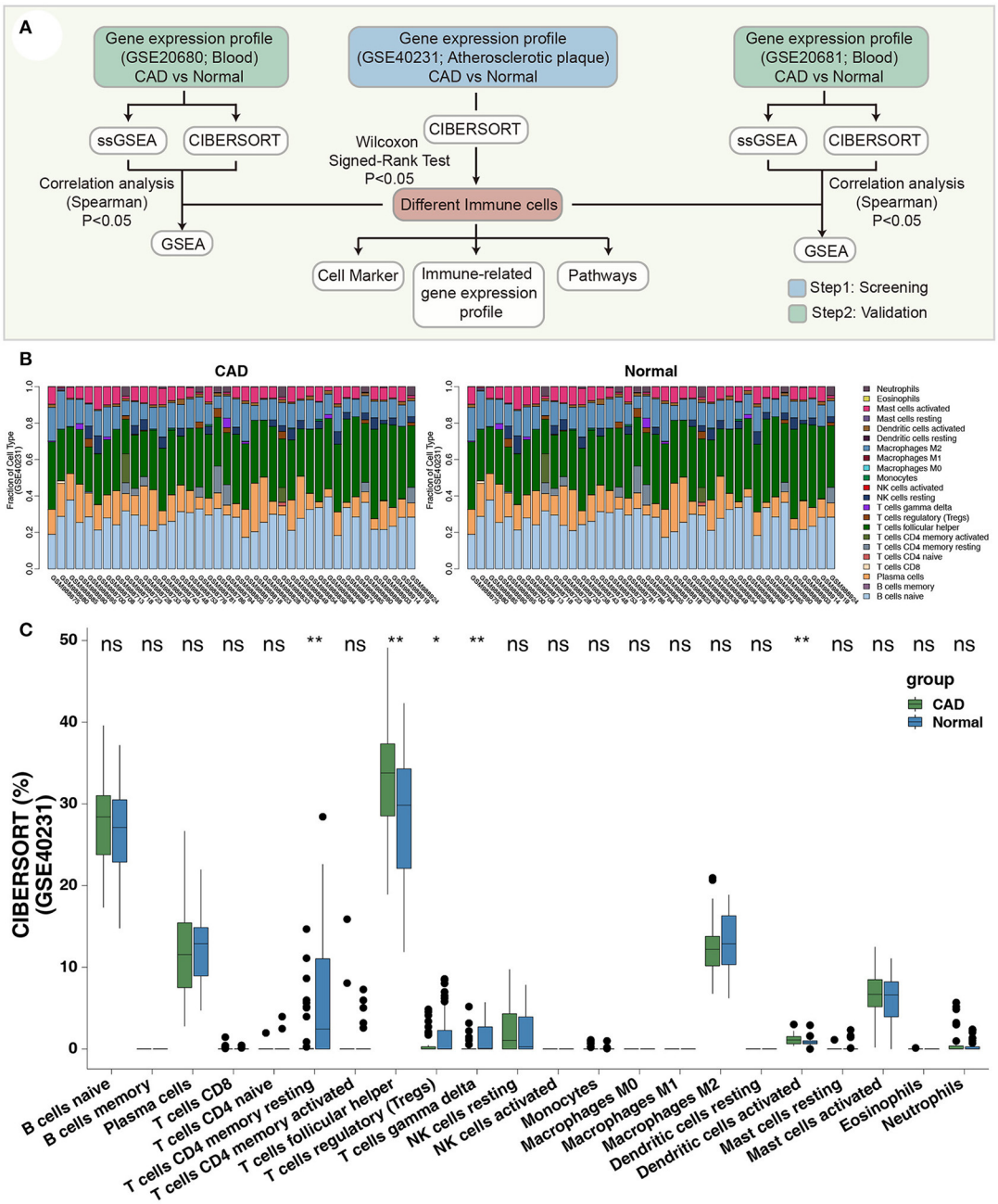

FIGURE 1 | (A) The workflow of the present study. **(B)** The bar plot indicates the proportions of the 22 immune cells of each sample in GSE40231. **(C)** The proportions of immune cells of the CAD cases vs. the healthy controls in GSE40231 using CIBERSORT analysis. Wilcoxon signed-rank test (paired): $^{**}P < 0.01$; $^{*}P < 0.05$. CAD, coronary artery disease.

group. These results suggest that CAD exhibits inflammatory microenvironment patterns.

Different GEPs Related to Immune Cells, CYT, Antigen Presentation, Chemokines, and Cytokines

We focused on the comparison of five differential immune cell markers between the CAD and control groups. Two cell types in CAD (activated DCs and Tfhs) exhibited increased expression levels of immune cell-related genes (**Figures 2A,B**; all Wilcoxon signed-rank Ps < 0.05). Tregs, γδ T cells, and resting CD4+ T cells in the control group had low expression levels of immune cell-related genes (**Figures 2C–E**; all Wilcoxon signed-rank Ps < 0.05). We performed the Wilcoxon signed-rank test on the expression levels of genes related to immune function between the CAD and control groups and found that the GEPs related to CYT (CD8A),

antigen presentation (HLA-A, HLA-DQA1, HLA-DQA2, and HLA-DQB1), chemokines (CCL5, CX3CL1, and CXCL10), and cytokines (IFNA1 and IFNA2) were significantly stronger in the CAD group than in the control group (all Wilcoxon signed-rank Ps < 0.05).

Identification of Abnormal Pathways Related to Five Immune Cell Types in CAD

To uncover the different signaling signatures between the CAD and control groups, we performed ssGSEA to calculate the scores of the pathways of each sample using the GSVA R package. Spearman's correlation analysis was performed to determine the relationship between the score of the ssGSEA and the fraction of each of the five immune cells. The proportion of activated DCs and the activity of lipid clearance–related signatures, including LDL and plasma lipoprotein clearance pathways (**Figure 3A**), showed a significant negative correlation (Spearman's R $= -0.23$; $P = 0.04$, and R $= -0.23$; $P = 0.04$, respectively; **Figure 3A**). Patients with a high proportion of activated DCs showed lower activity of the negative regulation of muscle hypertrophy (**Figure 3A**). To further explore whether activated DCs negatively correlated with pathways related to lipid clearance/muscle hypertrophy, we performed the above analyses in two datasets (GSE26081 and GSE26080). Notably, the content of activated DCs also negatively correlated with the signature of lipid clearance and muscle hypertrophy (**Supplementary Figure 2A**). The increase in Tfhs significantly correlated with the upregulated lipid biosynthetic process (Spearman's R $= 0.3$; $P = 0.0073$; **Figure 3B**). In contrast, this increase negatively correlated with the negative regulation of cellular extravasation (Spearman's R $= -0.26$; $P = 0.022$; **Figure 3B**). The score for negative regulation of glucocorticoid metabolic process negatively correlated with an increased proportion of Tfhs (Spearman's R $= -0.24$, $P = 0.31$; **Figure 3B**). These results showed a similar trend in these datasets (GSE26081 and GSE26080; **Supplementary Figure 2B**). **Supplementary Figures 2C,D** shows the correlation between the fractions of the two immune cell types and the activity of abnormal pathways. Overall, these results further indicated that CAD was associated with increased activation of DCs and Tfhs, the downregulated activity of lipid clearance and glucocorticoid metabolism, and the upregulated activity of cellular extravasation (**Figure 3C**). Notably, the proportion of Tregs and the score of these pathways, including metabolism of angiotensinogen to angiotensins, lipopolysaccharide, and smooth muscle cell migration, exhibited negative Spearman's correlations (R $= -0.27$, $P = 0.017$; R $= -0.23$, $P = 0.036$; and R $= -0.25$, $P = 0.024$, respectively; **Figure 4A**). We observed that the increased resting CD4+ T cells negatively correlated with the activity of some pathways related to ossification in bone remodeling and glutathione synthesis (R $= -0.32$; $P = 0.0033$; R $= -0.34$; $P = 0.036$; R $= -0.25$; $P = 0.024$; **Figure 4B**). There was a negative correlation between the fraction of γδ T cells and inflammation-related pathways, such as IL-23 signaling (R $= -0.25$; $P = 0.025$; **Figure 4C**) and NOTCH2 signaling (R $= -0.25$; $P = 0.027$; **Figure 4C**). Spearman correlation analysis showed similar results between these immune cell types (Tregs, resting CD4+ T cells and γδ T cells) and abnormal signaling signatures (**Figure 4D** and **Supplementary Figures 4A–C**). These results demonstrated that differentially abundant immune cells that are associated with abnormal signaling signatures may play an important role in the pathogenesis of CAD.

Identification of Differential GEPs and Key Pathways in CAD

We extracted the GEPs of hub genes in key pathways (related to **Figures 3**, **4**) and compared these GEPs between the CAD and control groups in GSE40231. Genes related to lipid biosynthesis were significantly upregulated in the CAD group compared to the control group, and genes associated with lipid clearance signaling were significantly downregulated in the CAD group compared to the control group (**Figure 5A**). GSEA in three independent datasets indicated that immune cell-related signatures, such as natural killer cells (NKs), B cells, myeloid leukocytes, neutrophils, Th2, mononuclear cells, and CD8+ T cells were significantly enriched in the CAD group compared to the control group in all datasets (**Figure 5B**). Similarly, cytokine-related pathways, such as IL-1, IL-4, IL-6, IL-8, IL-13, IL-18, and IFN-γ signaling were significantly enriched in the CAD samples compared to the control group (**Figure 5C**). Endothelial cell morphogenesis, regulation of glomerular filtration, ROS metabolic process, insulin resistance, positive regulation of vascular smooth muscle cell proliferation, ERK/MAPK targets, and regulation of angiotensin levels in blood were significantly upregulated in the CAD group (**Figure 5D**).

DISCUSSION

We analyzed gene expression data of CAD patients and healthy controls from three independent datasets—a discovery dataset (GSE40231) and two validation datasets (GSE20681 and GSE60280)—to examine the immune microenvironment of coronary artery disease (CAD). CIBERSORT analysis revealed significant differences in five immune cells: activated DCs, Tfhs, resting CD4+ T cells, Tregs, and γδ T cells. Cell markers of these immune cells and immune-related functional genes were used for downstream analyses. Immune transcriptome analysis indicated that higher expression levels of inflammatory markers (CYT, antigen presentation, chemokines, and cytokines) were detected in the CAD group than in the controls. The ssGSEA algorithm based on the GSVA R package was used to calculate the activity of pathways that may be associated with the pathogenesis of CAD. The proportion of activated DCs and the lipid clearance signaling score showed significant negative correlations. We also observed a positive correlation between the fraction of Tfhs and the lipid biosynthetic signature. Spearman's correlation analysis revealed that the increased proportion of Tregs correlated with the downregulated activity of some pathways, such as metabolism of angiotensinogen to angiotensins, lipopolysaccharide, and smooth muscle cell migration. Notably, resting CD4+ T cells and the activity of some pathways related to ossification in bone remodeling and glutathione synthesis showed a negative

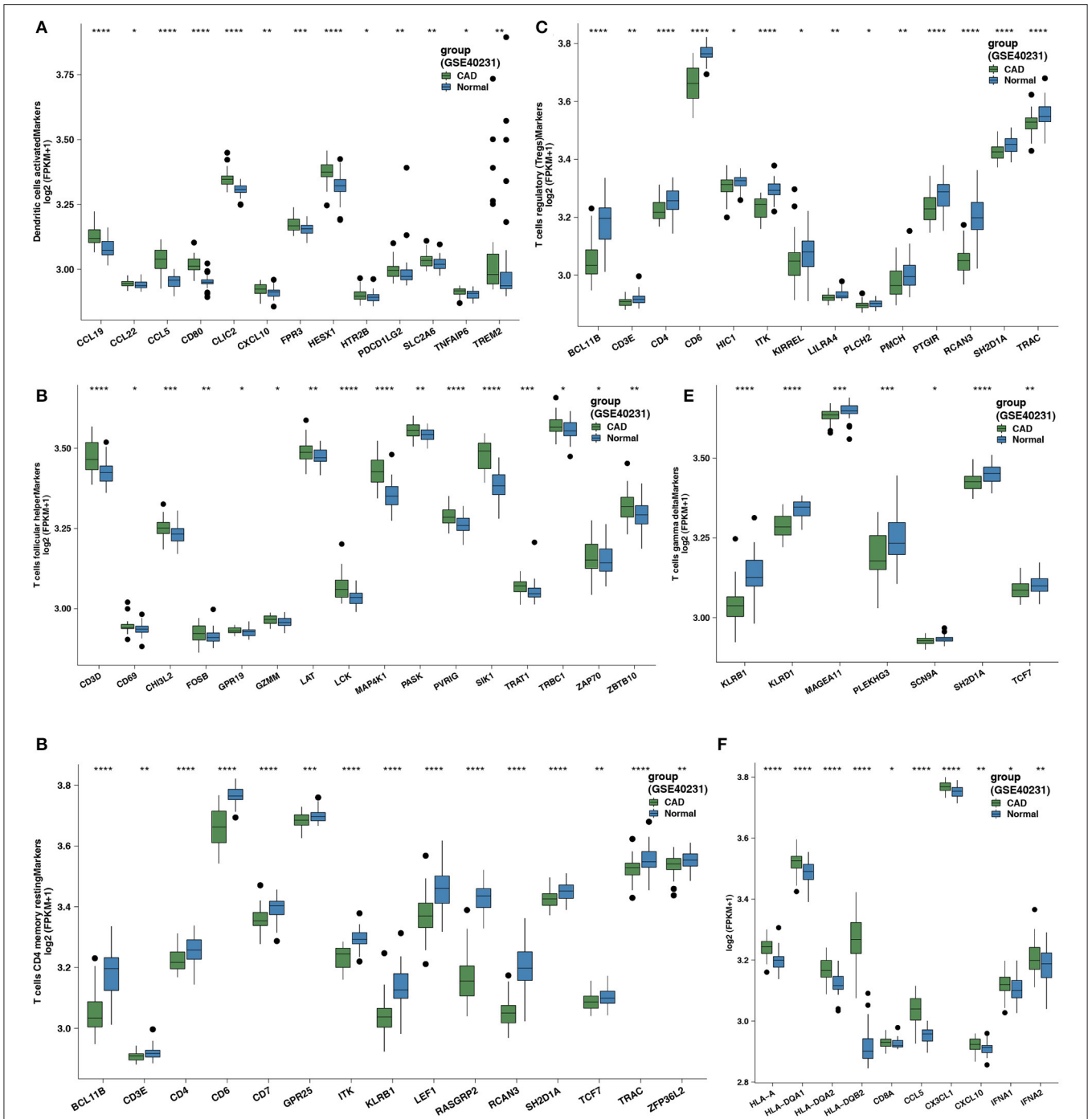

FIGURE 2 | (A) Comparison of the expression of cell markers of activated DCs between the CAD cases vs. the healthy controls in GSE40231. **(B)** Comparison of the expression of cell markers of Tfhs between the CAD cases vs. the healthy controls in GSE40231. **(C)** Comparison of the expression of cell markers of Tregs between the CAD cases vs. the healthy controls in GSE40231. **(D)** Comparison of the expression of cell markers of resting CD4+ T cells between the CAD cases vs. the healthy controls in GSE40231. **(E)** Comparison of the expression of cell markers of γδ T cells between the CAD cases vs. the healthy controls in GSE40231. **(F)** Comparison of the expression of CYT-related, antigen-presentation, and proinflammatory-related genes (chemokine and cytokine) between the CAD cases vs. the healthy controls in GSE40231. DCs, dendritic cells; CAD, coronary artery disease; CYT, cytochrome; Tfhs, T follicular helper cells; Tregs, regulatory T cells; γδ T cells, gamma delta T cells. Wilcoxon signed-rank test: $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

correlation. The γδ T cells negatively correlated with IL-23 signaling activity. GSEA revealed a close association between the inflammatory immune microenvironment, including activated immune cells, chemokines and cytokines, and CAD. Therefore, we identified the immune microenvironment associated with inflammatory signaling based on the expression profiles of CAD

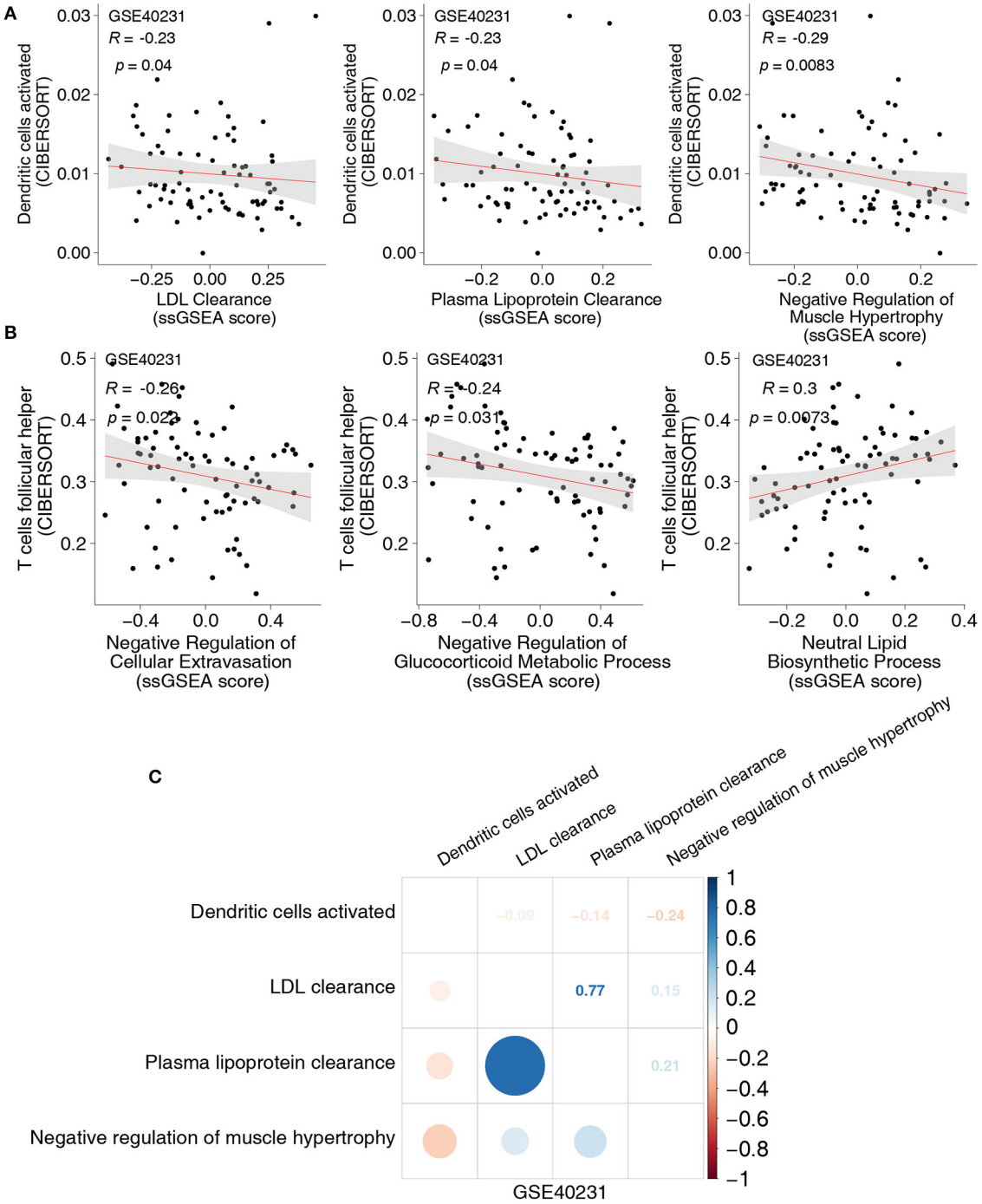

FIGURE 3 | (A) The correlation between the proportions of activated DCs and the score of ssGSEA in GSE40231. **(B)** The correlation between the proportions of Tfhs and the score of ssGSEA in GSE40231. **(C)** The correlation heatmap of the proportions of activated DCs and the score of ssGSEA in each three-gene expression dataset (GSE40231). DCs, dendritic cells; Tfhs, T follicular helper cells; ssGSEA, enrichment analysis.

cases. We summarized the possible mechanisms underlying the immune microenvironment in CAD (**Figure 6**).

DCs are the most powerful known antigen presenting cells and are a key link between innate immunity and acquired immunity. DCs play an important role in the occurrence and development of atherosclerosis (33). The release of proinflammatory cytokines, such as CCL5, CX3CL1, and CXCL10 promotes the migration of DCs (34–36). Mature and activated DCs accumulate locally with the progression of Aps (37). Liu et al. reported that the decreased expression of CX3CR1 and CX3CL1 and the downregulation

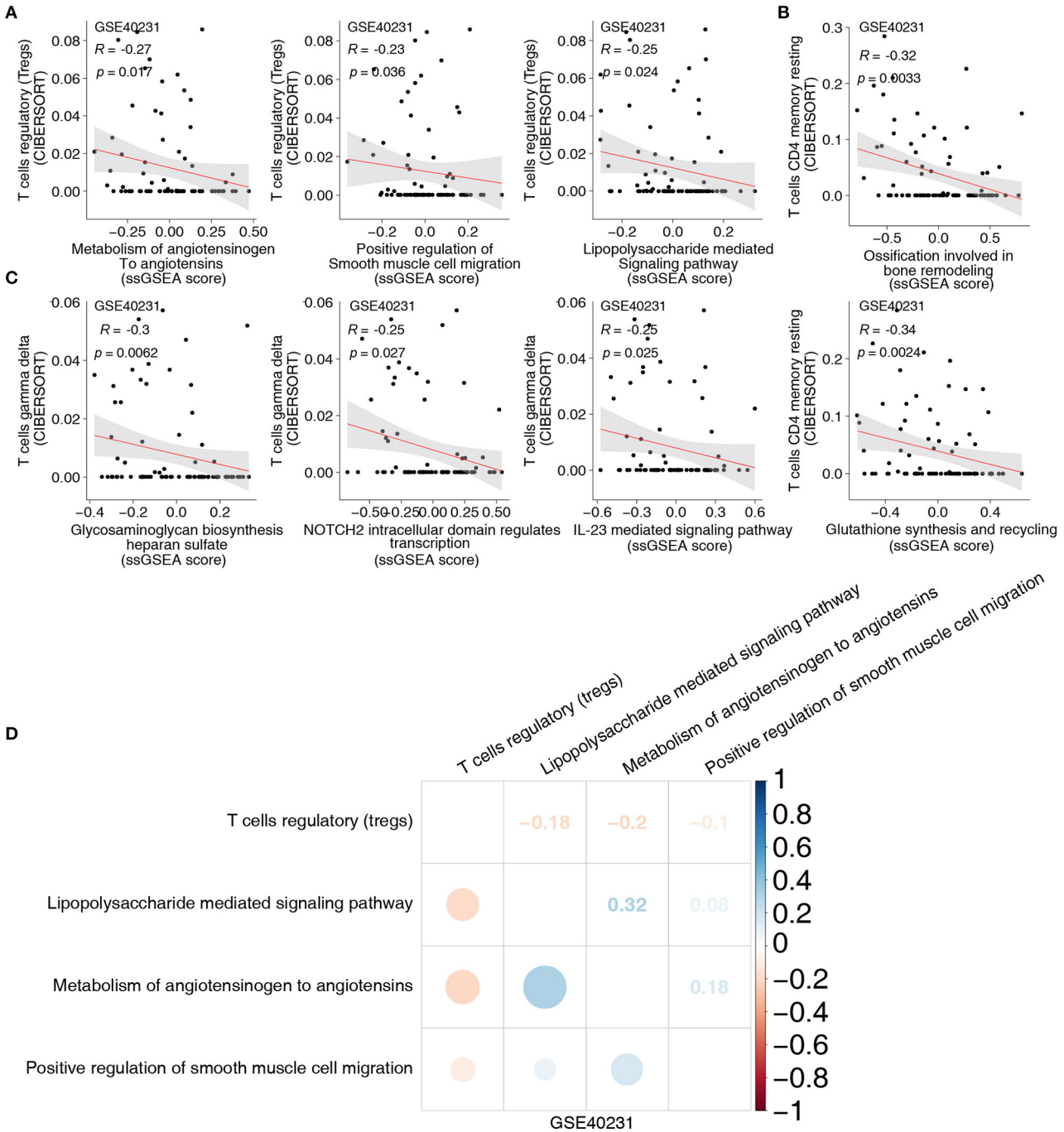

FIGURE 4 | (A) The correlation between the proportions of Tregs and the score of ssGSEA. **(B)** The correlation between the proportions of resting CD4+ T cells and the score of ssGSEA. **(C)** The correlation between the proportions of γδ T cells and the score of ssGSEA. **(D)** The correlation heatmap of the proportions of Tregs and the score of ssGSEA in each three-gene expression dataset (GSE40231). Tregs, regulatory T cells; ssGSEA, enrichment analysis; γδ T cells, gamma delta T cells.

of DCs narrowed plaques in CX3CR1$^{-/-}$ApoE$^{-/-}$ mouse models compared to healthy controls (38). The migration of DCs may also be impaired in hyperlipidemia (39), but the ability to stimulate these is not weakened (40), which may lead to a local accumulation of DCs and ultimately increase the inflammatory response, DC maturation, T cell activation, cytokine secretion (IL-1, IL-6, IL-8, INF-γ, and TNF), and TNF-α participation in the formation of atherosclerosis (41). Several recent trials focused on pro-inflammatory mediators in patients with CAD. The CANTOS (Canakinumab Anti-Inflammatory

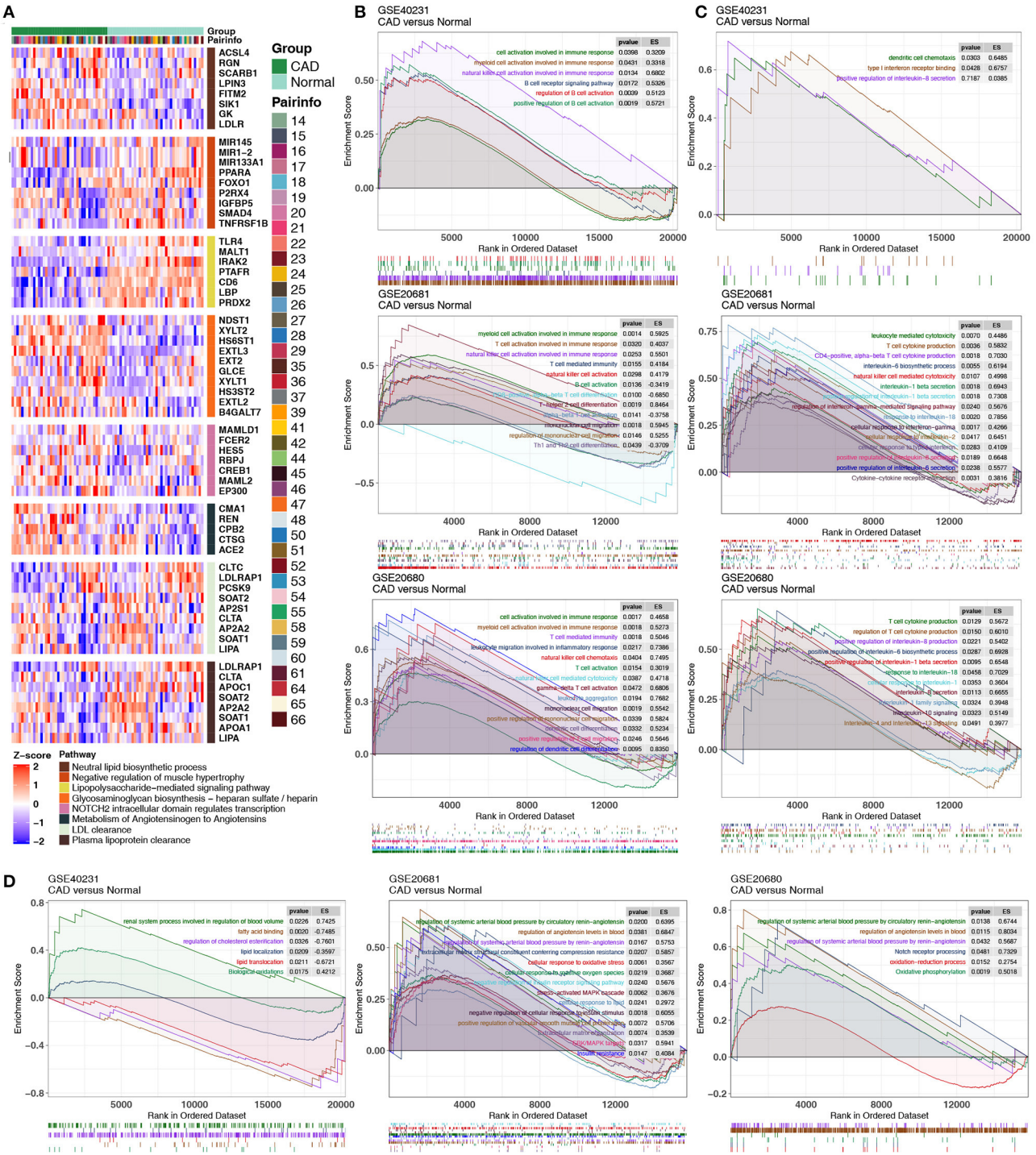

FIGURE 5 | (A) Heatmap of core genes in significantly enriched pathways between the CAD cases vs. the healthy controls in GSE40231. **(B–D)** GSEA of hallmark gene sets in the CAD cases and the healthy controls downloaded from MSigDB (GSE40231, GSE20681, and GSE20680). CAD cases were associated with activated immune cell- **(B)**, cytokine- **(C),** and inflammation-related pathways **(D)**. All transcripts were ranked as the $\log_2$ (fold-change) between the CAD cases and the healthy controls. Each run was performed with 1,000 permutations. CAD, coronary artery disease; MsigDB, Molecular signatures database.

Thrombosis Outcome Study) trial was the most noteworthy of the studies and targeted IL-1β (42). The CANTOS trial provided favorable evidence that inhibition of the IL-1β/IL-6 signaling cascade led to a significant reduction in cardiovascular risk, independent of a lipid-lowering effect, but with an increased risk of serious infection (42). Experimental data in hyperlipidemic

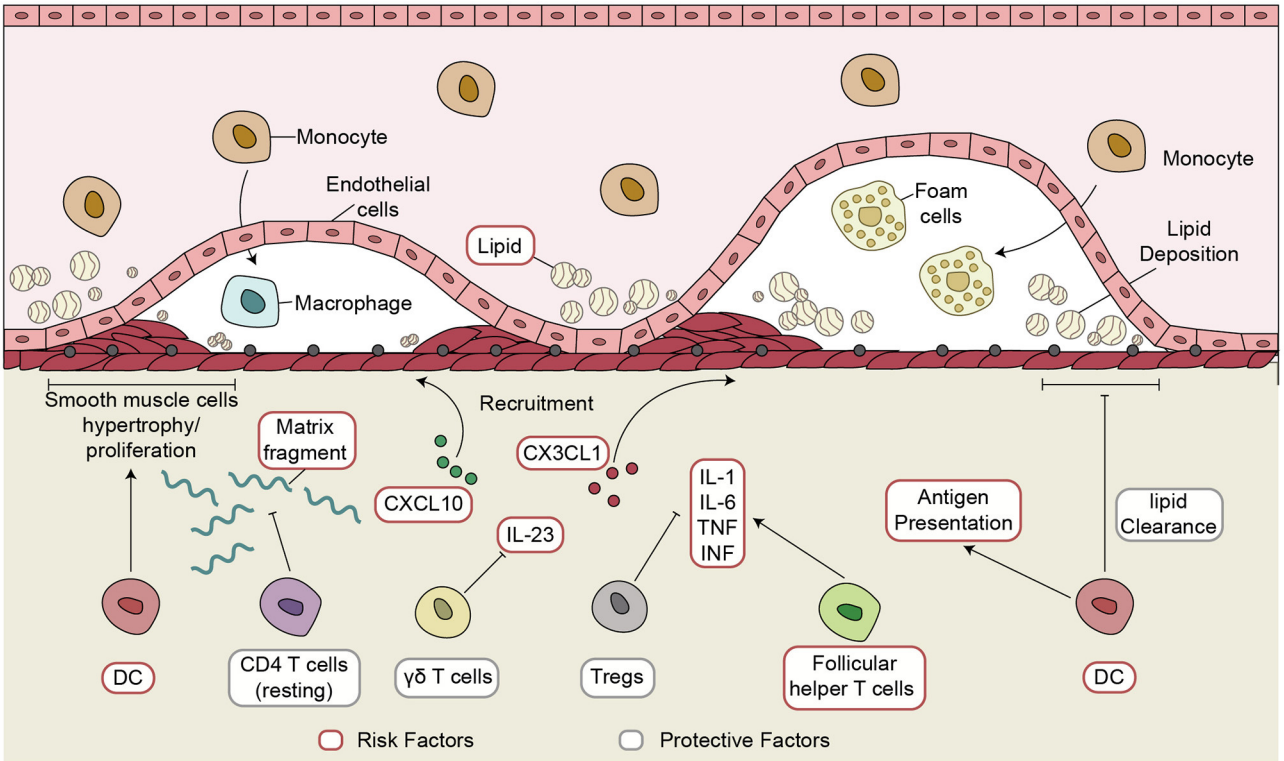

FIGURE 6 | The possible inflammatory immune microenvironment in CAD. CAD, coronary artery disease.

mice also revealed that inhibition of IL-6 or its receptor achieved atheroprotective effects (17). DCs may play an important role in the occurrence and development of atherosclerosis *via* lipid accumulation. Paulson et al. investigated the formation of early APs in Ldlr$^{-/-}$ mice using immunofluorescence (43). After a high cholesterol diet, lipid accumulation in the blood vessels of CD11c+ DCs was induced. These DCs exhibited foam cell morphology and may participate in the early formation of plaques. The antigen presentation function of DCs plays an important role in the occurrence and development of atherosclerosis (44). MHC-I and MHC-II are known antigen-presenting molecules of DCs (44). DCs present antigens to T cells *via* MHC-I and MHC-II, which leads to the activation and proliferation of T cells (45). A previous study found that atherosclerosis was significantly decreased in Ldlr$^{-/-}$CD74$^{-/-}$ mice, and the activated T cells were also decreased. This finding may be related to the decrease in the presenting function of the DCs (46). DCs mainly take up and present specific atherosclerotic antigens in atherosclerosis. Arterial DCs activate DCs *via* ox-LDL uptake and increase the presentation of lipid and polypeptide antigens to T cells, thereby participating in the development of atherosclerosis (44). DCs regulate the process of atherosclerosis by controlling the activation of T cells (47). Koltsova et al. observed that DCs in ApoE$^{-/-}$CD11c–YFP+ mice interacted with CD4+ T cells and resulted in T cell activation, proliferation, and the secretion of TNF-α and IFN-γ, which accelerated the process of atherosclerosis (47). Foam cells are one of the hallmarks of atherosclerotic plaques and develop when smooth muscle cells within the arterial wall take up ox-LDL *via* scavenger receptors (48). Consistent with previous findings (47), the results of the present study also suggest that activated DCs are associated with downregulated lipid clearance, increased antigen presentation, activation of cytotoxic responses, recruitment, and migration. Therefore, a critical protection mechanism toward avoiding immune cell infiltration into the vessel wall stems from wall-embedded DCs (49).

Tregs are a negative immune regulator that play an important anti-atherosclerotic role *via* inhibition of the autoimmune response and the maintenance of homeostasis of the body's immune response (50–52). Wigren et al. reported that individuals with low levels of Tregs were at increased risk of a first coronary event (53).

Hermansson et al. showed that an increase in Tregs was associated with a significant decrease in AP size (∼70%), and a decrease in CD4+ T cell infiltration and systemic inflammation was observed (54). A previous study investigating the distribution of Tregs in human atherosclerotic lesions found that low numbers of Tregs were present during all developmental stages (55). Our data were consistent with the observation of the deficiency of Tregs in the experimental atherosclerosis model (56–58) and suggest decreased CD4+CD25+ Treg participation in plaque destabilization.

Tfhs cells promote B cell proliferation, differentiation, class conversion, and antibody production and the occurrence and

development of atherosclerosis. Taghaviemoghadam et al. found decreased Tfhs and plasma cells in the spleen and aortic arch of STAT4$^{-/-}$Ldlr$^{-/-}$ mice compared to Ldlr$^{-/-}$ mice, but CD8+ Tregs were increased, and the size of the APs decreased (59). A recent study showed that the expression of Foxp3 in Tregs and the immunosuppressive function were lost during the process of atherosclerosis, which resulted in the transformation of some Tregs into Tfhs. Tfhs promote atherosclerosis, and Tfh depletion alleviates atherosclerosis, and ApoAI prevents Tregs from transforming into Tfhs, which affects the development of atherosclerosis (60). Tfhs in the peripheral blood of CAD patients are significantly higher than healthy populations and have a stronger proinflammatory function. An *in vitro* co-culture revealed a significant increase in the expression of INF-γ, IL-17, and IL-21 in coronary heart disease patients, and an increase in the expression level of B cell inflammatory genes (61). Our results suggest that CAD patients have a decreased proportion of γδ T cells, which negatively correlated with IL-23 signaling. A previous study showed that the expression level of IL-23 in the peripheral blood of CAD patients was significantly higher than that in healthy people. The high expression level of IL-23 was associated with a higher mortality of CAD patients (5). These results provide potential targets of inflammation to improve outcomes in atherosclerotic cardiovascular disease.

Although the findings in the present study systematically summarized the immune microenvironment of CAD cases based on their expression profiles using multiple bioinformatics methods, the results should be validated in prospective studies with larger populations. The present study also had some limitations. First, cell experiments and animal experiments are lacking. Second, this study was based solely on gene expression arrays (bulk transcriptome). All datasets in this study lacked clinical data, which may have potential confounders. This report lacks other omics analyses to further validate our results, and the traditional bulk array may obscure the heterogeneity of various immune cells in CAD. Third, there were limited tissue data for CAD, and the present study used two blood dataset and one tissue dataset of CAD to further validate the immune microenvironment. Fourth, the related clinical characteristics are lacking, a subgroup analysis will be conducted in the future using prospective cohorts.

CONCLUSIONS

The present study revealed that CAD patients may have an inflammatory immune microenvironment. The study also provides a timely update on inflammatory components in the immune microenvironment of patients with CAD.

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

Conceptualization, formal analysis, supervision, and visualization: HKH and RSD. Software: HKH, RSD, and PC. Writing–original draft: HKH, RSD, PC, JCZ, YC, and GL. Writing–review & editing: HKH, RSD, YC, and GL. 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.2020.602345/full#supplementary-material

Supplementary Figure 1 | The boxplot of gene expression profiles in each of the three datasets before and after normalization.

Supplementary Figure 2 | The correlation between the proportions of activated DCs and the score of ssGSEA in the two validation GEO datasets (GSE20681 and GSE20680). The correlation between the proportions of Tfhs and the score of ssGSEA in each validation GEO dataset (GSE20681 and GSE20680). The correlation heatmap between the proportions of activated DCs and the score of ssGSEA in the two validation GEO datasets (GSE20681 and GSE20680). The correlation heatmap between the proportions of Tfhs and the score of ssGSEA in each validation GEO dataset (GSE20681 and GSE20680). DCs, dendritic cells; ssGSEA, enrichment analysis; GEO, Gene Expression Omnibus database; Tfhs, T follicular helper cells.

Supplementary Figure 3 | The correlation between the proportions of Tregs and the score of ssGSEA in the two validation GEO datasets (GSE20681 and GSE20680). The correlation between the proportions of resting CD4+ T cells and the score of ssGSEA in the two validation GEO datasets (GSE20681 and GSE20680). The correlation between the proportions of γδ T cells and the score of ssGSEA in GSE20681. Tregs, regulatory T cells; ssGSEA, enrichment analysis; GEO, Gene Expression Omnibus database; γδ T cells, gamma delta T cells.

Supplementary Figure 4 | The correlation heatmap between the proportions of Tregs and the score of ssGSEA in the two validation GEO datasets (GSE20681 and GSE20680). The correlation heatmap between the proportions of resting CD4+ T cells and the score of ssGSEA in the two validation GEO datasets (GSE20681 and GSE20680). The correlation heatmap between the proportions of γδ T cells and the score of ssGSEA in GSE20681. ssGSEA, enrichment analysis; GEO, Gene Expression Omnibus database; γδ T cells, gamma delta T cells.

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REVIEW
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The Genetic Pathways Underlying Immunotherapy in Dilated Cardiomyopathy

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Heart failure (HF) is a global public health threat affecting 26 million individuals worldwide with an estimated prevalence increase of 46% by 2030. One of the main causes of HF and sudden death in children and adult is Dilated Cardiomyopathy (DCM). DCM is characterized by dilation and systolic dysfunction of one or both ventricles. It has an underlying genetic basis or can develop subsequent to various etiologies that cause myocardium inflammation (secondary causes). The morbidity and mortality rates of DCM remains high despite recent advancement to manage the disease. New insights have been dedicated to better understand the pathogenesis of DCM in respect to genetic and inflammatory basis by linking the two entities together. This cognizance in the field of cardiology might have an innovative approach to manage DCM through targeted treatment directed to the causative etiology. The following review summarizes the genetical and inflammatory causes underlying DCM and the pathways of the novel precision-medicine-based immunomodulatory strategies to salvage and prevent the associated heart failure linked to the disease.

Keywords: dilated cardiomyopathy, immunomodilation, growth factors, precision medicine, immuno suppression

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INTRODUCTION

Dilated Cardiomyopathy (DCM) is a common cause of Heart Failure (HF) and is the primary indication for heart transplantation. DCM is characterized by progressive dilatation and impaired contraction of one or both ventricles. The incidence of DCM has been estimated to be one case per 250 individuals, with a prevalence of >0.4% in the general population which accounts for 36% of HF cases (1, 2). DCM is responsible for about 10,000 deaths and 46,000 hospitalization each year in the United States making it amongst the top common causes of fatalities and a burden on health care system. These numbers might be an underestimation of the disease frequency because many affected patients have incomplete disease penetrance and expression (3, 4).

The World Health Organization (WHO) defines DCM as a serious cardiac disorder in which structural or functional abnormalities of myocardium that leads to cardiac malfunction, substantial morbidity and mortality, and complications such as heart failure and arrhythmias. The European Society of Cardiology (ESC) classifies cardiomyopathy into familial or non-familial (non-genetic) forms while the American Heart Association (AHA) committee classifies cardiomyopathies into three categories: "hereditary," "mixed," and "acquired" (5, 6). In respect to DCM, it is classified as a "mixed" disease and is best regarded as a complex trait with genetic and acquired/environmental components that promote cardiomyocyte injury or loss. DCM can occur due to a primary cause

or in association with diverse range of conditions such as coronary artery disease, autoimmune disorders, inflammatory/infectious agents, chemotherapeutic drugs, toxins, alcohol excess or nutritional deficiencies. In about 50% of DCM cases, there is no known identifiable cause; this has been traditionally termed "idiopathic" DCM. Most of the times, the management of DCM aims at reducing symptoms, improving cardiac function, and prolonging survival. However, this approach is untenable for the health care system and has a 40% failure rate at 2 years and might cause HF requiring heart transplant. During the last two decades, substantial research and progress were made resulting in a shift in focus from disease treatment to disease prevention and etiology-driven personalized approach. This approach has improved substantially the prognosis of DCM (1, 7, 8).

This review will briefly address the diagnosis, etiologies, and pharmacological and non-pharmacological management of DCM. The main focus will go to the emerging potential immunomodulator agents being developed to treat DCM. The current and future clinical practice through guided individualized treatment strategies bring promises to improve the patients' outcomes and reduce treatment costs.

DIAGNOSIS

The signs and symptoms of DCM may be fulminant, acute, subacute or chronic, as they are related to the extent of left ventricular or biventricular systolic dysfunction. The diagnosis of DCM is evident by chamber dilation and reduced systolic function of one or both ventricles with an ejection fraction <50%. A thorough evaluation and wide array of non-invasive and invasive techniques are needed to ascertain DCM diagnosis. Non-invasive imaging techniques like electrocardiogram, echocardiogram, chest X-ray screens and cardiac magnetic resonance (CMR) imaging are used to detect and assess enlarged cardiac silhouette, chamber size dimensions, ventricular dysfunction, strain abnormalities, contrast enhancement, the presence of oedema and/or fibrosis, abnormalities ranging from isolated T-wave changes to adverse myocardial remodeling as featured in DCM cases (9, 10). Contrast agents, mainly gadolinium, have been used to more efficiently evaluate fibrosis and subsequently the information is being used as a predictor of future hospitalization and all-cause mortality. Invasive methods such as coronary catheterization is used to rule out any other coronary artery diseases.

In patients with idiopathic DCM, genetic/familial reasons should be considered. Familial DCM diagnosis is ascertained when the proband has two or more first-degree relatives who experienced premature sudden cardiac death aged <35 or heart failure without a definitive cause or by three-generation history of DCM (1). Thus, genetic testing and sequencing the entire coding region of the gene in particular is a hallmark to identify the disease-causing mutations, along with detailed family history (10, 11). Such techniques would help to determine the disease cause and have a tailored risk stratification for etiology-driven therapeutic options for patients.

Additional considerations should however be added to other subcategories of DCM, mainly inflammatory cardiomyopathy. Inflammatory DCM (DCMi) or inflammation of the myocardium is best regarded as any heightened humoral or cellular immune response in the heart with various symptoms such as chest pain, mild dyspnea, or acute cardiogenic shock (12, 13). For definitive diagnosis of myocarditis (myocardium inflammation) and inflammatory DCM (DCMi), Endo-Myocardial-Biopsy (EMB) is used because it detects viral and non-viral causes in the acute and chronic stage of the disease.

Before highlighting standard and personalized approaches to treat DCM (sections Targeted Treatments for DCM and Emerging Immunomodulator Therapeutic Strategies), we will first provide an overall picture on the environmental and genetic etiologies of the disease (sections Environmental and Genetic Etiologies of DCM and Treatment With Conventional Medications).

ENVIRONMENTAL AND GENETIC ETIOLOGIES OF DCM

Environmental Etiologies

The cellular changes that result in DCM begin first as an adaptational response but are then transformed into a detrimental cellular, and organ "malaise" as a consequence of accumulated uncontrolled molecular events. We hereby refer to the microenvironment as the environment that surrounds the myocyte. This includes the surrounding cells and their secreted proteins and growth factors in the myocardial interstitium: endocardial, fibroblast, blood cells. In DCM, the myocardial interstitium is constantly subjected to an increase in the extracellular matrix content and reduction in collagen linkage, resulting in faulty matrix and Left Ventricular (LV) dilation (14). Studies have shown that any increase in the production of matrix metalloproteinases (MMPs), or Galectin-3, can be sensed as an early marker for DCM (15). In parallel, cytokines secreted from inflammatory cells and/or oxidative stressors that cause an increase in oxygen reaction species production could directly affect myocardial function and subsequently lead to DCM. As such, fibrosis is a common feature for both genetic and non-genetic dilated cardiomyopathies and constitutes a converging focal point to develop novel drugs to stop the progression of the disease.

In contrast, the macro-environment refers to the overall body adaptation/response to extra-cardiac "insults" and is sensed through a hemodynamic and/or hormonal overload on the heart (14). Examples of such conditions could be obesity, diabetes, infection, drug intolerance, toxicity, viruses, and autoimmune diseases. **Table 1** summarizes some of the etiologies of DCM, along with the tailored-diagnostic approaches and specific treatment options, and the following section will highlight some of the direct and indirect "environmental stressors" that leads to DCM.

Diabetes

Diabetes is a known risk factor for cardiovascular diseases (16, 17). Development of diabetes causes systolic and diastolic

TABLE 1 | Etiologies, diagnosis, and targeted treatments for DCM.

Etiologies	Diagnosis	Targeted treatments
Genes: titin, myosin 7/β-myosin heavy chain, cardiac muscle troponin T, RNA-binding motif protein 20	Family history, Gene testing, whole exome sequencing	If LVEF of <35%, use implantable cardioverter-defibrillators (ICDs).
Toxins: alcohol, cocaine, and cytostatics	Urine test, patient history, family interview, abuse history	Avoid exposure to toxins, pharmacological management for abstinence along with standard therapy
Cardiotropic viruses: like parvovirus B19 (B19V), human herpes virus-6 (HHV-6), and enterovirus Coxsackie B (CVB3)	Endo Myocardial Biopsy (EMB)	Antiviral therapies: IVIG administration, Betaferon, Ganciclovir, and Telbivudine
Medications - Antineoplastic drugs like Anthracyclines (Doxorubicin, Daunorubicin), alkylating agents (Cyclophosphamide), antimicrotubular molecules (Paclitaxel, docetaxel and Vinca alkaloids) and antimetabolites (Capecitabine, Cytarabine, 5-Flourouracil).	Based on prescription and patient history	Avoid usage or using alternative medications with same mechanism of action
Auto immune diseases: systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, Kawasaki disease–related myocarditis, Lupus erythematosus, Cardiac sarcoidosis, and giant cell myocarditis	Endo Myocardial Biopsy (EMB), cardiac imaging, blood tests	Immunoadsorption therapy
Endocrine or metabolic diseases: diabetes	Blood tests-Hbalc, glucose tolerance test, cardiac tests and imaging, symptoms	Glycemic control, medications, diet modification, diabetology consultation
Nutritional deficiency in: carnitine, thiamine and selenium	Blood tests and detailed examination	Follow balanced diet

dysfunction, thus leading to dilated cardiomyopathy. This development is related to insulin resistance, metabolism of fatty acids, hyperglycemia, and excessive activation of renin angiotensin system. Hyperglycemia, the main driving force of diabetic cardiomyopathy triggers various responses leading to heart failure. Glucose uptake causes increased oxidation and lipotoxicity of the myocardium as well as insulin resistance leading to the initiation of the renin angiotensin aldosterone system. This results in fibrosis and hypertrophy causing myocardial oxygen demand and alteration of calcium storage in the sarcoplasmic reticulum. These series of events eventually lead to a decrease in cardiac contractility, thus causing DCM (18, 19). Recent studies also showed that DCM associated with diabetes could result from the T helper (Th)-driven inflammatory functional and biomolecular changes bestowed on the cardiomyocytes (17). Moreover, a study on 206 DCM patients showed that the disease prognosis in DCM patients with type 2 diabetes is worse than patients without diabetes, in which 15 deaths, 43 hospitalizations and a new onset of atrial fibrillation were reported (19).

Autoimmunity

DCM can result from autoimmune diseases such as systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, Kawasaki disease–related myocarditis, lupus erythematosus, cardiac sarcoidosis, and giant cell myocarditis (20, 21). In patients with DCM of autoimmune etiologies, B cells produce cardiac-specific autoantibodies (AABs) such as the ones targeting the β1-adrenergic receptor, the muscarinic M2-acetylcholine receptor, the Na-K-ATPase pump, and Troponin I. These AABs form immune complexes with self-antigens and complement components. AABs influence myocytes function directly as pathogenic agents secondary to tissue aggression and are known to be present in 60% of patients with DCM (22, 23).

Toxic Environment

Some of the toxins that can cause DCM are alcohol, cocaine, and cytostatics (10). Left ventricular dysfunction and DCM have been related to heavy drinking with increased rate of cardiac morbidity and mortality. In regards to cocaine, it was shown that high cocaine doses cause reduction in left ventricular ejection fraction (LVEF) leading to dilated cardiomyopathy (24).

Drugs

Several anti-neoplastic drugs are cardiotoxic like Anthracyclines (Doxorubicin, Daunorubicin), alkylating agents (Cyclophosphamide), antimicrotubular molecules (Paclitaxel, docetaxel and Vinca alkaloids) and antimetabolites (Capecitabine, Cytarabine, 5-Flourouracil). The function, and metabolic activity of the heart are drastically perturbated by these agents (25, 26). Doxorubicin causes cardiotoxicity by generating oxygen reactive species that affect the whole contractile machinery leading to Doxorubicin-induced cardiomyopathy (DiCM). Antiretroviral agents like azidothymidine have cardiotoxic properties, as in the case of Doxorubicin, through increased generation of reactive oxygen species. Finally, immune check point (ICI) therapies like Programmed Death-Ligand 1/2 (PDL1 and PDL2) inhibitors have been successful in the improvement of advanced cancer stages. However, recent studies have shown they cause cardiac toxicity, myocarditis, decreased LV function, and immune related adverse events (27).

Viruses

Cardiotropic viral infections induce cardiac dysfunction and may lead to DCM. The predominant viral cause seems to change with every decade (coxsackievirus in the 1980s, adenovirus in the 1990's, and parvovirus B19 since 2000) (9). In developed countries, adenoviruses and enteroviruses were mostly recognized until the 1990's. However, in recent years cardiotropic

viruses like, parvovirus B19 (B19V), human herpes virus-6 (HHV-6), and enterovirus coxsackie B (CVB3) are significantly increasing in the population with cardiomyopathy. Overall, enteroviral genomes were found in 3–53%, cytomegalovirus in 3–40%, and adenoviruses in 3–23% of the myocardium (28). In viral infection, overexpression of the inflammatory cytokines like Tumor Necrosis Factor alpha (TNFα) causes initiation of the immune system response cascade that directly affects the function of cardiomyocytes and their survival. Infiltrating immune cells have a key role in eliminating infected myocardial cells and limiting viral replications in the heart but as such they contribute to the worsening of the phenotype by eliminating cardiomyocytes through apoptosis. Cytotoxic T lymphocytes (CTLs) are responsible for lysing virus-infected cardiomyocytes which leads to further myocardial cell damage. They recognize virus-derived peptides presented in the groove of the major histocompatibility complex (MHC) molecule class 1 antigen through T-cell receptors and play a key role in the pathogenic immune mechanism in viral myocarditis and DCM (29). Overall, the viruses might cause direct myocardial damage or a secondary virus-initiated myocardial injury where the end-results combine a series of molecular and cellular events like myocyte necrosis and fibrosis that lead to DCM (30).

Inflammatory Response: Cytokine Storm

As discussed above, TNF-α overexpression causes a series of cellular events that causes cardiomyocyte inflammation/necrosis. TNF-α and other cytokines such as interleukin-1 (IL-1), interleukin-6 level (IL-6), interleukin-10 (IL-10), and interleukin-18 (IL-18) are an essential part of the inflammatory process. Cytokines are produced by several immune cells like the innate macrophages, dendritic cells, natural killer cells and the adaptive T and B lymphocytes. Accumulating evidence suggest that patients with DCM suffer from a "cytokine storm." The "cytokine storm" results from a sudden acute overexpression in circulating levels of IL-6, IL-1, IL-10, IL-18, TNF- α, and interferon. This cytokines overexpression results in influx of various immune cells such as macrophages, neutrophils, and T cells from the circulation into the site of infection with destructive effects in cardiovascular cells resulting to capillary damage and myocardial fibrosis leading to DCM (31–33). Further studies are needed to establish the mechanisms of the cytokine storm/dysregulation concretely, which can explain the pathogenesis/prognosis of DCM.

Genetic Etiologies of DCM

A spectrum of genetic heterogeneity underlies DCM and accounts for half of the cases. At current, a list of 42 genes is used worldwide as a blueprint for all cases of cardiomyopathies including DCM. Most of these genes encode proteins implicated in the structure of the muscle heart cells like the sarcomeres, the Z-disks, and sarcolemma; consequently, alterations in their structure and/or function would affect muscle contraction. Examples of these genes are *LMNA* or *SCN5A*, *BAG3*, *FLNC*, *PLN*, *RBM20*, and *TTN*. Identifying the heredity can be difficult due to the incomplete penetrance and variable expressivity of DCM and genetic variation. Thirty-five percent of DCM cases have a family history, inherited in Autosomal Dominant manner (AD), in some cases autosomal recessive or X-linked inheritance traits (3, 34). We will cover herein the known genetic variants in genes that encode for sarcomeric, Z-disc and laminal membrane proteins.

Genes Encoding Sarcomeric Proteins-TTN (Titin) Mutations

TTN encodes Titin, one of the largest proteins in humans and harbors the most frequent genetic variants associated with DCM. The protein plays a structural role in maintaining the thick filaments stability within the sarcomere by avoiding the filaments overstretching. *TTN* genetic variants show a consistent high penetrance trait for familial DCM cases. Due to the enormous size of the gene along with the frequency of *TTN* variants in the reference population, it is challenging to interpret the variant as pathogenic, pathogenic, singleton or familial (35). The most frequent variant is a founder mutation leading to truncation of the C-terminal part of the protein (36). The proteotoxic effect of such misfolded and/or non-functional aggregates of TTN proteins in cardiomyocytes is the direct cause of DCM (37). The clinical phenotype of *TTN* mutations involves a tendency for left ventricular remodeling and dysfunction, atrial fibrillation, frequent ventricular ectopy, and non-sustained ventricular tachycardia, and malignant ventricular arrhythmia (38, 39).

Moreover, reduced expression of titin is believed to be associated with the pathophysiology of DCM. A significant decrease in titin and dystrophin mRNA and protein levels was seen in endomyocardial biopsy of DCM patients as compared to control, the severity of the disease correlated with this decrease.

The study suggested that TNF-α might modulated the expression of titin and dystrophin levels via nuclear factor kappa B (NF-kappaB) pathway. To confirm that, TNF-α was used as a treatment and resulted in a dose- and time-dependent decrease in mRNA levels of dystrophin and titin (40). Other studies supported this hypothesis, where they revealed that TNF-α gene polymorphism (G-308A) might play a key role in the susceptibility to dilated cardiomyopathy (41).

Genes Encoding Nuclear Laminal Proteins-LMNA Mutations

L*MNA* encodes Lamin A/C, an envelope protein that acts as a support element to intermediate filaments and regulates gene expression by stabilizing the inner nuclear membrane (42). After *TTN* variants, *LMNA* are the second most common DCM-causative mutations. Mutations in *LMNA* are inherited in AD manner and might be associated with other autosomal dominant disorders such as Emery-Dreifuss muscular dystrophy. *LMNA* variants increase the risk of sudden cardiac death up to 46%, cardiac muscular atrophy, premature aging, systolic dysfunction and high prevalence of arrhythmias, disturbance of signal transduction in non-dividing cells and disturbance of chromatin organization in dividing cells (38, 43, 44). Pathogenesis of LMNA-associated DCM includes disturbance of signal transduction in non-dividing cells and disturbance of chromatin organization in dividing cells. The common features associated

with *LMNA* mutations in DCM patients are the coexistence of a defect in mechano-transduction and laminopathy development with conduction system abnormalities resulting in diverse phenotypes. Phenotypes such as lipodystrophy, skeletal and/or cardiac muscular atrophy, dysplasia, premature aging, systolic dysfunction and high prevalence arrhythmias and other neuromuscular diseases which result in poor prognosis and response to medical treatment (38, 42).

Genes Encoding RNA Binding Proteins-RBM20 Mutations

Mutations in the gene encoding the RNA-binding motif 20 (RBM20), a nuclear phosphoprotein mainly expressed in the cardiac myocytes have been emerging as one of the latest causes of familial DCM cases despite being first linked to arrhythmogenic cardiomyopathies (45, 46). The link to the DCM phenotype has been recently explored, and as such the role of RBM20 as a master regulator of alternative splicing of genes involved in the contractile machinery namely Titin and Lamin has been pointed out (47, 48).

With all the etiologies being exposed, the following sections will first provide a current summary of the ongoing and proposed clinical trials that use conventional treatment and etiology-driven therapeutic treatments.

TREATMENT WITH CONVENTIONAL MEDICATIONS

Conventional medications are the first line drug treatment that have been studied in large clinical scale trails and shown survival improvement and reduction in hospital admission. Conventional treatment is based on the classification of the patients as per "measured" clinical criteria. The New York Heart Association (NYHA) classified DCM patients into five groups based on their heart failure. Class I: patients with cardiac disease but without resulting limitations of physical activity, and ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain. Class II: patients with cardiac disease resulting in slight limitation of physical activity, are comfortable at rest and ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain. Class III: patients with cardiac disease resulting in marked limitation of physical activity, are comfortable at rest, and less-than- ordinary physical activity causes fatigue, palpitation, dyspnea, or anginal pain. Class IV: patients with cardiac disease resulting in inability to endure physical activity without discomfort. Symptoms of cardiac insufficiency or of the anginal syndrome may be present even at rest. If any physical is undertaken, discomfort is increased. Overall, the treatment of each class varies between the use of Angiotensin Converting Enzyme Inhibitors (ACEIs) (49–51), Angiotensin Receptor Antagonists Losartan (52, 53), β blockers (54–57), Aldosterone antagonists (58, 59), Ivabradine (60), Angiotensin receptor-neprilysin inhibitors (61). **Table 2** summarizes the pharmacological management of DCM based on NYHA and the American Heart Association (AHA) class of recommendation (63, 72).

TARGETED TREATMENTS FOR DCM

In general, with conventional therapies, ∼25% of DCM patients with recent onset symptoms of HF will have spontaneous improvement, but patients with symptoms lasting >3 months who show severe clinical presentations generally have less chance of recovery, thus treatments will be recommended to optimize heart function, reduce the risk of worsening disease, prevent complications, and/or reduce symptoms caused by heart failure. Non-pharmacological treatment is strongly recommended for etiologies that can be controlled such as avoiding exposure to toxins (e.g., alcohol, cocaine). In alcoholic cardiomyopathy (aCM), abstinence from alcohol has shown to improve the LVEF in patients. A study conducted for 82 months on 101 aCM patients showed a noticeable improvement in LVEF function (i.e., QRS duration <120 ms) for 42% of them (73). Also, following a balanced healthy cardiac die or treating endocrine disorders (e.g., diabetes, thyroid disease) should be considered (6). Patients with DCM and diabetes should be educated on glycemic control and the importance of adhering to medication to order to avoid complications (74). For patients with genetic causes, the clinical management is based on the clinical features associated with the genetic information. The first approach is to improve the clinical outcomes based on a definite genetic mutation. The primary preventive method for patients with genetic background with LVEF of <35% is to use implantable cardioverter-defibrillators (ICDs) to reduce sudden cardiac death (SCD). To reduce the risk of instabilities in cardiac rhythm and SCD in DCM patients with LMNA mutation, the threshold frequency for implanted cardiac defibrillators should be lowered (75). Bi-ventricular pacing is recommended for symptomatic bradycardia that show left bundle block in DCM patients. Studies on DCM patients undergoing cardiac resynchronization therapy showed improved survival, quality of life along with reduced hospital admissions (76).

Targeted Therapy for Genetic Causes

Known Drugs

The second approach is targeting the defect gene mutations at the molecular level directly by affecting the structure and function of the encoded proteins. Currently, an allosteric modulator was developed to directly bind in the same region on myosin, increasing actin affinity and cross-bridge formation, as well as enhancing sarcomere force production. This modulator is well-studied in patient with reduced LVEF from heart failure, which can benefit DCM patients since they have reduced LVEF. CK-1827452 also known as Omecamtiv mecarbil (INN) accelerates the transition of actin-myosin complex from weakly to strongly bound and increases the number of myosin heads engaged with the thin filament. These effects are independent of calcium transients because CK-1827452-treated cardiomyocytes with isoproterenol augment contraction, whereas β-adrenergic inhibition does not diminish contractility (77) ATOMIC (78). Additional studies indicated that CK-1827452 traps some myosin heads in a weak actin affinity state with slow force development and at high concentrations prolonged cellular relaxation in hiPSC-CMs (79). A randomized, parallel-group, double-blind,

TABLE 2 | Pharmacological treatment of DCM.

References	(n)Targeted population	Study design	Drug	Assessment of outcome	Outcome	Class of recommendation by AHA
Conventional drugs						
ACEIs						
Group (49)	(253) NYHA IV; 38 (15%) with non-ischaemic cardiomyopathy	Double-blind, placebo-controlled, parallel group	Enalapril (120 mg twice a day)	1 year	Improved survival and NYHA class; mean dose 18.4 mg/day	I
Investigators et al. (50)	(2569) NYHA II–III; 469 (18%) with non-ischaemic cardiomyopathy	Double-blind, placebo-controlled, parallel group	Enalapril (10 mg twice a day)	3 years	Improved survival and fewer hospital admissions; mean dose 11 mg/day	I
Investigators et al. (51)	(4228) NYHA I; 396 (10%) with non-ischaemic cardiomyopathy	Double-blind, placebo-controlled, parallel group	Enalapril (10 mg twice a day)	3 years	Improved survival, fewer hospital admissions, and less HF progression; mean dose 12.7 mg/day	I
Angiotensin receptor antagonists						
Pitt et al. (52)	(3152) NYHA II-IV; ≥60 years; 1,292 (41%) with non-ischaemic cardiomyopathy	Double-blind, active-controlled	Losartan (50 mg once a day) vs. captopril (50 mg three times a day)	1.5 years	There were no significant differences in all-cause mortality	I
Granger et al. (53)	(2028) NYHA II-IV; β blocker; 396 (20%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor intolerant	Double-blind, placebo-controlled	Candesartan Cilexetil (32 mg once a day)	2 years	Reduced cardiovascular mortality and morbidity in patients with symptomatic chronic heart failure	I
β blockers						
Packer et al. (54)	(1094) NYHA II-IV; 570 (52%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker	Double-blind, placebo-controlled	Carvedilol (various dosing)	6–12 months	Mortality rate was 7.8 percent in the placebo group and 3.2 percent in the carvedilol group; the reduction in risk attributable to carvedilol was 65 percent (95% confidence interval, 39–80%; $P < 0.001$)	I
(55)	(2674) NYHA class III-IV; 317 (12%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker	Double-blind, placebo-controlled	Bisoprolol (10 mg once a day)	1.3 years	All-cause mortality was significantly lower with bisoprolol than on placebo (156 [11.8%] vs. 228 [17.3%] deaths)	I
(56)	(3991) NYHA II-IV; 1,385 (35%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker	Double-blind, placebo-controlled	Metoprolol controlled release (200 mg once a day)	1 year	The total mortality or all-cause hospitalizations was lower in the metoprolol CR/XL group than in the placebo group (641 vs. 767 events; risk reduction, 19%; 95% confidence interval [CI], 10–27%; $P < 0.001$)	I
Packer et al. (57)	(2289) NYHA III-IV; 755 (33%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker	Double-blind, placebo-controlled	Carvedilol (25 mg twice a day)	4 years	Carvedilol reduced the risk of death by 39 percent (95 percent confidence interval, 11–59 percent; $P = 0.009$) and decreased the combined risk of death or hospitalization by 29 percent (95 percent confidence interval, 11–44 percent; $P = 0.003$).	I

(Continued)

TABLE 2 | Continued

References	(n)Targeted population	Study design	Drug	Assessment of outcome	Outcome	Class of recommendation by AHA
Aldosterone antagonists						
Pitt et al. (58)	(1663) NYHA III-IV; 765 (46%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker	Double-blind, placebo-controlled	Spironolactone (25 mg once a day)	2 years	Reduced mortality and hospital admissions	I
Zannad et al. (59)	(2737) NYHA II; 846 (31%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker + β blocker	Double-blind, placebo-controlled	Eplerenone (25–50 mg once a day)	21 months	Reduced mortality;18.3% of patients in the eplerenone group as compared with 25.9% in the placebo group (hazard ratio, 0.63; 95% confidence interval [CI], 0.54–0.74; $P < 0.001$)	I
Ivabradine						
Swedberg et al. (60)	(6558) NYHA II-IV; sinus rhythm with heart rate of >70 beats per min; 2,087 (33%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker + β blocker	Double-blind, placebo-controlled	Ivabradine (5–7.5 mg twice a day)	18–28 months	24% patients in the ivabradine group had cardiovascular death or hospital admissionand, compared to 937 (29%) of those taking placebo (HR 0.82, 95% CI 0.75–0.90, $p < 0.0001$)	IIa
Angiotensin receptor-neprilysin inhibitors						
McMurray et al. (61)	(8442) NYHA II-IV; 3,363 (40%) with non-ischaemic cardiomyopathy; angiotensin converting enzyme inhibitor or angiotensin receptor blocker; β blocker	Double-blind, active-controlled with enalapril	Sacubritil valsartan (200 mg twice a day)	27 months	As compared with enalapril, sacubritil valsartan reduced the risk of hospitalization for heart failure by 21% ($P < 0.001$) and decreased the symptoms and physical limitations of heart failure ($P = 0.001$)	I
Targeted therapy for viral causes						
McNamara, et al. (62)	(63) Patients with recent onset (</= 6 months of symptoms) of dilated cardiomyopathy and LVEF </ = 0.40	Prospective randomized, placebo-controlled, double-blind	Intravenous immunoglobulin(IVIG2) 2 g/kg	2 years	LVEF improved from 0.25+/−0.08 to 0.41+/−0.17 at 6 months ($P < 0.001$) and 0.42+/−0.14 ($P < 0.001$ vs. baseline) at 12 months	
Dennert et al. (64)	(17)Patient with Parvovirus B19 DCM	Uncontrolled pilot study	(IVIg) (2 g/kg) for 6 months	9 months	Decrease in EMB viral load ($P = 0.004$) and improved LVEF ($P = 0.001$).	
Zimmermann et al. (65)	(110) patients with chronic viral DCM	Open trial with untreated control group	Interferon β-1b for 6 months	3 years	No benefit of interferon β-1B treatment observed.	
Schultheiss et al. (66)	(143) patients with symptoms of heart failure and biopsy-based confirmation of the enterovirus (EV), adenovirus, and/or parvovirus B19 genomes)	Double-blind treatment	Interferon β-1b for 6 months	1 year	Improvement in quality of life	
TARGETED THERAPY FOR NON-VIRAL (INFLAMMATORY) CAUSES						
Immunosuppresive drugs						
Parrillo et al. (67)	(102) patients with Idiopathic Cardiomyopathy	Prospective, randomized, controlled	Predinsone (60 mg a day)	3 months	Marginal clinical benefit, and should not be administered as standard therapy for dilated cardiomyopathy.	

(Continued)

TABLE 2 | Continued

References	(n)Targeted population	Study design	Drug	Assessment of outcome	Outcome	Class of recommendation by AHA
Wojnicz et al. (68)	(67) DCM patient with increased HLA expression	Randomized, placebo-controlled	Prednisone + azathioprine (3 months)	2 years	At the end of the follow-up period, 71.4% patients from the immunosuppression group vs. 30.8% patients from the placebo group were improved ($P = 0.001$).	
Frustaci et al. (69)	(70) Myocarditis and chronic (>6 months) heart failure patients, unresponsive to conventional therapy, with no evidence of myocardial viral genomes	Randomized, double-blind, placebo-controlled	Prednisone + azathioprine (6months)		improvement of LV-EF and decrease in LV dimensions and volumes compared with baseline with no major adverse reaction	
Escher et al. (71)	(114) Chronic myocarditis or inflammatory cardiomyopathy following Caforio et al. (≥14 infiltrating inflammatory cells/mm^2)	Retrospective case series	Immunosuppresion (6 months)	3 years	Improvement of LV-EF compared to baseline after 6-mo period (LV-EF rising from 44.6 ± 17.3% to 51.8 ± 15.5%; $P = 0.006$)	
Merken et al. (70)	(209) Inflammatory cardiomyopathy following Caforio et al. (≥14 infiltrating inflammatory cells/mm^2)	Retrospective case series	Immunosuppresion (1 year)	≤10 years	A significant larger increase of LV-EFmproved long-term outcome (e.g., heart transplantation-free survival)	

phase II conducted over 87 sites in 13 countries (The Chronic Oral Study of Myosin Activation to Increase Contractility in Heart Failure COSMIC-HF trial) showed that CK-1827452 administered to patients with chronic stable symptomatic heart failure increased stroke volume and modestly reduced left ventricular end-diastolic diameter, heart rate, and serum levels of N-terminal brain natriuretic factor, increased systolic ejection time, and it may have improved dyspnea in the high-dose group, though it did not meet the primary endpoint of dyspnea improvement. Many other trails have been conducted and are still under investigation to study this drug (**Table 2**) (77, 78, 80).

Novel Drugs

Specific inhibitors to the pathogenic effects bestowed by the pathogenic mutations are postulated to be beneficial in treating DCM. Pre-clinical trials using novel small molecules did yield encouraging results. The LMNA mouse model was treated with ARRY-371797, an oral medication that inhibits the p38 MAP kinase: LV dilation was prevented, and EF substantially improved. To investigate the benefits of ARRY-371797 in DCM patients with LMNA mutations, patients with LMNA mutations, a clinical trial is currently ongoing to evaluate its effectiveness based on changes in the 6 min-walk tests over a 24 weeks-time period (NCT03439514). With the increasing focus on the role of RBM20 as a master regulator of alternative splicing, recent data suggest that in patients with reduced RBM20 activity, all-trans retinoic acid (ATRA) could be used to restore RBM20 levels and efficiently curb down the deleterious effects of loss of function mutations in this gene (81).

A novel myosin activator is Danicamtiv (formerly known as MYK-491). It is a new targeted myosin activator under development for the treatment of DCM. It accelerates and activate myosin contractility directly by increasing cross-bridge formation with no effects on the calcium transient. This has the potential to improve the hemodynamic profile of patients with systolic heart failure while avoiding the energetic consequences of adrenergic agonists and phosphodiesterase inhibitors. It was characterized in *in vitro* and *in vivo* (in mice, rats, dogs, and monkeys) preclinical studies and this led to the support of its advancement into clinical investigations (82).

Targeted Therapy for Viral Causes

Antiviral therapy treatment is suggested for viral causes of DCM and currently there are many trials ongoing to understand their beneficial effects at distinct phases. In summary, patients with enterovirus are treated with Interferon beta, Parvovirus B19 with immunoglobulins and Telbivudine and Human Herpesvirus Type 6-Positive Patients with Ganciclovir and this will be further explained below in details (**Table 2**) (83).

Immunoglobulin

Immunoglobulins reduces oxidative stress by preventing the development of proinflammatory cytokines; thus, they have both antiviral and anti-inflammatory properties (84). Intravenous Immunoglobulin (IVIG) treatment for DCM patients has been controversial Improved LVEF function with reduced amount of virus load and lower hospitalization rates was noted in DCM

patients receiving IVIG (64). Studies have shown that children and infants with DCM infected by human parechoviruses benefited from IVIG therapy (85, 86). DCMi patients treated with Ig-Therasorb and immunoglobulin G (IgG) showed a continued improvement in the cardiac index (87). In patients with virus positive, the therapeutic dosage for treatment with IVIG (IgG, IgA, and IgM- Pentaglobin) is 10–15 g (variable according to weight) on day 1 and day 3. High doses of intravenous 1 g of dose (2.0 g/kg body weight) were administered for patients with B19v for viral load reduction. There was no improvement in cardiac function and quality of life after this 4-day treatment. This negative finding could be related to the number of days of treatment with IVIG in this patient population (88). This finding also emphasizes on the fact that multifactorial causes of DCM make it difficult to have a unidirectional treatment.

Telbivudine

Telbivudine, an analog of thymidine is an antiviral nucleoside that has immunomodulatory-antiapoptotic properties. The PreTopic Study assessed the effect of Telbivudine on B19V-positive patients (83). Patients with less B19V DNA load having inflammation of the cardiac cells have benefited from the drug while those with high viral loads did not show significant improvement (89). Telbivudine inhibited viral replication, and reduced inflammation while improving cardiac function. In addition, Telbivudine did reduce cardiomyocyte apoptosis (71, 83); however, there are no randomized clinical studies to evaluate the results of Telbivudine, and as such it cannot be used as a standard treatment.

Interferon Beta (IFN-ß)

Anti-viral treatment with type I interferons was shown to be a suitable choice for viral positive cardiomyopathies. A non-randomized study showed administration of INF-ß results in improved survival by reducing the virus induced injury to the myocardium. The study showed that patients who received IFN-ß had higher virus elimination rates, improved quality of life and fewer adverse cardiac associated occurrences (66).

Targeted Therapy for Inflammatory Causes-Immunosuppressants

Treatment guidelines specific to inflammatory causes of DCM are scare. Extensive multicenter studies need to be conducted to prove the effectiveness of the treatment options (28). Current studies suggest that treatment need to be initiated at the early phase before the symptoms becomes worse and chronic (12). Early studies (1980's and 1990's) concluded that the use of immunosuppressive like prednisone, cyclosporine and azathioprine has a minimal clinical benefit and should not be used as standard therapy for DCM. The improvement was observed at the beginning of the trails, but it was not constant on extended period of time and there was no differences in the disease progression upon using these immunosuppressant agents (67). This might be due to the limitation in diagnosis and initiating this therapy to patient with virus positive DCM, thus determining the etiology of DCMi is important before initiating an immunosuppressive treatment regimen. There is no evidence of a guideline directed therapy but there are recent studies showing treatment with Immunosuppressants to beneficial. Studies showed the higher efficacy of immunosuppressive therapy when combined with regular heart failure medications in patients with biopsy-proven, virus-negative inflammatory cardiomyopathy when initiated before irreversible remodeling occurs (70, 71) (**Table 2**). In virus negative patients with inflammatory DCM, prednisolone and azathioprine was given for 3 months and were evaluated for 2 years in a randomized placebo-controlled study. Results showed that 71.4% patients from the immunosuppression group vs. 30.8% patients from the placebo group were improved ($P = 0.001$) (68). A larger retrospective case series from the Innsbruck and Maastricht Cardiomyopathy Registry showed that immunosuppressive therapy (azathioprine, prednisone, and cyclosporine) is associated with heart transplantation-free survival as compared with standard heart failure therapy alone (70). Other retrospective analysis showed that immunosuppressive treatment of patients with virus-negative inflammatory cardiomyopathy showed an improvement of LVEF with no major adverse reaction (69).

Targeted Therapy for Autoimmunity-Immunoadsorption

The assumption that disease associated auto antibodies (AAB) play a role in DCM pathogenesis, entail that removing such antibodies will result in improving the patients' cardiac parameters. Few studies have been conducted and some are still underway. There was an improvement in the pulmonary capillary output pressure, cardiac index, pulmonary resistance, reduction in AAB levels when immunoadsorption therapy was done for 5–7 cycles on 9 patients with DCM and increased anti-beta adrenergic receptor AAB levels (84). The results of other studies that followed up with patients for 3 months and 1 year showed significant improvement in the LVEF function (69.9%) (87). Patients who can benefit from this intervention can be identified using a combination of assessment of negative inotropic activity of antibodies and expression of gene patterns (90). In a study 4 courses of IA therapy administered for 3-month period intervals showed improvement in LVEF along with reduction of LV factor. Recently, Aptamer BC 007, a 15-mer single-strand DNA oligonucleotide drug (5′GGTTGGTGT GGTTGG-3′), was developed to neutralize AAB that bind to the extracellular domains of G protein-coupled receptors (GPCR-neutralization), amongst which beta 1 adrenergic receptors. Aptamer BC 007 was shown to improve cardiac function and prolong survival of Doberman Pinschers (DP) with DCM. These results promise that aptamer BC 007 might be effective in human patients with DCM (91). S100A8/A9 might serve as therapeutic targets in inflammatory cardiomyopathies. IA is still in an experimental period and use of it in clinicals require double blinded large multicenter studies (92).

EMERGING IMMUNOMODULATOR THERAPEUTIC STRATEGIES

Substantial emerging anti-inflammatory agents are entering early phase clinical evaluation (**Figure 1**). These strategies

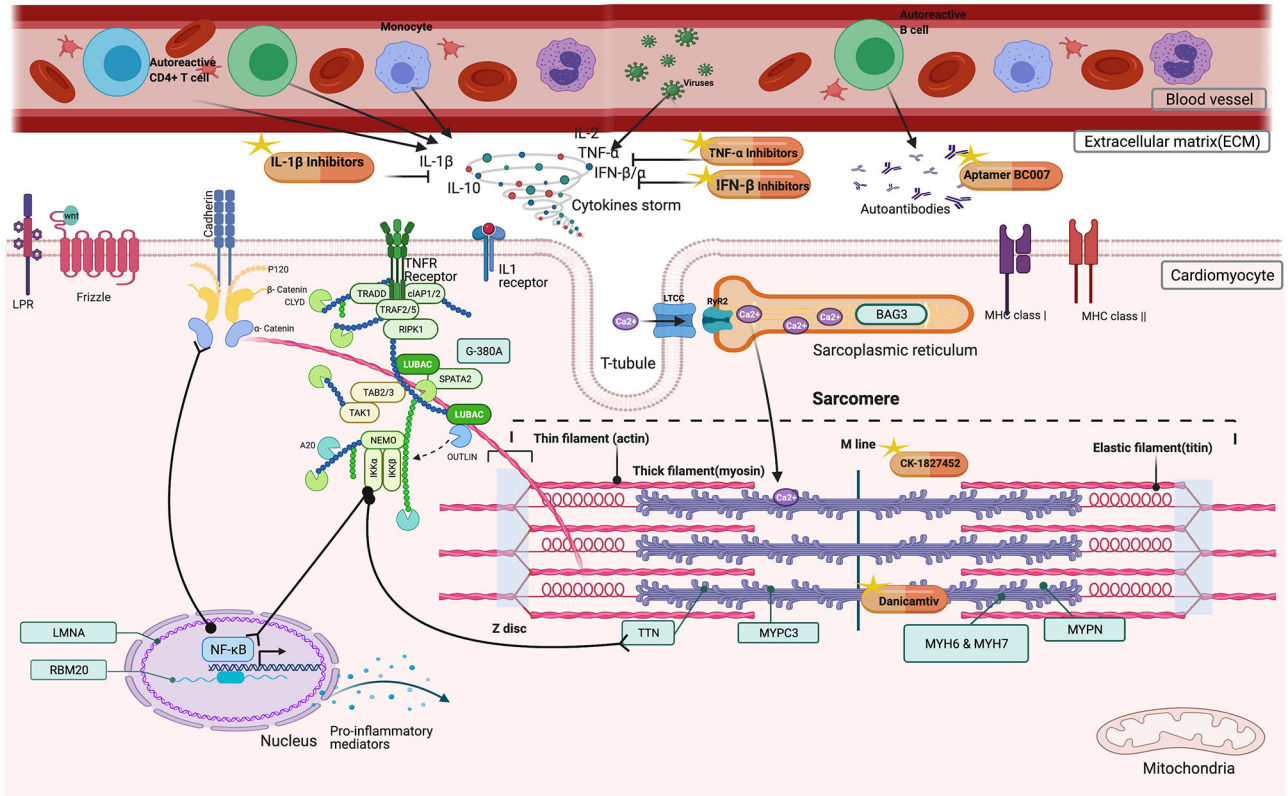

FIGURE 1 | Possible beneficial effects of immunomodulators in DCM patients. Inside the cardiomyocyte, CK-1827452, Danicamtiv accelerates the transition of actin-myosin complex in the sarcomere. In the extracellular matrix (ECM), TNF-α, IL-1β Inhibitors, and IFN β, Interferon beta downregulates the cytokine storm by inhibiting several factors (TNF-α,Tumor Necrosis Factor alpha, IL-1β Inhibitors, Interleukin−1 beta, IL-2, Interleukin-2, IL-10 Interleukin-10, IFN β, Interferon beta, IFN α, Interferon alpha). Aptamer BC007 acts on AAB neutralization.

are pathway-specific which include TNF-α inhibitors, IL-1β inhibitors, or immunomodulation by cellular components.

TNF-α Inhibitors

Up until now, Etanercept and Pentoxifylline are the most studied agents, but there is a lack of recent studies of their effectiveness. The use of Etanercept, a TNF-α antagonist lowered the levels of biologically active TNF in patients with moderate heart failure. The treatment was safe and well-tolerated for 3 months; it led to a significant dose-dependent improvement in left ventricular (LV) ejection fraction and LV remodeling (93). Other studies showed LVEF improvement and a considerable TNF-α production reduction in patients with ischemic cardiomyopathy after receiving pentoxifylline to conventional medications for 6 months (94). These studies have limitations due to small number of patients and short duration of follow-up. More importantly, some contradictions have been raised as to their beneficial effects, and most of the studies have been halted since 2010. Finally, Qiliqiangxin another promising drug that acts on regulating the balance between TNF-alpha and IL-10 showed improvement in cardiac function in rats with myocardial infarction (NCT01293903) (95). Unfortunately, the molecular mechanisms underlying the mode of action of this traditional Chinese medicine were not dissected and studies have been shifted to its usage in heart failure of all causes and not restricted to DCM (96).

IL-1 Inhibitors

As such, biological treatments that block the IL-1 pathway are potential agents to treat myocarditis such as the IL-Ra (IL-1 receptor antagonist) anakinra, and canakinumab.

Interlukin-1a Receptor Antagonists

The IL-Ra antagonist (Anakinra) blocks the acute inflammatory response accompanied with ST-segment elevation in acute myocardial infarction. A study on Anakinra receiving patients showed a lower incidence of heart failure (90). In recently decompensated systolic heart failure, a benefit of prolonged anakinra treatment was suggested by observing improved peak Vo2, with anakinra treatment for 12 weeks, but not for 2 weeks (97). In patients with colchicine resistance and corticosteroid-dependent recurrent pericarditis, over a median of 14 months, anakinra reduced the risk of recurrence (98). In heart failure with preserved ejection fraction, the use of anakinra for 14 days reduced the systemic inflammatory response and improved the aerobic exercise capacity. Conversely, in a group of patients with heart failure with preserved ejection fraction, treatment

with anakinra for 12 weeks deteriorated the peak Vo2 (97, 99). Two patients with fulminant myocarditis have recovered after Anakinra was administered along with standard therapy (100, 101). Moreover, the ACTION Study Group in France initiated a Phase 2B double blind randomized controlled trial evaluating ARAMIS (anakinra vs. placebo for the treatment of acute myocarditis) (NCT03018834). The study is estimated to be completed in 2021. Furthermore, Anakinra administration for 4 weeks to a DCM patient showed improvement in LVEF, arrhythmias, ventricular ectopic beat, and myocardial edema. It also resulted in interleukin-6 levels serum reduction, which measures the inflammation induced by interleukin-1 (102).

Canakinumab

Canakinumab which is an anti–IL-1β monoclonal antibody has demonstrated reduction of highly sensitive c reactive proteins and 1β, and IL-6 in patients with CAD (CANTOS [Canakinumab Anti-inflammatory Thrombosis Outcome Study] trial). However, its possible benefits in DCM need to be studied further (103).

Immunomodulation by Cellular Components

Mesenchymal stromal cells cardioprotective and immunomodulatory properties have been well-established and have been shown myocarditis improvement in experimental models of CVB3-induced myocarditis, autoimmune-induced DCMi and chronic Chagas cardiomyopathy (89, 104, 105). In DCM patients, transendocardial injection of autologous and allogeneic mesenchymal stromal cells in non-ischemic have been shown to be safe and clinical efficient in the randomized POSEIDON-DCM trial, through the involvement of the cardiosplenic axis- the homing of immune cells from the spleen to the heart and then subsequent involvement in cardiac remodeling in myocarditis or DCMi (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis-DCM) (104).

CONCLUSION

There is a necessity to better understand DCM pathogenies and etiologies to tailor the treatment for patients. Many immuno-based therapies are currently available, but refinement of these novel drugs need to be done to better understand the clinical efficacy in patients. Large cohort studies and advanced animal model experimentation need to be carried out prior conducting clinical trials to validate the significance of these etiology-based treatments. A challenge to apply specific tailored therapies would be the stratification of patients based on the causative factors. Effective stratification of patient into virus positive, negative, and inflammatory causes using EMB and imaging methods will allow for causative agent identification that can be used in the diagnosis, prognosis, and initiation of immuno-modulators agents. Thus, more clinical research needs to be conducted to prove the effectiveness of stratification, pharmacological use of immuno-modulators and the significance of it in hospital settings.

AUTHOR CONTRIBUTIONS

AK, FM, and GN have made significant contributions to writing this manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: TNFα Antagonists Are an Effective Therapy in Cardiac Sarcoidosis

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Introduction: Cardiac sarcoidosis (CS) is a life-threatening disease in which clear recommendations are lacking. We report a case series of CS successfully treated by tumor necrosis factor (TNF)α antagonists.

Methods: We conducted a single-center retrospective study of our patients with CS treated by TNFα antagonists.

Results: Four cases (4/84, 4.7%) were found in our database. Mean age was 40 years (range 34–53 years), and all were Caucasian men. Mean follow-up was 54.75 months (range 25–115 months). All patients received corticosteroid therapy (CT) and immunosuppressive therapy (IT). TNFα antagonists (infliximab or adalimumab) were started after the first or second CS relapse under CT and IT. One patient experienced relapse under TNFα antagonists (isolated decreased left ventricular ejection) and responded to a shorter interval of TNFα antagonist infusion. CT was discontinued in three patients treated with TNFα antagonists without relapse or major cardiac events during follow-up. No serious adverse event occurred in our case series, possibly due to dose sparing and frequent arrest of CT.

Conclusion: TNFα antagonists were effective in refractory and/or relapsing CS treated by corticosteroids and/or immunosuppressive agents, without serious adverse events, and should be considered earlier in CS treatment scheme.

Keywords: cardiac sarcoidosis, corticosteroids, immunosuppressive therapy, TNF antagonist, case series

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INTRODUCTION

Sarcoidosis is a rare multisystemic granulomatous disease of unknown etiology, which most frequently involves the lungs, lymph nodes, skin, eyes, liver, and spleen (1). Cardiac sarcoidosis (CS) is a rare condition, with symptomatic cardiac features reported in 2.3–39% of patients with sarcoidosis (2, 3). Cardiac involvement in sarcoidosis ranges from 27 to 50% in morphological studies (4, 5). Although CS is rare, it can be a life-threatening condition, mainly with left ventricular (LV) systolic failure, ventricular arrhythmias, and atrioventricular conduction abnormalities, which can lead to disability or cardiac sudden death (6). Research, diagnosis, and management of CS have all seen great progress in the last few years (7, 8). Corticosteroid therapy (CT) remains the mainstay for CS, despite a lack of prospective controlled studies, and CT should be started early after CS diagnosis (9). Treatment is recommended based on clinician experience, expert opinion,

and observational cohorts. To our knowledge, only two studies have investigated the impact of adjunctive immunosuppressive therapy (IT) on CS (10, 11).

In sarcoidosis, a key feature of granuloma is the interaction of CD_4^+ T cells with antigen-presenting cells to initiate and maintain the development of granuloma (1). CD_4^+ T cells differentiate into type 1 auxiliary T cells that secrete interleukin-2 and interferon-γ and increase production of TNFα, proinflammatory cytokines that amplify the cellular immune response (12). Therefore, TNFα appears to be an indicated therapy target. In severe or refractory disease, TNFα antagonists are effective in ocular (13), neurological (14), osseous (15), and pulmonary (16, 17), sarcoidosis. In CS, a few cases reports (18–22) and five cohort studies (23–27) have shown benefits of CT with or without IT in patients with severe and/or refractory cardiac involvement. Although there has been no randomized controlled study, some articles and expert opinions have suggested that TNFα antagonists for severe or refractory CS might be an option in case of CT or IT failure (28–30). We report our case series of CS treated by TNFα antagonists as adjunctive therapy.

METHODS

We conducted a single-center retrospective study of CS treated by TNFα antagonists using a systematic search of the Clermont-Ferrand CHRU CIM10 database in the Department of Internal Medicine, using code D868 "Sarcoidosis of other localizations and associated," between January 2000 and January 2020. Inclusion criteria were CS diagnosis by endomyocardial biopsy positive for myocardial granuloma compatible with CS, or Heart Rhythm Society (HRS) criteria for probable CS (31), and use of TNFα antagonists in follow-up for CS. We collected data retrospectively concerning baseline demographic characteristics, previous diseases, sarcoidosis diagnosis, CS features, and follow-up. The critical endpoints were (1) clinical and/or imaging relapse defined as onset of a new CS manifestation or worsening of preexisting CS manifestation; (2) major adverse cardiovascular events [MACEs: defined as cardiac death, ventricular fibrillation, sustained ventricular tachycardia (sVT), and hospitalization for heart failure]; and (3) adverse medical or drug events. Treatment efficacy was defined by the absence of critical endpoint during follow-up with a CT dose of 5 mg or below. Treatment failure was defined by the presence of a critical endpoint during follow-up. Investigation of the data was approved by the local ethics committee *Comité de Protection des Personnes Sud-Est 6* (number 2020/CE 75). Patients gave oral consent for retrospective collection of their medical data.

Abbreviations: AZA, Azathioprine; CS, Cardiac sarcoidosis; CT, Corticosteroid therapy; CYC, Cyclophosphamide; FDG, Fluorodeoxyglucose; HRS, Heart Rhythm Society; IT, Immunosuppressive therapy; LVEF, Left ventricular ejection fraction; MACEs, Major adverse cardiovascular events; MMF, Mycophenolate mofetil; MRI, Magnetic resonance imaging; MTX, Methotrexate; PET, Positron emission tomography; VT, Ventricular tachycardia; sVT, sustained ventricular tachycardia; TNFα, Tumor necrosis factor α.

RESULTS

We screened 84 patients and four met the inclusion criteria. Baseline characteristics are presented in **Table 1** and follow-up outcomes in **Figure 1**. Mean age was 40 years (range 34–53 years), and all four patients were Caucasian men. Mean follow-up was 54.75 months (range 25–115 months). All four patients were treated with corticosteroids and immunosuppressive agents, including cyclophosphamide (CYC), azathioprine (AZA), methotrexate (MTX), and mycophenolate mofetil (MMF). Cardiac treatment was managed by the referent cardiologist. TNFα antagonists (infliximab and adalimumab) were given after the first or second CS relapse under CT and IT.

All four patients experienced at least one relapse under IT alone (cases 2 and 3) or associated with CT (cases 1 and 4) before TNFα antagonists' initiation. In cases 2 and 3, relapses were a high number of premature ventricular contractions and a cardiac fluorodeoxyglucose positron emission tomography (FDG-PET) uptake, respectively. In each case, CT was tapered off within 12–14 months from initiation under MTX as immunosuppressive adjunctive therapy. Relapse occurred 2 months after CT discontinuation and led to TNFα antagonist initiation, without CT resumption. The chosen TNFα antagonist was infliximab at 5 mg/kg, at 0 and 2 weeks, and every 4 weeks. In each of those two cases, infliximab was changed for adalimumab 40 mg subcutaneously every 2 weeks, for the patient's personal convenience. These two patients did not show any evidence of relapse on a 19-month follow-up under adalimumab, without CT.

In cases 1 and 4, relapses were magnetic resonance imaging (MRI) late gadolinium enhancement with increasing angiotensin-converting enzyme and LVEF decreases, respectively. In case 1, MRI relapse occurred under prednisone at 10–15 mg/d and AZA, which led to infliximab initiation at 5 mg/kg, at 0 and 2 weeks, and every 4 weeks. CT was tapered to reach 5 mg within 9 months and was stopped within 14 months from infliximab initiation. In case 4, CS activity was defined by LVEF decrease. At diagnosis, CYC, mycophenolate mofetil and AZA permitted CT tapering to 10 mg/d of prednisone. First relapse/MACE (hospitalization for heart failure) was treated by increasing CT dose to 60 mg/d and new CYC pulses (1,000 mg/month for six pulses) relayed by MTX. Twelve months later, a second relapse/MACE occurred (hospitalization for heart failure) while the patient was under MTX and prednisone 15 mg/d. MTX was discontinued and infliximab was introduced, at 3 mg/kg, at 0 and 2 weeks, and every 8 weeks. Nineteen months later, under prednisone 15 mg/d and infliximab every 8 weeks, a third relapse/MACE (hospitalization for heart failure) occurred, leading to strengthening of the dose to 5 mg/kg and shortening of the infliximab infusion interval to 6 weeks. Thereafter, CT was slowly tapered down to reach 5 mg within 39 months after the third relapse, without any other relapse.

In those four patients (**Table 2**), a total of six relapses, considered as therapeutic failure, were recorded, in which three MACEs occurred in case 4. Five relapses occurred under standard IT associated or not with CT: three relapses under CT associated with IT, two relapses under IT alone. One relapse occurred under

TABLE 1 | Baseline characteristics of patients in our case series.

	Case 1	Case 2	Case 3	Case 4
Age at sarcoidosis diagnosis, yr	34	36	38	53
Male	+	+	+	+
Caucasian	+	+	+	+
Habitus				
Alcohol	0	0	0	0
Tobacco	+	+	+	+
BMI (kg/m²)	19.7	28.7	22.1	26.1
Previous diseases				
Cardiovascular disease	0	0	0	0
Diabetes mellitus	0	0	0	+
Hyperlipidaemia	0	0	0	0
Organ involvement				
Lungs	0	0	0	+
Lymph nodes	+	+	+	+
Skin	0	0	0	0
Ear, nose, throat	0	0	0	0
Eyes	0	+	0	0
Liver/spleen	+	+	0	0
Central nervous system	0	0	0	0
Peripheral nervous system	0	+	0	0
Kidney	0	0	0	0
Pathological evidence of granuloma	Salivary glands, lymph node	Lymph node	Lymph node	Lymph node
Angiotensin-converting enzyme (UI/L, *N* < 60)	67	120	-	62
Hypercalcaemia	0	0	0	0
Gammaglobulin level (g/L)	12.5	16.3	9.8	12.1
Time from diagnosis to CS diagnosis (months)	2	1	0	6
Initial evaluation				
VES/24 h	3,213	3,227	0	0
sVT	0	0	0	0
nsVT	0	0	0	0
AV block	0	0	0	0
Bundle branch block	+	0	0	0
LVEF (%)	60	66	30	26
Wall motion abnormalities	0	0	+	+
NT-pro-BNP (ng/mL, *N* < 150)	170	7	791	130
Troponin (pg/mL, *N* < 0.015)	N	0.044	0.24	N
Cardiac PET	0	+	0	+
Cardiac LGE on MRI	+	0	+	0

BMI, body mass index; LGE, Late gadolinium enhancement; LVEF, left ventricle ejection fraction; MRI, magnetic resonance imagery; N, Normal; nsVT, nonsustained ventricular tachycardia; PET, positron emission tomography; sVT, sustained ventricular tachycardia; VES, ventricular extrasystoles.

infliximab therapy in case 4 under infliximab 3 mg/kg every 8 weeks, successfully treated by increased dose of infliximab and shorter infusion interval. Adalimumab 40 mg every 2 weeks was used in two patients without any evidence of relapse or MACE.

Standard IT, with or without CT associated, failed five times whereas TNFα antagonists failed once. TNFα antagonists demonstrated efficacy defined by the absence of critical endpoint during follow-up with a CT dose of 5 mg or below in all cases, with a follow-up under TNFα antagonists of 16–80 months. In case 4, TNFα antagonists failed to demonstrate efficacy with one relapse under prednisone 15 mg/d and infliximab 3 mg/kg every 8 weeks, but succeeded to demonstrate efficacy at 5 mg/kg every 6 weeks.

Five infectious adverse events were retrieved in cases 1, 2, and 4, consisting in a pharyngitis under prednisone 3 mg/d, azathioprine 150 mg/d, and infliximab 300 mg every 4 weeks in case 1; a pharyngitis under prednisone 75 mg/d, CYC 1,000 mg/month in case 2; and three sigmoiditis treated by oral antibiotics under prednisone 22.5–8 mg/d and infliximab in

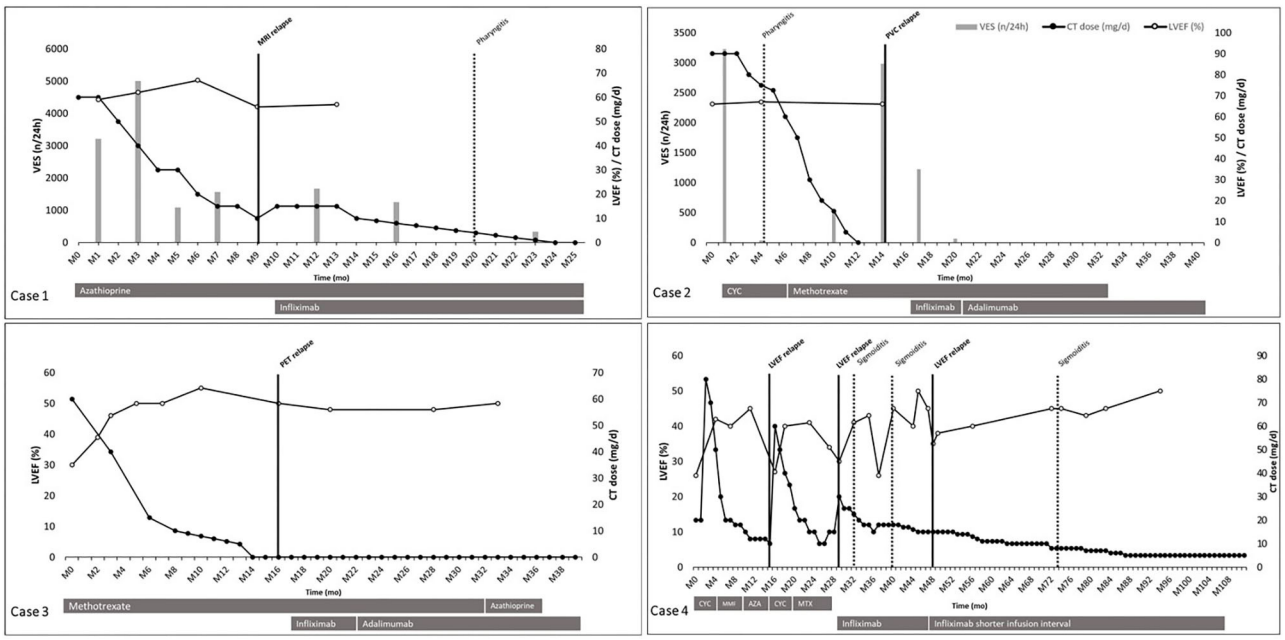

FIGURE 1 | Patients' follow-up outcomes. Individual case presented from diagnosis (MO) to the end of follow-up for the study. Time in month on abscissa. Prednisone dose (mg/d), left ventricular ejection fraction (%), number of ventricular extrasystole (n/24 h) on Ordinate. Used therapies are represented in gray bars below each timeline. Black dots correspond to prednisone dose (mg/d). White dots correspond to left ventricular ejection fraction (%). Vertical gray bars correspond to number of ventricular extrasystole (n/24 h). Vertical black bars correspond to relapse. Vertical black dotted line corresponds to adverse events. Case 1: azathioprine was used 150 mg/d; infliximab was used 5 mg/kg at week 0 and 2, and every 4 weeks. Case 2: cyclophosphamide was used 1,000 mg/mo; methotrexate was used 15–20 mg/w; infliximab was used 5 mg/kg at week 0 and 2, and every 4 weeks; adalimumab was used 40 mg every 2 weeks, subcutaneously. Case 3: methotrexate was used 10–15 mg/w; azathioprine was used 100 mg/w; infliximab was used 5 mg/kg at week 0 and 2, and every 4 weeks; adalimumab was used 40 mg every 2 weeks, subcutaneously. Case 4: cyclophosphamide was used 1,000 mg/mo; mycophenolate mofetil was used 720 mg/d; azathioprine was used 150 mg/d; methotrexate was used 10–15 mg/w; infliximab was used 3 mg/kg at week 0 and 2, and every 8 weeks, then 5 mg/kg every 6 weeks. AZA, azathioprine; CI, corticosteroid therapy; CYC, cyclophosphamide; LVEF, left ventricular ejection fraction; MMF, mycophenolate mofetil; MRI, magnetic resonance imaging; MIX, methotrexate; PET, positron emission tomography; PVC, pre-mature ventricular contraction; VES, ventricular extrasystole.

case 4. No drug-related adverse event or serious adverse event was recorded.

DISCUSSION

This case series included all patients treated with the TNFα antagonist (infliximab or adalimumab) for CS in our center. TNFα antagonists show an effectiveness in all patients as rescue treatment in CS relapsing under IT and/or CT, in long and close follow-up without serious infectious or drug-related adverse events. As reported by Ballul et al. (10), our patients were Caucasian men who relapsed even with IT, although they suggested that IT associated with CT may reduce the relapse rate. Only one patient experienced relapse under TNFα antagonists, but without other IT, and responded to a stronger dose and shorter TNFα antagonist infusion interval. CT was discontinued in three patients treated with TNFα antagonists without relapse or MACEs during long-term follow-up and only one patient had a long-term low-dose prednisone of 5 mg/d. Adalimumab and infliximab were the chosen TNFα antagonists in our patients, because of their effectiveness in extra-cardiac sarcoidosis (32) and because of the convenience of subcutaneous administration of adalimumab and reassuring data on switching infliximab to adalimumab (33). In our patients, adalimumab did not show any evidence of inferiority compared to infliximab since we did not record any relapse or adverse event under this therapy. In two patients, CT was discontinued before TNFα antagonists' initiation, and relapses were treated with infliximab only, without resumption of CT.

Standard therapy including IT and/or CT failed in all our cases, whereas one patient relapsed under infliximab but succeeded with a stronger dose and shorter infusion interval. In this case, infliximab dose was lower (3 mg/kg) and interval infusion was greater (8 weeks) than the other cases, due to referral physician decision and lack of standard protocol in CS. Moreover, this patient received infliximab 28 months after CS diagnosis with already two relapses. This remark raises the question of early "optimal" treatment in CS in order to avoid myocardial scar formation after each relapse, which are probably not responsive to intensive treatment. In three out of four cases, IT was used in combination with TNFα antagonists, as corticosteroid-sparing agents and as demonstrated by Ballul et al. (10) as CS specific treatment. This attitude should be highlighted because of the effectiveness of IT in CS, especially in relapsing or in CT-dependent patients. Although all our cases failed under IT alone

TABLE 2 | Relapses and MACEs in our patients.

Events	Case 1	Case 2	Case 3	Case 4	Overall
Relapses	1	1	1	3	6
IT					
CT associated	1	0	0	2	3 (50%)
IT alone	0	1	1	0	2 (33%)
TNFα antagonists	0	0	0	1	1 (16%)
MACEs	0	0	0	3	3
IT					
CT associated	0	0	0	2	2 (66%)
IT alone	0	0	0	0	0
TNFα antagonists	0	0	0	1	1 (33%)
Overall events	1	1	1	3	6

CT, corticosteroid therapy; IT, immunosuppressive therapy; MACEs, major adverse cardiovascular events; TNFα, tumor necrosis factor alpha. In case 4, MACEs were also considered as relapses.

or associated with CT, IT utility, when used aside from TNFα antagonists, remain unclear in CS, and further studies might be interesting.

No serious infectious or drug related adverse event occurred in our case series, possibly due to dose sparing and frequent stopping of CT. In fact, 3 out of 4 cases discontinued CT 11–23 months after CS diagnosis and last patient had a 49 months follow-up under the threshold of prednisone 10 mg.

Until 2019, there were no randomized trials on TNFα antagonists in CS, and only a few cases reports or series had shown the potential benefit of TNFα antagonists (17–22). The ATTACH (Anti-TNF Therapy Against Congestive Heart Failure) trial (34) found worsening of heart failure in patients treated by high-dose infliximab for congestive heart failure with other causes than sarcoidosis limiting its use in cardiac inflammatory conditions such as CS. However, Drent et al. (29) have suggested using TNFα antagonists, especially infliximab, if there is no response to conventional treatment, in the presence of active CS and if CS is identified as the only cause of heart failure. Adler et al. conducted a systematic review of efficacy and safety of TNFα antagonists in sarcoidosis (32). They showed similar rates of adverse events (88.2 vs. 91%) and severe adverse events (18.5 vs. 14.8%) in five randomized control trials comparing TNFα antagonists with placebo. Rates of malignancy and death were comparable between groups (1.1 vs. 0.8% for malignancies and 1.1 vs. 1.6% for deaths, TNFα compared to placebo).

In September 2019, Rosenthal et al. (25) recommended adalimumab as second-line therapy after high-dose prednisolone (>30 mg/day for 4–8 weeks) and MTX (20 mg/week) if patients experienced clinical relapse, did not achieve FDG-PET remission, or experienced adverse effects of previous immunosuppression. This retrospective, single center study included 29 patients with CS according to the 2017 criteria of the Japanese Cardiology Society. The study found that discontinuation of immunosuppression was significantly associated with FDG-PET SUV increase and VT recurrence, and steroid-sparing immunosuppressive agents such as MTX or adalimumab were effective in suppressing inflammation in CS. In November 2019, Harper et al. (24) reported 36 patients treated by infliximab for CS refractory to conventional treatment. Twenty-four (66%) patients were categorized as "responders" after infliximab initiation in at least one of the three outcomes categories (steroid-sparing dose, LVEF, and dysrhythmia). Similar to our case series concerning sparing the dose of CT, Baker et al. (23) reported 20 patients with CS treated by TNFα antagonists. Seventeen patients had complete resolution of disease imaging activity within 12 months, and LVEF did not change (44 vs. 47%). TNFα antagonists permitted a decrease in mean dose of CT (23–4 mg/day) at 6 months and complete discontinuation of CT after a mean of 9 months of treatment with TNFα antagonists. In 2020, Gilotra et al. (26) reported 38 patients with CS treated with TNFα antagonists (infliximab or adalimumab), with a significant decrease in CT dose within 6 or 12 months of treatment, and CT discontinuation in 10 out of 38 patients. The increase in LVEF after treatment was not significant (45 before vs. 47% after treatment), and four (11%) patients suffered from infection as an adverse effect of IT. Finally, in 2021, Bakker et al. (27) reported 22 patients with CS treated by infliximab, with a mean follow-up of 18.9 months. Eighteen patients (82%) were classified as "responders," with a significant decrease in myocardial SUVmax on FDG-PET and a significant increase in LVEF after TNFα antagonist initiation.

Comparing our case series to these recent data (**Table 3**), we found similar indications of TNFα antagonists use in CS suffering patients, particularly in patients with relapse occurring under IT. All studies described were retrospective. Mean follow-up in our series was higher than in the other series. Our study endpoints were relapses and MACEs as defined in our method whereas the other studies chose specific parameters such as prednisone dose, LVEF, myocardial FDG-PET uptake and dysrhythmias. CT and IT use at TNFα antagonists' initiation were similar throughout studies, as were CT mean doses and tapering schemes after TNFα antagonist initiation. Most used IT was methotrexate. Most used TNFα antagonists were infliximab at 5 mg/kg at week 0 and 2, and every 4 weeks and subcutaneous adalimumab 40 mg every 2 weeks. In two studies (24, 35), 66% (24/36) and 82% (18/22) TNFα antagonist's treated patients were classified as "responders." Imaging endpoints were chosen in three studies (23, 25, 27) with good outcomes on disease activity on myocardial FDG-PET uptake. Data on CT discontinuation after TNFα antagonists' initiation were scarce, but studies found significant decrease in CT dose in the follow-up under TNFα antagonists (24, 26). Data on risk of worsening heart failure under TNFα antagonists were reassuring in two studies, with a global LVEF stability before and after treatment initiation (26) and significant LVEF increase from 45 to 55% (27). Nonetheless, a specific attention toward worsening heart failure under TNFα antagonists should be mentioned, as four patients experienced worsening in LVEF in Gilotra et al. cohort (26), especially in patients with severely impaired pre-treatment LVEF (20–35%). A few major drugs associated adverse events were recorded, such as sepsis, *Cryptococcus* infection, aseptic meningitis, *C. difficile* diarrhea,

TABLE 3 | Outcomes in recent studies upon TNFα antagonists' efficiency in CS.

Endpoints	Baker et al. (23)	Harper et al. (24)	Rosenthal et al. (25)	Gilotra et al. (26)	Bakker et al. (27)	Our series
TNFα antagonist treated of patients (*n*)	20	36	19	38	22	4
Mean follow up (mo)	Data not available	<12	49.2	15.9	18.9	54.7
Evaluation criteria	Prednisone dose, LVEF	LVEF, dysrhythmias, prednisone dose	FDG-PET uptake	Clinical composite criteria†, FDG-PET uptake	FDG-PET uptake, device interrogation, TTE, biomarker, treatment dose	Relapse, MACEs
CT and IT used at TNFα antagonist introduction, number of treated patients (*n*)	MTX, $n = 20$ Prednisone and MTX, $n = 13$	Prednisone 10–30 mg/d, $n = 35$ MTX, $n = 25$ LEF, $n = 9$ AZA, $n = 1$ HCQ, $n = 2$	Data not available	Prednisone alone, $n = 2$ Prednisone and MMF (mean dose 2 g/d), $n = 16$ Prednisone and MTX (mean dose 16 mg/w), $n = 11$ Prednisone and AZA (mean dose 171 mg/d), $n = 8$	Prednisone, $n = 12$ MTX, $n = 10$ AZA, $n = 6$ MMF, $n = 3$ HCQ, $n = 1$	Prednisone 0–20 mg/d ($n = 2$) and AZA 100–150 mg/d ($n = 2$) or MTX 10–20 mg/w ($n = 2$)
Mean time from CS diagnosis to TNFα antagonist introduction (mo)	16	Data not available	Data not available	16.1	22.8	17.25 (9–28)
TNFα antagonist treatment scheme, number of treated patients (*n*)	IFX (data not available), $n = 10$ ADA (data not available), $n = 10$ GOL (data not available), $n = 1$	IFX 5–10 mg/kg every 4–8 weeks, $n = 36$	ADA 40 mg every 2 weeks $n = 19$	IFX 3–10 mg/kg (interval not shown), $n = 30$ ADA 40 mg every 2 weeks, $n = 8$	IFX 5 mg/kg week 0 and 2, and every 4 weeks	IFX 3–5 mg/kg every 4–8 weeks n=4 ADA 40 mg every 2 weeks $n = 2$
Prednisone tapering scheme after TNFα antagonist initiation	Mean of 23 mg tapered to mean of 4 mg over 6 months	Mean of 20 mg/d tapered to mean of 5 mg/d over 12 months	Data not available	21.7 ± 17.5 mg/d tapered to 7.3 ± 7.3 mg/d over 12 months	Data not available	0–30 mg/d tapered to 0–5mg/d Discontinuation before ($n = 2$) Discontinuation 13 months after ($n = 1$)
CT discontinuation after TNFα antagonist initiation (*n*, %)	Data not available	Data not available	Data not available	10 (26%)	Data not available	$n = 1$ (25%), 13 months after
Adverse events under TNFα antagonist (*n*)	0	6	1	8	5	3
Mean follow-up under TNFα antagonists (mo)	12	<12	Data not available	<12	18.9	33.8 (16–80)
Relapse (*n*)	Data not available*	Data not available*	Data not available*	Data not available*	Data not available*	6
MACE (*n*)	Data not available*	Data not available*	Data not available*	Data not available*	Data not available*	3
Relapse under TNF (*n*, %)	Data not available*	8 (22%)	Data not available*	Data not available*	2 (9%)	1 (25%)
MACE under TNF (*n*, %)	Data not available*	Data not available*	Data not available*	Data not available*	Data not available*	1 (25%)

†*Incidence of ventricular arrhythmias, worsening heart failure, heart transplantation, left ventricular assist device implantation and death from any cause.* **Relapse and MACEs were not defined in each study's methodology. ADA, adalimumab; AZA, azathioprine; CT, corticosteroid therapy; GOL, golimumab; HCQ, hydroxychloroquine; IFX, infliximab; LVEF, left ventricular ejection fraction; MACE, major adverse cardiac event; MMF, mycophenolate mofetil; MTX, methotrexate; TNFα, tumor necrosis factor alpha; TTE, transthoracic echocardiography.*

hepatitis, and allergic reaction during infusion. In overall cohort studies, 23 adverse events in 139 TNFα antagonists treated patients were retrieved. All these data taken together emphasize TNFα antagonists' efficacy and safety but highlight the lack of homogeneity in study design and prevent us from drawing any clear recommendations.

In 2021, Kouranos et al. published a state-of-the-art review on CS (35), emphasizing early use of CT in CS but without established scheme. Also, initial dosage remain unclear. Use of IT such as methotrexate is mentioned as of interest in avoiding steroid related adverse events and dysrhythmias such as ventricular extra systole and non-sustained ventricular tachycardia. Nonetheless, duration and dose remain unknown in this specific condition. Unfortunately, TNFα antagonists were not mentioned as possible part of CS treatment, including as rescue treatment in severe of refractory CS to conventional treatment.

TNFα antagonists are effective in CS cases that failed or relapsed under conventional IT, including CT or non-biological IT, and represent a good steroid-sparing therapy with an overall good tolerance profile in our case study and in the literature data.

Our case study had some limitations. Only four patients received TNFα antagonists for CS at our institution, which limited interpretation of the usefulness of this medication. Moreover, evolution of each patient could be attributed to CS natural course, due to the lack of a control group. The retrospective nature of the study meant that our patients had different treatment regimens that were adapted by referral physicians to the specific condition of each patient. The initial starting dose of CT was 1 mg/kg/day in each case, which was higher than some proposed regimens in the literature but without long-term maintenance dose of CT because of the use of IT and TNFα antagonists. In retrospective studies, CT schemes varied a lot as shown in Sadek et al. systematic review on CT in CS (9), probably due to physicians' decision toward each patient conditions and retrospective design of most of the studies.

In order to clarify TNFα antagonists' position in the treatment scheme of CS, further studies are needed, with control groups, standardized treatment schemes on CT and IT use, and strong clinical endpoints such as relapse or MACEs. However, in the absence of such studies, we recommend TNFα antagonists use early in severe or relapsing CS, because of the life-threatening issues in this specific condition, and to avoid long and/or high CT cumulative exposure in frequently young people.

CONCLUSION

We reported a case series of four patients successfully treated by TNFα antagonists for relapsing CS under CT and IT, with good long-term outcomes regarding relapse rate, steroid-sparing dose and adverse drug events. Treatment regimens and standardized approaches are lacking in CS, showing that a tailored approach is needed for each patient to achieve remission, but with the possibility of TNFα antagonists use in case of severe or relapsing CS under IT and/or CT.

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.

ETHICS STATEMENT

Investigation of the data was approved by the local ethics committee Comité de Protection des Personnes Sud-Est 6 (number 2020/CE 75). The patients/participants provided their oral and written informed consent to participate in this study, and oral consent to publication of their anonymous data.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Focus on Autoimmune Myocarditis in Graves' Disease: A Case-Based Review

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The manifestations of hyperthyroidism-related myocardial damage are multitudinous, including arrhythmia, dilated cardiomyopathy, valvular diseases, and even cardiogenic shock. Acute myocarditis induced by thyrotoxicosis had been reported in a few studies. However, attention on its prevalence and underlying mechanisms is sorely lacking. Its long-term harm is often ignored, and it may eventually develop into dilated cardiomyopathy and heart failure. We report a case of Graves' disease with a progressive elevation of hypersensitive cardiac troponin-I at several days after discontinuation of the patient's anti-thyroid drugs. Cardiac magnetic resonance imaging (CMRI) showed inflammatory edema of some cardiomyocytes (stranded enhanced signals under T2 mapping), myocardial necrosis (scattered enhanced signals under T1 late gadolinium enhancement) in the medial and inferior epicardial wall, with a decreased left ventricular systolic function (48%), which implied a possibility of acute myocarditis induced by thyrotoxicosis. The patient was then given a transient glucocorticoid (GC) treatment and achieved a good curative effect. Inspired by this case, we aim to systematically elaborate the pathogenesis, diagnosis, and treatment of hyperthyroidism-induced autoimmune myocarditis. Additionally, we emphasize the importance of CMRI and GC therapy in the diagnosis and treatment of hyperthyroidism-related myocarditis.

Keywords: Graves' disease, thyrotoxicosis, autoimmune myocarditis, cMRI, glucocorticoid

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INTRODUCTION

Cardiovascular disease remains one of the largest causes of death worldwide. Thyroid hormones are closely related to the cardiovascular system (CVS) in both physiological and pathological situations. Dating back to ontogeny, the thyroid and the CVS are derived from the same embryological origin. The former modulates each component of the latter for a normal function during the developmental stage (1). Thyroid hormone dysfunction has been shown to be devastating to the heart, and both all-cause and cardiovascular mortality are increased in hyperthyroidism (2).

Classic hyperthyroidism cardiomyopathy was defined as a range of heart diseases caused by hyperthyroidism, mainly manifesting as arrhythmia, atrial fibrillation, cardiac enlargement, heart failure, and valvular diseases (3). Its severity is second only to hyperthyroidism crisis, which is an important cause of death in hyperthyroidism patients (4). Currently, an interesting case with

Graves' disease (GD) was admitted to our hospital, shifting our attention to acute myocarditis induced by hyperthyroidism. Progressive elevation of high-sensitivity cardiac troponin I (hs-cTnI) without any discomfort was her main clinical feature. The features of cardiac magnetic resonance imaging (CMRI) met the upgraded Lake Louise criteria (LLC) in 2018 for myocarditis rather than coronary ischemia.

Myocarditis is broadly defined as an inflammatory process invading cardiomyocytes (5). Viral myocarditis is the most frequent type, often with fever or other symptoms of infection, chest pain, dyspnea, electrocardiogram (ECG) changes, and troponin elevation (6). There is strong evidence that autoimmune diseases are also involved in the occurrence of acute myocarditis (7). Furthermore, 7.2% of myocarditis patients and 15% of fulminant myocarditis had autoimmune diseases (8). Although GD is a common autoimmune thyrotoxicosis and well-known to be related to chronic heart failure (especially dilated cardiomyopathy), hyperthyroidism-associated acute myocarditis is rarely reported. However, retrospective studies reported that 9–16% of unexplained non-ischemic dilated cardiomyopathy cases have a histological evidence of myocarditis (9). Thus, there may exist huge omissions about hyperthyroidism-related myocarditis in GD patients.

Additionally, our patient showed a good response to transient anti-inflammatory therapy with glucocorticoid (GC). Based on the treatment experience from this patient, we will systematically elaborate the following scientific issues: (1) the potential mechanisms and characteristics of thyrotoxic-related myocarditis, (2) the diagnosis and antidiastole about thyrotoxic-related myocarditis, (3) the value of CMRI in the diagnosis of inflammatory cardiomyopathy, and (4) potential treatment strategies of thyrotoxic-related myocarditis.

TABLE 1 | Laboratory parameters of thyroid function (FT3, FT4, and TSH).

Date	FT3 (pg/L)	FT4 (ng/L)	TSH (μIU/L)
Normal range	2.0–4.4	9.32–17.09	0.27–4.2
21 July	16.19	51.27	<0.005
25 July	12.35	50.44	<0.005
28 July	7.65	38.1	<0.005
30 July	6.89	33.79	<0.005

TABLE 2 | Clinical indicators about infection.

Respiratory symptoms	None	Anti-CMV IgM	Negative
Clinical indicators			
Temperature (°C)	36.1 (36.1–37)	Anti-HSV-I IgM	Negative
Neutrophils (10^9/L)	2.10 (1.80–6.30)	Anti-HSV-II IgM	Negative
Lymphocytes (10^9/L)	1.38 (1.10–3.20)	Anti-EBV IgM	Negative
Hemoglobin (g/L)	118 (115.0–150.0)	Anti-PVB19 IgM	Negative
Platelets (10^9/L)	223 (125.0–350.0)	Anti-CVB IgM	Negative
hsCRP (mg/L)	3.8 (<10)	Anti-CA16 IgM	Negative
ESR (mm/H)	6 (0–15)	Anti-ECHO IgM	Negative
TG (μg/L)	0.33 (3.5–77)	Anti-MV IgM	Negative
TG-Ab (IU/ml)	>4,000 (0–115)	Anti-VZV IgM	Negative
TPO-Ab (IU/ml)	>600 (0–34)	Anti-RV IgM	Negative
TRAb (IU/L)	7.08 (0–1.58)	Anti-TOX IgM	Negative

hsCRP, high-sensitivity C-reactive protein; ESR, erythrocyte sedimentation rate; TG, thyroglobulin; TG-Ab, thyroglobulin antibody; TPO-Ab, thyroid peroxidase antibody; TRAb, thyrotropin receptor antibody; CMV, cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein–Barr virus; PVB19, parvovirus B19; CVB, coxsackievirus B; CA16, coxsackievirus A16; ECHO, enteric cytopathic human orphan virus; MV, measles virus; VZV, varicella-zoster virus; RV, rubella virus; TOX, toxoplasma.

CASE PRESENTATION

A 31-year-old woman with 2-month pregnancy was diagnosed with hyperthyroidism 3 years ago and mainly complained with palpitation and excessive sweating. Then, she accepted propylthiouracil treatment orally and persisted 6 months after her delivery. Her above-mentioned symptoms were significantly improved, and she gained healthy birth outcomes. The doctors later switched her medication to methimazole (5 mg, b.i.d.). Both drugs showed good control for her disease without any adverse events. On July 10, 2020, she voluntarily discontinued the medications without any medical consultation. At 10 days later (July 20), she was re-admitted at our hospital for further treatment because her thyroid function examination showed obvious abnormalities without any discomfort. Except for laparoscopic surgery for ovarian cyst in 2015, she denied any other history of surgery, chronic diseases, inherited diseases, and allergy.

Upon admission, the physical examination showed that the heart rate was 96 beats/min and the thyroid gland was of grade II enlargement. The laboratory workup showed that the patient had decreased thyroglobulin (Tg), 0.33 μg/L, with positive thyroglobulin antibody (Tg-Ab) >4,000.00 IU/ml, positive thyroid peroxidase antibody (TPO-Ab) >600.00 IU/ml, and positive thyroid-stimulating hormone (TSH) receptor antibody (TR-Ab) 7.08 IU/L. Her thyroid function showed decreased TSH (0.005 μIU/ml) and increased free thyroxine (FT4, 51.27 ng/L) and free triiodothyronine (FT3, 16.19 pg/ml; **Table 1**). No obvious abnormality was found in the blood routine test, liver and kidney function, electrolyte, urine routine test, D-dimer, ESR, hsCRP (**Table 2**), and NT-proBNP. Surprisingly, the hs-cTnI was increased by 101.5 pg/ml (normal range, <15.6 pg/ml) (**Figure 1**). In addition, the 12-lead ECG showed poor progression of R waves in leads V1–V3 (**Figure 2A**). The echocardiography showed that the heart morphology was normal without valvular disease and segmental wall motion, and the value of ejection fraction (EF) was 70%. No abnormality was found by color Doppler ultrasound about the liver, gallbladder, spleen, pancreas, kidney, and bladder, except for multiple calculi in the intrahepatic duct.

At the following 2 days, the level of hs-cTnI was gradually increased to 1,516.3 pg/ml. The results of a re-examination of both ECG and echocardiography were similar to the previous results. Although the patient did not express any discomfort, the elevation of hs-cTnI and abnormal ECG suggested that myocardial necrosis existed. In addition to prophylactic antiplatelet (aspirin), statins, and anti-hyperthyroidism drug

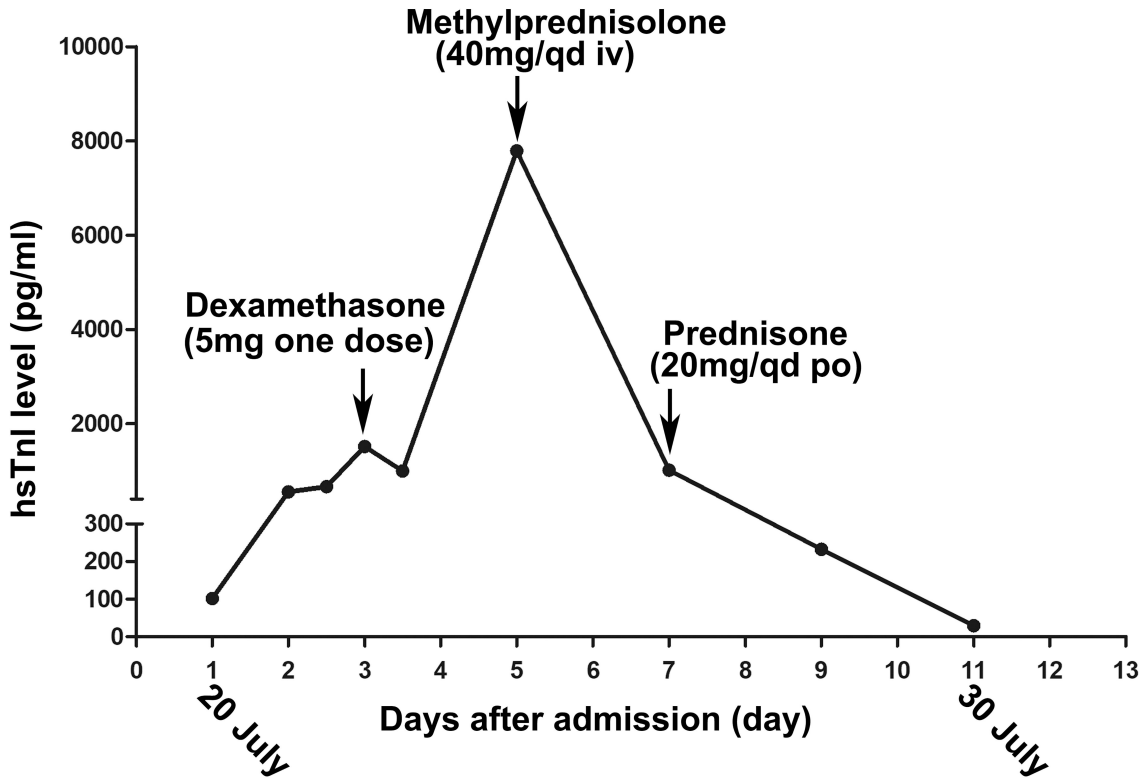

FIGURE 1 | The changing trend of the high-sensitivity cardiac troponin I (hs-cTnI) during her hospitalization. The arrow indicates the times when the patient accepts glucocorticoid treatment.

methimazole, 5 mg dexamethasone sodium phosphate (once) was tried intravenously at 10:28 on July 22. At 16:27 on the same day, the hs-cTnI was surprisingly found to drop to 994.6 pg/ml, rapidly. However, the level of hs-cTnI rose dramatically to its peak 7,785.9 pg/ml for the next 2 days (**Figure 1**). During this period, the only changed treatment was that the glucocorticoids were discontinued. Moreover, 24-h Holter ECG captured the occasional premature ventricular contractions (**Figure 2B**). Although the patient still had no abnormal symptoms about the heart, percutaneous coronary angiography (PCA) and CMRI were recommended. She rejected the invasive PCA. The CMRI showed an inflammatory edema of the myocardium [stranded enhanced signals under T2 mapping and ventricular septal enhancement signal in T2 black blood fat suppression (T2-BB-FS) sequences], myocardial necrosis [scattered enhanced signals under T1 late gadolinium enhancement (LGE)] in the medial and inferior epicardial wall, with a decreased left ventricular systolic function (LVEF, 48%), which are consistent with the change of acute myocarditis (**Figure 3**). Additionally, she does not appear to have been infected by any pathogens since the results of serotype antibodies for several viruses showed that the IgM of all selected viruses are negative, including CMV, HSV-I, HSV-II, EBV, PVB19, CVB, CA16, ECHO, MV, VZV, RV, and TOX. Thus, methylprednisolone (40 mg/d) was given intravenously. Considering no risk factors and evidence for coronary heart disease, antiplatelet and statins were withdrawn in the meantime. At the sixth day after admission (26 July), her hs-cTnI was significantly decreased to 1,014.8 pg/ml. Then, intravenous methylprednisolone was changed to oral prednisone (20 mg/day) for the next 4 days. The patient's myocardial injury markers gradually decreased and fell to the normal range (29.4 pg/ml) when she was discharged from our hospital on July 30.

DISCUSSION

Although direct evidence to exclude coronary artery occlusion in this patient is lacking, the CMRI result seemed not consistent with the characteristics of myocardial infarction. Interestingly, hs-cTnI decreased within hours after the first temporary administration with 5 mg dexamethasone but rapidly increased by 7.8-fold in the next 2 days once GC was withdrawn. When the GC treatment became continuous, the myocardial injury markers decreased steadily. These features are more in line with the characteristics of myocarditis. In addition, the facts that the patient did not have fever and respiratory symptoms and her blood routine, inflammatory markers, and serum IgM of several viruses were normal ruled out bacterial or viral infection (**Table 2**). Therefore, we conjectured that this patient suffered from acute autoimmune myocarditis caused by thyrotoxicosis. However, current evidence for the concept of thyrotoxicosis-induced myocarditis seem to be limited. There are only some scattered reports and few studies with small sample

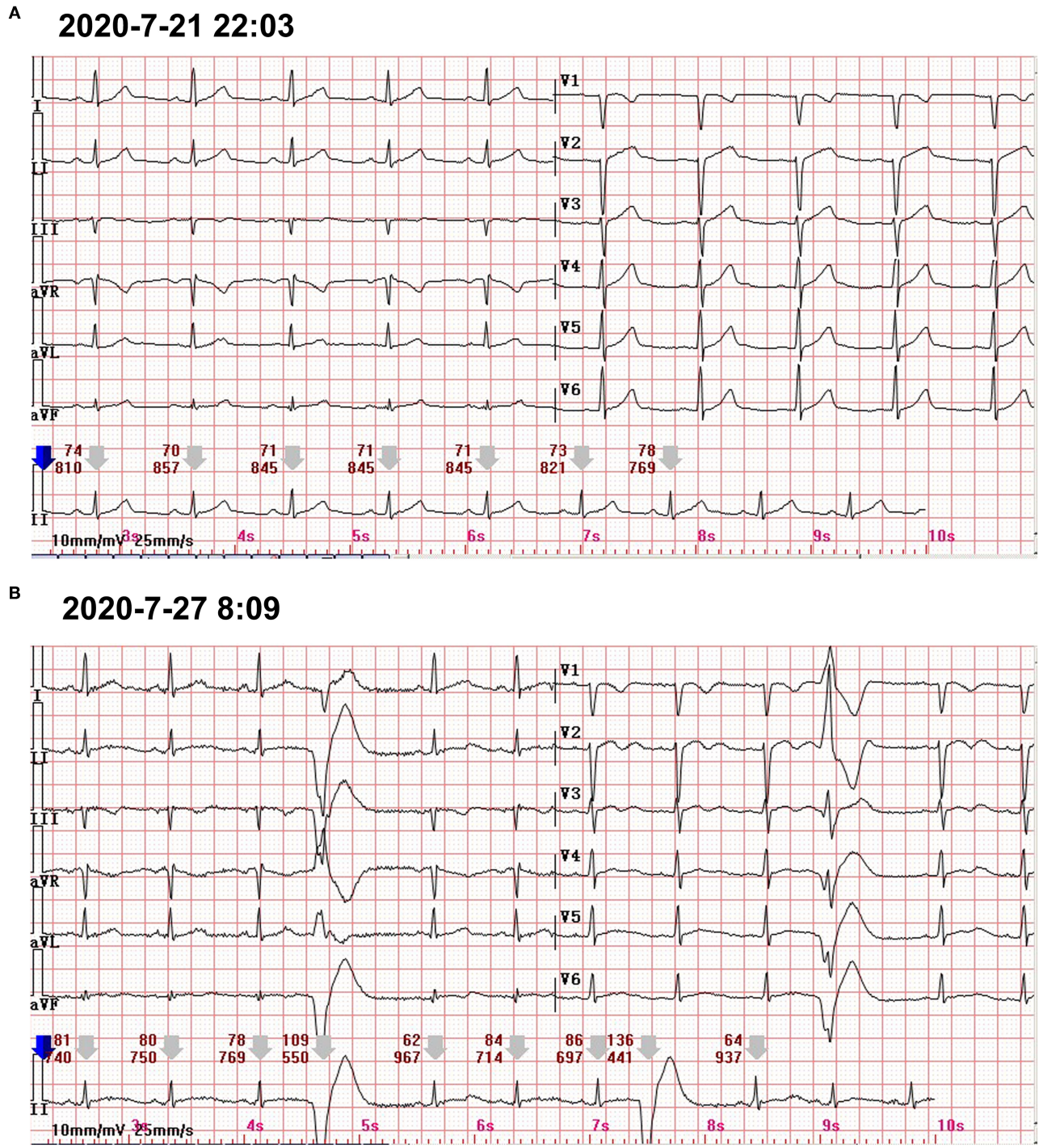

FIGURE 2 | (A) The 12-lead standard ECG only showed the poor progression of R waves in V1–V3. **(B)** A 24-h Holter ECG captured the occasional premature ventricular contractions.

sizes about myocarditis caused by hyperthyroidism (10–12). Inspired by the successful experience of the patient, we believe that it is necessary and meaningful to deeply elaborate this disease and summarize the current related research. Although more clinical studies are urgently need, we try to introduce the epidemiological data, mechanism, diagnostic methods, and treatment strategies for thyrotoxicosis-induced myocarditis in this study.

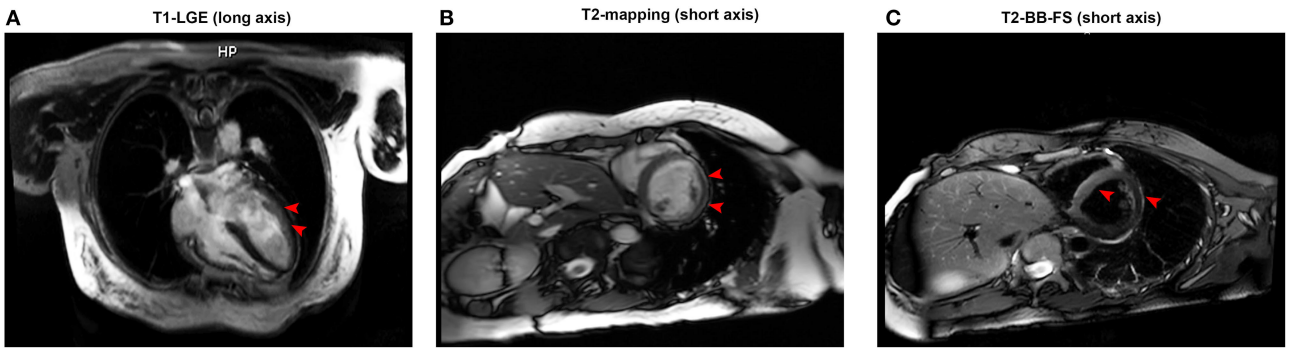

FIGURE 3 | (A) Representative late gadolinium enhancement (red arrow) in the left ventricular anterior wall under T1 imaging (long axis). **(B)** An enhancement signal (red arrow) appeared in the inferior wall and inferolateral wall of the middle segment of the left ventricle under T2 mapping (short axis). **(C)** An enhanced signal (red arrow) appeared in the interventricular septum in T2 black blood fat suppression sequences (short axis).

Epidemiological Characteristics

Hyperthyroid cardiomyopathy is a general term for a series of heart diseases, including arrhythmia, cardiac hypertrophy, heart failure, and valvular diseases, which are precipitated by Graves' disease (GD) (3). Thyrotoxicosis-related myocarditis can be differently defined as the inflammatory response damaging the muscular tissues of heart caused by thyrotoxicosis (13). Thyrotoxic cardiomyopathy has been reported as an initial presentation in 6% of hyperthyroidism patients (14). Compared with arrhythmia, congestive heart failure, and dilated heart disease, thyrotoxicosis-related myocarditis is relatively rare and occasionally reported in few studies (15). Mavrogeni et al. investigated 250 patients with hyperthyroidism, 50 of whom had persistent cardiac symptoms, including chest pain, dyspnea, and palpitations. Fifteen of 50 (30%) had been confirmed as inflammatory edema of the cardiomyocytes by CMRI. In addition, three patients were further confirmed by myocardial (or endomyocardial) biopsy as lymphocyte infiltration, but not any evidence of viral infection was found (10). Although accurate epidemiological information is lacking, we can speculate that the incidence rate of thyrotoxicosis-related myocarditis may be seriously underestimated. On the one hand, many cases of myocarditis are likely underdiagnosed due to subclinical or non-specific symptoms; on the other hand, CMRI or myocardial biopsy, which has an important value in the diagnosis of myocarditis, is not widely used in the clinic due to their limitations. In 2000, WD Edwards and his colleagues analyzed 11 biopsied patients with GD with reduced ejection fraction. The results showed that 18.2% (2/11) of the patients presented with lymphocytic myocarditis, and the other important manifestations were dilated cardiomyopathy (6/11) and arrhythmogenic right ventricular dysplasia (3/11) (12). In addition, a recent study which analyzed 173 patients with myocarditis, as confirmed by cardiac biopsy, found that about 23/173 (13.3%) of them had hyperthyroidism, and the main cause was autoimmune myocarditis (19/23 = 82.6%) (16). These results suggest that thyrotoxicosis-related myocarditis needs be paid more attention.

Underlying Mechanism

Myocarditis is an inflammatory disease of the myocardium and associated with immune dysfunction which may frequently lead to the development of dilated cardiomyopathy (17). Three distinct forms of myocarditis are recognized: idiopathic, autoimmune, and infectious. Some autoimmune and auto-inflammatory diseases, such as sarcoidosis, Behçet's disease, eosinophilic granulomatosis, myositis, and systemic lupus erythematosus, had been well-documented to cause myocarditis in previous researches (18, 19). In recent years, some studies found that Graves' disease, another autoimmune disease, can also develop into inflammatory cardiomyopathy (8, 20). In 1999, Yagoro et al. firstly identified lymphocyte infiltration by endomyocardial biopsy in a patient with autoimmune thyroiditis and confirmed the presence of anti-cardiac antibodies in the plasma or myocardium (21). Later, some studies had confirmed its pathological change as lymphocyte-dominated autoimmune myocarditis (12, 22). Due to the lack of animal models of myocarditis caused by thyrotoxicosis, the mechanism of myocarditis is poorly understood. What is clear, however, is that the crosstalk between cardiomyocyte injury and inflammation dysfunction should be the key steps.

The Pathophysiological Mechanism of Myocardial Damage

The damage of hyperthyroidism on the cardiovascular system had long been well-recognized (23), involving hyperdynamic, hypermetabolism, genomic, and non-genomic molecular regulation. Clinical findings indicate that excessive elevated thyroid hormone increased the heart rate and myocardial contractility and relaxed the peripheral vessels, leading to a significant increase in cardiac output. However, a long period of hyperdynamic state is not good for the heart (3), which was also described as thyrotoxicosis. Firstly, a significant increase of cardiac load can eventually lead to compensatory cardiac hypertrophy and gradually develop into heart failure. Secondly, increased myocardial metabolism and oxygen consumption lead to mitochondrial dysfunction and oxidative stress injury (24). Thirdly, heart relative ischemia aggravates cardiomyocyte injury

for shortening of the coronary artery filling time. Myocardial injury can trigger inflammatory responses that have been demonstrated in several basic studies, such as in transverse aortic constriction mice or myocardial infarction mice (25, 26).

Additionally, thyroid hormone also changes myocardial function by regulating gene expression and ion channel state, which provides a molecular biological perspective for us to understand the myocardial damage of hyperthyroidism (27). Both thyroid hormone (TH) receptors and two T3-binding nuclear receptors, TRa1 and TRb1, are expressed in the cardiac myocyte (2). The latter mediates the binding of TH to TH response elements in the promoter regions of TH-responsive genes. Those genes encoding important structural and regulatory proteins, including myosin heavy chain isoforms α and β, sarcoplasmic reticulum calcium-activated ATPase (SERCA2), phospholamban, the β-adrenergic receptor, adenylyl cyclase V and VI, and various membrane ion channels, regulate the reuptake and release of calcium from the sarcoplasmic reticulum, thereby regulating the systolic and diastolic capacity of the myocardium (3, 28, 29). The non-genomic effects are usually receptor independent and largely occur at the plasma membrane, regulating ion transporter activity, and are responsible, in part, for the ability of T3 to increase the heart rate (30). Although a hyperdynamic and hypermetabolic state induced by hyperthyroidism seems be more associated with chronic heart remodeling and heart failure, inflammatory infiltration, and network have been revealed as both the cause and the outcome of heart damage (31–33). Clinically, thyroid storms can induce acute heart failure and eventually lead to dilated cardiomyopathy, confirming that direct toxicity, and ion channel regulation by thyroxine accelerate myocardial inflammation (34, 35).

The Mechanism of Inflammation Dysfunction

The infiltration of inflammatory cells and the production of a large number of inflammatory factors aggravate the myocyte necrosis (8). Thus, we elaborate here on the underlying mechanism and characteristic of inflammation dysfunction in the heart induced by hyperthyroidism. Endomyocardial biopsy has documented that lymphocytic-dominated myocarditis is the commonest histological subtype of GD-related myocarditis, characterized by a dominant component of T lymphocytes and a variable number of macrophages (12, 22, 36). The lymphocytes are divided into several functional subtypes based on their surface markers: T lymphocyte [including CD4+ T effector cells, T helper cells (Th), regulatory T cells (Tregs), CD8+ T cell, B lymphocyte, and NK cells] (37). Infiltrated lymphocyte subpopulations contribute to the progression of myocarditis and subsequent cardiac remodeling. Using CD4 and CD8 knockout mice improves the prognosis and confirms their vital role in virus myocarditis (37). Furthermore, the transfer of cTnI-specific CD4+ effector T cells to healthy recipients causes severe inflammation, fibrosis, and cardiac dysfunction in animal models, suggesting an exclusive role for CD4+ T cells in myocarditis development and progression (38, 39). Th cells including Th1, Th2, and Th17 and FOXP3+ Treg cell may also contribute to this pathological process *via* complex regulatory networks (40). Song et al. found that a high frequency of Th2 cells and an increase of Th2 cytokines were characteristics of myocarditis patient hearts during the end stages of heart failure (41). The alteration of thyroxine influences the recruitment and activity of T lymphocytes (42). The enhanced expression of ICAM-1, VCAM-1, and the tissue inhibitors of metalloproteinases has been observed in GD patients, which promotes the infiltration of lymphocytes in the injured organs, including the heart (43). A hyperthyroid state leads to an increased activation of lymphocytes mediated by various factors including NF-κB, protein kinase C signaling pathways, and β-adrenergic receptor (44). However, the distribution and functional characteristics of these lymphocyte subsets in the occurrence and development of hyperthyroidism myocarditis are still not very clear and deserve further exploration in the future.

Additionally, attack by autoantibodies against the cardiomyocytes has been demonstrated to be the spark for the inflammatory disorders in autoimmune myocarditis (45). The discovery of heart reactive antibodies in the plasma or myocardium in autoimmune thyroiditis demonstrates that autoimmune-mediated tissue destruction in GD possibly contributes to autoimmune myocarditis (21). We have to ask how autoimmune antibodies against self-myosin are produced. In fact, cardiac myosin is well-concealed within the intracellular compartment. Its antigens, such as myosin heavy chain α (α-MHC), are expressed on thymic medullary epithelial cells as part of T cell selection under ischemia or toxic stimulation, which, in turn, launches an autoimmune sensitization (46, 47). A correlation between anti-myosin autoantibodies and deterioration of systolic and diastolic left ventricular function had been documented in patients with chronic myocarditis (48). Thus, more confirmed myocardial autoantibodies and their pathogenic mechanisms are worthy of identification in patients with GD.

Additionally, innate immune cells (macrophages, killer cells, dendritic cells, etc.) are also present in varying degrees in autoimmune myocarditis. Among them, monocyte–macrophage lineages are predominant in human and experimental myocarditis (49). Cardiac injury upon myocarditis results in an early recruitment of $Ly6C^{hi}$ inflammatory monocytes from the circulation (50). Monocytes stimulated by Th1 cells tend to differentiate into pro-inflammatory M1 macrophages (51). Th2-associated cytokines, mainly IL-4 and−13, activate the anti-inflammatory M2 macrophage phenotype, which blunts the inflammatory response and promotes cardiac fibrotic healing (52, 53). Growing evidences have documented that excessive thyroxine enhances the proliferation and pro-inflammatory function of multiple cells in the innate immune system (44).

Conclusion

In conclusion, direct myocyte damage through hormonal effects or ion channel regulation and uncontrolled autoimmune response may be two coexisting mechanisms in the development of GD-induced myocarditis (**Figure 4**). However, their contributions vary at different stages of pathological development. In the acute phases, myocyte damage/necrosis

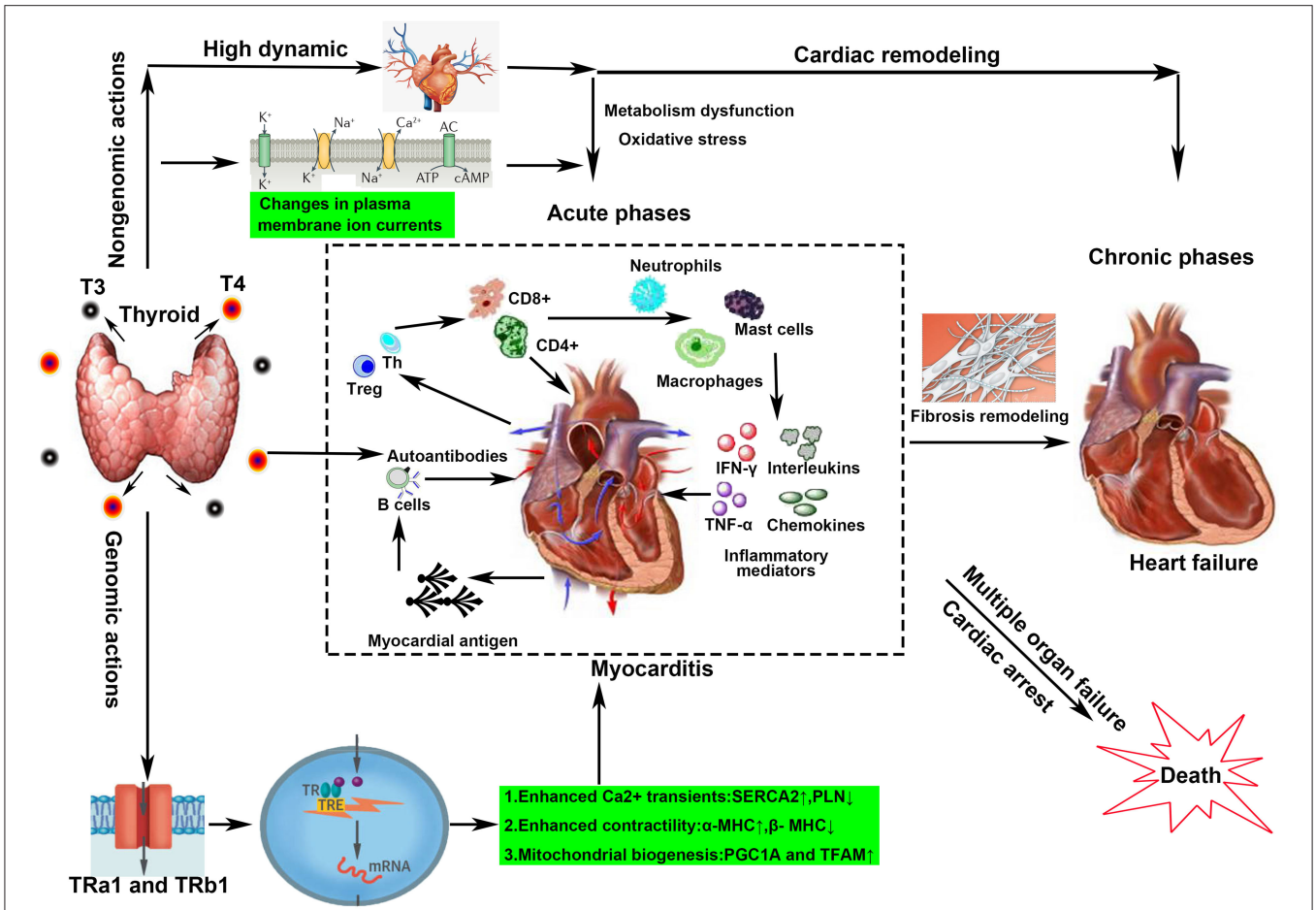

FIGURE 4 | Overview of the possible mechanisms of hyperthyroidism-induced myocarditis. Both genomic and non-genomic actions contribute to the development of Graves' disease-induced myocarditis. Genomic actions mainly refer to binding to TH response elements in the promoter regions of TH-responsive genes. Non-genomic actions include high dynamic damage, ion channel alteration, and auto-attack from autoantibodies. All lead to a complex cascade of inflammation and sustained destruction of the heart muscle in the acute phases. In the chronic stage, fibrous repair, and structural remodeling are the main changes.

is pronounced and promotes the production of inflammatory cascades and storms. In healing stages, myocyte damage becomes localized, and the release of inflammatory cytokines calms down (54). Lymphocytes give way to macrophages, and mesenchymal reparative tissue appears and is gradually substituted by replacement fibrosis (**Figure 4**).

Animal Model

The establishment of animal models is of great significance for better understanding and elucidating the mechanisms behind Graves' disease-associated myocarditis. TH had been directly injected into different mice strains to investigate its association with cardiac structure and function. Short-term TH treatment (10 days) appeared to affect the heart rate primarily, with no change in heart size or function. However, moderate-length (2 months) and longstanding TH stimulation (10 months) in F1b hamsters resulted in significant cardiac hypertrophy and deleterious cardiac remodeling characterized by myocyte lengthening, chamber dilatation, decreased relative wall thickness, increased wall stress, and increased left ventricular (LV) interstitial fibrotic deposition (55, 56). However, whether myocarditis is involved in these processes has not been revealed in the above-mentioned study. In a hyperthyroid Wistar rat model induced by intraperitoneal injection with exogenous thyroxine for 4 weeks, cardiac lipid peroxides and serum endothelin-1 were increased, whereas cardiac superoxide dismutase, catalase, glutathione, and matrix metalloproteinase-2 were reduced in the hyperthyroid group. Unfortunately, myocardial inflammation (MPO level) and fibrosis (Masson trichrome staining) did not change significantly compared with the controls (57). Interestingly, hypothyroidism rats seem to be related with significant sterile inflammatory changes (leukocyte infiltration) in cardiac tissue (58). As mentioned above, the development of GD-associated myocarditis is associated not only with hyperthyroidism but also with the production of autoantibodies. A mouse model for GD was successfully established by three immunizations with recombinant adenovirus expressing the human TSHR, giving

TABLE 3 | Overview of various autoimmune myocarditis models.

Virus or peptide	Mouse strain	Brief methods (dose/times/injection)	Days	References
CVB3	A.CA/SnJ, A.SW/SnJ	0.1 ml of 10^5 TCD_{50}/once/intraperitoneally	15–21	(61)
EMCV	BALB/c	0.1 ml of 100 TCD_{50}/once/intraperitoneally	14	(62)
Myosin	A/J, A.SW/SnJ, A.CA/SnJ	100 μg/twice*/subcutaneously	21	(63, 64)
α-Myosin	BALB/c	100 μg/twice/subcutaneously	21	(65)
Myosin fragment (1–1,032)	BALB/c	100 μg/twice/subcutaneously	56	(66)
Myosin fragment (1,074–1,646)	A/J	250 μg/once/subcutaneously	21	(67)
Myosin (334–352)	A/J	100 μg/twice/subcutaneously	21	(68)
Myosin (614–629)	BALB/CJ	100 μg/twice/subcutaneously	25–30	(69)
Myosin (614–643) Myosin (735–747) Myosin (947–960)	BALB/c	100 μg/twice/subcutaneously	21	(64)
Cardiac troponin I (cTnI)	A/J BALB/c	120 μg/twice/subcutaneously	21	(70)
cTnI (105–122) cTnI (131–148)	A/J	120 μg/twice/subcutaneously	28	(38)

EMCV, M variant of encephalomyocarditis virus; TCD_{50}, 50% tissue culture infective dose; twice, a week between the two injections.*

us hope to study the development of this myocarditis (59, 60). In a word, we have to admit that no animal model has been definitively considered suitable for the study of hyperthyroidism-associated myocarditis so far.

However, several murine models have been developed to simulate the development of autoimmune myocarditis (**Table 3**), which may help us to deeply track the mechanics of GD-associated myocarditis. Coxsackievirus B3 (CVB3), a non-enveloped single-strand positive-sense RNA virus, is often used to investigate viral myocarditis (71). Interestingly, heart-specific autoantibodies, especially anti-myosin IgG autoantibodies, are discovered in CVB3-infected mice (72, 73) and in patients with post-CVB3 myocarditis (74). Therefore, CVB3-infected mice were also used as a virus-induced autoimmune myocarditis model. Myosin and myosin peptides are also used to induce an autoimmune myocarditis model (**Table 3**). In 1987, Neu et al. described the immunization of mice with myosin-inducing myocarditis paralleled by high titers of myosin autoantibodies (63). Segments/smaller peptides from myosin also possess antigenicity and induce myocarditis. So far, at least seven different segments have been identified to induce autoimmune myocarditis (**Table 3**). Additionally, Göser et al. reported that immunization of mice with cardiac troponin I (cTnI) induced severe cardiac inflammation, fibrosis, and impaired LVEF (70).

Diagnosis and Antidiastole

The proper diagnosis of hyperthyroidism-associated myocarditis is challenging due to a heterogeneous clinical presentation that ranges from no symptom, left ventricle systolic dysfunction, to mimicking the symptoms of acute myocardial infarction (AMI). Additionally, takotsubo myocardiopathy (TM), a rare transient abnormality of heart function with clinical manifestations like AMI but without a coronary artery disease, has been reported to be linked with hyperthyroidism, which also increases the difficulty of differential diagnosis (75, 76). Thus, AMI, takotsubo myocardiopathy, and myocarditis should be taken into consideration during the making of a differential diagnosis in this kind of patients.

Hyperthyroidism is prone to acute ischemic heart disease (77, 78). The proposed mechanisms responsible for coronary events in hyperthyroidism include a hypercoagulable and hypofibrinolytic state as well as hyperthyroidism-associated coronary vasospasm and increased myocardial oxygen demand (79, 80). Although many hyperthyroidism patients with typical chest pain, elevated levels of myocardial injury, and abnormal electrocardiograms have been diagnosed with myocardial infarction (MI) in the past, most of these patients are then shown to be free of coronary disorder as documented by both angiographic and postmortem studies (81–84). Thus, an important question haunts us as to whether these patients really suffered from myocardial infarction.

In order to comprehensively and carefully analyze the characteristics and incidence of patients in these reports, we reviewed and summarized the previous literatures in **Supplementary Table 1**. According to Takeshi's study, publications in English written before 1986 contained reports of 28 cases of concurrence of thyrotoxicosis and myocardial infarction (85). Seven of the patients showed normal coronary arteries, and the rest had been confirmed to have varying degrees of coronary disorders. In fact, only one patient was suspected of hyperthyroidism with myocarditis (86). She was a 22-year-old Navajo woman who experienced fatigue and dizziness after admission to a hospital. The ECG showed a progressive atrioventricular block,

premature ventricular beat, and, eventually, cardiac arrest. Although she was suspected of hyperthyroidism-associated myocarditis, cardiac pathology was not performed. After 1986, we retrieved 43 cases of hyperthyroidism complicated with myocardial infarction in PubMed (**Supplementary Table 1**). Most of them (39/41) presented with active chest pain, palpitation, and other discomforts. Elevated markers of myocardial injury in circulation and myocardial infarction like ECG features, such as ST-segment elevation, depression, and pathological Q wave, linked them with MI. However, coronary angiography revealed that 23 patients had completely normal coronary arteries, seven patients presented with a reversible coronary artery spasm, and one patient had a myocardial bridge in the left anterior descending artery. One 25-year-old patient was suspected to be related to myocardial infarction because of old necrosis of the left ventricle and fresh ischemic necrosis of the ventricular septum after an autopsy. The other patients had different degrees of coronary atherosclerotic lesions, while only four patients had true occlusive atherosclerotic lesions. Obviously, most patients with normal coronary artery and myocardial injury are difficult to be explained by coronary ischemia. Coronary angiography is very important for ruling out AMI or coronary spasm. Unfortunately, our patient verbally rejected the recommendation for coronary angiography.

TM, also named as stress cardiomyopathy, transient left ventricular ballooning, or broken heart syndrome usually occurs following an emotional or physical stress. TM is more common in postmenopausal women with an average age of 66.8 years, accounting for as much as 89.8% of cases (87). Our patient is a young woman without any misfortune before being admitted to our hospital. Clinically, chest pain and dyspnea are the most common symptoms of TM. Moreover, the apical motility disorder shown in the echocardiogram and a ventriculogram with normal coronary arteries are the most common characteristics of TM. Moreover, transient akinesis, hypokinesis, or dyskinesis of ventricular myocardial segments in the echocardiogram, usually accompanied by a decrease of EF and an increase of BNP/NT-proBNP, is one of the major diagnostic criteria (88–90). However, the results of repeated echocardiographic examinations in July 21 and 23 are normal in our patient, and BNP is also in normal range. CMRI may be a useful tool to differentiate between TM and AMI but not suitable for TM and myocarditis. In TM, the MRI may show an isolated mid-wall or subepicardial pattern of LGE, which is similar to the MRI features of myocarditis but different to either subendocardial or transmural LGE observed in AMI. However, TM is an exclusive diagnosis, and the exclusion of ischemia, myocarditis, toxic damage, and tachycardia is one of the necessary criteria (88–90). In conclusion, our patient does not meet the diagnostic criteria for stress cardiomyopathy.

Myocarditis can also be manifested as a significant increase in myocardial injury markers and ischemia-like ECG changes (91). We only found five patients diagnosed as hyperthyroidism with myocarditis (**Supplementary Table 1**). One of them was diagnosed as acute myopericarditis. Two of these patients were diagnosed as having an extensive interstitial inflammation and necrosis of the myocardium at autopsy. The remaining two patients were diagnosed as having myocardial edema and delayed gadolinium enhancement by CMRI. Obviously, acute myocarditis complicating hyperthyroidism or thyrotoxicosis is underestimated. The main reason is that myocardial biopsy and CMRI are used less frequently in these patients with myocardial injury. Our patient is relatively young, without coronary heart disease risk factors and genetic predisposition. CMRI also indicates delayed gadolinium enhancement in T1 imaging and striped enhancement in T2 mapping. Thus, it is more likely to consider myocarditis with hyperthyroidism.

Cardiac Biopsy or Cardiac Magnetic Resonance Imaging?

Myocardial biopsy is known to be the gold standard for the diagnosis of myocarditis, but it is rarely used in the clinic because of its invasive, serious complications, and false-negative results (92). CMRI can accurately display the pathological characteristics of acute myocarditis (AM) and has been widely used in the clinic (93, 94). Additionally, the non-radiation, non-invasive, high-resolution features, coupled with the emergence of various new technologies, make CMRI diagnosis more convenient and accurate (95). In 2009, the CMRI diagnostic criteria for AM, namely, LLC, was published (96, 97), which mainly targets the three core pathological features of AM: edema, congestion, and necrosis or fibrosis. The criteria include T2-weighted imaging, early gadolinium enhancement, and myocardial LGE. If more than two items are positive, AM can be diagnosed. The upgraded LLC in 2018 requires the following two requirements at the same time to diagnose AM: at least one sequence (T2-weighted imaging or T2 mapping) sensitive to edema and at least one T1 sequence (T1 mapping, extracellular volume, and myocardial delayed enhancement imaging) sensitive to necrosis (98, 99).

The differences between AM and MI on CMR are mainly as follows (100): (1) the T2-weighted images of AM show diffuse myocardial tissue edema that fails to match the distribution of coronary arteries, and it usually occurs in the epicardium or middle myocardium; MI is characterized by edema of the myocardial tissue in the area corresponding to the distribution of coronary arteries and is usually subendocardial or transmural; and (2) there is no abnormality in the resting myocardial perfusion of AM. The imaging of delayed myocardial enhancement shows multiple and scattered delayed enhancement of the epicardium or the middle myocardium, which is not consistent with the distribution of the coronary artery. It could exist alone or simultaneously in the interventricular septum and the anterior wall of the left ventricle, and the degree of enhancement gradually decreases with time. MI is characterized by subendocardial myocardial perfusion defect and delayed myocardial enhancement, consistent with the distribution of the coronary arteries, and the delayed enhancement usually does not go away.

However, CMRI also has its inevitable limitations. First of all, image quality can be limited by trigger problems (e.g., rhythm disturbances) and other artifacts (e.g., breath-holding and motion artifacts). In addition, the diagnostic

sensitivity of borderline myocarditis is much lower than that of biopsy-proven active myocarditis, which may lead to omissions (44 vs. 84%) (101). They even recommended that only patients with myocardial necrosis of more than 2 g [corresponding CK levels: median, 229; range, 146–709 (U/L)] were eligible for CMRI (102). However, the correct detection rate of myocarditis was similar between the endomyocardial biopsy (EMB) (72/82, 88%) and CMRI (66/82, 80%; P ¼ 0.31) groups for all patients who were troponin-positive but without a coronary artery disease (103). Some researchers believe that the combination of CMR and EMB is probably the best option in improving diagnostic sensitivity because the combined use is more accurate than any of them alone (103, 104).

Treatment

The clinical manifestations of myocarditis in patients are broad, ranging from the asymptomatic to minimal exertional dyspnea or palpitation, acute left heart failure, cardiogenic shock, and even sudden death (105). Therefore, the management of this condition must be personalized according to the severity in each case. The conventional treatment for hemodynamically stable patients emphasizes standard anti-heart failure regimens, including positive inotropic drugs, vasoactive drugs, beta-blockade, diuretics, ACEi/ARBs, and also aldosterone antagonists. It is also necessary to prevent and quickly respond to malignant arrhythmias such as malignant tachycardia and high atrioventricular block (106). Here we focus on the following three aspects on the treatment of hyperthyroidism-induced myocarditis: (1) restraint of thyrotoxicosis, (2) active response to cardiac damage, especially acute circulatory failure in fulminant myocarditis, and (3) immunosuppression or immunomodulatory therapy for excessive inflammatory storms.

Treatment of Thyrotoxicosis

Rapid blocking of thyrotoxicosis is the critical beginning to suppress the progress of hyperthyroidism-related myocarditis. Several mature drugs, including thioamides, iodine, β-blockers, and corticosteroids, are widely used to inhibit the synthesis and release of thyroid hormone, inhibit the peripheral effects of thyroid hormone, and increase thyroid hormone clearance (35). They can also alleviate heart injury and promote cardiac function by suppressing the thyrotoxic state and correcting the hemodynamic disturbances (75, 107). In one study, seven thyrotoxic patients with congestive heart failure showed an increase of mean LVEF from 28 to 55% after treatment for thyrotoxicosis (108). However, whether anti-thyroid drugs alone can be successful in the treatment of hyperthyroidism-induced myocarditis still needs more research.

Acute Supportive Treatment of Fulminant Myocarditis

Fulminant myocarditis (FM) is the most serious type of myocarditis, which is characterized by a rapid progressive decline in cardiac function and a high mortality rate. FM usually responds poorly to conventional vasoactive drug therapies as well as to standard heart failure, refractory heart failure, and cardiogenic shock treatments but relies on mechanical circulation support (MCS). The application of MCS devices, including intra-aortic balloon pump (IABP), peripheral venous–arterial extracorporeal membrane oxygenation (ECMO) or Impella, has resulted in better efficacy in FM patients from being <20 to 40–70% (106). Recently, the "life support-based comprehensive treatment regimen" proposed by our center has been verified to further reduce FM mortality from ∼50 to <5% and shorten the hospitalization period to <2 weeks (106). The core idea of this treatment regimen is to strive for more recovery time for the exhausted heart by reducing or temporarily replacing its pumping function by using MCS devices. IABP is the most commercially available MCS device, can lower LV afterload, and can increase the tissue perfusion of important organs. However, due to the limited size of the balloon and less power of the pump, IABP can only provide about 15% of extra circulation support compared with the total circulation demand (109). ECMO is another useful MCS device which provides a more powerful circulation support or better oxygenation to venous blood and meets the basic demand of the body circulation. Impella is a small pump sent into the LV to drain blood and decrease the load. Although several reports had reported the benefit of Impella in treating cardiogenic shock, recent studies revealed that Impella might be associated with a higher risk of major bleeding and in-hospital mortality, accompanied by a decreased cost-effectiveness value (110, 111). Taken together, mechanical circulation support, especially IABP and ECMO, should be initiated if FM is the primary manifestation of hyperthyroidism-induced myocarditis.

Immunomodulatory Therapy

With the consensus of the importance of inflammatory mechanisms or inflammatory storms in myocarditis (112), the prospect of immunosuppressive agents in the treatment of myocarditis has aroused the researchers' enthusiasm. Our clinical practice in virus-induced fulminant myocarditis suggested that "immunomodulatory therapy" may be more appropriate instead of "immunosuppressive therapy," highlighting caution in the use of immunosuppressive agents such as azathioprine, cyclosporine, etc., but position on GC and immunoglobulin. In this study, we focus on the benefits of GC in myocarditis. For viral myocarditis, some people may worry that GC leads to viral replication at the first phase and aggravate the disease. This concern, though, seems to have been unfounded, as many large studies have shown that GC benefits from viral myocarditis with or without other immunosuppressants (113–115). Differently, the benefit of GC for autoimmune myocarditis is considered definite since it not only controls the severity of autoimmune diseases but also protects the heart through a pluripotent mechanism (65, 116, 117). A case–control study of 811 patients showed that early glucocorticoid treatment significantly reduced the in-hospital mortality of patients with thyroid storm (118). Additionally, we found that the early administration of dexamethasone can significantly reduce myocardial necrosis and inflammatory cell infiltration in α-MHC-induced autoimmune myocarditis animal model (**Figure 5**).

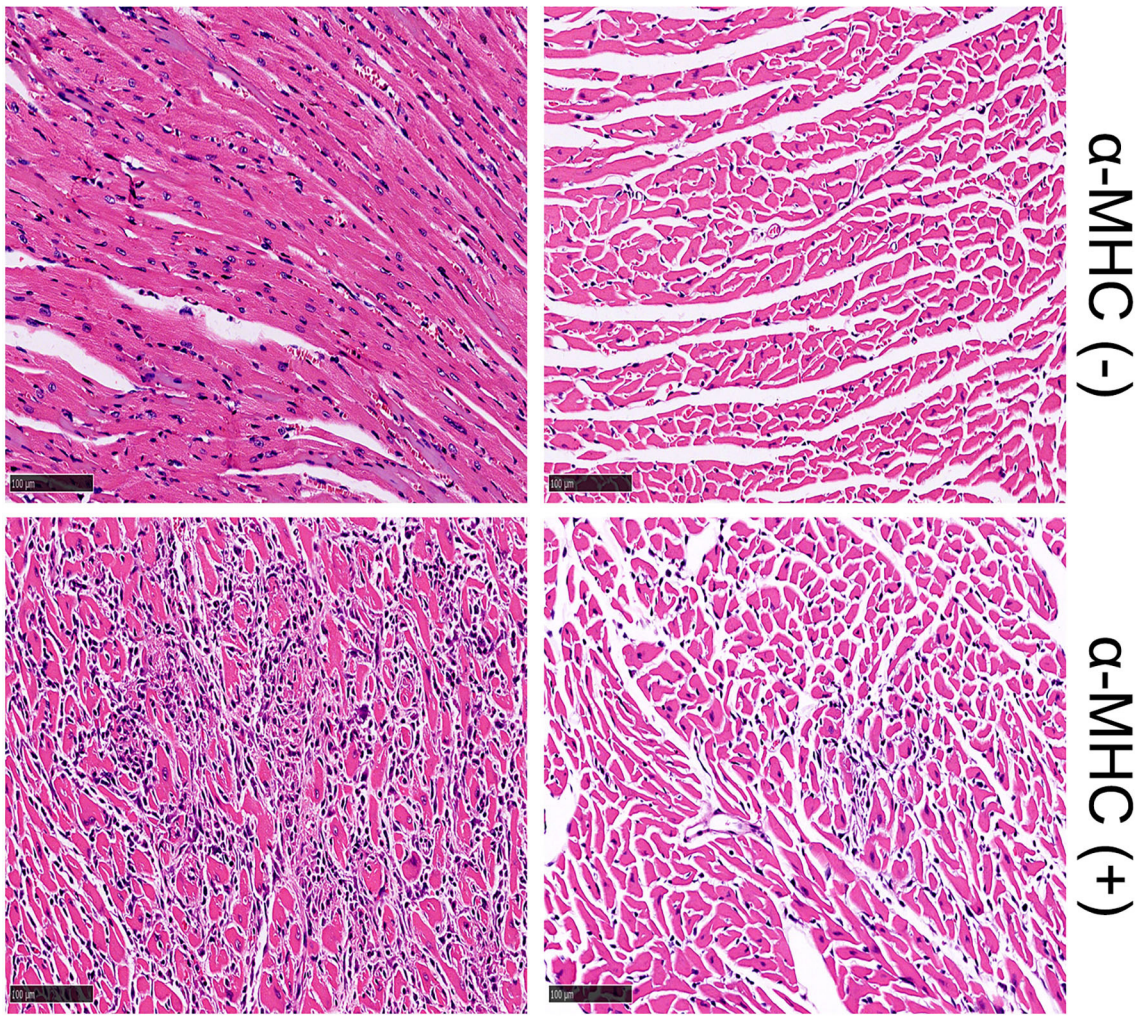

FIGURE 5 | Representative heart sections of dexamethasone-treated autoimmune myocarditis induced by α-MHC immunization (α-MHC+). Establishment and treatment methods: in 0 and 7 days, respectively, 300 μg α-MHC (Ac-RSLKLMATLFSTYASADR-OH) was subcutaneously injected into BALB/C mice. Then, 0.75 mg/kg/day dexamethasone was given for intervention on the 16th to 20th day *via* intraperitoneal injection. The mice were sacrificed and their hearts were fixed with formalin for H&E staining on day 21.

GC seems to be associated with cardio-protection and a range of possible favorable effects on inflammation regulation, heart metabolism, function, and survival (119). Firstly, GC translocates into the nucleus *via* a cytosolic glucorticoid receptor, binding to specific DNA binding sites (glucocorticoid-responsive elements), which promotes the production of anti-inflammatory and regulator proteins and inhibits the transcription of pro-inflammatory genes (120). Suppressing inflammation-related nuclear transcription factors such as activator protein 1, nuclear factor kB, interferon regulator factor 3, JAK, and STAT (121, 122) controls the expression of many inflammatory factors and adhesion molecules, such as interleukin (IL)-1, IL-2, IL-3, IL-6, IL-8, and IL-12, tumor necrosis factor-alpha, interferon-γ, granulocyte–macrophage colony-stimulating factor, MCP-1, VCAM-1, and ICAM-1 (123). Additionally, GC inhibits the activity of phospholipase A2 and cyclooxygenase, decreasing the production of inflammatory mediators, such as prostaglandins, leukotrienes, prostaglandin endoperoxides, and thromboxane (124). GC also causes programmed cell death in monocytes, macrophages, and T-lymphocytes via the upregulation of CD95 expression (125) but protects the myocardium from apoptosis by blocking pro-apoptotic signals (126). Moreover, GC's direct and rapid effects on cellular membranes (plasma and mitochondrial) result in the impairment of inflammatory and immune cell functions, such as phagocytosis, migration, antigen processing, and presentation via a non-genomic model (120, 127). On the other hand, GC also plays an important role on the control of thyrotoxicosis and regulation of T lymphocytes in GD. GC seems to decrease T4 secretion from the thyroid, although the efficacy and duration of this effect are unknown (128), and inhibit the

peripheral conversion of T4 to T3 (129). Dexamethasone could effectively improve the function of Treg cells and sets up a new balance of T-helper1/T-helper2 in Graves' disease patients (130).

On the whole, except for direct relief of thyrotoxicosis, the pharmacological effects of corticosteroids include inhibition of immunological reactions, prevention of myocardial apoptosis, and reduction of cardiotoxicity by thyrotoxicosis and inflammatory cytokines. Our patient also proved that the use of GC can significantly reduce myocardial cell necrosis caused by hyperthyroidism.

AUTHOR CONTRIBUTIONS

LW collected and analyzed the data and wrote this manuscript. QL, WW, and NT helped to collect the data and treat this patient. DW designed the experiments and guided this study. NZ, YW, and DW checked this manuscript. All the authors have read and approved the 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.2021.678645/full#supplementary-material

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MINI REVIEW
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Unraveling the Immunopathogenesis and Genetic Variants in Vasculitis Toward Development of Personalized Medicine

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Leukocytoclastic vasculitis (LCV) is a systemic autoimmune disease characterized by the inflammation of the vascular endothelium. Cutaneous small vessel vasculitis (CSVV) and anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) are two examples of LCV. Advancements in genomic technologies have identified risk haplotypes, genetic variants, susceptibility loci and pathways that are associated with vasculitis immunopathogenesis. The discovery of these genetic factors and their corresponding cellular signaling aberrations have enabled the development and use of novel therapeutic strategies for vasculitis. Personalized medicine aims to provide targeted therapies to individuals who show poor response to conventional interventions. For example, monoclonal antibody therapies have shown remarkable efficacy in achieving disease remission. Here, we discuss pathways involved in disease pathogenesis and the underlying genetic associations in different populations worldwide. Understanding the immunopathogenic pathways in vasculitis and identifying associated genetic variations will facilitate the development of novel and targeted personalized therapies for patients.

Keywords: vasculitis, autoimmune disorder, immunopathogenesis, susceptibility loci, personalized medicine

INTRODUCTION: VASCULITIS EPIDEMIOLOGY AND CLASSIFICATION

Vasculitides are a group of multi-system diseases characterized by inflammation of blood vessels, endothelial injury and tissue damage (1). Referring to the Chapel Hill Consensus Conference (CHCC) nomenclature system, vasculitides are classified according to the size of the affected vessels, lesion histopathology and other clinical findings (2). Leukocytoclastic vasculitis (LCV) refers to

a type of small vessel vasculitis, where it can be characterized based on several histopathological findings including presence of neutrophil infiltrates, leukocytoclasis (fragmented nuclear debris from neutrophils), fibrinoid necrosis, and damaged vessel walls at the affected vessels (3, 4). In this review, we focus on two forms of LCV, namely the cutaneous small vessel vasculitis (CSVV) which describes small vessel vasculitis that is usually confined to the skin, and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), which is usually a severe and systemic condition. Currently, there is a lack of consensus on whether AAV should be classified as a form of CSVV or be treated as a distinct form of vasculitis; in this review, however, we discuss these two forms separately. Both CSVV and AAV are of interest because their incidences have been steadily increasing over the years, most likely due to greater awareness in clinicians and having more definitive diagnostic criteria for each condition. Insights from this review can bridge gaps in knowledge for the development of personalized medicine to treat these two types of vasculitis in patients who do not respond to conventional therapeutic strategies.

CSVV is the most common type of vasculitis in dermatology, mainly affecting the post-capillary venules of the skin (5). The incidence of CSVV ranges between 15 and 38 per million/year, with a prevalence between 2.7 and 29.7 cases per million people (6). In the United States, a population-based study determined an incidence of 4.5 per 100,000 person-years in biopsy-proven cases of LCV (7). The trigger for CSVV may either be idiopathic or due to defined causes such as medications, infections and underlying rheumatologic diseases (8). These vasculitides often involve superficial dermal vessels and manifest as purpuric macules, petechiae or hemorrhagic vesicles and urticarial lesions mainly on the lower legs (5, 8). The cutaneous manifestations are sometimes associated with burning sensations, itchiness or pain (9). However, there is a lack of evidence to say that CSVV impairs the mobility and mobility of affected individuals. CSVV can be diagnosed using skin biopsy, based on the presence of pathological features of LCV when evaluated histologically. However, these features of LCV are found in different subtypes of CSVV such as cryoglobulinemic vasculitis, IgA vasculitis (Henoch-Schonlein purpura, HSP) and hypocomplementemic urticarial vasculitis (anti-C1q vasculitis, HUV), as well as in other forms of vasculitis (6). Hence, apart from using histological findings, specific diagnosis must be accompanied by the evaluation of clinical features by clinicians. While cutaneous signs of CSVV are sometimes accompanied by systemic symptoms such as fever, joint and muscle aches, systemic progression and multi-organ inflammation is not seen and if present, often requiring differential diagnosis for other systemic vasculitides, such as AAV (5).

AAV is characterized by microvascular endothelial inflammation, leading to extravascular inflammation, progressive injury, tissue destruction, fibrosis and loss of function in affected tissue (1). AAV is classified as a rare disease, with an estimated historical prevalence of 48–184 cases per million people (1). However, in the past 30 years, the incidence and prevalence of AAV have increased, with increased peak age of onset and geographical variations in female-to-male incidence ratios (10). More recently, the global prevalence rate has been reported to be 300–421 cases per million persons (1). Increased number of AAV cases may be attributed to factors such as having better classification criteria and definitions, greater awareness amongst clinicians, improved patient survival and prognosis, and wider availability of serological assays for diagnosis (1, 10). AAV is diagnosed by the presence of ANCA targeting perinuclear myeloperoxidase (p-ANCA) and cytoplasmic protease-3 (c-ANCA) (11, 12). Several subtypes of AAV have been identified, including microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), and eosinophilic GPA (EGPA, formerly Churg-Strauss syndrome) (2, 11, 13).

Abbreviations: AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; ARPC1, actin-related protein complex-1; BAFF, B cell activating factor; CCL17, CC chemokine ligand 17; CD, cluster of differentiation; COVID-19, coronavirus disease 2019; CSVV, cutaneous small vessel vasculitis; CTLA-4, cytotoxic T lymphocyte antigen 4; DMARD, disease-modifying anti-rheumatic drug; EGPA, eosinophilic granulomatosis with polyangiitis; FcγR, receptor for Fc fragment of IgG; FOXP3, forkhead box P3; GC, glucocorticoid; GPA, granulomatosis with polyangiitis; GWAS, genome-wide association studies; HLA, human leukocyte antigen; HSP, Henoch-Schonlein purpura; GUV, hypocomplementemic urticarial vasculitis; GBM, glomerular basement membrane; IC, immune complex; ICAM-1, intercellular adhesion molecule-1; IFNγ, interferon gamma; Ig, immunoglobulin; IL, interleukin; JAK, janus kinase; LCV, leukocytoclastic vasculitis; Lyp, lymphoid tyrosine phosphatase; MCP, monocyte chemoattractant protein; MHC, major histocompatibility complex; MPA, microscopic polyangiitis; MPO, myeloperoxidase; MS, multiple sclerosis; MyD88, myeloid differentiation primary response 88; NE, neutrophil elastase; NET, neutrophil extracellular trap; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; PEXIVAS, plasma exchange and glucocorticoids for treatment of ANCA-associated vasculitis; PM, personalized medicine; PR3, proteinase-3; RANTES, regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; scRNA-seq, single cell RNA sequencing; SLE, systemic lupus erythematosus; TCR, T cell receptor; TF, tissue factor; Th, T helper cell; TNFα, tumor necrosis factor alpha; VCAM-1 vascular cell adhesion molecule-1, α1AT, α1-antitrypsin.

VASCULITIS: IMMUNOPATHOGENESIS AND ALTERATIONS IN CELLULAR SIGNALING PATHWAYS

Cutaneous Small-Vessel Vasculitis (CSVV)

The major pathogenetic mechanism in CSVV is the Gell and Coombs type III immune complex-mediated reaction (5) (**Figure 1A**). The latent period between the trigger and manifestation of CSVV can range from seven days to more than 2 weeks, depending on the time required to produce sufficient quantities of antibodies and antigen-antibody complexes upon encountering a stimulus (14). Immune complexes (ICs) circulating in the patients can activate the complement system, generating C3a and C5a anaphylatoxins, which initiate neutrophil chemotaxis and release of vasoactive amines causing endothelial cell retraction (5). Pro-inflammatory cytokines and chemokines, such as IL-1, TNF-α, IFN-γ, IL-8, MCP-1, and RANTES produced by macrophages increase the expression of endothelial selectins, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which promotes neutrophil diapedesis. After the neutrophils exit

the blood vessel, neutrophil degranulation releases collagenases and elastases, and generates reactive oxygen species (ROS), resulting in sustained inflammation and fibrinoid necrosis of neighboring vessel walls (5). ICs can also deposit directly on endothelial cells and trigger localized inflammation, such as the deposition of IgA-ICs in glomerular capillaries as seen in cases of Henoch-Schonlein purpura with glomerulonephritis (5). As the disease progresses, lymphocytes gradually become more abundant over time and may also be involved in the pathogenesis and disease progression, eventually becoming the most abundant cell type in lesion histopathology (4). $CD4^+$ helper T cells secrete cytokines, notably IL-1, IFN-γ, and TNF-α, and recruit $CD8^+$ cytotoxic T cells, B cells and natural killer cells to the affected site, further promoting inflammation (5). Unlike in systemic vasculitides such as GPA, CSVV is not associated with abnormal circulating Treg counts, indicating that CSVV usually does not have systemic involvement (4).

Previous studies have identified various stimuli that can trigger the production of pathogenic antibodies in CSVV patients, which subsequently lead to immunopathology. Hepatitis C virus infection can induce the production of cryoglobulins (immunoglobulins that precipitate at temperature below 37°C), leading to cryoglobulinemic vasculitis where cryoglobulin immune complexes precipitate and deposit on affected small vessels. This subsequently activates the classical complement pathway and causes endothelial damage (15). Besides infection, several drugs have also been known to trigger cutaneous hypersensitivity vasculitis, often presenting as superficial neutrophil or lymphocytic small vessel vasculitis. Drug-induced CSVV accounts for ∼20% of all CSVV cases (16). Examples of drugs that may induce CSVV include TNF-α inhibitors and levamisole (17, 18). CSVV may also be triggered directly by endothelial damage due to infections by vasculotropic viruses such as the COVID-19 causative agent, SARS-CoV-2 (14); or indirectly by antibodies generated against exposed autoantigens, such as antiphospholipid antibodies and anti-neutrophil cytoplasm antibodies (ANCA) (5). Although possible, ANCA are rarely found in patients with CSVV, with cases being defined in a distinct subgroup as ANCA-associated vasculitides (AAV).

ANCA-Associated Vasculitis (AAV)

Although the pathogenesis of AAV is multifaceted and multifactorial, it shares many aspects of that in CSVV, involving the activation of both cytokine-primed neutrophils and alternative complement pathways (11) (**Figure 1B**). ANCAs are autoantibodies generated by the immune recognition of autoantigens, such as neutrophil myeloperoxidase (MPO), proteinase-3 (PR3) and neutrophil elastase (NE) (19). While most MPO and PR3 are localized in the cytoplasm of unstimulated neutrophils, small amounts of these antigens can be found on the cell surface, even in healthy individuals. However, healthy individuals have only low levels of circulating natural autoantibodies against these auto-antigens, and the epitope specificity of their MPO-ANCA differs from that of pathogenic MPO-ANCA (2). In AAV patients, several mechanisms such as apoptosis and NETosis (release of neutrophil extracellular traps) can further promote the release of cytoplasmic antigens into the extracellular space or increase cell surface expression of cytoplasmic antigens by neutrophils. It was shown *in vivo* that neutrophils from AAV patients with active disease were more likely to undergo apoptosis and their cells had higher expression of surface PR3 and MPO (20). In kidney biopsies from AAV patients, NETs comprising DNA, histones, granule proteins MPO, PR3, LL37, and NE were found in the glomeruli (20). In addition, levels of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and C5a were increased in AAV patients and these can also promote the migration of intracellular MPO and PR3 from the cytoplasm to the cell membrane or into the extracellular space (21, 22).

ANCAs play various roles in the pathogenesis of AAV, contributing to disease development and progression. They are able to induce NETosis by binding to FcγRIIA on neutrophils and their ability to induce NETosis correlated with disease activity (20, 23). ANCAs can also bind to cytoplasmic antigens exposed on neutrophil surface leading to respiratory burst, neutrophil degranulation and release of inflammatory mediators such as pro-inflammatory cytokines, ROS and lytic enzymes, which can damage the vascular endothelium (11, 24). ANCA-activated neutrophils can induce injury in nearby microvascular beds, with NETosis leading to the release of neutrophil autoantigens for presentation by antigen-presenting cells. In individuals with active disease, increased numbers of defective Tregs and Bregs may be found (25, 26). Functional Bregs are potent immunosuppressors and inducers of Tregs, where Breg deficiency has been demonstrated in various autoimmune diseases such as SLE and MS, and most likely in AAV (26). The loss of an exon-2-deficient FOXP3 in Tregs due to aberrant splicing results in the loss of downstream protein sequestration involved in immunosuppressive pathways disrupts adaptive immune tolerance, leading to activation of neutrophils and subsequent inflammation of the vessel walls (1, 2, 11, 25, 27). Defective Treg function is thought to be associated with exaggerated neutrophil activity, although the underlying mechanisms are not fully understood (28).

In a mouse model of MPO-ANCA vasculitis, neutrophil depletion prevented disease progression, highlighting the pivotal role of neutrophils in the development of AAV (11). In AAV patients, there is a higher proportion of autoantigen-presenting neutrophils, which can trigger the production of ANCAs through effect T cell recruitment and subsequent B cell activation, resulting in disease (1). Circulating neutrophils isolated from patients with active disease have been shown to generate more basal superoxide, suggesting that they have been primed *in vivo* (24). In addition, surface expression of PR3 on apoptotic cells acts as a signal to initiate efferocytosis by macrophages. PR3-expressing apoptotic neutrophils increase production of pro-inflammatory cytokines, chemokines and nitric oxide (NO) via the IL-1R1/MyD88 signal pathway (11). The internalization of PR3 results in diminished anti-inflammatory macrophage reprogramming, leading to sustained inflammation in a positive feedback loop (29).

Activation of the complement system through the alternative pathway also has a central role in the development of AAV,

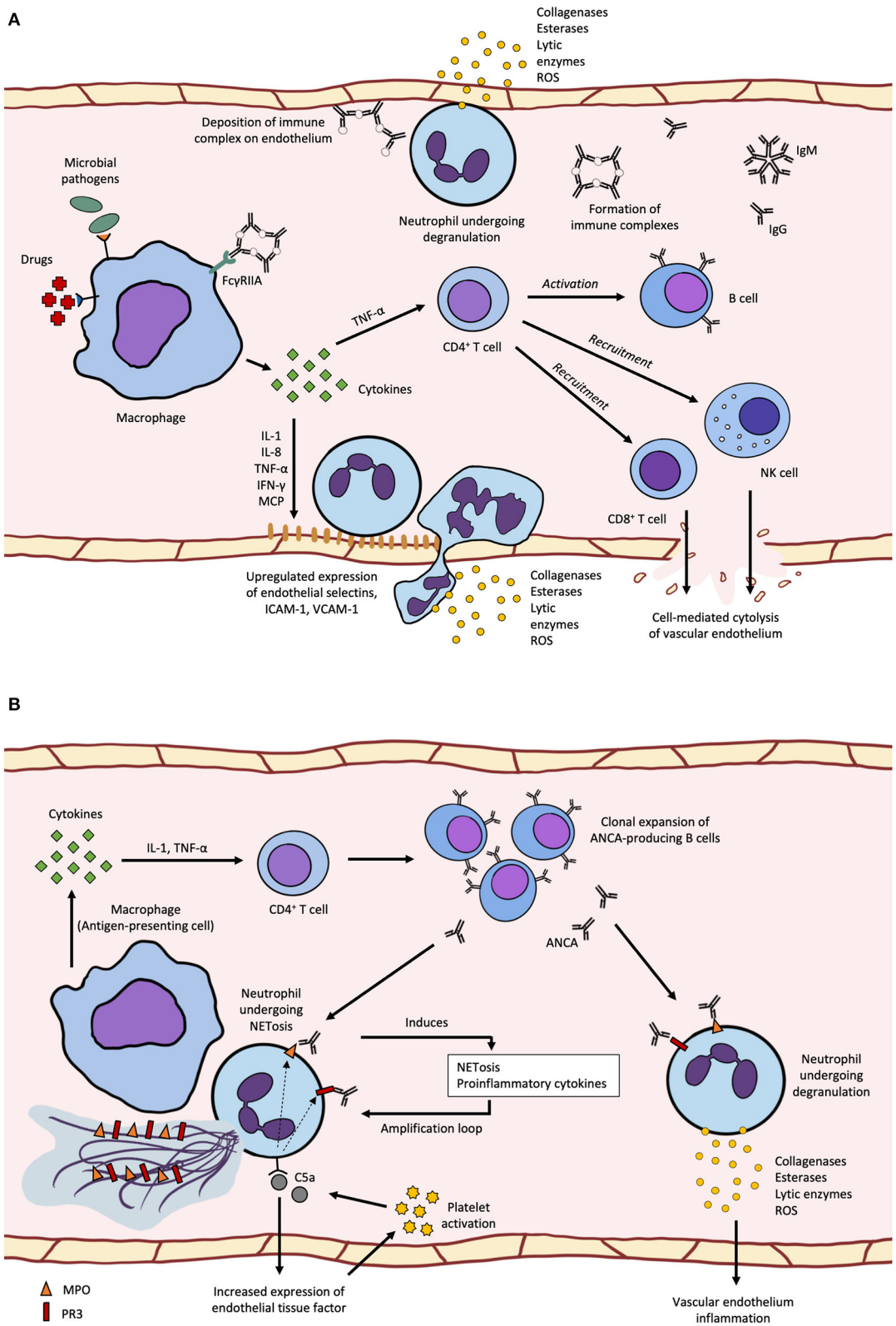

FIGURE 1 | (A) cutaneous small-vessel vasculitis (CSVV) begins with pre-exposure to microbial pathogens or certain drugs can induce pro-inflammatory cytokine secretion by macrophages leading to increased endothelial selectin, ICAM-1 and VCAM-1 expression, neutrophil diapedesis and degranulation that damages the vascular wall and surrounding tissues. Activation of the adaptive immune system leads to recruitment of cytotoxic lymphocytes and production of IgG and IgM

(Continued)

FIGURE 1 | immune complexes that deposit along the endothelium and induce neutrophil degranulation; **(B)** ANCA-associated vasculitis (AAV) can be caused by APC recognition of surface or secreted neutrophil autoantigens, MPO and PR3, which leads to the production of anti-neutrophil cytoplasmic antigen antiboodies (ANCAs). ANCA can bind to primed neutrophils expression MPO or PR3 on the surface and induce degranulation or NETosis, which forms an amplification loop of antigen release and recognition. Activation of the alternative complement pathway through C5a-mediated upregulation of tissue factor and platelet activation also forms an amplification loop, where C5a binding on neutrophils results in migration of cytoplasmic MPO and PR3 to be displayed on the cell surface.

bridging the inflammatory and coagulation processes found in active disease (30). C5a induces expression of tissue factor (TF) on neutrophils and endothelial cells, triggering the extrinsic coagulation pathway. Increased expression of TF, which may result in hypercoagulability, has been reported in AAV patients with active disease (31). Various components of the coagulation and fibrinolytic cascades can cleave C3 and C5 to generate C3a and C5a, respectively. Activated platelets express receptors for C3a and C5a, while C5b-9 induce the release of alpha-granules and microparticles which further activate the complement system. Platelet counts are often elevated in patients with active AAV and these correspond to disease severity, though the actual crosstalk and interactions are not fully understood (30). In a mouse model of MPO-ANCA vasculitis, C5-deficient mice were completely protected from developing the disease (32), suggesting that C5a can be a potential therapeutic target.

Although less common, eosinophilia has also been identified in cases of AAV, most commonly in EGPA (33). EGPA is characterized by three phases: (1) asthma and allergy symptoms, (2) tissue and blood eosinophilia, and (3) necrotising vasculitis (33). The presence of ANCAs have been reported in 30–40% of EGPA patients (33). While the direct pathogenic mechanisms of eosinophils in EGPA are unclear due to a lack of a suitable animal model, it has been considered to be a T_H2-mediated disease. CCL17 (a chemokine that recruits T_H2 cells) and IL-25 (a cytokine that induces and enhances T_H2 responses) are amongst the mediators that have been implicated in EGPA pathogenesis (33).

GENETIC VARIANTS AND THEIR ASSOCIATION WITH IMMUNOPATHOGENESIS IN VASCULITIS

The human leukocyte antigen (HLA) region, also known as the major histocompatibility complex (MHC), is a region within the human genome with the highest density of genes that encode for several important molecules involved in immune responses, and has been frequently associated with autoimmune diseases (34). HLA molecules encoded by this region present autoantigens to T cells, resulting in the development of pro-inflammatory or suppressive T cells depending on how the autoantigens are presented, subsequently leading to either autoimmunity or protection from disease (35). Antigen specificity is determined by specific pockets in the antigen-binding groove of the HLA molecule, and therefore the amino acid sequence of these pockets is crucial to understanding the risk for certain autoimmune diseases (35). While the MHC constitutes the strongest association in vasculitis, there are several loci outside the MHC, including *SERPINA1*, *PRTN3*, *SEMA6A*, *PTPN22*, *CTLA4, FCGR3B*, and *ARPC1B* that have also been established as genetic risk factors for these diseases, coding for immunological molecules that increase susceptibility to autoimmunity (36) (**Table 1**).

Genetic Variants Associated With Cutaneous Small-Vessel Vasculitis

Currently, there is not much literature on the genetic factors that contribute to CSVV pathogenesis. However, some have suggested that the gene *ARPC1B* may play a role in predisposition to this disease (36, 37). The *ARPC1B* gene encodes ARPC1B, which is an isoform of ARPC1 (actin-related protein complex-1) and one of the regulatory subunits of the Arp2/3 complex involved in actin polymerization and cellular motility (35). Genetic defects in the proteins regulating cytoskeletal rearrangements often cause syndromes involving the blood and immune systems (50). Arp2/3, in particular, is believed to have a critical role in immune cell synapses formation and T-regulatory cell function (51). A homozygous complex frameshift mutation in *ARPC1B* was identified in a 7-year-old Moroccan boy who presented with a novel combined immunodeficiency involving recurrent infections, mild bleeding tendency, vasculitis (including leukocytoclastic vasculitis), and allergic reactions (52). This mutation had resulted in a complete lack of the ARPC1B protein in the boy's neutrophils. Kahr et al. also described three patients suffering from CSVV who showed homozygous mutations in the *ARPC1B* gene (51). As a result of this mutation, complete loss of or minimal ARPC1B protein in their platelets led to defects in Arp2/3 actin filament branching associated with a range of diseases including inflammatory diseases and cutaneous vasculitis. Thus, the *ARPC1B* gene may be identified as a possible susceptibility locus contributing to CSVV pathogenesis.

Genetic Variants Associated With ANCA-associated Vasculitis

MHC Associations

With regards to the HLA region, the alleles *DPA1*, *DPB1*04:01*, *DPB1*04:02*, *DPB1*23:01*, *DQB1*02*, and *DRB1*03* were associated with the development of PR3-ANCAs, whereas *DQA1*03:02*, *DQB1*03:03*, and *DRB1*09:01* were associated with the MPO-ANCAs (38, 53). These risk factors were identified based on previous association studies (35, 37, 38, 53, 54).

Merkel et al. found a strong association between the *DPB1* risk haplotype allele with AAV in European populations, suggesting that the allele gives rise to a β chain polymorphism in the antigen-binding pocket of the HLA-DP molecule, which may impact T cell allorecognition and thus affect susceptibility to autoimmune disease (37). Furthermore, the association between *DQA1*03:02* and *DQB1*03:03* with MPO-ANCA pathogenesis

TABLE 1 | Relationship between vasculitis immunological mechanisms and susceptibility loci in different populations.

Type of vasculitis	Immunological target	Susceptibility loci	Associated population	Mechanism	References
ANCA-associated vasculitis	HLA-DP	*DPB1*04:01*, *DPB1*04:02*, *DPB1*23:01*	European	The risk haplotype allele underpins a β chain polymorphism in the antigen-binding pocket of HLA-DP, influencing T cell allorecognition.	(37)
	HLA-DQ	*DQA1*03:02*, *DQB1*03:03*	Chinese	*DQA1*03:02* codes for Asp160 on the DQ α chain. Asp160 forms a salt bridge with His111 on the DQ β chain, stabilizing the HLA homodimer and better priming the CD4+ T cells.	(38, 39)
	HLA-DR	*DRB1*09:01*	Japanese	Binds to MPO and forms an MPO/HLA-DR complex to transport the MPO protein to the cell surface. Exposed MPO associated with HLA-DR on cell surface induces autoantibody production.	(40)
	α1AT	*SERPINA1* (rs28929474/null allele)	European	Inhibits PR3 and thus inhibits inflammatory responses induced by PR3. Under-expression of the gene causes increased levels of circulating PR3, resulting in synthesis of PR3-directed ANCA.	(37, 41–43)
	PR3	*PRTN3* (rs62132293)	European	A proteinase which leads to proteolytic vessel damage. Risk variant causes overexpression of gene by neutrophils.	(37, 41, 43, 44)
	SEMA6A	*SEMA6A* (T allele of rs26595)	European	Involved in the immune response related to autoimmune disorders.	(42, 45, 46)
	Lyp	*PTPN22* (rs2476601)	European	Base substitution at Lyp620W makes Lyp more susceptible to proteolytic degradation, impairing its inhibitory effects on T cell activation and reducing immune tolerance.	(37, 47)
	CTLA-4	*CTLA4* (rs231726, G allele of rs3087243, rs3096851)	European	Individuals with the risk variant (G allele of rs3087243) have T cells with lower activation threshold and thus an increased risk for autoimmune diseases.	(42, 48)
	CD16B	*FCGR3B* (NA1, NA2)	Caucasian	Engaged by anti-PR3 antibodies to activate the ANCA-effector response, causing respiratory burst, phagocytosis, and neutrophil degranulation, thus affecting the severity of disease.	(49)
Cutaneous small vessel leukocytoclastic vasculitis	ARPC1B	*ARPC1B*	Inconclusive	Homozygous complex frameshift mutation causes under-expression of the gene. ARPC1B deficiency in neutrophils and platelets leads to defects in Arp2/3 actin filament branching, resulting in blood and immune-related diseases.	(50–52)

that was found in Chinese subjects may be due to the formation of stable HLA class II $\alpha\beta$ homodimers (38). These stabilized homodimers facilitate CD4+ T cell priming, whereby CD4+ T cells can only be primed if an immune complex is formed, made up of the stabilized $\alpha\beta$ homodimer, T cell receptor, and the antigen (39). The $\alpha\beta$ homodimer is stabilized through the formation of a salt bridge between Asp160 on the DQ α chain, encoded by the risk allele *DQA1*03:02*, and His111 on the DQ β chain (38). On the other hand, the role of *DRB1*09:01* in AAV pathogenesis for the MPO-ANCA subgroup in Japanese populations has been attributed to the association between MPO proteins and HLA-DR molecules encoded by *DRB1*09:01* (40). A major role of HLA class II molecules is to present antigens to T cells, where HLA-DR, a type of HLA class II molecule, had a high affinity to MPO. HLA-DR binds to intracellular MPO to form an MPO/HLA-DR complex in order to transport the MPO protein to the cell surface, thus initiating the production of autoantibodies against this complex (40). This suggests that MPO associated with HLA-DR are structurally different from native MPO, likely due to cryptic autoantibody epitopes on MPO being exposed by binding with HLA-DR. These structurally different MPO proteins are recognized as "neo-self" antigens by immune cells, therefore inducing autoantibody production (55).

Non-MHC Associations

A susceptibility locus associated with AAV targeting PR3, a proteinase causing proteolytic vessel damage, was identified as *SERPINA1* with haplotype rs28929474 in European populations

(37, 41). This gene encodes for α1-antitrypsin (α1AT), an inhibitor of PR3 and PR3-induced inflammatory responses (37). As for the risk variant, it causes under-expression of the gene, suggesting that it leads to increased levels of circulating PR3, thus resulting in the synthesis of ANCA directed to PR3 (41). Furthermore, the gene *PRTN3* (haplotype rs62132293) encoding PR3 was identified as a susceptibility locus for AAV in individuals of European descent, with the risk variant causing overexpression of this gene by neutrophils and thus increased expression of PR3 (37, 41).

Another susceptibility locus is *SEMA6A* (rs26595 T risk allele), found to be significantly associated with GPA in a genome-wide association study involving subjects of European descent (42). The functions of semaphorin 6A, encoded by this gene, are not well-characterized, and its role in the risk for GPA remains unclear, but a possible link points to the involvement of semaphorins in the immune response in autoimmune disorders (42, 45, 46).

Meanwhile, the gene *PTPN22* with haplotype rs2476601 has been associated with AAV pathogenesis in European populations due to the link between the risk variant to the aberrant increase in dendritic cell activation and lymphocyte antigen receptor signaling (37). The risk variant encodes Lyp620W which leads to dendritic cell and lymphocyte hyper-responsiveness, increasing the risk for autoimmune diseases (47). Lyp (lymphoid-tyrosine phosphatase) downregulates T cell antigen receptor (TCR) signaling, and risk variants are associated with multiple autoimmune diseases including rheumatoid arthritis (47, 56, 57). Lyp620W has a loss-of-function effect; at the site of the Lyp620W variant, the arginine to tryptophan substitution causes Lyp to become more susceptible to proteolytic degradation, reducing Lyp levels and impairing its inhibitory effects on T cell activation, thus compromising immune tolerance (47). However, the mechanism by which this loss-of-function effect of the Lyp620W variant impacts AAV pathogenesis has yet to be identified.

CTLA4 was confirmed as a genetic risk factor in CSVV (48). Three haplotypes of this gene—rs231726, the G allele of rs3087243, and rs3096851—were identified in European populations (42, 48). *CTLA4* encodes the protein CTLA-4 (cytotoxic T lymphocyte antigen 4), expressed on activated T cells, which represses T cell activation by associating with CD80 and CD86 on antigen-presenting cells (48, 58, 59). CTLA-4 competes with CD28, a T cell co-stimulant, for CD80 and CD86 binding, and CTLA-4 levels increase when T cells are activated via T cell receptor and CD28 (58). Patients with GPA were found to have increased levels of CTLA-4, a sign of T cell activation (60). Steiner et al. suggested that elevated levels of CTLA-4 are involved in the development of Th1 cells (the primary T cell subpopulation in GPA), due to the role of CTLA-4 in the differentiation of T cells into Th1 cells in TcR transgenic mice. It is also elucidated that individuals carrying the G allele of rs3087243 possess T cells with a lower activation threshold, thus leading to a higher risk for autoimmune diseases (48). The complete role of *CTLA4* in AAV pathogenesis, however, is not fully understood and requires further investigation (48).

The gene *FCGR3B* could also be considered a susceptibility locus for AAV. The role of its genetic variants, namely NA1 and NA2, in AAV pathogenesis can be explained through their involvement in the ANCA-effector response (49). The ANCA-effector response was associated with inflammatory necrosis of small blood vessels (61). ANCA-induced effector mechanisms are triggered when anti-PR3 antibodies bind to granular PR3 presented on activated neutrophils (49). These anti-PR3 antibodies then engage IgG Fc receptors such as FCGR3B (CD16B), encoded by *FCGR3B*, which further activate the ANCA-effector response, leading to respiratory burst, phagocytosis, and neutrophil degranulation, and this affects the strength of ANCA-induced activation of neutrophils and thus the severity of AAV (49).

TREATMENT STRATEGIES FOR VASCULITIS

Due to genetic factors playing a major role in vasculitis pathogenesis, targeting associated genes and their corresponding molecular pathways is the suitable approach. Over the years, monoclonal antibodies have gained much attention as a promising therapeutic strategy for the management of vasculitis. Monoclonal antibodies can be a suitable alternative to the first-line drugs based on glucocorticoids (GC), which have limitations such as GC resistance and severe complications (62). Here, we discuss recent findings obtained from studies on potential therapies for both AAV and CSVV.

Cutaneous Small-Vessel Vasculitis

Treatment of CSVV is clinically driven, perhaps due to the lack of understanding of the genetic background of this disease. Approach to therapy depends on the etiology and severity of the disease (63, 64). If the underlying etiology can be identified, such as due to an infection or a known drug, eliminating the cause would be the best course of action (64). If the CSVV is a result of a systemic vasculitis (e.g., AAV), treatment will be determined by the severity of internal organ involvement, and will usually require a combination of steroids and an immunosuppressive drug, for example rituximab for the treatment of AAV (64, 65). Idiopathic CSVV, on the other hand, has an excellent prognosis, with 90% of cases resolving within weeks to months of onset (66). Therefore, conservative treatment like bed rest, elevation of lower extremities, warming, analgesics, non-steroidal anti-inflammatory drugs (NSAIDs) and antihistamines can be used to alleviate symptoms such as burning or pruritus (63). If the condition extends to an ulcerative, nodular, or vesicobullous form or it becomes recurrent, additional aggressive systemic medications are necessary (63).

Diet, especially those involving a specific food allergen, is commonly cited as an inciting agent of CSVV, and adjusting to bland, low-antigenic diets is usually recommended to prevent recurrences of the condition (63). With regards to medication, the corticosteroid prednisone is the most widely used treatment for idiopathic CSVV (63, 64). The anti-gout agent colchicine

(0.6–1.8 mg per day) has been shown to resolve CSVV within 1–2 weeks (63, 67–69). If colchicine proves ineffective, dapsone, an anti-inflammatory and antineutrophilic agent, can be substituted or added (64). If CSVV is persistent, daily azathioprine, a steroid-sparing agent, may be prescribed (63, 70).

In 2017, a case was reported whereby a woman in her 40s suffering from CSVV, with a history of intermittent purpuric lesions for 20 years, was successfully treated with leflunomide (71). Leflunomide is a pyrimidine synthesis inhibitor and is an inexpensive, effective treatment for psoriatic and rheumatoid arthritis and several types of vasculitis. The patient was reported to remain free of new skin lesions and other cutaneous symptoms following 4 months of leflunomide therapy. Further investigation through prospective clinical trials is needed to assess the efficacy of leflunomide for CSVV treatment.

A recent study explored the role of oxidative stress in CSVV pathogenesis, suggesting that damage to blood vessels so often seen in CSVV could be caused by an imbalance in redox homeostasis (72). Recruitment of neutrophils to the affected tissues triggers ROS production, leading to lipid peroxidation in skin tissues and forming acrolein, a type of reactive aldehyde (73). The study showed that the concentration of acrolein-protein adducts in the skin of small-vessel vasculitis patients is proportional to disease severity (72). Therefore, CSVV might be treated through the mitigation of oxidative stress using antioxidative pharmacologic agents, while disease activity could be assessed using immunohistochemical assessments of acrolein content in the patient's skin, thus allowing for more targeted treatments (72). One such promising antioxidative therapeutic agent is peoniflorin, the main component of total glucosides of peony derived from the root of *Paeonia lactiflora* Pall. (74–76). The results from a study revealed that peoniflorin reversed the oxidative damage in human umbilical vein endothelial cells caused by hydrogen peroxide, hence suggesting that peoniflorin may be a candidate therapeutic strategy for oxidative stress-related vascular diseases (76).

ANCA-Associated Vasculitis

Based on findings derived from genetic studies, it has been elucidated that ANCA-mediated neutrophil activation and B cells are crucial to AAV pathogenesis, and therefore potential treatments can include B cell-depleting drugs (36, 77). In terms of the induction of AAV remission, rituximab represents a very promising contender (65). Rituximab is a murine / human chimeric monoclonal antibody against CD20, a B cell marker, and was licensed in 2011 for remission induction of AAV (65, 77, 78). During the first phase of an international randomized controlled trial known as RITAZAREM, rituximab was shown to be very effective in reinducing remission in relapsed AAV patients when taken in combination with relatively low doses of glucocorticoids (77).

One of the classical features of AAV is represented by granulomatous inflammatory lesions, which are initiated by macrophages and CD4+ T cells (79). T cell activation requires a costimulatory receptor (CD28) and CTLA-4 acts as a negative regulator of CD28, preventing the binding of CD28 with its ligand, thus blocking T cell activation (80). Abatacept, a disease-modifying anti-rheumatic drug (DMARD), is made up of the CTLA-4 ligand-binding domain and a modified Fc domain derived from IgG1, hence can be a possible therapeutic option for AAV treatment (80). An open label clinical trial involving patients with relapsing GPA showed that abatacept resulted in remission in 80% of patients (81). There is also an ongoing phase III clinical trial (NCT02108860) that aims to assess the efficacy of abatacept in achieving sustained remission in patients suffering from a non-severe relapse of GPA.

Despite the promise of these biological agents as therapeutics for AAV, they are still limited by adverse effects such as infections (81, 82). One study found that severe pulmonary infections were the major infectious complication observed in AAV patients treated with a low dose of rituximab (82). They attributed the B cell-depleting role of rituximab as a cause of these infections and identified renal dysfunction and old age as risk factors of such adverse effects. Given that both rituximab and abatacept are immunosuppressants, it is not surprising that patients treated with these drugs are more vulnerable to infections, and therefore this adverse event should be assessed accordingly during follow-up appointments with the clinicians (81, 82).

Plasma exchange, an effective treatment against thrombocytopenic purpura, a disorder which causes low platelet count, is also considered as a course of treatment for AAV patients (83–85). Plasma exchange may benefit patients by removing pathogenic ANCAs in the plasma, as well as clotting factors involved in the coagulation cascade (85, 86). However, the PEXIVAS trial, the largest study of plasma exchange in AAV, did not show that adjunctive plasma exchange benefits patients with severe AAV (87, 88). Nevertheless, it was suggested that patients with both ANCA and anti-glomerular basement membrane (GBM) antibodies should still be treated with adjunctive plasma exchange. Treatments for patients with anti-GBM disease involve immunosuppression and adjunctive plasma exchange (87). Since both patients with single anti-GBM positivity and double positivity for anti-GBM antibodies and ANCA experience aggressive pulmonary and renal disease, plasma exchange seems appropriate for treating double positivity patients (87).

DEVELOPING PERSONALIZED MEDICINES FOR VASCULITIS

Personalized medicine (PM) seeks to deliver targeted therapies to patients who have otherwise been unresponsive to previous treatments, based on a well-informed understanding of the mechanisms of the disease within the individual patient, and is currently the desired treatment of choice for rheumatic diseases (89, 90). As a primary step toward the success of PM, actionable biomarkers are required to assess disease pathophysiology and the molecular pathways involved (90). One technology advance in the field of PM in terms of pathophysiology characterization is Big Data, a multi-dimensional approach that enables the generation of vast amounts data and involves genomics, proteomics, metabolomics, and epigenetics in areas of epidemiology and healthcare delivery (90–95). An example of

Big Data technology is single cell RNA-seq (scRNA-seq) which details the transcriptional products of an individual cell, allowing researchers to define diseases functionally instead of clinically based on signs and symptoms (90–94). As Big Data technology has been applied in rheumatic diseases such as rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis (90), it is reasonable to think that Big Data can also be applied in studies on vasculitis pathophysiology. Hence, as mentioned throughout this review, genetic studies play a crucial role in identifying these mechanisms as well as in determining the genetic predisposition of each unique patient.

Literature on the development of PM for CSVV is considerably less compared to that for AAV, possibly due to the lack of publications on the genetic factors that may contribute to the disease. Nevertheless, scRNA-seq has been shown to be a promising strategy in identifying CSVV risk factors. Using scRNA-seq, the distribution of signaling molecules as well as HLA-II molecules in vascular endothelial cells across different tissues could be assessed (59). It was found that HLA-II genes exhibited higher expression levels in vascular endothelial cells from the skin than that from the thyroid, trachea, and brain, perhaps leading to the higher rate of dermal vasculitis in CSVV (59, 69). Furthermore, tofacitinib has been proposed as a possible therapy for CSVV patients in a recent case report (96). Tofacitinib is a Janus kinase (JAK) 3/1 inhibitor used in the treatment of rheumatoid arthritis, which suppresses inflammation by interfering with inflammatory cytokine signaling (5, 97). The case report presented a 29-year-old Chinese woman with a 5-year recalcitrant CSVV, who upon treatment with tofacitinib, achieved complete recovery, possibly due to tofacitinib interfering with the signal transduction of proinflammatory cytokines (96). However, more investigation into the efficacy and safety of tofacitinib in CSVV treatment is greatly needed since this is the first case of its kind.

Currently, AAV treatment is based primarily on organ involvement and severity of disease, as seen in the use of rituximab in the induction of remission and plasma exchange in patients with severe or refractory AAV suffering from severe renal involvement (serum creatinine $>$ 500 μmol/L) (65, 98–100). Over the years, PM in AAV has been gaining attention due to the increasing understanding of its immune pathology (65). This understanding has identified distinct AAV categories based on the immunological markers PR3 and MPO (100). Treating AAV patients based on their ANCA type (PR3 or MPO) may be more effective than treating them based on their clinicopathologic disease definitions (EGPA, MPA, or GPA), due to the stronger correlation between the differences in pathogenesis, genetics, and treatment responses with the ANCA type (101, 102). For example, the PR3-ANCA subgroup is associated with the variants of HLA-DP, α1AT, and PR3, while the MPO-ANCA subgroup is associated with the variants of HLA-DQ (43). Conversely, differences in genetic associations were found to be weaker when AAV patients were grouped following the traditional GPA/MPA classification (100). Overall, further understanding of the immunopathology of ANCA type may provide a basis for PM for AAV, addressing issues related to cost and unnecessary drug toxicity (100, 103).

Hence, treatments focusing on immunological targets in PR3-ANCA and MPO-ANCA subgroups are being investigated, such as anti-CD20 therapy (rituximab) which reduces in AAV patients the levels of ANCA, shown to stimulate the release of pro-inflammatory cytokines and ROS from monocytes and neutrophils (77, 100). Furthermore, treatments that target the pro-inflammatory cytokines IL-17, IL-21, and IL-23, namely anti-IL-12/IL-23, anti-IL-17, and anti-IL-17R treatments, could be effective for AAV therapy as AAV patients show elevated levels of these cytokines (100). IL-17, IL-21, and IL-23 are involved in the development of Th17 cells, and IL-17 and IL-21 are said to assist autoreactive B cells in patients suffering from systemic autoimmune diseases (104). The cytokine B cell activating factor (BAFF), which stimulates PR3- and MPO-specific B cell differentiation into antibody-secreting cells, may also be targeted (105). In a multicenter, double-blind, placebo-controlled trial, anti-BAFF therapy (belimumab) administered to AAV patients in remission did not reduce the risk of relapse (106). Belimumab did, however, maintain remission in patients who were treated with rituximab prior to the trial. Thus, belimumab as a potential targeted treatment for AAV requires further investigation.

Personalized medicine for AAV can be taken a step further with autoantigen-specific or ANCA-specific treatment (100). A study reported a promising strategy using cytotoxic T cells conjugated with a chimeric autoantibody receptor that recognizes antibodies against PR3 or MPO on B cells in AAV patients (107). These cytotoxic T cells would kill PR3- or MPO-specific B cells in the AAV patient. In addition, the use of peptibodies or peptides linked to an antibody backbone to target pathogenic ANCA has also been suggested for the mitigation of self-reactive B cells in AAV patients (108). Of course, the safety and efficacy of these treatments as well as their feasibility as AAV targeted therapies need to be further assessed.

CONCLUSION

It is clear that a deeper understanding of the immunopathogenesis of vasculitis and its associated genetic risk factors has allowed for recent developments in treatment and promising ideas for personalized medicine. However, the specific genetic variants which predispose individuals to CSVV as well as their pathogenic mechanisms have yet to be fully explored. Therefore, genome-wide association studies (GWAS) and population studies are necessary to identify risk alleles associated with CSVV, while functional analyses are crucial to pinpoint the direct causal effects of the risk alleles to aid in the development of targeted treatments. Further investigations into the immune pathology of ANCA types and into prospective targeted therapies are also recommended for the optimization of personalized medicine for AAV.

AUTHOR CONTRIBUTIONS

WY and CC conceptualized the project. BY and AL-F wrote the manuscript. BY designed the figures. AL-F prepared the table. All authors contributed to manuscript revision, read, and approved the submitted version.

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Complement Inhibition Targeted to Injury Specific Neoepitopes Attenuates Atherogenesis in Mice

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Rationale: Previous studies have indicated an important role for complement in atherosclerosis, a lipid-driven chronic inflammatory disease associated to oxidative stress in the vessel wall. However, it remains unclear how complement is activated in the process of atherogenesis. An accepted general model for complement activation in the context of ischemia reperfusion injury is that ischemia induces the exposure of neoepitopes that are recognized by natural self-reactive IgM antibodies, and that in turn activate complement.

Objective: We investigated whether a similar phenomenon may be involved in the pathogenesis of atherosclerosis, and whether interfering with this activation event, together with inhibition of subsequent amplification of the cascade at the C3 activation step, can provide protection against atherogenesis.

Methods and Results: We utilized C2scFv-Crry, a novel construct consisting of a single chain antibody (scFv) linked to Crry, a complement inhibitor that functions at C3 activation. The scFv moiety was derived from C2 IgM mAb that specifically recognizes phospholipid neoepitopes known to be expressed after ischemia. C2scFv-Crry targeted to the atherosclerotic plaque of *Apoe*$^{-/-}$ mice, demonstrating expression of the C2 neoepitope. C2scFv-Crry administered twice per week significantly attenuated atherosclerotic plaque in the aorta and aortic root of *Apoe*$^{-/-}$ mice fed with a high-fat diet (HFD) for either 2 or 4 months, and treatment reduced C3 deposition and membrane attack complex formation as compared to vehicle treated mice. C2scFv-Crry also inhibited the uptake of oxidized low-density-lipoprotein (oxLDL) by peritoneal macrophages, which has been shown to play a role in pathogenesis, and C2scFv-Crry-treated mice had decreased lipid content in the lesion with reduced oxLDL levels in serum compared to vehicle-treated mice. Furthermore, C2scFv-Crry reduced the deposition of endogenous total IgM in the plaque, although it did not alter serum IgM levels, further indicating a role for natural IgM in initiating complement activation.

Conclusion: Neoepitope targeted complement inhibitors represent a novel therapeutic approach for atherosclerosis.

Keywords: atherosclerosis, oxidization, natural antibody, animal–mouse, complement

INTRODUCTION

Clinical histological studies indicate that the complement system plays a critical role in atherogenesis (1–3). Recently, we (4) and others (5–7) suggested an atherogenic role for the membrane attack complex (MAC), the terminal product of complement activation, and an anti-atherogenic role for CD59, a MAC inhibitor. The complement system is usually activated via one of three pathways (classical, alternative and lectin pathways) which converge at C3 cleavage, leading to the formation of C3 and C5 convertases, and finally assembly of the MAC (8, 9). The MAC is a cytolytic macromolecular pore that can insert into host cell membranes in pathological conditions (8, 10, 11). Of note, since self-nucleated cells express various complement regulators and have intrinsic anti-lytic mechanisms (12), the MAC may mediate sub-lytic effects in the cells in disease conditions. The sub-lytic MAC formed on cell membranes may activate signaling cascades (13–21), leading to an inflammatory response (22–24). More than 10 plasma- and membrane-bound inhibitory proteins have been identified that restrict complement activation at different stages of the cascade and protect self cells from MAC attack (8, 9). CD59 is the most important membrane inhibitor for specifically restricting MAC formation (25). Previously, we showed that overexpression of human CD59 (hCD59) or inhibition of MAC formation protected against atherogenesis in mice (4, 26). Deficiency of C6, a component necessary for MAC formation, attenuated atherogenesis (6, 27–29). We have also shown that CR2-Crry, an inhibitor of all complement pathways at the C3 activation step and which is targeted to sites of C activation, protects $Apoe^{-/-}$ mice against the development of atherosclerosis (26). Recent human studies indicate that some complement components or complement activation products are associated with the occurrence and development of coronary heart disease (30) and subclinical atherosclerosis in systemic lupus erythematosus patients (31). These clinical results further support the critical atherogenic role of the complement system. However, it remains unclear how complement is activated in the process of atherogenesis, and whether inhibition of earlier complement activation products together with the MAC would provide optimum protection against atherogenesis. In this context, it has been shown that C5a inhibition also reduces atherogenesis, whereas C5a supplementation accelerates it (32, 33).

Atherosclerosis is a lipid-driven chronic inflammatory disease. Accumulation of oxidized low-density lipoprotein (oxLDL) in vessel walls is the central event in the initiation and progression of atherosclerotic plaque (34). OxLDL results in the formation of neoepitopes from lipid peroxidation and contributes to plaque development via several mechanisms, including endothelial dysfunction, foam cell formation, and activation of an inflammatory response (35, 36). Previous studies also suggest oxLDL plays a role in complement activation. In $Apoe^{-/-}$ mice, immune complexes of oxidized lipids and immunoglobulins (Igs) activate the classical complement pathway, leading to leukocyte infiltration of atherosclerotic lesions (37). *In vitro* studies also suggest that human oxLDL-IgG immune complexes activate complement via the classical pathway (38). Meanwhile, cholesterol crystals derived from oxLDL (39) present in early atherosclerotic plaque (40) and induce complement activation via both the lectin and classical pathways, which may represent the initial complement activation trigger in the plaque (41). Natural self-reactive IgM antibodies present in plaque is another potential complement activator. It has been demonstrated that natural IgM antibodies recognizing injury-induced phospholipid neoepitopes activate complement in an ischemia reperfusion injury model (42). However, whether a similar phenomenon exist in atherosclerosis is not clear.

To examine these questions, we utilized a novel complement inhibitor that is specifically targeted to injury induced phospholipid neoepitopes, in atherosclerosis mouse models. The inhibitor, C2scFv-Crry, consists of single-chain variable fragment (scFv) derived from a natural IgM mAb (C2) linked to Crry, a murine inhibitor of C3 activation (43). C2scFv-Crry recognizes a subset of phospholipids exposed on injured cells post-ischemia injury (42), and it has been demonstrated that C2scFv-Crry is protective in arthritis (44) and lung transplantation (45) mouse models. Since C2scFv specifically recognizes phospholipids, which are components of oxLDL (46), we sought to determine the targeting and therapeutic effect of C2scFv-Crry in a model of atherosclerosis. We found that C2scFv-Crry treatment significantly attenuates atherosclerosis by targeting the plaque. which is associated with decreased complement activation, lipid and IgM deposition.

METHODS

Production and Purification of C2scFv-Crry

C2scFv-Crry was constructed and purified as previously described (45). The purity of C2scFv-Crry was determined by SDS-PAGE and complement inhibitory activity confirmed by zymosan assay.

Abbreviations: scFv, Single chain fragment variable; HFD, High-fat diet; MAC, Membrane attack complex; oxLDL, Oxidized low-density lipoprotein; hCD59, Human CD59; ORO staining, Oil-red-O staining; SMC, Smooth muscle cell; SMA-α, Alpha smooth muscle actin; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Animal Treatment and Characterization of Atherosclerotic Lesions

Apoe$^{-/-}$ mice (JAX 002052) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in Lewis Katz School of Medicine at Temple University and Tulane University School of Medicine. To investigate the effect of C2scFv-Crry in the development of atherosclerosis, 8-week-old male *Apoe*$^{-/-}$ mice were treated with C2scFv-Crry (intravenously, 0.25 mg/mouse, twice per week) or the same volume of PBS (intravenously, twice per week) and maintained on a high-fat diet (HFD) (C12108; Research Diets, Inc.) for 2 or 4 months. At the end of the experiment, mice were euthanized by CO_2 asphyxiation. Serum was collected and the atherosclerotic lesion was characterized as previously described (26, 47). Briefly, the entire aorta from the heart outlet to iliac bifurcation was collected and stained with Oil Red O. The percentage of plaque area (%) was calculated as (Oil Red O-stained area/total aorta area) × 100. The aortic root was snap frozen in optimal cutting temperature (OCT) compound and sectioned (5 μm). Slides were stained with either hematoxylin and eosin (H&E) or Oil Red O, and the plaque area and lipid deposition were calculated. To determine the target deposition of C2scFv-Crry in the plaque, sections of aortic root from PBS or C2scFv-Crry-treated mice were stained with either Alexa Fluor-488 (AF488) isotype antibody or AF488 anti-His antibody (Invitrogen, 4E3D10H2/E3). All animal experiments were reviewed and approved prior to commencement of activity by the Institutional Animal Care and Use Committee at Temple University.

Immunofluorescence and Immunohistochemistry Staining

Frozen sections of aortic root (5 μm) were stained with 1) rat anti-mouse C3, IgG2a (clone: 3/26, Hycult Biotechnology), which recognizes mouse complement protein C3 as well as activated C3 fragments C3b, iC3b, and C3c; 2) rabbit anti-rat C9, which cross-reacts with mouse C9 (kindly provided by Dr. P. Morgan, University of Wales); 3) rat anti-mouse CD68, IgG2a (clone: FA-1, AbD Serotec) for mononuclear phagocytes; 4) goat anti-mouse IgM (Bio-rad); and 5) rabbit anti alpha smooth muscle actin (SMA-α) (Abcam). All primary antibodies were detected using corresponding secondary antibodies and compared with negative controls, which were stained with the secondary antibody alone. For IgG staining, the sections were directly stained with Alexa Flour 594-conjugated anti-mouse IgG antibody (Invitrogen). For TUNEL staining, the sections were stained with in situ cell death detection kit, Fluorescein (Roche) according to the manufacture's instruction. We quantified immunofluorescence and histological results from three serial sections from each mouse using Image ProPlus 6.0 software as described previously (48). The means of the quantitative results of three sections obtained from each mouse were used to perform the statistical analysis.

Serum Lipid and oxLDL Measurement

Serum cholesterol and triglyceride profiles were measured at NIH/NIAAA as previously described (49). Serum oxLDL level was determined by oxLDL ELISA kit (Mybiosource) according to the manufacturer's instructions.

OxLDL Uptake Assay

Mouse primary peritoneal macrophages were isolated at 3 days after i.p. injection of 3% thioglycollate broth medium as previously described (50). For oxLDL uptake assay, 50 μg/ml Dil-labelled oxidized LDL (Dil-oxLDL) (Thermo Fisher) was pre-incubated with PBS or 20 μg/ml C2scFv-Crry for 30 min, followed by incubation with peritoneal macrophages for 16 h. The uptake of Dil-oxLDL was determined by the intensity of Dil (PE channel) in macrophages measured by flow cytometry analysis. In some experiments, macrophages were treated with PBS, oxLDL+PBS, oxLDL+C2scFv-Crry for 24 h and RNA was extracted. The mRNA level of IL-1β was determined by qRT-PCR as previously described (50).

Statistical Analysis

Experimental results are shown as the mean ± SEM. The difference between the two groups was examined with a nonparametric Mann-Whitney test. All statistical results with $p < 0.05$ were considered significant.

RESULTS

C2scFv-Crry Targets Atherosclerotic Plaque

To determine whether C2scFv-Crry specifically localizes to the atherosclerotic plaque in a therapeutic paradigm, we administered C2scFv-Crry (0.25 mg/mouse) or PBS to 2-month-old *Apoe*$^{-/-}$ mice twice per week for 2 months and maintained the mice on a high-fat diet (HFD). We then immunostained aortic root sections with anti-His antibody, which recognizes the 6xHis tag conjugated to the N-terminus of C2scFv-Crry. Immunofluorescent (IF) staining showed extensive deposition of the His tag in aortic root of C2scFv-Crry-treated mice, but not in PBS-treated mice (**Figures 1A,B**, right), Of note, there was no positive staining in mice with an isotype control antibody (**Figures 1A,B**, left). Thus, the C2 neoepitope is expressed in atherosclerosis plaque, which facilitates the targeting of C2scFv-Crry to the plaque.

C2scFv-Crry Treatment Attenuates the Development of Atherosclerosis in *Apoe*$^{-/-}$ Mice

To determine the therapeutic effect of C2scFv-Crry in the development of atherosclerosis, we treated 2-month-old *Apoe*$^{-/-}$ mice with C2scFv-Crry (0.25 mg/mouse) or PBS (twice per week) for either 2 or 4 months and maintained the mice on a high-fat diet (HFD), following which we examined atherosclerotic plaque in the whole aorta and aortic root. *En face* Oil Red O (ORO) staining of the aorta showed that C2scFv-Crry-treated mice had significantly less plaque area on the aortic surface than PBS-treated mice after both 2 and 4 months of treatment (**Figures 2A,B**). Of note, although C2scFv-Crry treatment did not affect body weight at either time point, the weight of abdominal fat tissue was reduced in mice

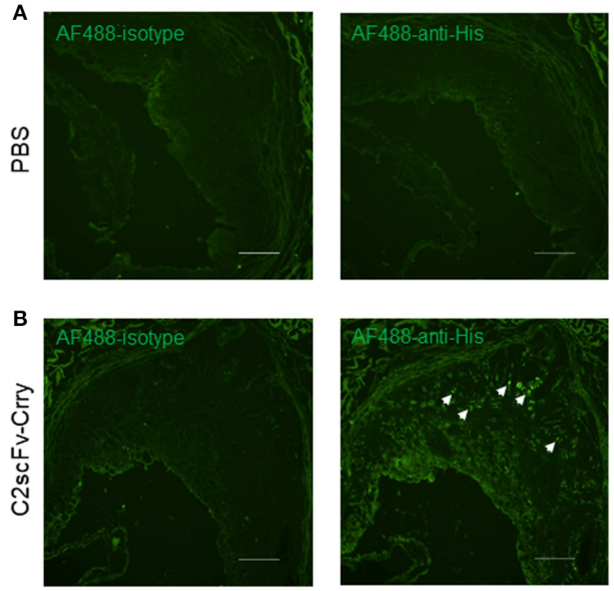

FIGURE 1 | Deposition of C2scFv-Crry in the atherosclerotic plaque of Apoe$^{-/-}$ mice. Aortic root cryosections from Apoe$^{-/-}$ mice treated with PBS **(A)** or C2scFv-Crry **(B)** were stained with Alexa Fluor 488 (AF488)-Isotype (left) or anti-His antibody (right). Representative image shows the deposition in the plaque of C2scFv-Crry-treated mice. Scale bar 100 um.

treated with C2scFv-Crry for 2 months as compared to PBS (**Supplementary Figures 1A,B**). Further, H&E staining of aortic root sections showed reduced plaque area in aortic roots of C2scFv-Crry-treated mice compared to PBS-treated mice at both time points (**Figure 2C**). Together, these results demonstrate that C2scFv-Crry treatment attenuates the development of atherosclerosis in mice.

C2scFv-Crry-Treated Mice Have Significantly Less Complement Activation in the Plaque Than PBS-Treated Mice

To investigate whether the anti-atherogenic effect of C2scFv-Crry is associated with the targeted inhibition of the complement system, we first analyzed C3 activation in the plaque of these mice. Immunostaining using an antibody specific for deposited C3 activation products (C3b/iC3b/C3c) showed significantly less C3 deposition in aortic roots from *Apoe*$^{-/-}$ mice treated with C2scFv-Crry for 4 months compared to PBS controls (**Figure 3A**). We previously demonstrated that the MAC has an atherogenic role. To this end, we further explored the functional connection between C2scFv-Crry treatment and MAC formation. Using an antibody to C5b-9/MAC, we found that C2scFv-Crry-treated mice had significantly less MAC deposition in the plaque of aortic roots than PBS-treated mice in both the 2- and 4-month groups (**Figure 3B**). To investigate whether the MAC may be inducing apoptosis, which may stimulate the formation of vulnerable plaque (51). We measured the apoptosis level in the plaque using TUNEL staining. We found that C2scFv-Crry-treated mice had very low, but similar levels of TUNEL positive area compared to PBS-treated mice in both the 2- and 4-month groups, suggesting C2scFv-Crry does not act through an effect from apoptosis in this model (**Supplementary Figure 2**). Furthermore, the area of CD68+ macrophage and SMA-α+ smooth muscle cell in the plaque was not changed by C2scFv-Crry (**Supplementary Figure 3**), suggesting that C2scFv-Crry may not function through affecting immune cell composition in the plaque. Altogether, these results demonstrate that the atheroprotective effect of C2scFv-Crry is associated with the targeted inhibition of complement activation in plaque, further supporting the therapeutic potential of complement inhibition in atherosclerosis.

C2scFv-Crry Inhibits Oxidized LDL-Induced Inflammation *in vitro* and Lipid Deposition in Plaque

Since the C2 mAb was originally characterized as an antibody that recognizes injury-exposed phospholipids normally recognized by natural circulating IgM, and phospholipids make up the outer shell of lipoproteins, we examined the effect of C2scFv-Crry on lipoprotein uptake, which is a critical event in the progression of atherosclerosis. To this end, we performed an oxLDL uptake assay in which peritoneal macrophages were incubated with fluorescence (Dil)-labeled oxLDL (Dil-oxLDL), followed by flow cytometry analysis of Dil fluorescence intensity in macrophages. We found that pre-incubation of C2scFv-Crry with Dil-oxLDL significantly inhibited the uptake of oxLDL by macrophages, as illustrated by reduced Dil intensity (**Figure 4A**). In addition, oxLDL has been demonstrated to promote inflammatory cytokine expression (26). We found that C2scFv-Crry counteracted oxLDL-induced IL-1β upregulation in macrophages (**Figure 4B**). *In vivo*, although C2scFv-Crry-treated mice had similar levels of serum cholesterol and triglycerides compared to PBS-treated mice, serum oxLDL levels were significantly reduced in mice treated with C2scFv-Crry for 4 months (**Figure 4C**), suggesting that long-term administration of C2scFv-Crry can lower the oxidization of LDL. Finally, ORO staining revealed that C2scFv-Crry-treated mice had significantly lower lipid content in aortic root plaques than PBS-treated mice at both time points (**Figure 4D**). Together, these data suggest that in addition to inhibiting complement activation, C2scFv-Crry also attenuates lipid deposition, oxidization, and uptake in plaques, possibly mediated by its C2 moiety.

C2scFv-Crry Reduces Endogenous IgM Deposition in the Plaque

A previous study demonstrated that natural IgM recognizing oxidized phospholipids deposit in plaques and play a protective role in atherosclerosis (52). To determine whether the infusion of C2scFv-Crry has any effect on endogenous natural antibody deposition, we analyzed the level of total IgM and IgG in the plaque after C2scFv-Crry treatment. Using an antibody specific for total IgM or IgG, but not single-chain fragments, we found that mice treated with C2scFv-Crry for 2 and 4 months had reduced levels of endogenous IgM, but not IgG, in the aortic root plaque compared to mice treated with PBS (**Figures 5A–D**).

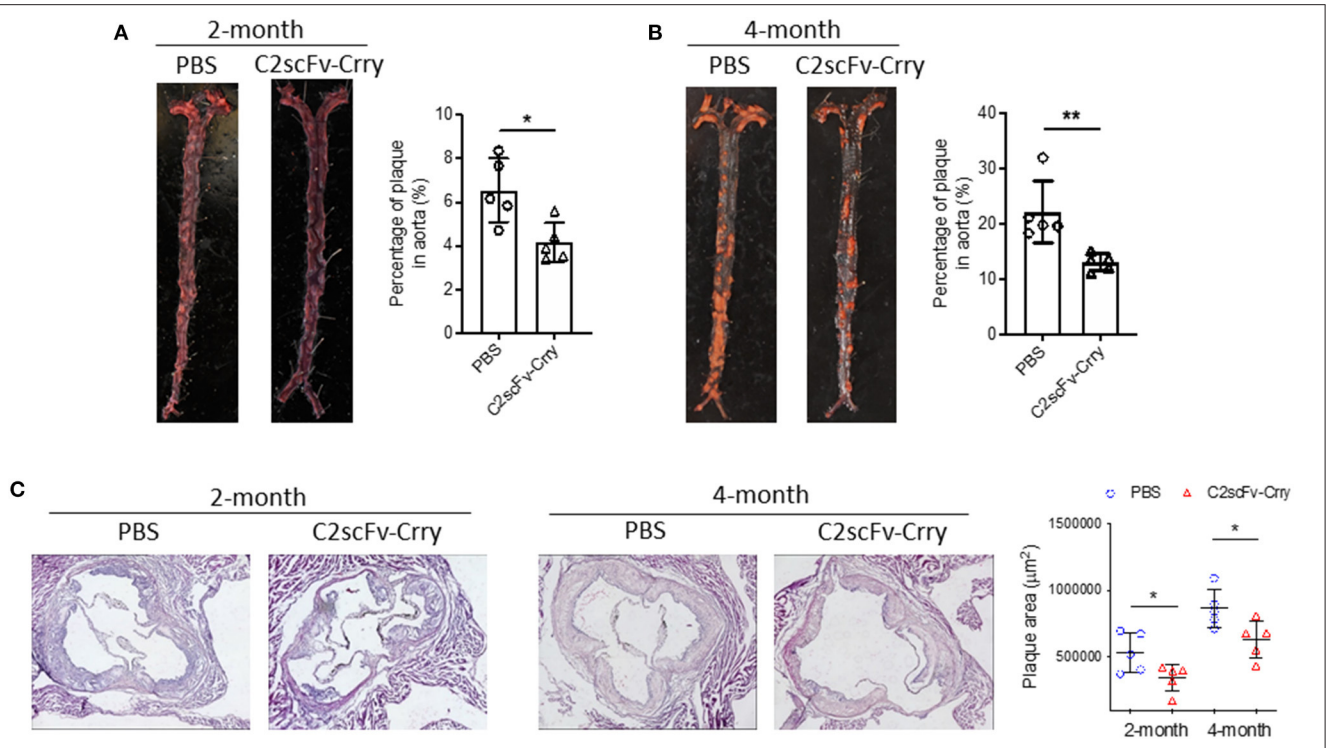

FIGURE 2 | C2scFv-Crry attenuates atherosclerosis plaque in Apoe$^{-/-}$ mice fed with high-fat diet (HFD) for 2 or 4 months. Apoe$^{-/-}$ mice were fed a high-fat diet (HFD) and treated with PBS or C2scFv-Crry (0.25 mg/mice, twice a week) for 2 or 4 months, followed by quantification of atherosclerosis plaque. **(A,B)** Representative images (left) and quantification (right) of aortic en face plaque area determined by Oil Red O (ORO) staining for 2-month **(A)** and 4-month groups **(B)**. Quantification represents the percentage of ORO-stained plaque areas in the entire aorta. **(C)** H&E staining of cross-sections of aortic root in Apoe$^{-/-}$ mice treated with PBS or C2scFv-Crry. Left: representative images. Right: quantification plaque aera in aortic root (*$p < 0.05$, **$p < 0.01$ using unpaired Student's *t*-test).

This effect is plaque-specific, since IgM levels in serum is not changed (**Figure 5E**). These results indicate that C2scFv-Crry treatment impairs the deposition of endogenous phospholipid-specific IgM in the plaque, which may result from the competitive binding of phospholipids by the single-chain fragment of C2. The atheroprotective effect of C2scFv-Crry is thus likely due to the combined inhibition of complement by Crry and lipid uptake by single-chain of C2.

DISCUSSION

Here we report a novel complement inhibitory strategy to protect against the development of atherosclerosis. A potential mechanism for the protective role of C2scFv-Crry is localized inhibition of complement activation and reduced lipid deposition at site of pathology and the subsequent dampening of a sterile inflammatory response. The C2 IgM mAb, from which the targeting moiety was derived, was initially identified as binding to a subset of phospholipids displayed specifically on injured cells following cerebral ischemia. Here we demonstrate that similar phospholipid neoepitopes are expressed in atherosclerotic plaques, and that targeting a complement inhibitor to these neoepitopes is anti-atherogenic and reduces local complement activation as evidenced by decreased C3 deposition and MAC formation. The targeting of C2scFv-Crry was confirmed by immunostaining showing the localization of C2scFv-Crry in the plaque. Since the C2scFv targeting moiety recognizes neoepitopes exposed in the plaque, phospholipids, which are a typical component of plaques, represent epitopes that could be used for the targeted delivery of therapeutics to treat atherosclerosis. It is important to note that our current study mainly focuses on the effect of C2scFv-Crry, and the role of IgM and complement, in the development of the plaque, and as such does not represent a therapeutic examining the effect of C2scFv-Crry on establishment of the plaques. Thus, the therapeutic effect of C2scFv-Crry in established atherosclerosis remains unclear and requires further investigation.

In addition to complement inhibition, we also demonstrate that C2scFv-Crry inhibits oxLDL uptake *in vitro* and lipid content in the plaque. A critical event in the early stage of plaque development is the deposition and oxidization of LDL (53). OxLDL accumulates with the progression of plaque and contributes to plaque development (35, 36). A recent study showed that oxidized phospholipids, active components of oxLDL, are proinflammatory and proatherogenic in mice (46), suggesting the therapeutic potential of targeting oxLDL. Indeed, it has been recognized that natural IgM against oxidized phospholipids plays an anti-atherogenic role in the context of atherosclerosis (54–56). For example, the natural IgM antibody T15/E06, which was isolated from cholesterol-fed apoE mice

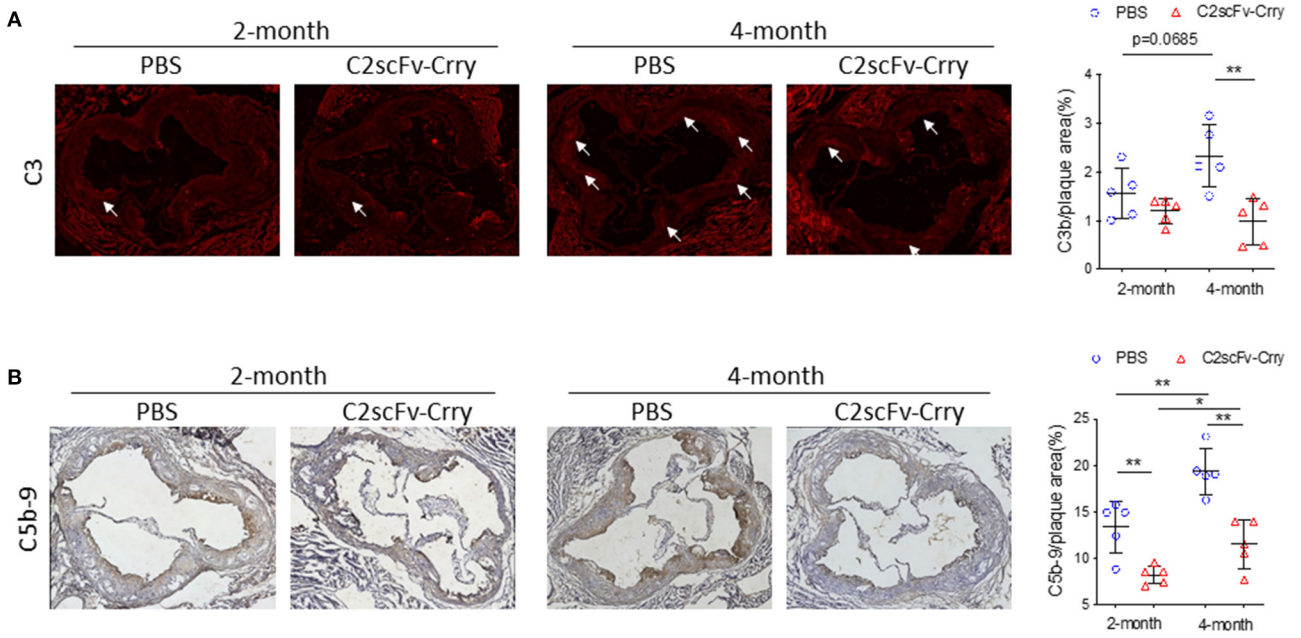

FIGURE 3 | C2scFv-Crry attenuates complement activation in the plaque of Apoe$^{-/-}$ mice. **(A)** Representative C3b (left) staining in the aortic root from Apoe$^{-/-}$ mice treated with PBS or C2-Crry and quantification of C3b positive area in plaque (right). **(B)** Representative C5b-9 (left) staining in the aortic root from Apoe$^{-/-}$ mice treated with PBS or C2-Crry and quantification of C5b-9 positive area in plaque (right) (*$p < 0.05$, **$p < 0.01$ using unpaired Student's *t*-test).

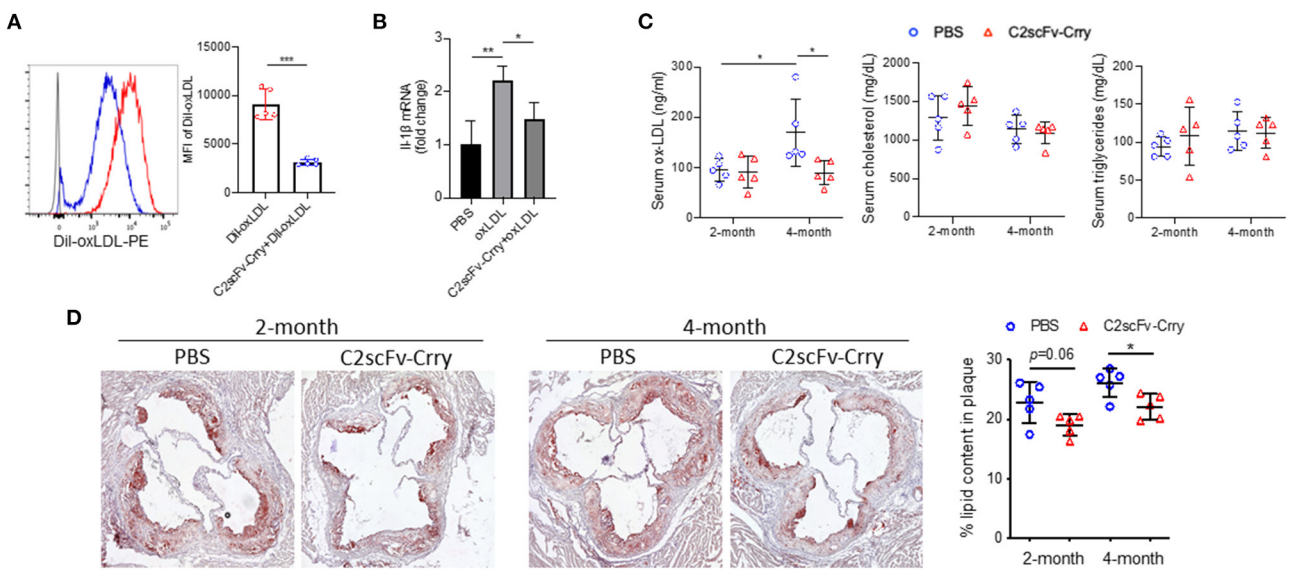

FIGURE 4 | C2scFv-Crry inhibits oxidized low-density-lipoprotein uptake by macrophage and lipid deposition in the plaque. **(A)** Dil-labelled oxidized low-density lipoprotein (Dil-oxLDL) (50 ug/ml) was pre-incubated with PBS or 20 ug/ml C2scFv-Crry for 30 min, followed by incubation with peritoneal macrophage for 16 h. The uptake of Dil-oxLDL was determined by the intensity of Dil in macrophages. Left: Representative histogram of Dil. Right: Mean fluorescence intensity (MFI) of Dil. **(B)** IL-1ß mRNA level in peritoneal macrophage treated with pbs, oxLDL or oxLDL+C2scFv-Crry for 24 h. **(C)** Serum oxLDL, cholesterol and triglyceride levels in Apoe$^{-/-}$ mice treated with PBS or C2scFv-Crry. **(D)** Representative image and quantification of Oil Red O staining in aortic root of Apoe$^{-/-}$ mice treated with PBS or C2scFv-Crry (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ using unpaired Student's *t*-test).

and binds to oxidized phospholipids, blocks the uptake of oxLDL by macrophages and reduces vascular inflammation (57). In addition, passive infusion of T15/E06 reduced vein-graft atherosclerosis in *Apoe*$^{-/-}$ mice (58). In our study, we found that C2scFv-Crry significantly attenuated oxLDL uptake and inflammation, and reduced lipid content in plaques, suggesting that in addition to complement inhibition mediated by Crry, C2scFv-Crry may also act on lipid deposition in the plaque

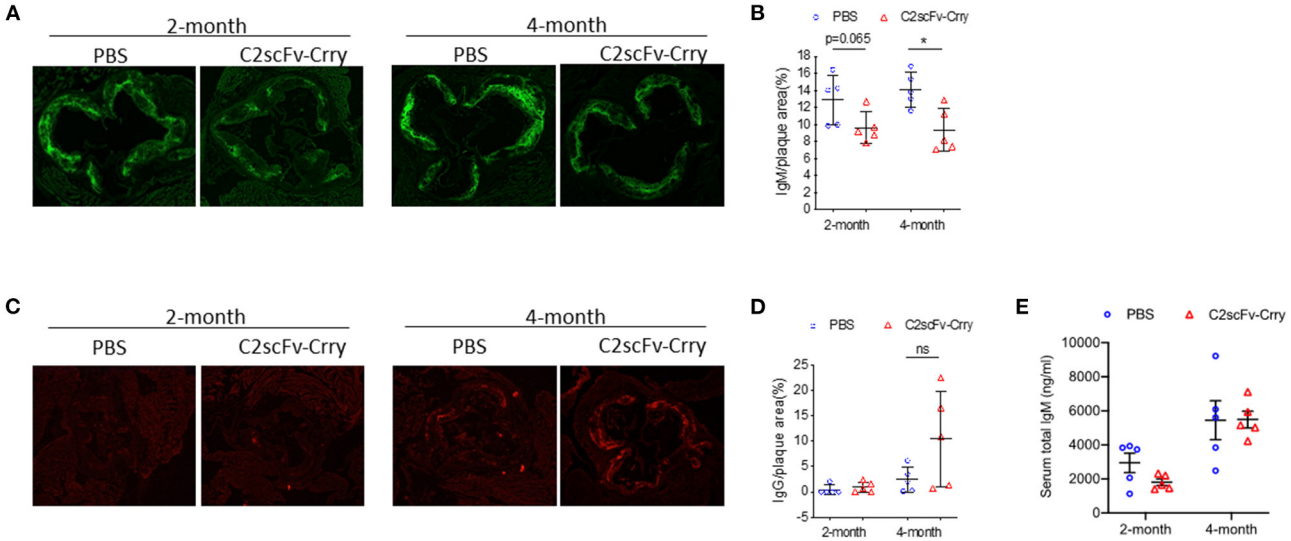

FIGURE 5 | C2scFv-Crry specifically decreases IgM levels in the plaque but not the serum of Apoe$^{-/-}$ mice. **(A)** Representative total IgM staining in the aortic root of Apoe$^{-/-}$ mice treated with PBS or C2scFv-Crry. **(B)** Quantification of total IgM positive area in the plaque. **(C)** Representative total IgG staining in the aortic root of Apoe$^{-/-}$ mice treated with PBS or C2scFv-Crry. **(D)** Quantification of total IgG positive area in the plaque. **(E)** Serum total IgM level in Apoe$^{-/-}$ mice (*$p < 0.05$ using unpaired Student's *t*-test).

through its C2scFv component. Interestingly, we also found that 4 months (but not 2 months) of C2scFv-Crry treatment reduced serum oxLDL levels, an indicator of LDL oxidization in plaques (59). These results indicate that long-term C2scFv-Crry treatment inhibits the oxidization of LDL, which may further contribute to its atheroprotective effect. However, further study is required to determine the exact mechanism.

It is known that natural IgM recognizing neoepitopes on stressed and injured cells can activate complement and drive pathology in various disease conditions. Although a previous study has demonstrated an overall beneficial role of lipid-targeting natural IgM in atherosclerosis, the role of IgM in complement activation has not been determined in atherosclerosis. Whereas the C2 mAb can induce complement activation via an Fc fragment, as demonstrated in a lung transplant injury model (45), the C2scFv-Crry construct does not contain an Fc fragment and thus lacks the ability to activate complement. An area for future study will be the role of natural antibody in complement activation in driving atherosclerosis. In this context, we observed decreased deposition of IgM in the plaque following C2scFv-Crry treatment. A potential explanation for this is competitive binding of C2scFv-Crry to oxLDL in the plaque, which attenuates the deposition of endogenous IgM. It will be interesting to investigate the therapeutic effect of other oxidized phospholipid-targeted IgM-mediated complement inhibitors in atherosclerosis, and which may have additive effects by blocking IgM binding to multiple epitopes. Interestingly, C2scFv-Crry treatment does not change the level of total IgG in the plaque, suggesting IgG may not involve in complement activation in the current model. Furthermore, we did not observe any change in macrophage and SMC content in the plaque. It has been shown that oxLDL uptake induces macrophage polarization toward alternative M2-like types, which take up a higher level of oxLDL than M1-like macrophages *in vitro* (60, 61). The dynamic change of macrophage phenotype and activation status *in vivo* after C2scFv-Crry treatment and whether there are other cell types involved requires further investigation.

In summary, we have demonstrated that C2scFv-Crry protects mice against atherosclerosis by targeting inhibition of complement activation and pro-inflammatory oxLDL uptake and deposition, which represents a novel targeted approach of complement inhibition for the treatment of atherosclerosis.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Tulane University and Temple University.

AUTHOR CONTRIBUTIONS

SD, FL, X-FY, HW, ST, and XQ developed the concept. SD, FL, MR, ZQ, NR, and XQ contributed to perform the experiments and analyze the results. ST provided the complement inhibitors. SD, FL, NR, X-FY, HW, ST, and XQ wrote the manuscript, and all authors participated in the review and critique of the manuscript. HW, ST, and XQ interpreted the results and supervised the experiments.

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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.2021.731315/full#supplementary-material

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Conflict of Interest: ST is a consultant for Q32 Bio, a company developing complement inhibitors, and is an inventor on a licensed patent for targeting constructs based on natural antibody. ST is a co-founder of Q32 Bio and owns equity in the company.

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

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Isoproterenol-Induced Cardiomyopathy Recovery Intervention: Amlexanox and Forskolin Enhances the Resolution of Catecholamine Stress-Induced Maladaptive Myocardial Remodeling

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The increasing incidence of stress-induced cardiomyopathy is due to the complexities of our modern-day lives, which constantly elicit stress responses. Herein, we aimed to explore the therapeutic potential of Amlexanox and Forskolin in promoting the recovery from stress-induced cardiomyopathy. Isoproterenol-induced cardiomyopathy (ICM) models were made, and the following treatment interventions were administered: 5% v/v DMSO as a placebo, Amlexanox (2.5 mg/100 g/day) treatment, Forskolin (0.5 mg/100 g/day), and Amlexanox and Forskolin combination, at their respective aforementioned dosages. The effects of Amlexanox and Forskolin treatment on ICM models were assessed by eletrocardiography and echocardiography. Also, using histological analysis and ELISA, their impact on myocardial architecture and inflammation were ascertained. ICM mice had excessive myocardial fibrosis, hypertrophy, and aggravated LVSDs which were accompanied by massive CD86+ inflammatory cells infiltration. Amlexanox treatment attenuated the myocardial hypertrophy, fibrosis, and inflammation and also slightly improved systolic functions. Meanwhile, forskolin treatment resulted in arrhythmias but significantly enhanced the resolution of myocardial fibrosis and inflammation. Intriguingly, Amlexanox and Forskolin combination demonstrated the most potency at promoting the recovery of the ICM from LVSD by attenuating maladaptive myocardial hypertrophy, fibrosis, and inflammatory responses. Our findings highlight the Amlexanox and Forskolin combination as a potential therapeutic intervention for

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enhancing cardiac function recovery from stress-induced cardiomyopathy by promoting the resolution of maladaptive cardiac remodeling.

Keywords: stress, amlexanox, forskolin, isoproterenol-induced cardiomyopathy, left ventricular systolic dysfunction, myocardial inflammation, myocardial fibrosis

INTRODUCTION

The incidence of cardiovascular diseases (CVDs) keeps increasing due to the enormous amount of risk factors (sex-gender difference, heredity, and unhealthy lifestyles) which hastens their progression. Typically, stress resulting from the demands and complexities of our modern-day lives adversely affects overall cardiac health if it remains chronic (1–4). During chronic stress, elevated levels of circulating catecholamine overstimulate β-adrenergic receptors (βARs), which induces their dysregulation and causes the initiation of cardiomyopathies (1, 5, 6). Recent studies have associated the development of hypertrophied hearts and left ventricular systolic dysfunctions (LVSD) with chronic stress-induced cardiomyopathy (7).

Isoproterenol (an agonist of β_1AR and β_2AR), besides being used for bradycardia treatment, has been extensively used to mimic catecholamines in modeling chronic stress-induced cardiomyopathy [hereafter referred to as Isoproterenol-induced cardiomyopathy (ICM)] so as to be able to elucidate its underlying pathomechanisms (8, 9).

Despite considerable efforts in elucidating the disease mechanisms of ICM, which have mainly implicated the maladaptive stimuli signal mediation of β_2AR (5), very few treatment interventions aimed at attenuation of the pathological cardiac remodeling have been demonstrated in animal models. Conversely, there are overwhelming numbers of studies demonstrating potential therapeutic intervention for acute myocardial infarction (AMI). Like AMI, ICM is characterized by exacerbated myocardial inflammation and LVSD (7, 10, 11). Hence, this study sought to explore if amlexanox (AMLX), which was recently demonstrated to aid in the recovery of cardiac function from AMI, could do the same in ICM models. Also, cyclic adenosine monophosphate (cAMP) plays essential roles in modulating cardiac and inflammatory responses adaptively. However, cAMP is reported to be downregulated in most CVDs (1, 12); hence forskolin (FSKN) was utilized as a treatment intervention to ensure its bioavailability and explore the possible therapeutic outcomes.

Herein, we demonstrated that AMLX and FSKN combination is the most potent at attenuating the progression of LVSD and enhancing cardiac function recovery in ICM models. We also showed that this treatment intervention facilitates cardiac fibrosis resolution and adaptively modulates myocardial inflammatory.

Abbreviations: AC, Adenylyl cyclase; AMLX, Amlexanox; cAMP, cyclic adenosine monophosphate; FSKN, Forskolin; GRK, G protein-coupled receptor kinases; ISO, Isoproterenol; LPS, Lipopolysaccharide; LVSD, Left ventricular systolic dysfunction; PM_Φ, Peritoneal macrophage; PbT, Placebo treatment.

MATERIALS AND METHODS

Experimental Animals and Models

Mice (FVB males aged 2–3 months) were randomly grouped for *in vivo* experiments. 0.5 mg/100 g/day of ISO were injected subcutaneously (s.c.) as done previously (2, 13), for 21 days to induce ICM in the mice. The 5% v/v dimethyl sulfoxide equivalents were injected (s.c.) to the placebo (Pb) group.

ICM recovery was initiated after day 21 of ISO administration. The recovery groups included; placebo treatment (PbT) [5% v/v dimethyl sulfoxide (DMSO)], 2.5 mg/100 g/day of AMLX (Abcam; ab142825) treatment and 0.5 mg/100 g/day of FSKN (Tocris Bioscience, UK; 1099) treatment. Also, AMLX and FSKN combine treatments were employed (**Figure 1A**). The dosages of AMLX and FSKN employed were based on their efficacies demonstrated in previous studies (2, 11).

Euthanization of mice for heart and peritoneal macrophage (PM_Φ) isolations were done by cervical dislocation.

Electrocardiography and Echocardiography

Mice (n = 6–8 per treatment group) were sedated with 0.5% isoflurane and secured with echo gel. The body temperatures were maintained at 37°C to enable electrocardiography (ECG) data acquisition using Vevo 2100 Ultrasound system (VisualSonics, Canada). Simultaneously in M-mode, systolic cardiac function indexes; Ejection fraction (EF), and fractional shorten (FS) were assessed as previously described (14). Also, left ventricle internal diameters, posterior wall, and interventricular septal wall thicknesses were assessed at end-systole and end-diastole.

Histological Analysis of Hypertrophy, Interstitial Fibrosis, and Immune Cells Infiltration

Excised hearts (n = 6–8 mice per group) were rinsed, fixed overnight in 10% formaldehyde, embedded in paraffin, and sectioned. Wheat germ agglutinin (WGA) (Thermo Fisher; W11261), Masson's trichrome (Solarbio; G1340), and H&E (Solarbio; G1120) staining were done to ascertain cardiomyocyte hypertrophy and interstitial fibrosis deposition. Also, CD68 (Abcam; ab955), CD86 (Abcam; ab53004), and CD206 (Abcam; ab8918) immunohistochemical (IHC) staining were then performed to evaluate myocardial inflammatory cells infiltration. Microscopies were performed at x400 magnification and analyzed using ImageJ (1.53 h version; National Institute of Health).

Enzyme-Linked Immunosorbent Assay

Sera (n = 4–8 mice per group) were used to evaluate concentrations of inflammatory mediators and cytokines; iNOS (JL20675; Jianglai Bio, Shanghai), Arg-1 (JL13668; Jianglai Bio, Shanghai), IL-1β (Abcam; ab197742), IL-6 (Abcam; ab222503), TNFα (Abcam; ab208348), IL-10 (Abcam; ab255729), TGF-β (Proteintech; KE10005). cAMP bioavailability was assessed with cAMP ELISA kits (JL13362; Jianglai Bio, Shanghai). Also, myocardial lysates (n = 6–8 mice per group) were used to evaluate ANP (JL20612; Jianglai Bio, Shanghai) and BNP (JL12884; Jianglai Bio, Shanghai) concentrations. The assays were performed as per the manufacturer's instructions and in triplicates.

Western Blot

Concentrations of lysates obtained from apical myocardia (n = 4 hearts per group) were normalized, treated with loading buffer, and proteins denatured at 100°C for 10 min. The proteins were separated were SDS-PAGE gels, transferred on PVDF membranes which were blocked and blotted overnight with the following antibodies: Collagen Type I (Proteintech; 14695-1-AP), Collagen Type III (Proteintech; 13548-1-AP), ANP (Santa Cruz Biotechnology; sc-515701), BNP (Santa Cruz Biotechnology; sc-271185), Cleaved Caspase-3 (Cell Signaling Technology; 9661T) and GAPDH (Proteintech; 10494-1-AP). Western blots were performed in triplicates and normalized with their respective loading controls.

Immune Cells Isolation and Culture

Macrophages ($n > 1^{*}10^{6}$ cells per group) were isolated from the mice peritoneal as previously described with slight modifications (15). Prewarmed (37°C), 5–10 ml of 3% fetal bovine serum (FBS) were carefully injected into the peritoneal cavity. After 5 min of softly massaging the peritoneum, the fluids were collected from the cavity and centrifuged at 1,500 rpm for 10 min. The cell pellets were resuspended and cultured with 10% FBS for 48 h. Macrophage identifications were done with F4/80 (BioLegend; 123116) and CD11b (BioLegend; 101206). Subsequently, the cultured PM_{Φ} were treated with 10 μM/ml of ISO and/or 1 μg/ml LPS for 12 h, and the following treatment interventions were employed for the next 12 h. (i) 0.5 % v/v DMSO as placebo (ii) 35 μM/ml of AMLX, (iii) 10 μM/ml FSKN, and (iv) AMLX and FSKN combination, at the respective doses. These treatment interventions were explored in the absence of ISO (stress), and

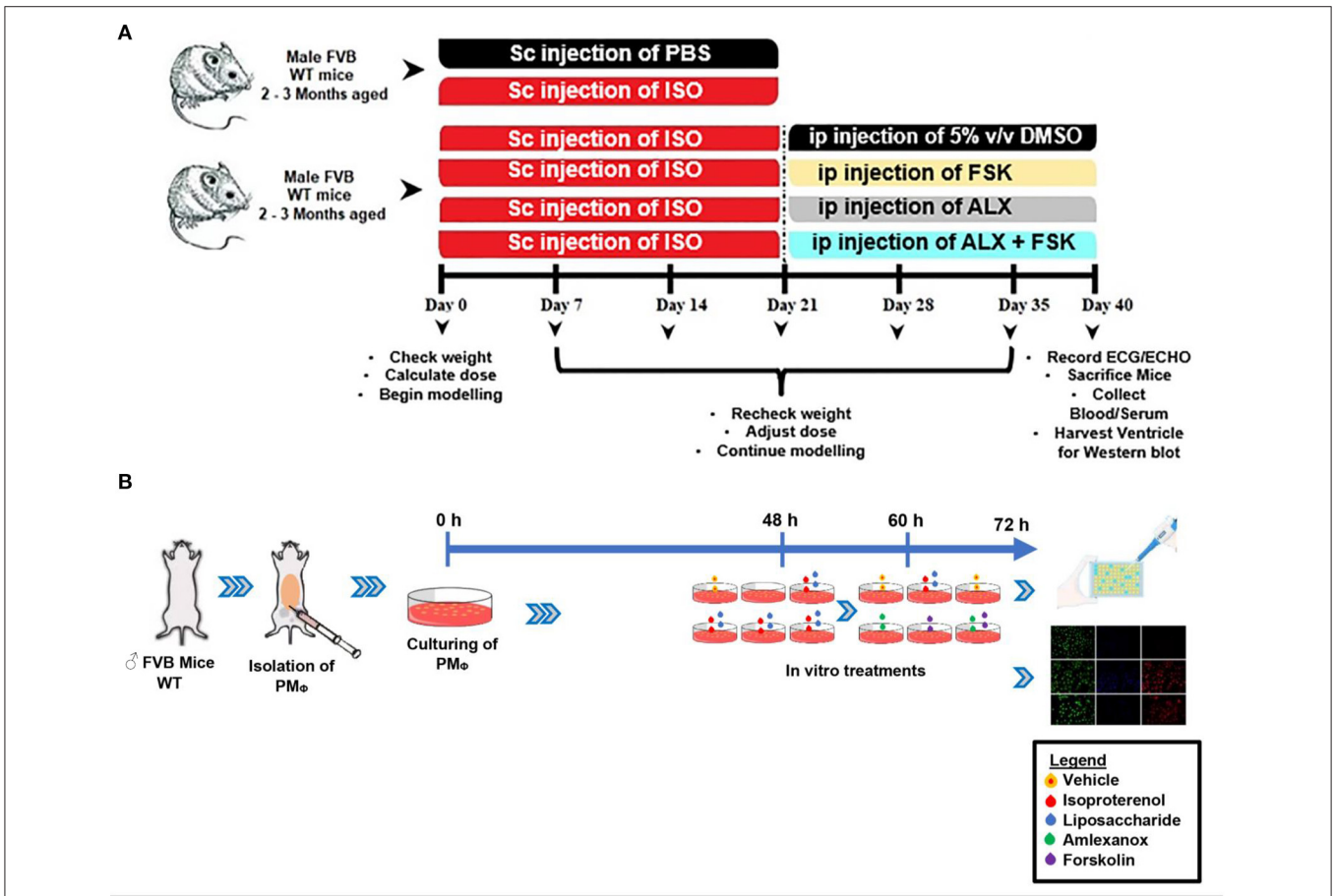

FIGURE 1 | (A,B) Schematic of the experiment design timeline for making *in vivo* and *in vitro* models, respectively. ISO, Isoproterenol; Sc, Subcutenous; ip, intraperitoneal; FSK, forskolin; ALX, Amlexanox.

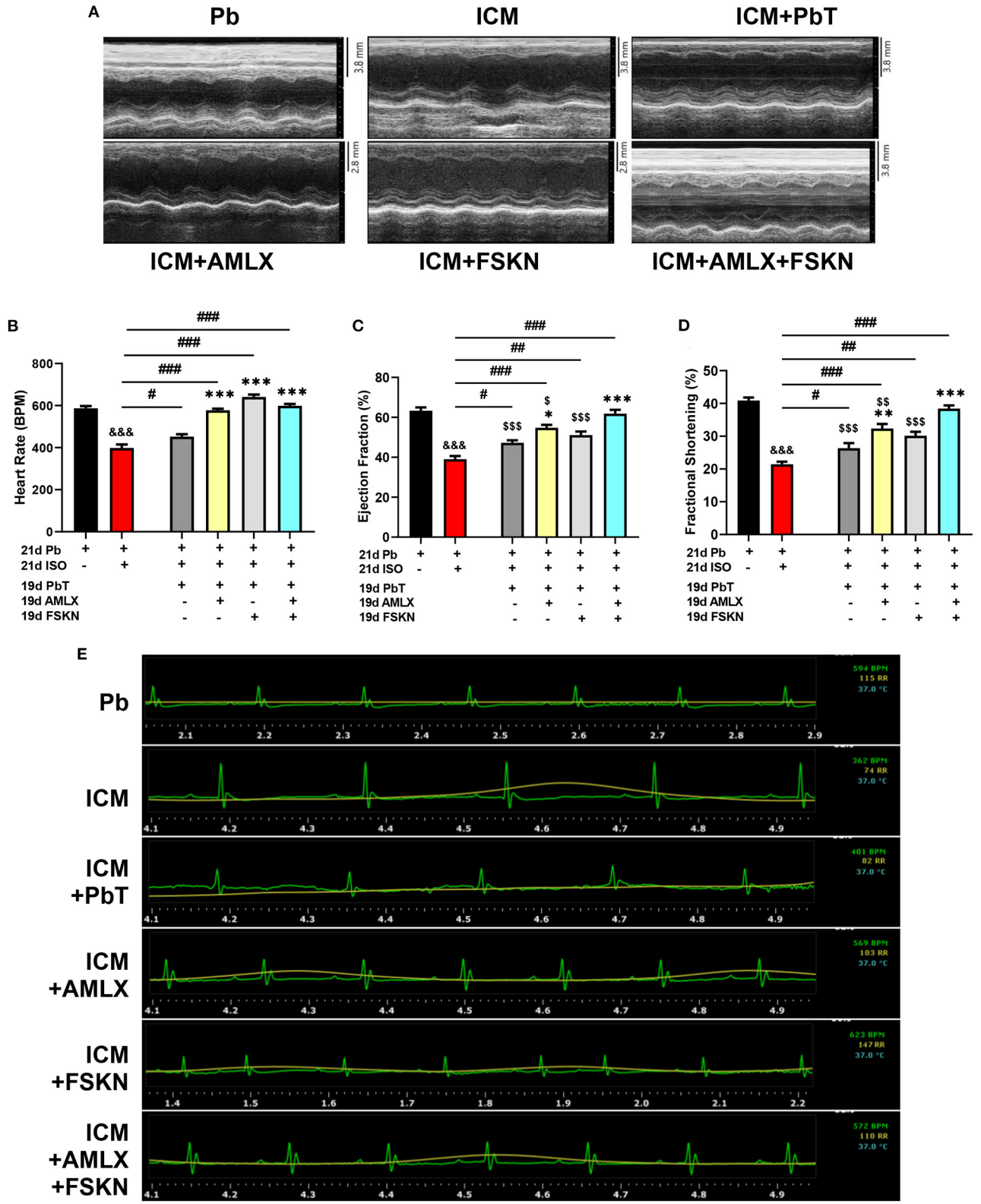

FIGURE 2 | Amlexanox and Forskolin ameliorates isoproterenol-induced LVSD. **(A)** Representative echocardiogram imaging obtained in short-axis view M-mode from all the experimental groups. **(B)** Graphical presentation of heart rates assessments from electrocardiography. **(C,D)** Graphical presentation of cardiac systolic function

(Continued)

FIGURE 2 | indexes (Ejection fraction and Fractional shortening, respectively), assessed at the end of treatments (n = 6–8 mice per treatment group). **(E)** Representative electrocardiogram imaging from Pb, ICM, ICM+PbT, ICM+AMLX, ICM+FSKN, and ICM+AMLX+FSKN groups displaying heart rate (Green), respiration rate (yellow) and temperature (turquoise). $^{\&\&\&}p < 0.001$ vs. Pb; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. ICM+PbT; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. ICM+AMLX+FSKN. Data are expressed as mean ± SEM. Data were analyzed using one-way ANOVA and Tukey's *post-hoc* analysis.

the dosages employed were based on their efficacy in previous studies (2, 16). Supernatants and PM_{Φ} were used for ELISA and immunofluorescence, respectively (**Figure 1B**).

Immunofluorescence Staining

Fixation and permeabilization of pre-treated PM_{Φ} ($n > 1^{*}10^{6}$ cells per group) were done using pre-chilled methanol-acetone (ratio 1:1). The cells were blocked with 1% BSA, incubated overnight at 4°C with GRK5 antibody (ab64943; Abcam), and probed with R-PE-conjugated antibody (Proteintech; SA00008-2) at room temperature for 1 h. Next, the cells were wash and conditioned with 0.5% BSA in Hanks' balanced salt solution. Cholera toxin B (CTxB) (Thermo Fisher. C34775) was used to stain the cytoplasmic membranes for 30 min at 4°C and then counterstained with DAPI. GRK5 localization and expression ratios were ascertained using ImageJ (n = 12–15 cells per 4 mice).

Statistical Analysis

Using GraphPad Prism (Prism Version 8.0.2), one-way ANOVA was used to analyze data among experimental groups and followed by Tukey's multiple comparisons test. All data were expressed as mean ± SEM. $P < 0.05$ were assigned statistical significance.

RESULTS

Amlexanox and Forskolin Ameliorates Isoproterenol-Induced Left Ventricular Systolic Dysfunction

Results obtained from systolic function assessment using echocardiography demonstrated that the chronic administration of ISO induced LVSD as depicted in the ICM group. The LVSD observed in the ICM group is characterized by a significant decrease in heart rates (HR), EF, and FS (**Figures 2A–E**). Although these indexes are observed to have slightly improved in the placebo recovery treatment group (ICM+PbT), which were only given the vehicle for 19 days after the ISO injections were discontinued, cardiac functions were not fully restored as HR, EF, and FS were below their stated normal ranges (17). However, treatment of the ICM models with AMLX improved recovery from the LVSD by increase HR, EF, and FS. Contrarily, FSKN treatment results in tachycardia and arrhythmias even though EF and FS were increased in the ICM+FSKN group. Ultimately, the treatments of the ICM mice with AMLX and FSKN combination significantly enhanced the attenuation of the LVSD and hastened cardiac function recovery as HR, EF, FS, and other cardiac function indexes were all restored to their normal ranges (**Figures 2A–E** and **Supplementary Table 1**).

Amlexanox and Forskolin Attenuates Isoproterenol-Induced Maladaptive Cardiac Hypertrophy and Promotes Fibrosis Resolution

Cardiac morphometrics (**Supplementary Table 1**), histological assessment of cardiomyocyte diameter (**Figures 3A,B** and **Supplementary Figures 1A,B**), and ELISA and immunoblots of hypertrophy biomarkers (ANP and BNP) (**Figures 3C,D** and **Supplementary Figures 1C–E**) showed that chronic administration of ISO induced cardiac hypertrophy markedly. Cardiomyocyte diameters, ANP, and BNP levels were significantly increased in the ICM group. The placebo treatments (PbT) given to ICM models failed to significantly decrease the cardiomyocyte diameters after day 19; however, AMLX treatment in the AMLX+ICM group showed overt attenuation of the maladaptive cardiac hypertrophies that were observed in the ICM mice. Similarly, cardiomyocytes diameters and expression of the natriuretic peptides were observed to have decreased the FSKN treatment group, however, with lesser significance ($p < 0.05$) as compared to AMLX treatment ($p < 0.001$) and its combined treatment with FSKN ($p < 0.001$).

Also, trichrome staining of tissue sections from the ICM hearts shown typical distortions of the myocardial architecture as cardiomyocyte apoptosis and interstitial fibrosis were massively increased (**Supplementary Figures 1C,F** and **Figures 3E–G**). The placebo treatments in the ICM+PbT group showed less significance in resolving the cardiomyocyte deaths and fibrosis. However, similar to AMLX, FSKN administration to the ICM mice significantly decreased both apoptosis and collagen I and III depositions in the myocardia (**Supplementary Figures 1C,F** and **Figures 3H–J**). Intriguingly, AMLX and FSKN combination demonstrated trends of improved apoptosis attenuations and fibrosis resolutions, although there were no statistical significances compared with the single therapeutic groups.

Amlexanox and Forskolin Enhances Attenuation of Isoproterenol-Induced Maladaptive Myocardial Inflammation

IHC assessment of CD68 (total inflammatory cells), CD86 (proinflammatory cells), and CD206 (anti-inflammatory cells) from myocardial sections revealed enormous inflammatory cells infiltration into the hearts of ICM mice (**Figures 4A,B**).

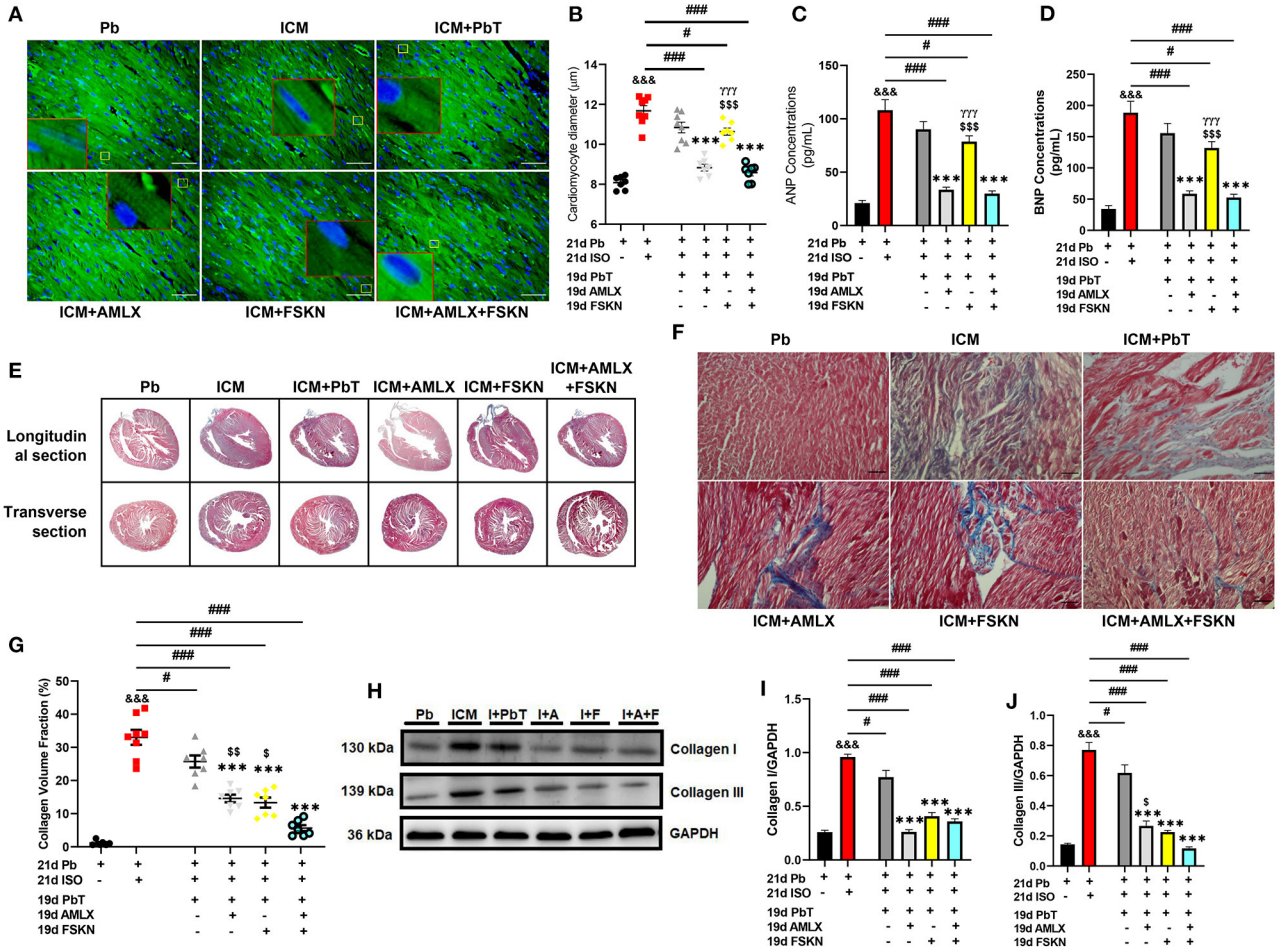

FIGURE 3 | Amlexanox and Forskolin attenuates isoproterenol-induced maladaptive cardiac hypertrophy and promotes fibrosis resolution. **(A,B)** Representative wheat germ agglutinin (WGA) merged with DAPI staining and graphical presentations of measured cardiomyocyte diameter from all treatment groups (n = 10–12 cells per 5 field of view per 5 sections per 6–8 hearts per group). Representative cardiomyocytes are outlined in yellow boxes, and their zoomed-in (7x) inserts to show hypertrophy are outlined in red boxes. **(C,D)** Graphical plots of ELISA evaluations of cardiac hypertrophy markers (ANP and BNP, respectively), from obtained myocardial lysate. The ELISA were performed in triplicates (n = 6–8 mice per treatment group). **(E,F)** Representative Masson's trichrome stained longitudinal and transverse sections with detailed microscopic images showing interstitial collagen deposition. **(G)** Graphical plots collagen volume fraction from all groups (n = 6–8 field of view per 5–7 sections per 6–8 hearts per group). **(H–J)** Representative immunoblots and graphical presentations of Collagen I and Collagen III from Pb; placebo, ICM; isoproterenol-induced cardiomyopathy, I+PbT; ICM + placebo treatment, I+A; ICM + AMLX treatment, I+F; ICM + FSKN treatment and I+A+F; ICM + AMLX + FSKN combine treatment groups (n = 4 hearts per treatment group). $^{\&\&\&}p < 0.001$ vs. Pb; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$; $^{***}p < 0.001$ vs. ICM+PbT; $^{\text{ŸŸŸ}}p < 0.001$ vs. ICM+AMLX; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. ICM+AMLX+FSKN. Data are expressed as mean ± SEM. Data were analyzed using one-way ANOVA and Tukey's *post-hoc* analysis.

It was observed that ICM hearts had biased proinflammatory cell infiltrations while anti-inflammatory cell infiltrations were significantly reduced. After ISO discontinuation, the placebo treatment administration (in the ICM+PbT group) did not attenuate the maladaptive inflammatory responses as AMLX, FSKN and their combination treatment did. AMLX, FSKN, and their combination increase anti-inflammatory (CD206+) cells and decrease the infiltration of proinflammatory (CD86+) cells into the myocardial. ELISA of proinflammatory mediator, inducible nitric oxide synthase (iNOS) and cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNFα) validated the occurrence of maladaptive inflammatory responses in the ICM model. The results also showed that AMLX and FSKN were potent at downregulating the proinflammatory responses (**Figures 4C–F**). Furthermore, the anti-inflammatory markers, arginase-1 (Arg-1), transforming growth factor-beta (TGF-β), and IL-10 were downregulated in ICM mice. However, AMLX and FSKN treatments upregulated these anti-inflammatory markers more significantly than was observed in the placebo treatment group (**Figures 4G–I**). Individually, AMLX and FSKN treatments effectively modulated inflammatory responses; however, their combination showed trends of further enhanced immunoregulatory potencies but without statistical significance.

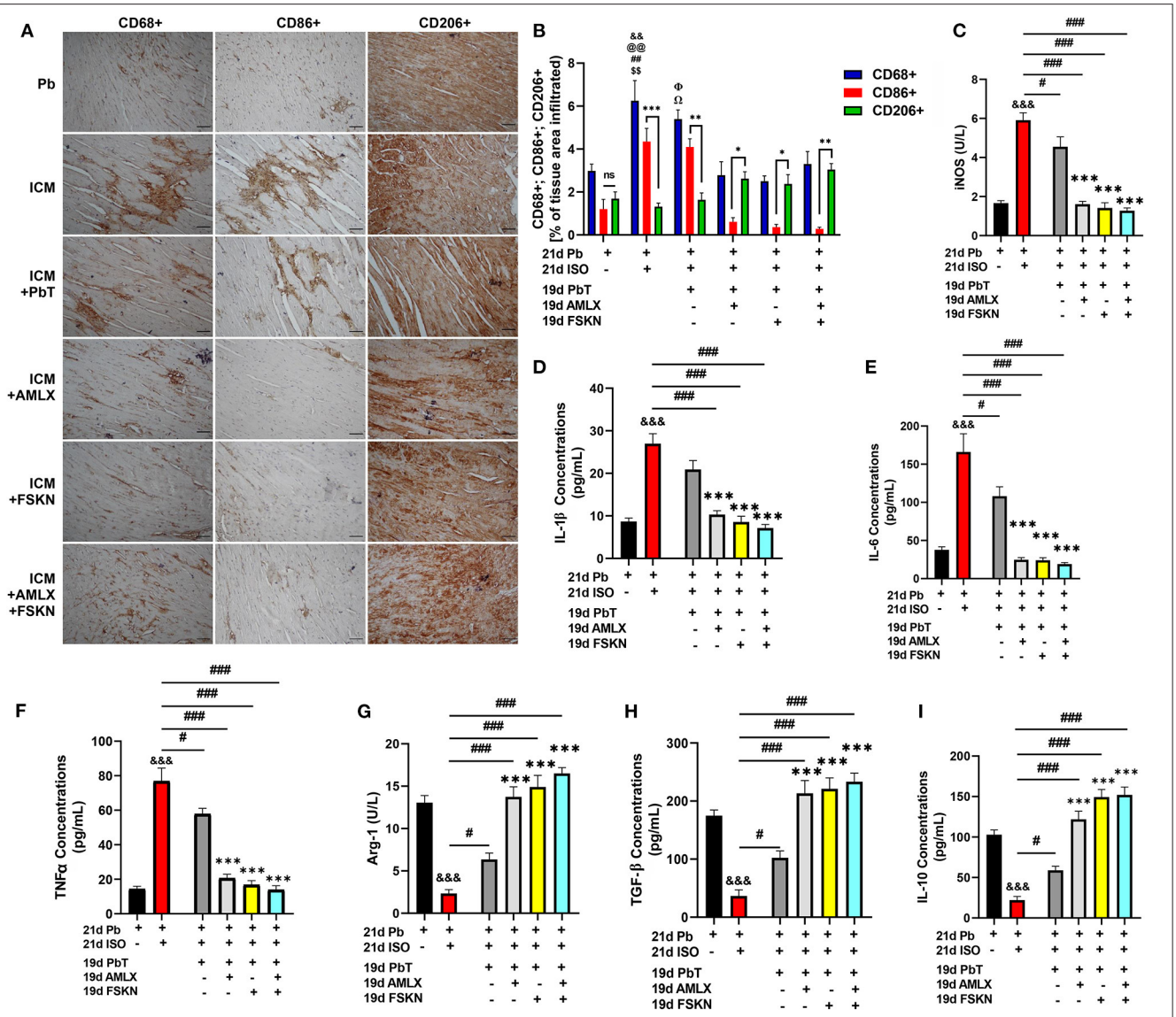

FIGURE 4 | Amlexanox and Forskolin attenuates isoproterenol-induced myocardial inflammation by repressing proinflammatory mediators and responses. **(A,B)** Representative CD68+, CD86+, and CD206+ IHC staining and graphical presentations of their evaluations demonstrate the extent of inflammatory cell infiltrations into the myocardia and the ratio between pro and anti-inflammatory cells evading the myocardia among the experimental groups. $^{ns}p \geq 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $^{\&\&}p < 0.01$ vs. Pb; $^{\$\$}p < 0.01$ vs. ICM+ AMLX; $^{\#\#}p < 0.01$ vs. ICM+ FSKN; $\Omega p < 0.05$ vs. ICM+ AMLX; $\Phi p < 0.05$ vs. ICM+FSKN; $@@p < 0.01$ vs. ICM+FSKN+AMLX **(C–F)** Graphical plots of proinflammatory mediator (iNOS) and cytokines (IL-1β, IL-6 and TNFα). **(G–I)** Graphical plots of the anti-inflammatory mediator (Arg-1) and cytokines (TGF-β and IL-10). The sera ELISA were performed in triplicates ($n = 6$–8 mice per treatment group). $^{\&\&\&}p < 0.001$ vs. Pb; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$; $^{***}p < 0.001$ vs. ICM+PbT. Data are expressed as mean ± SEM. Data were analyzed using one-way ANOVA and Tukey's *post-hoc* analysis.

ALX and FSK Inhibits Proinflammatory Responses *via* G Protein-Coupled Receptor Kinase 5 Inhibition and Synergistic Upregulation of Cyclic Adenosine Monophosphate

Elucidations of the mechanisms employed by the treatment interventions were explored using PM_{Φ}. After 12 h of ISO+LPS treatment, the PM_{Φ} were incubated with AMLX (35 μM/ml) and/or FSKN (10 μM/ml) for the next 12 h. GRK5 nucleic-cytosolic expression ratios assessed showed its upregulation and increased translocation to the nuclei after LPS+ISO treatments. Unlike the placebo (PbT) and FSKN treatment, AMLX inhibited GRK5 expression and translocation. Similarly, GRK5 activities were inhibited in the AMLX and FSKN combination treatment group (**Figures 5A,B**). Assessing the impact of these treatments on cAMP bioavailability demonstrated its significant upregulation in the AMLX and

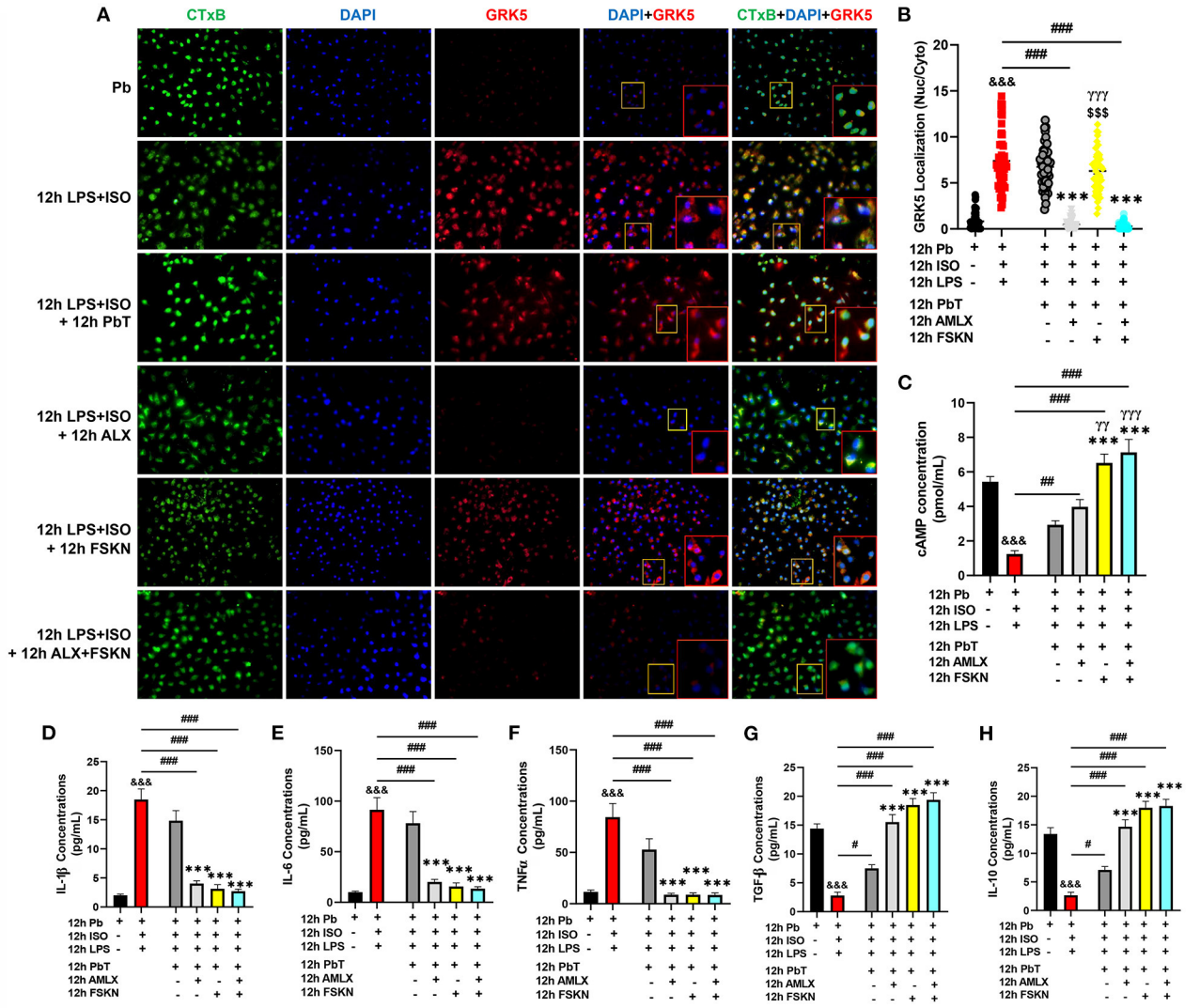

FIGURE 5 | Amlexanox and Forskolin inhibits hyperactive proinflammatory responses *via* G protein-coupled receptor kinase 5 (GRK5) inhibition and synergistic upregulation of cyclic adenosine monophosphate (cAMP). **(A)** Representative immunofluorescence of GRK5 localizations, cytoplasmic membrane (CTxB), and nuclei (DAPI). **(B)** The plotted values are the GRK5 (nuclear/cytoplasm) expression ratios assessed from each PM Φ (n = 12–15 cells per 4 mice per group). Color channels were adjusted in the merged images to enhance the visualization of all the respective fluorescence dyes. **(C)** Graphical plots of evaluated cAMP concentrations to assess the effect of the treatment interventions cAMP bioavailability. **(D–F)** Graphical plots of ELISA evaluated proinflammatory cytokines (IL-1β, IL-6, and TNFα) concentrations. **(G,H)** Graphical presentations of ELISA evaluated anti-inflammatory cytokines (TGF-β and IL-10) concentrations. The sera ELISA were performed in triplicates ($n \geq 1*10^6$ cells per 4 mice per group). $^{\&\&\&}p < 0.001$ vs Pb; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; $^{***}p < 0.001$ vs. ICM+PbT; $^{\gamma\gamma}p < 0.01$, $^{\gamma\gamma\gamma}p < 0.001$ vs. ICM+AMLX; $^{\$\$\$}p < 0.001$ vs. ICM+AMLX+FSKN. Data are expressed as mean ± SEM. Data were analyzed using one-way ANOVA and Tukey's *post-hoc* analysis.

FSKN combination treatment group, followed by FSKN and AMLX treatments groups. cAMP concentrations were also increased in the LPS+ISO+PbT group but exhibited no statistical significance compared to the LPS+ISO group (**Figure 5C**). Additionally, cytokines analyses showed sustained significant upregulation of proinflammatory cytokines (IL-1β, IL-6, and TNFα) in LPS+ISO and LPS+ISO+PbT groups while the anti-inflammatory cytokines (TGF-β and IL-10) were downregulated (**Figures 5D–H**). Meanwhile, the contrasts of these phenomena were observed in the ISO+LPS+AMLX, ISO+LPS+FSKN, and ISO+LPS+AMLX+FSKN groups, as these treatment interventions adaptively heightened the anti-inflammatory and dampened the proinflammatory cytokines secretions. Hence, it is inferred that AMLX attained its potency via inhibiting GRK5-mediated proinflammation response activation and facilitating cAMP-mediated immunoregulation, while FSKN treatment employed only the latter. As such, the potencies attained by the AMLX and FSKN combination in the absence of stress were *via* GRK5 inhibition and the synergistic upregulation of cAMP to adaptive immunoregulatory responses.

DISCUSSION

Chronic stress, a resultant from the demands of our modern-day human societies, induces ICM, which ultimately results in heart failure (HF). This study aimed to explore the therapeutic potentials of AMLX in attenuating LVSD and facilitating recovery from ICM, as demonstrated in the AMI model (11). Also, we sought to, for the first time, explore if FSKN could enhance the recovery rates of the ICM model, as it stimulates the synthesis of cAMP, which is an essential modulator of cardiac and inflammatory responses (1, 2, 12, 18).

The results obtained from cardiac function assessments using ECG and ECHO revealed that HR, EF, and FS were significantly reduced in the ICM groups, which are indicative of the occurrence of LVSD. Comparing the obtained values for these clinically relevant cardiac function indexes with other similar studies (19), it could be remarked that the hearts of mice from the ICM group were progressing into HF. The administration of 5%v/v DMSO as a placebo treatment for 19 days after discontinuing ISO injection did not restore HR, EF, and FS to their normal ranges. However, AMLX treatment improved HR and systolic functions, which are consistent with Mo et al.'s findings (11). Nonetheless, our previous study found that when AMLX treatments were given simultaneously with ISO injections, AMLX's efficacy in attenuating LVSD was dampened (2). Hence, it is speculated that for AMLX treatment to be effective at attenuating LVSD in ICM, the sources of stressors must be obliterated. Also, FSKN treatments resulted in tachycardia and arrhythmias. In conformity with these findings, Christ et al. and Huang et al. had early demonstrated the arrhythmic side effects of FSKN treatment (20, 21). Intriguing, AMLX, and FSKN combination demonstrated the most potency in restoring systolic functions in the ICM mice.

Furthermore, assessments of cardiac morphometric data, cardiomyocyte diameter from histological analysis, and expressions of hypertrophy biomarkers (ANP and BNP) provided additional evidence that ICM is characterized by a pathologically hypertrophied heart (8, 22). Again, treatment of the ICM mice with placebo demonstrated an insignificant decrease in cardiomyocytes' diameters. In contrast, AMLX treatment showed overt attenuation of the hypertrophy, which were validated as the natriuretic peptides ANP and BNP expressions well also downregulated, as previously reported (2, 23, 24). Consistent with the findings of Gesmundo and Miragoli (25), FSKN treatment *via* AC/cAMP/PKA exerted slight anti-hypertrophic effects, but these were less significant as compared to AMLX treatment. Hence, the AMLX and FSKN combine treatment attenuated maladaptive cardiac hypertrophy in ICM mice synergistically.

Clinically, the LVSD occurring in patients with ICM is attributed to increased interstitial fibrosis, which disrupts the typical myocardial architecture and causes stiffness of the heart (7, 26). The increased fibrosis ultimately impedes the heart from rapidly replenishing a sufficient amount of blood for the subsequent ejection (27). In conformity with the characterization of the myocardial remodeling occurring in ICM patients, histological assessments of hearts from the ICM models showed the occurrence of massive interstitial fibrosis. Therefore, the synergy of maladaptive ventricular hypertrophy and increased fibrosis in the ICM heart might constitute underlying factors contributing to the LVSD. Individually, AMLX and FSKN treatments promoted myocardial fibrosis resolution effectively compared to the placebo (in ICM+ PbT) (**Figures 3E–G**). Zhou et al. had previously shown the anti-fibrotic potencies of AMLX treatment (28). Similarly, consistent with our findings, FSKN treatments were shown by El-Agroudy et al. and Roberts et al. to exert anti-fibrotic effects by inhibiting fibroblast activation, proliferation, and differentiation (29, 30). As such, by coupling their anti-fibrotic effects, AMLX and FSK combination further enhanced myocardial fibrosis resolution significantly, as evidenced by the more decreases in collagen expression and deposition (**Figures 3E–J**).

Recent therapeutic interventions aimed at attenuating pathological remodeling of the heart are targeted at the adaptive modulation of myocardial inflammatory responses, besides preserving and sustaining cardiomyocyte function (11). This is due to the fact that maladaptive myocardial inflammatory responses have been implicated in expediting the adverse remodeling of the heart in most CVDs (1, 11, 31, 32). Therefore, the myocardia of ICM mice were histologically assessed to ascertain the extent of inflammatory cell infiltrations. As demonstrated in the aforementioned studies, enormous amounts of inflammatory cells (CD68 positive) were found in the ICM hearts, of which the majority were CD86 positive cells and accompanied by fewer CD206 positive cells. Individually, AMLX and FSKN treatments administered to the ICM models exert adaptive immunoregulatory effects in the myocardia. Both significantly downregulated CD86 positive cells and comparatively increased CD206 positive cells infiltration to facilitate timely resolution of the myocardial inflammation. In addition, it was observed that the AMLX and FSKN treatments decreased iNOS, IL-1β, IL-6, and TNFα while upregulating Arg-1, TGF-β, and IL-10. Likewise, their combination treatment synergistically exerted anti-inflammatory effects to timely resolve the observed myocardial inflammation in the ICM models. In conformity with these findings, previous studies have extensively demonstrated the anti-inflammatory effects of AMLX and FSKN (11, 18, 33). Again, it is worth mentioning that, in our previous study, where AMLX treatments were administered during ISO-induced stress, it failed to facilitate anti-inflammatory responses effectively (2). As such, the elimination of stressors is crucial as they affect the anti-inflammatory potency of AMLX.

Previously, we had reported that rather than the individual treatments of AMLX and FSKN, their combination was the most potent treatment intervention for preventing ICM during chronic stress (2). However, findings from this explorational study which sought to find the therapeutic potentials of AMLX and FSKN in promoting recovery from ICM, showed the following. (1) Comparatively, AMLX treatment improved cardiac functions in the ICM recovery model but failed to sustain these functions during stress in ICM preventive models. (2) AMLX treatment had failed to inhibit the upregulation of proinflammatory responses in ICM preventive models during chronic catecholamine stress but comparably

exerted adaptive immunoregulation in the myocardial of ICM recovery models in the absence of stress. (3) Lastly, although AMLX and FSKN combination treatment showed increased adaptive immunoregulation trends, there were no statistical significance among it and the individual treatments with AMLX and FSKN.

Hence, further experiments were performed to elucidate the possible underlying mechanisms for the observed outcomes. By mimicking cardiac damage-associated molecular patterns with LPS during stress, hyperactive proinflammatory responses were elicited from PM_{Φ}. The GRK5 nucleic–cytosolic expression ratios assessed in PM_{Φ} after followed-up treatments with AMLX and FSKN and their combination showed similar results as previous (2). Only AMLX in both single and combination treatment repressed GRK5 expression and translocation, while FSKN failed to do these similarly to the placebo. Additionally, evaluated cAMP concentration from the culture supernatants after the treatment intervention showed significant upregulations in AMLX and FSKN and their combination treatment groups, but not in the placebo (ISO+LPS+PbT) group. While FSKN is well-demonstrated to facilitate cAMP synthesis by directly activating adenylyl cyclase activity (20, 34), finding cAMP bioavailability being facilitated by AMLX was intriguing as this phenomenon was observed previously under catecholamine stress condition (2). However, consistent with the finding, Han et al. had previously shown that besides GRK5, AMLX non-selectively inhibits phosphodiesterase (PDE), which degrades cAMP (35). As such, by impeding the degradation of cAMP by PDE, AMLX sustained cAMP bioavailability in the absence of stress, as demonstrated here. Followed-up cytokines analyses revealed the effective attenuation of proinflammatory responses (IL-1β, IL-6, and TNFα) and the upregulation of TGF-β and IL-10 by AMLX and FSKN and their combination treatment. Therefore, inferences made from the findings in **Figure 5** confirm that ALX and FSK treatment enhanced the resolution of maladaptive myocardial inflammation by inhibiting GRK5-mediated inflammation and synergistic enhancement of cAMP bioavailability which exerted adaptive immunoregulation and promoted recovery from ICM.

Taken together, the findings from this study demonstrate that treating ICM models with AMLX and FSKN combination enhances the recovery outcomes by attenuating LVSD and timely resolving both myocardial inflammation and fibrosis. However, the source of stressors must be eliminated during the recovery treatment as it undermines the efficacy of AMLX. Also, due to the clinical significance of this study, it is recommended that the toxicity of AMLX and FSKN combination is determined, and its effect on diastolic function and lung congestion be evaluated to ascertain their therapeutic and translational potentials fully.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Centre of Xuzhou Medical University and the Animal Ethics Committee of the Medical University (permit number: xz11-12541).

AUTHOR CONTRIBUTIONS

GKA conceived the experiment ideas. With HS's supervision, GKA designed the experiments. HJH and AOA provided experimental animals. RR, JA-A, and WS assisted in making animal models. KL and Q-MD performed cardiac function assessments. GKA, JA-A, RM, and MLNN performed furthers experiments. GKA, WS, and Q-MD analyzed and interpreted data. GKA wrote the manuscript based on contributions from all authors. GKA, HJH, AOA, RR, JA-A, WS, RM, and MLNN proofread and all authors approved the 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.2021.719805/full#supplementary-material

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Remote Conditioning by Rhythmic Compression of Limbs Ameliorated Myocardial Infarction by Downregulation of Inflammation *via* A2 Adenosine Receptors

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Background: Remote ischemic conditioning (RIC) is a cardioprotective phenomenon, yet transient ischemia is not a requisite trigger for remote cardioprotection. In fact, RIC is a stimulus compound containing interruption of the blood vessel and tissue compression. In this study, we evaluate the effects of remote tissue compression on infarct size after myocardial infarction and explore its preliminary mechanisms.

Methods and Results: We used a murine model of myocardial infarction to assess ischemia injury and identified remote conditioning by rhythmic compression on forelimb as a novel cardioprotective intervention. We show that the cardioprotective signal transduction of remote conditioning from the trigger limb to the heart involves the release of adenosine. Our results demonstrate that A2a and A2b receptors are indispensable parts for cardioprotection of remote conditioning, which is linked to its anti-inflammatory properties by the subsequent activation of cAMP/PKA/NF-κB axis.

Conclusion: Our results establish a new connection between remote tissue compression and cardiovascular diseases, which enhances our cognition about the role of tissue compression on RIC cardioprotection.

Keywords: remote tissue compression, myocardial infarction, adenosine, A2 receptor, signal transduction, anti-inflammatory

INTRODUCTION

Acute myocardial infarction (AMI) is a condition of acute ischemic necrosis of myocardial tissues, which contributes significantly to morbidity and mortality on a global scale (1). Over the past 3 decades, various strategies have been developed to protect the heart against AMI (2). Remote ischemic conditioning (RIC), especially, has been established in many experimental studies and shown to be cardioprotective. Importantly, RIC by brief episodes of ischemic or reperfusion on an extremity reduces infarct size and improves the prognosis of AMI patients (3). Regarding RIC, a variety of evidence from a spectrum of models suggests that the interruption of blood flow is not an essential trigger for remote cardioprotection (3). Apart from the cycles of transit ischemia, RIC also involves mechanical stimuli through tissue compression (3). However, the role of tissue compression in cardioprotection of RIC is rarely studied.

Indeed, mechanical manipulation of body tissues by rhythmical compression involves the immune system and that this can be used as an adjunct treatment for the variety of mental and physical conditions (4, 5). The recent experimental evidence suggested that cycles of compression temper the increase in the number of cells expressing pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1(MCP-1) (6). It has been demonstrated that tissue compression acts on mechanoreceptors in the skin to influence the release of soluble messengers that are considered responsible for mediating the immune response (7). Specifically, chiropractic manipulation and massage might be associated with an efflux of cytosolic ATP that is sufficient to elevate extracellular adenosine, which is also a critical trigger and also a mediator in RIC-induced cardioprotection (8–10). Adenosine is a master regulator of energy metabolism in an emerging paradigm of immunity, where recognition of cell stress initiates and inhibits inflammation (11). In the previous studies, we found that the reduction of myocardial inflammation was associated with decreased infract size and improved cardiac function (12). Of the 4 adenosine receptor subtypes, A2 receptors are proposed to act as the triggers of the emergency downregulation of overactive inflammatory response (13). What is more, some studies directly showed that adenosine analogs selectively targeting A2 receptors are promising to protect the heart against myocardial infarction (14).

As mentioned above, it is reasonable to think that tissue compression could regulate cardioprotective effect of RIC by its immunomodulatory function. To test this hypothesis, we designed a murine model showing remote conditioning using rhythmic stimulation of limbs protect myocardial infarction by the downregulation of inflammation *via* adenosine-mediated activation of A2 adenosine receptors and consequent activation of cAMP/PKA signaling pathway inhibiting NF-κB-mediated expression of inflammatory cytokines, which in favor of developing an easy implement and efficient cardiac conditioning therapy with no or fewer adverse events.

MATERIALS AND METHODS

Experimental Animals

Adult male Sprague-Dawley (SD) rats weighing 260 ± 20 g were obtained from Charles River Laboratories (SCXK 2016-0006) and housed in a standard environment, with the temperature of 25 ± 2°C, relative humidity of 50 ± 5%, and light–dark cycle for 12 h. The study protocol was in accordance with the Guide for the Care and Use Committee at the Nanjing University of Chinese Medicine.

Establishment of AMI in Rats

Briefly, rats were anesthetized with 5% isoflurane and oxygen with a flow rate of 0.4 L/min until the loss of righting reflex. They were then placed supine on a temperature-controlled experimental board set at 37 ± 3°C and maintained by 2% isoflurane in 100% oxygen with a flow of 0.4 L/min by means of intubation connected to a small animal ventilator (R407, RWD, China) set at a respiratory rate of 60–70 breaths per minute. After disinfecting the surgical area, a longitudinal incision was made to expose the heart. Left anterior descending (LAD) coronary artery was ligated between the pulmonary artery and the left atrial appendage with 6.0 silk suture to induce ischemia. Decreased ventricular wall motion, pale left ventricular wall, and elevated ST-segment confirmed a successful establishment of AMI model in rats.

Remote Conditioning by Rhythmic Compression on Forelimbs of Rats

Forelimb-immobilized rats were anesthetized and laid at a supine position with their shoulder joints extended, so that their facies palmaris faced upward. Remote conditioning was applied by a pair of vertically placed weight units combined with 3-mm-diameter cylindrical rubbers. Remote conditioning magnitudes depended on the quality of the weight unit, totaling 150 g. The target surface was located on the anterior forelimb, 3 mm above to the wrist joint, between the ulnar and radial sutures. The rat forepaws remained ruddy after rhythmic compression. Remote conditioning was repeated using 5 min of tissue compression and 5 min of relaxation for 3 cycles daily for 3 days (**Figures 1A,B**).

Experimental Groups

All rats were randomly divided into the following groups: *Sham group*—Rats were subjected to identical surgical procedures, except for LAD ligation. *Model control group (CON)*—Rats were subjected to identical surgical procedures, which include LAD ligation. *Remote conditioning by rhythmic compression (RCRC) group*—Rats were subjected to LAD ligation plus 3 days of RCRC. *RCRC + SCH group*—Identical to RCRC group + administration of selective A2a antagonist SCH 58261(1 mg/kg/d, i.p.). *RCRC + MRS group*—Identical to RCRC group + administration of selective A2b antagonist MRS 1754 (1 mg/kg/d, i.p.). BAY + CGS group—Identical to CON group + selective A2b agonist BAY 60-6583 (1 mg/kg/d, i.v.) + selective A2a agonist CGS 21680 (0.5 mg/kg/d, i.v.). The dose of A2 receptor antagonists and agonist used here was based on the previous *in vivo* study (15–17).

Echocardiography in Rats

On the day of LAD ligation and after 3 days of remote conditioning, rats were maintained anesthesia with 2% isoflurane and fixed in a spine position. M-mode echocardiograph was performed using a small animal ultrasound (Esaote, Italy) on the long axis of the left ventricle. Left ventricular end-diastolic and end-systolic diameters (LVEDds and LVESds), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS) were recorded.

Measurement of Infarct Size

After 3 days of remote conditioning, triphenyltetrazolium chloride (Sigma-Aldrich) was stained to the heart, and the infarct size was measured. Briefly, after washing out the remaining blood, the heat below the ligature was cut into 5 pieces and stained with 1% triphenyltetrazolium chloride at 37°C for 15 min. The living aera is red, and the infarct area is stainless. The infarct area of the heart was calculated using digital planimetry software (ImageJ 2.1).

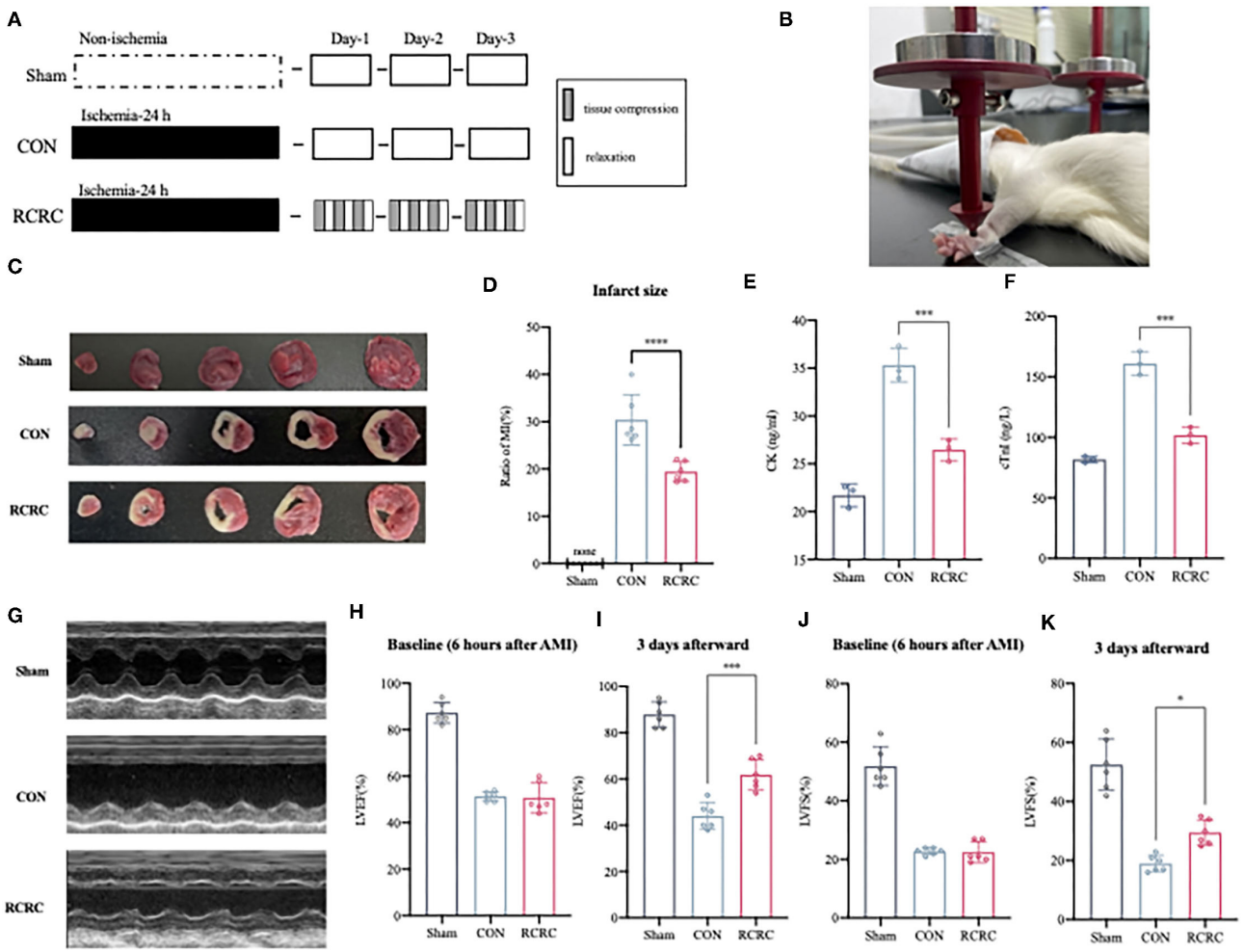

FIGURE 1 | Remote conditioning by rhythmic compression decreases infarct size and ameliorates ventricular dysfunction in a rat model of AMI. **(A)** Schematic showing the overall animal study design used to test the cardioprotection of remote conditioning in a rat model of AMI. **(B)** Presentation of remote conditioning on forelimbs of rats. **(C,D)** TTC staining representing infarct area on multiples slices of an infarct rat heart and quantitative analyses of infarct size after 3 days of remote conditioning, $N = 6$. **(E,F)** Serum concentration of CK and cTnI of each group were evaluated after 3 days of remote conditioning, $N = 3$. **(G–K)** Representative M-mode echocardiographic images showing LV wall motion of the hearts and LVEF, LVFS measured by echocardiography at 6 h (baseline) and 3 days (endpoint) after MI, $N = 6$. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. CON.

Enzyme-Linked Immunosorbent Assay

The serum levels of creatine kinase (CK), cardiac troponin I (cTnI), and cardiac levels of interleukin 1 beta (IL-1β), TNF-α, adenosine, and cyclic adenosine monophosphate (cAMP) were determined using enzyme-linked immunosorbent assay (ELISA) kit (Jin Yibai Biological Technology Co. Ltd. China), according to the manufacturer's protocol.

Western Blot

Radioimmunoprecipitation assay (RIPA) protein lysate (Beyotime, China) was used to extract the total protein in myocardial tissues. BCA protein assay (Thermo Fisher, USA) was performed using supernatants to determine the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat milk for 2 h, the membranes were incubated with primary antibodies (Affinity Biosciences, USA) overnight at 4°C. On the next day, the membranes were incubated with secondary antibodies (Proteintech Group, USA) for 2 h. β-Actin was used as the loading control.

Quantitative Real-Time Polymerase Chain Reaction

Total RNAs in myocardial tissues were extracted using TRIzol (Thermo scientific, USA). After reverse transcription into cDNA, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to amplify the target genes. The mRNA expressions of A2a and A2b receptors were determined through Lightcycle 96 real-time-PCR system (Roche, Germany) with GAPDH as an internal control. Each sample was analyzed 3 times and the

relative expressions of genes were determined using the $2^{-\Delta\Delta CT}$ method. The nucleotide sequences of primers used are shown in **Supplementary Table 1**.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 software. Quantitative data were represented as mean ± standard deviation ($\bar{X} \pm S$). Student's *t*-test and/or one-way analysis of variance with multiple comparison posttest (Bonferroni) was used to compare the means between experimental groups as indicated. If the data were not normally distributed, the Kruskal–Wallis H method was used for group comparisons. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Remote Conditioning by Rhythmic Compression Decreases Infract Size and Improves Cardiac Function in AMI Rats

To determine whether RCRC decreases infract size and improves cardiac function, rats were exposed to forelimb RCRC or CON. At 24 h post-ischemia, rats were subjected to 30-min remote conditioning (**Figures 1A,B**). As shown in **Figures 1C,D**, the infarct size was smaller in the RCRC group (19.44 ± 2.104%) than in the CON group (30.42 ± 5.331%). CK and cTnI are the important markers of AMI, so we detected serum CK and cTnI activity. Serum CK and cTnI activity was significantly increased at 3 days post-AMI that was decreased in RCRC group compared with the CON (**Figures 1E,F**). Therefore, remote conditioning displayed cardioprotective effect against ischemic injury. Additionally, we evaluated cardiac function by echocardiography as shown in **Figures 1G–K**. The baseline LVEFs and LVFSs were both similar for both CON and RCRC groups, which indicated a similar degree of initial injury (**Figures 1H,J**). The LVEF and LVFS were significantly declined to 44.00 ± 5.692 and 19.00 ± 2.757%, respectively, in the CON group, while they were increased to 61.83 ± 6.494 and 29.50 ± 4.231%, respectively, in the RCRC group (**Figures 1I,K**). The results strongly indicated that remote conditioning attenuates the deterioration of left ventricular function in AMI.

Remote Conditioning by Rhythmic Compression Abates the Inflammatory Response in the Hearts of AMI Rats

The growing evidence suggests that accentuation, prolongation, or expansion of the postinfarction inflammatory response result in worse remodeling and dysfunction after myocardial infarction (18). NF-κB is well known to be a key regulator of several kinds of inflammatory response, including postinfarction inflammation (19). Based on the observation that remote conditioning could protect myocardium against AMI, investigation of the NF-κB may help us to further explain the underlying mechanisms. Western blot analysis indicated that remote conditioning could significantly reduce NF-κB p65 phosphorylation induced by AMI (**Figures 2C,D**). A reduction in NF-κB activation suppresses the expression of various genes, which include TNF-α and IL-1β (20). In this study, we also found remote conditioning markedly dampened the cardiac expression of TNF-α and IL-1β, which were decreased by 42.4 and 21.57% at the day 3 (**Figures 2A,B**), respectively. These results confirmed that remote conditioning inhibits the postinfarction inflammatory response by inhibiting NF-κB-mediated expression of inflammatory cytokines.

Remote Conditioning by Rhythmic Compression Induces Serum and Cardiac Adenosine Levels

In response to all remote stimuli, there is humoral signal transfer to the target organ heart. Having established the phenomenon that remote conditioning inhibited the postinfarction inflammatory response and protected heart against AMI, we next asked whether remote conditioning induces the release of substances into the bloodstream that reach the heart to exert cardioprotective effect. Since adenosine has the potential to contribute to humoral transfer of remote conditioning and promotes tissue repair and regeneration with suppression of inflammation (21, 22), rats were exposed to sham, CON, and RCRC to determine whether remote conditioning regulates adenosine levels in the blood and heart. Serum and heart adenosine levels were measured after 3 days of remote conditioning (**Figure 3**). Remote conditioning applied by 5 min of tissue compression of 150 g weight unit every 10 min for a total of 30 min increased the serum adenosine levels (**Figure 3A**), while in the heart, remote conditioning also increased adenosine levels (**Figure 3B**), which suggested that upregulation of serum and cardiac adenosine levels may produce a strong cardioprotection.

Remote Conditioning by Rhythmic Compression Is Associated With Induction of Cardiac A2 Receptors Expression and Function

Considering the observation that adenosine-induced cardioprotection may be possibly related to decrease in inflammation (23), we first examined the expression of A2 adenosine receptors (AR) in the infarct myocardium after 3 days of remote conditioning, which were shown to decrease the levels of inflammatory mediators in the previous studies (24, 25). As shown in **Figure 4**, the message for A2a and A2b receptors was significantly increased in hearts of AMI rats treated with remote conditioning, which suggested the induction of A2 receptors in the hearts during remote conditioning.

Effect of Remote Conditioning by Rhythmic Compression on the cAMP/PKA Signaling Pathway in AMI Rats

A2a and A2b ARs are linked to Gαs proteins, which upregulate cAMP expression (22). As we know, cAMP elevation leads to the activation of serine kinase PKA, which phosphorylates the transcription factor CREB on S133 (26). CREB phosphorylation in the infarct zone of hearts was examined after 3 days of remote conditioning to determine the PKA activity. The phosphorylation signal was significantly increased

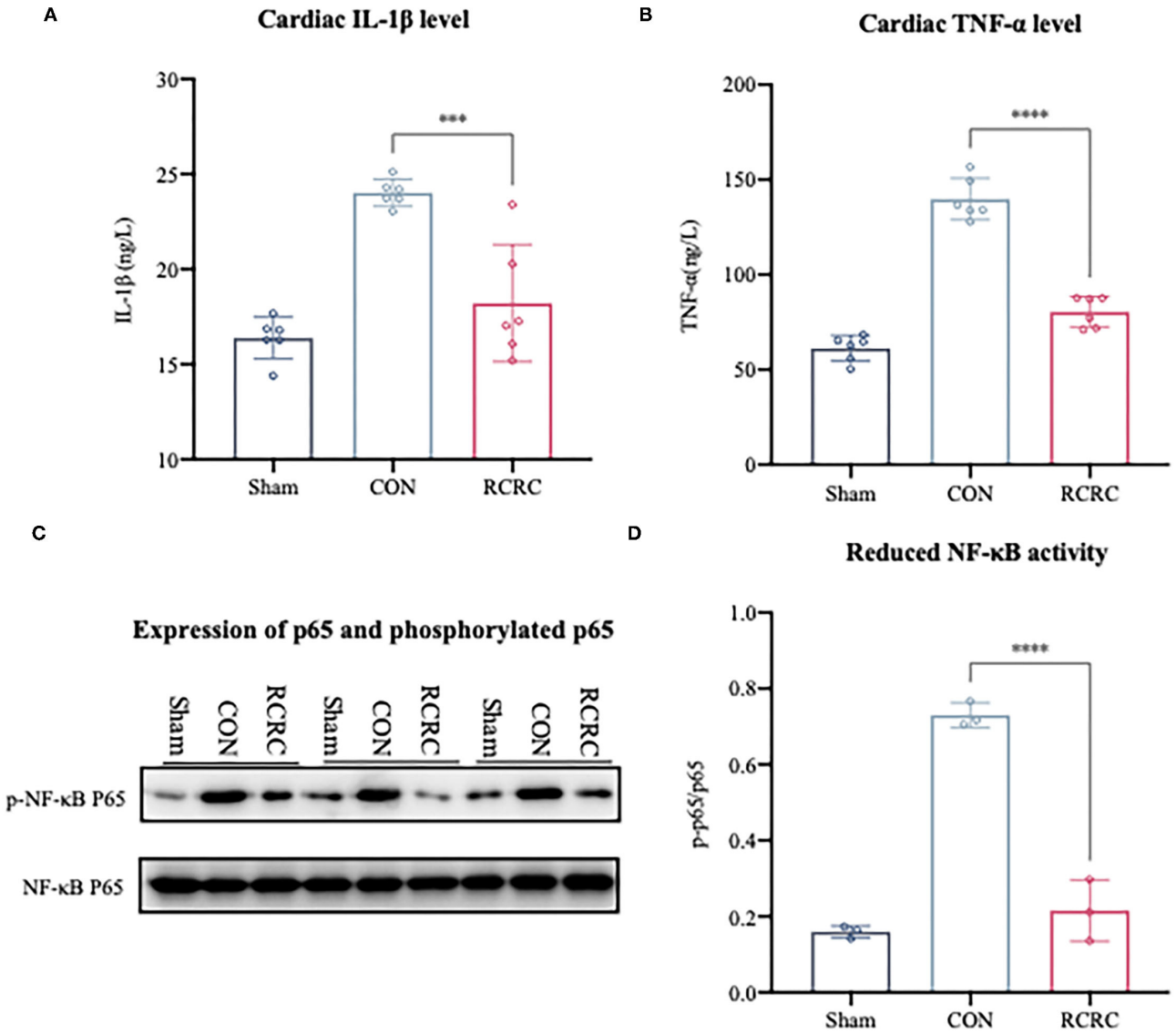

FIGURE 2 | Remote conditioning by rhythmic compression abates the inflammatory response in the hearts of AMI rats. **(A,B)** The concentrations of IL-1β and TNF-α in the heart were determined by ELISA, $N = 6$. **(C,D)** The levels of phosphorylated p65 (p-p65) and total p65 in heart were detected by western blot. The corresponding quantification data of p-p65 to p65 ratio are shown in right panel. All values represent as means ± SD. $N = 3$. ***$p < 0.001$, ****$p < 0.0001$ vs. CON.

in RCRC group (**Figures 5B,C**), and this increment was associated with a rise in cAMP production (**Figure 5A**), which suggested that remote conditioning is linked with cAMP/PKA signaling pathway.

The A2 Receptors Are Responsible for the Cardioprotective Effects of Remote Conditioning by Rhythmic Compression

To determine whether A2 receptors are critical for the cardioprotective effect of RCC, we then evaluated the impact of A2a and A2b receptors' blockage and activation on the cardioprotection of remote conditioning. A schematic diagram of the protocol is shown in **Figure 6A**. Combination treatment with remote conditioning and the selective A2a antagonist SCH 58261 or the selective A2b antagonist MRS 1754 reversed the anti-infarct effect compared with that in AMI rats treated with remote conditioning alone (**Figures 6B,C**), which indicated that both A2a and A2b receptors are involved in the action of remote conditioning. Based on the observation, we then treated AMI rats by combining the selective A2a agonist CGS 21680 and the selective A2b agonist BAY 60-6580, which reduced infarct size to 21.88 ± 1.288%, which was similar to remote conditioning (19.44 ± 2.104%), which implies that simultaneous activation of A2a and A2b receptors may produce a robust cardioprotection. The baseline (6 h after AMI) and

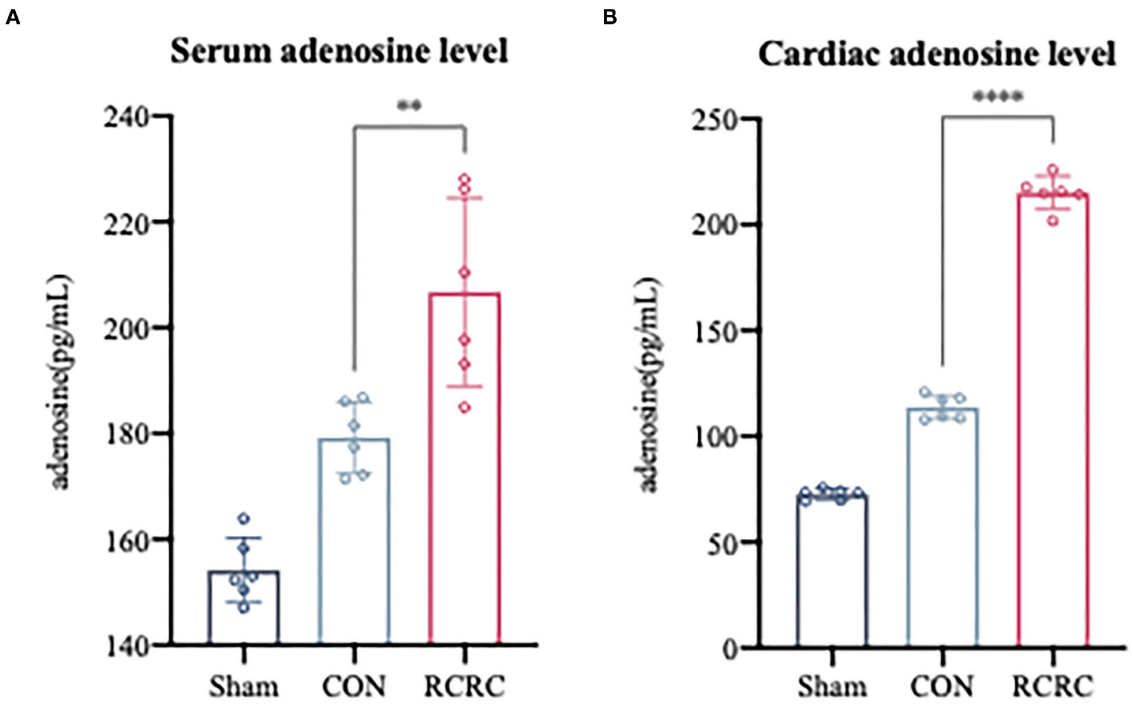

FIGURE 3 | Remote conditioning by rhythmic compression increases serum and myocardial adenosine concentrations in a rat model of AMI. **(A)** Serum adenosine concentration after 3 days of remote conditioning, $N = 6$. **(B)** Adenosine concentration in infarct zone after 3 days of remote conditioning. All values represent as means ± SD. $N = 6$. $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. CON.

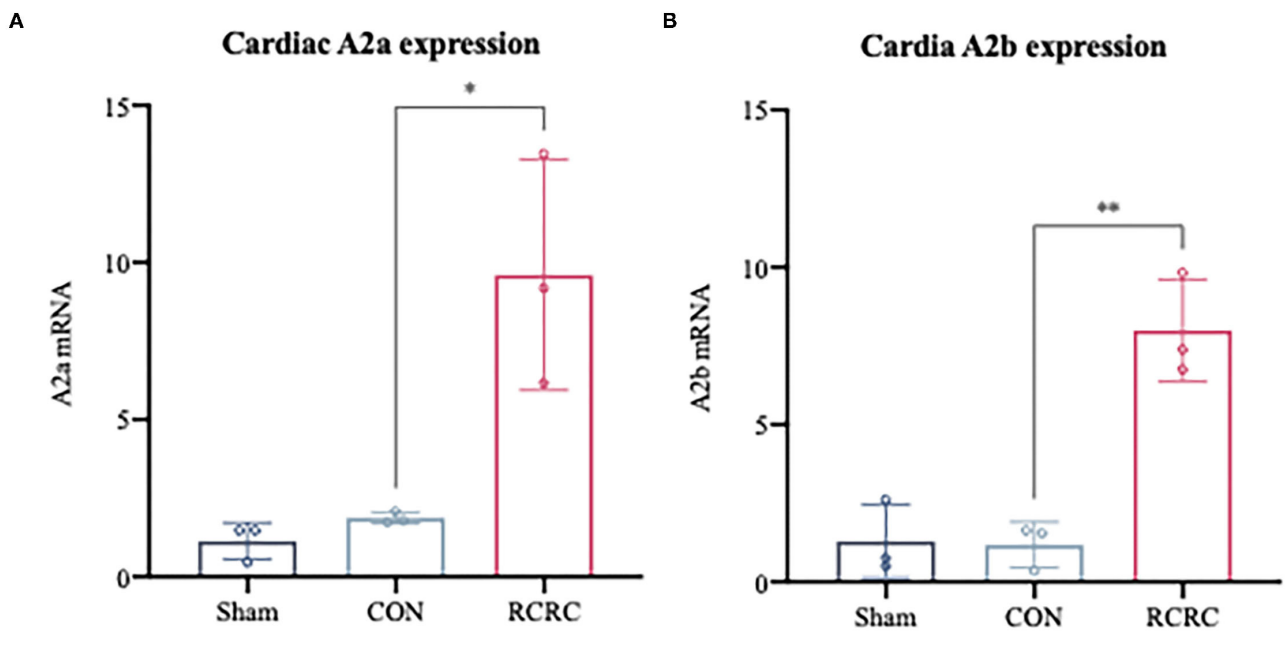

FIGURE 4 | A2 adenosine receptors mRNA expression in infarct myocardium after treatment with 3 days of remote conditioning. **(A)** Relative A2a AR mRNA levels were determined by RT-PCR after treatment with 3 days of remote conditioning, $N = 3$. **(B)** Relative A2b AR mRNA levels were determined by RT-PCR after treatment with 3 days of remote conditioning. All values represent as means ± SD. $N = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. CON.

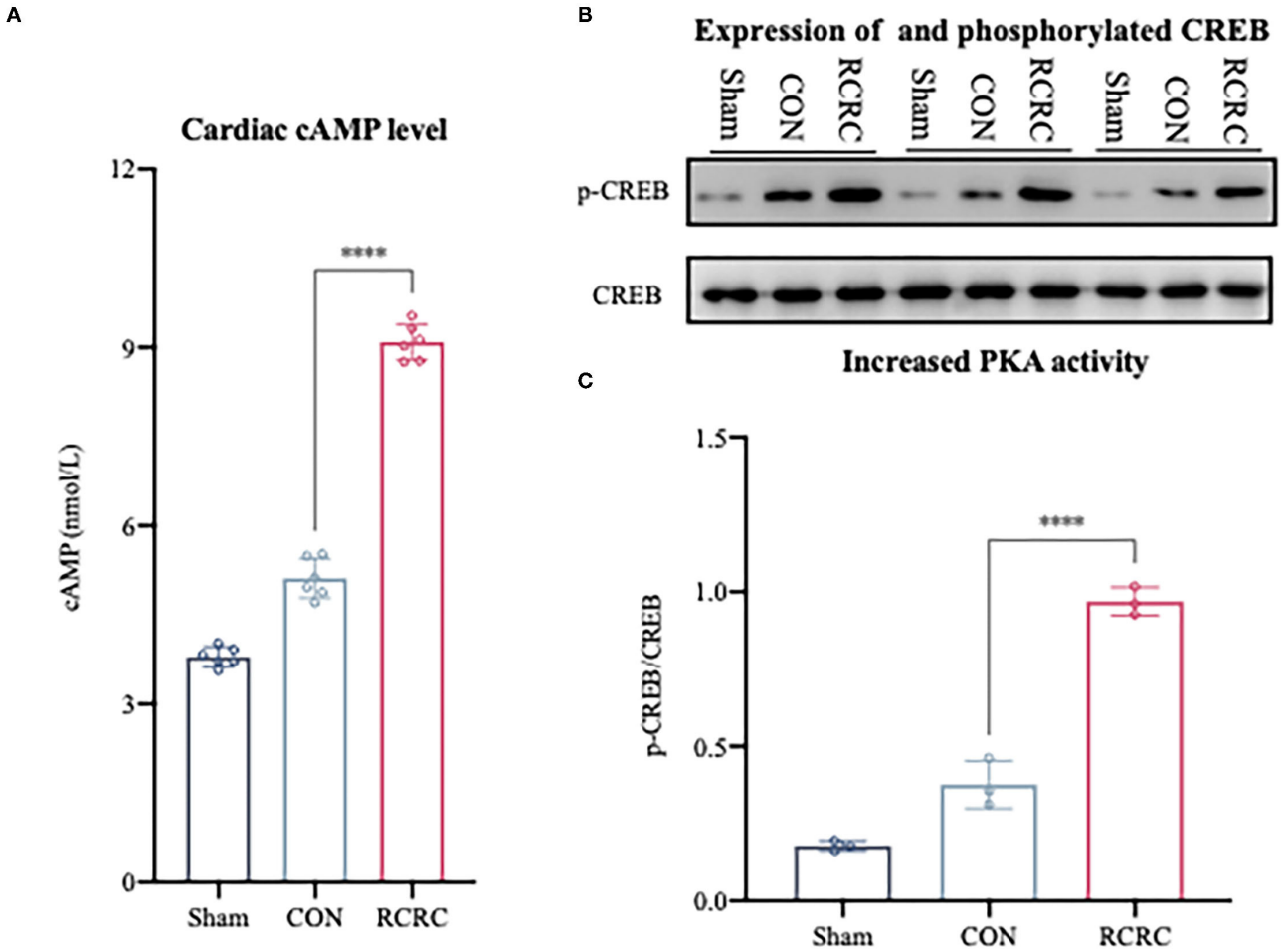

FIGURE 5 | Remote conditioning by rhythmic compression reduced cAMP content and PKA activity in heart of AMI rats. **(A)** Levels of cAMP in the heart of AMI rat were determined by ELISA, $N = 6$. **(B,C)** Levels of phosphorylated CREB (p-CREB) and total CREB in heart were detected by western blot. The corresponding quantification data of p-CREB to CREB ratio are shown in right panel. All values represent as means ± SD. $N = 6$. ****$p < 0.0001$ vs. CON.

endpoint (3 days afterward) LVEFs and LVFSs were measured as the indicators of cardiac functions in all groups. The baseline LVEFs and LVFSs were comparable for all the groups, which suggested a similar degree of initial ischemia injury (**Supplementary Figure 1**). Likewise, combined application of remote conditioning and SCH or MRS exhibited smaller LVEFs and LVFSs than those from the only remote conditioning group at the 3rd day (**Figures 6D,E,G**). However, CGS combined with BAY show a rise LVEFs and LVFSs, an effect that was equipotent with remote conditioning (**Figures 6D,E,G**). We also calculated treatment effect, that is, the changes of LVEFS and LVFSs from the baseline to the endpoint. While the RCRC + SCH group and RCRC + MRS group displayed a functional decline, CGS + BAY group preserved cardiac function (**Figures 6D,F,H**). Thus, these results suggest that A2a and A2b receptors are indispensable parts for cardioprotection of remote conditioning.

Activation/Inhibition of A2 Receptors Affects Remote Conditioning-Regulated Release of Pro-inflammatory Factor Following Myocardial Ischemia Injury

To further determine the causal relationship between remote conditioning and A2 receptors, we also identified the downstream signal pathway following antagonization and inhibition of A2 receptor. As shown in **Figures 7A–C**, treatment with the selective A2a antagonist SCH 58261 or the selective A2b antagonist MRS 1754 reduced cAMP expression and PKA activity caused by remote conditioning considerably, which suggested that remote conditioning plays its role through A2 receptors. Meanwhile, the selective A2a agonist CGS 21680 and the selective A2b agonist BAY 60-6580 mimicked the cAMP expression and PKA activity of remote conditioning,

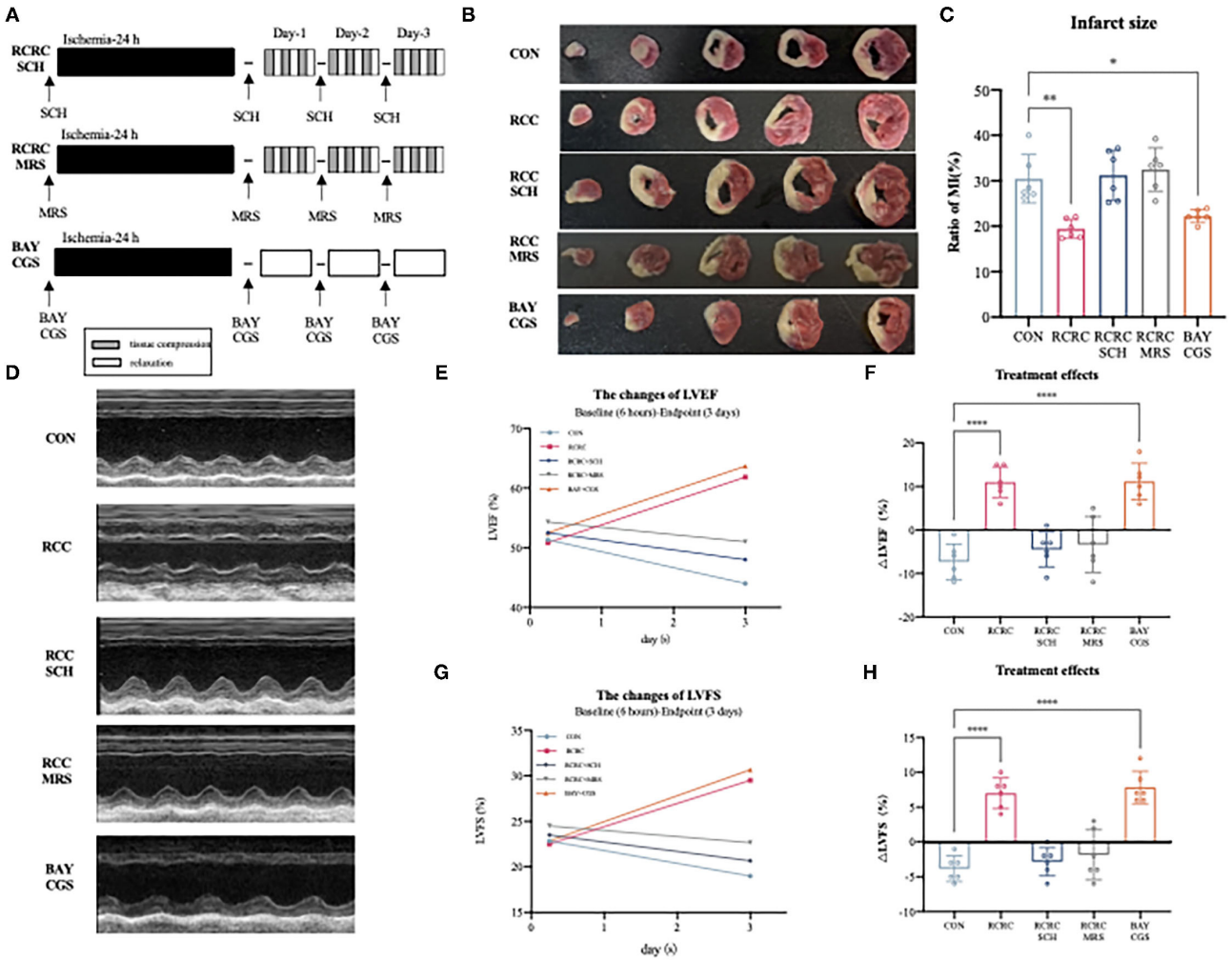

FIGURE 6 | Cardioprotection of remote conditioning by rhythmic compression is lost in SCH or MRS treated rats and mimicked by BAY and CGS treatment. **(A)** Schematic diagram of the experimental design. RCRC group were treated with the selective A2a antagonist SCH 58261, the selective A2b antagonist MRS 1706; CON group treated with the selective A2a agonist CGS21680 and the selective agonist A2b agonist BAY 60-6583. **(B,C)** TTC staining representing infarct area on multiples slices of an infarct rat heart and quantitative analyses of infarct size after 3 days of remote conditioning in combination with A2a and A2b receptor blockage and activation, $N = 6$. **(D–H)** Representative M-mode echocardiographic images showing LV wall motion of the hearts, the treatment effect was calculated as the changes of LVEFs and LVFSs from baseline to endpoint in combination with A2a and A2b receptor blockage and activation. All values represent as means ± SD. $N = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs. CON.

lending weight to the preceding deduction. These results demonstrate the functional activation of A2 receptors by remote conditioning.

In addition, treatment with either SCH 58261 or the MRS 1754 significantly blocked the anti-inflammatory action of remote conditioning as evidenced by the decreased expression of TNF-α, IL-1β, and NF-κB p65 phosphorylation, although this was not observed with A2 agonists (**Figures 7D–F**). According to the above study, the A2 ARs are involved in mediating cAMP/PKA signaling pathways that inhibit NF-κB-mediated expression of inflammatory cytokines. In summary, remote conditioning attenuated MI-induced inflammation and myocardial injury *via* A2 receptors.

DISCUSSION

A growing body of evidence points the cardioprotective effect of RIC and related signal transduction pathways (27). However, there is currently nothing known about the cardioprotection of mechanical tissue compression, which is involved in composing RIC. In this study, we first report to our knowledge, the cardioprotective effects of RCRC on forelimbs. We demonstrated that remote conditioning reduces infarct size and attenuates left ventricular dysfunction, by decreasing postinfarction inflammatory response. Then, we showed that remote conditioning increased plasma and cardiac adenosine levels after 3 days of remote conditioning. Meanwhile, remote

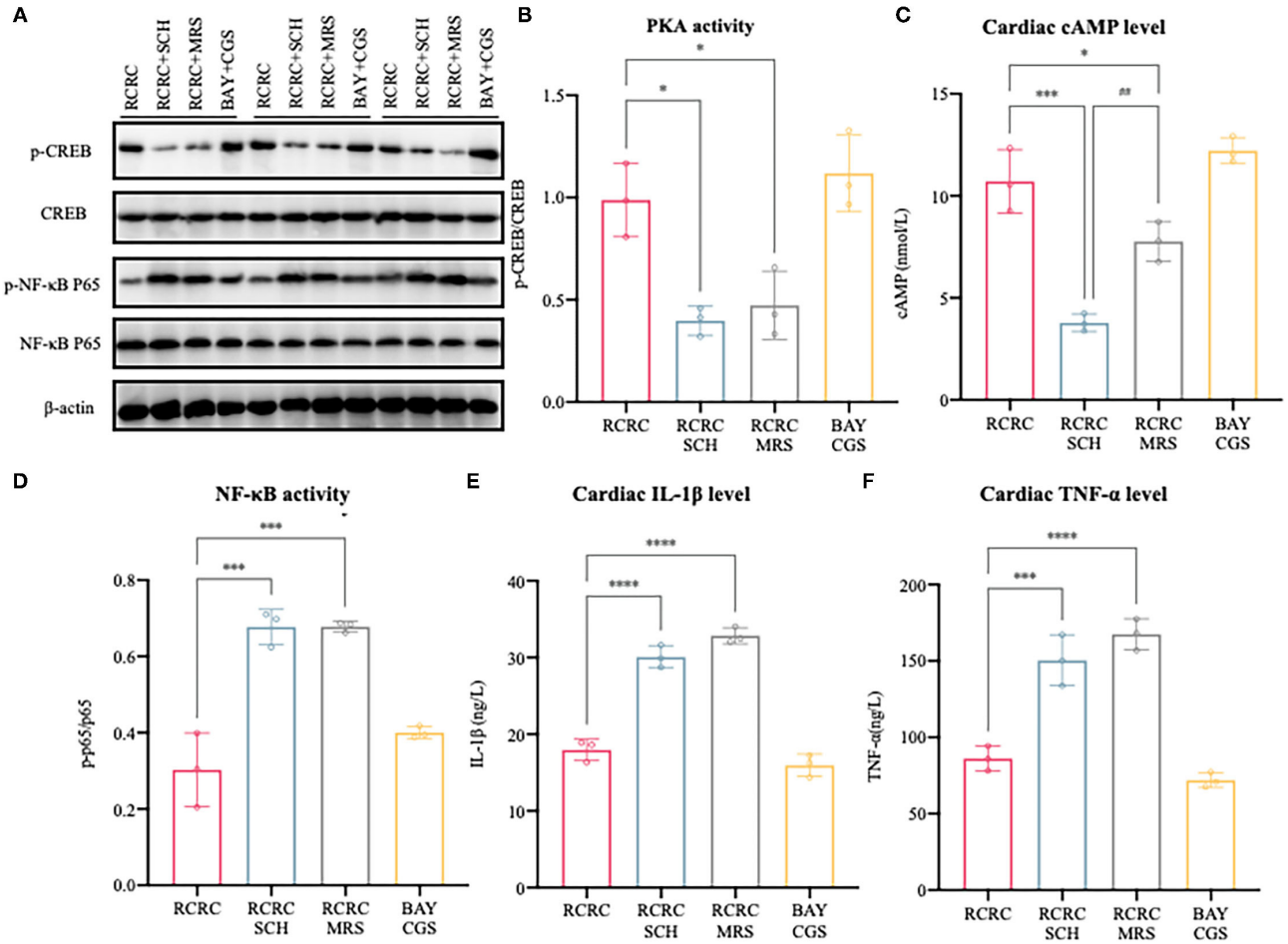

FIGURE 7 | Activation/inhibition of A2 receptors affects remote conditioning-regulated release of pro-inflammatory factor following myocardial ischemia injury. **(A)** Western blot was performed to determine p-CREB, CREB, p-NF-κB p65, and NF-κB p65. **(B)** Quantitative analysis of p-CREB and CREB expression and calculate the ratio of p-CREB/CREB, $N = 3$. **(C)** Levels of cAMP in the heart of AMI rat were determined by ELISA, $N = 3$. **(D)** Quantitative analysis of p-NF-κB p65 and NF-κB p65 expression and calculate the ratio of p-p65/p65, $N = 3$. **(E,F)** The concentrations of IL-1β and TNF-α in the heart were determined by ELISA, $N = 3$. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. RCRC; $^{\#\#}p < 0.01$ vs. RCRC+SCH, $^{****}p < 0.0001$.

conditioning also prompted the expression of cardiac A2 receptors and its subsequent cAMP/PKA/CREB signaling pathway. With the background, we shed light on the importance of A2 receptors on the cardioprotective effect of remote conditioning. The results showed that the inhibition of either A2a or A2b receptor alone abolished the cardioprotection of remote conditioning and simultaneous stimulation of both A2 receptor subtypes contributed to a robust cardioprotective effect against AMI, which collectively demonstrated that A2a and A2b receptors work in concert to confer cardioprotection of remote conditioning. Thus, our study has demonstrated for the first time that remote conditioning confers cardioprotection by upregulating levels of adenosine, as a humoral factor and activating cAMP/PKA/CREB signaling pathway through A2 receptors in the heart.

Remote ischemic conditioning by brief episodes of ischemia with reperfusion on an extremity displayed a robust reduction of infarct size and improvement of the prognosis in patients with AMI (3, 28). However, accumulating evidence suggests that transient ischemia is not a requisite trigger for remote cardioprotection (29). RIC is now considered as a systemic response, which could be mimicked by various stimuli (3). For instance, trauma by transverse abdominal skin incision, partly a mechanical stimulus, contributes to a myocardial infarct-limiting effect in rodents and dogs (30–32). In fact, RIC is a stimulus compound containing interruption of blood vessel, edema, and tissue compression. In this study, we separated the above factors and only focused on the tissue compression, to extend into the novel concept of remote protection in AMI. Our results presented for the first time that tissue compression, a form of mechanical stimulus involved in RIC (33), also showed cardioprotective effect against AMI.

Myocardial infarction induces an inflammatory response that removes the wound from dead cells and matrix debris, while

prolonged or expanded inflammation would contribute to the adverse outcome (18). To find out its cause, inflammation to injury is evolved to protect organisms against infectious pathogens, which may be excessive for the delicate requirements of the ischemic myocardium. In other words, cardiac repair is dependent on a terrifically orchestrated inflammatory reaction. Accumulating evidence suggests that appropriate and timely containment and resolution of inflammatory response are the determinants of the quality of cardiac repair (34, 35). As previously described, mechanical stimuli, such as massage, are generally recognized to be beneficial for reducing inflammation, particularly reflecting on tempering the increase of pro-inflammatory mediators and inflammatory cell infiltration (6, 36). As an important upstream regulator of the inflammation, activated NF-κB upregulates the transcription of target genes, for example, TNF-α and IL-1β, which are linked to the progression and prognosis of AMI (37). This study showed that remote conditioning inhibited the activation of NF-κB and lowered the expression of pro-inflammatory cytokines (TNF-α, IL-1β), which may help to ameliorate the susceptibility of AMI.

It is proposed that regulation of the immune responses needs at least two "danger" signals. Aside from the first danger signal leading to the activation of immune cells, there must be a second danger signal, which could downregulate the inflammation to protect against the excessive collateral damage and the destruction of normal tissues (13). Extracellular ATP accumulation frequently induces inflammation, which reflects metabolic alterations caused by cellular stress (38). The hydrolysis of ATP to adenosine, which represents such earliest "second danger signals," has immunosuppressive action on immune cells and hence protects tissues from excessive inflammation (13, 38). In fact, adenosine is a purine nucleoside that is broadly distributed in a variety of tissues and body fluids and plays a vital role in different physiological and pathological conditions (23, 39). Here, we demonstrated that remote conditioning induced adenosine liberate into the blood and increased the concentration of it in the heart, for defining the signal transduction of remote conditioning from the trigger limbs to heart.

Extracellular adenosine may, in a way, fulfill its supposed role as a "retaliatory" metabolite in protecting the heart and other tissues from, for example, ischemia damage as the first signal of danger that reports excessive immune damage to normal tissues by hyperactive immune cells (40). It has been reported that adenosine may be associated with ischemic conditioning and its release could confer cardioprotective effect (41). In mice, adenosine levels in arterial plasma were increased after persistent brain ischemia by bilateral ligation of the internal carotid arteries, and this increment was subsequently linked to infarct-limiting effect in isolated perfused mice hearts (42). It is also documented that exogenous of adenosine mimicked the cardioprotective effects of ischemic and remote preconditioning (43). Based on the previous findings and this study, we proposed that adenosine is the key humoral factor of remote cardioprotection, which could act as a "reporter" of metabolic changes.

Adenosine produces its pharmacological effects mainly through its interaction with AR: A1, A2a, A2b, A3, which are all members of the G-protein-coupled receptors family (44). A1 and A3 ARs are coupled to Gi/o, through which they reduce cAMP levels, while Gs protein coupled A2a and A2b ARs stimulate adenyl cyclase (AC) and cause accumulation of intracellular cAMP, which possesses immunosuppressive effects (13). In this study, we demonstrated that remote conditioning increases cardiac cAMP production, which suggested the activation of A2 receptors from a functional perspective. Follow this approach, we detected the expression of A2 receptors in the heart and witnessed a rise in the levels of A2 receptors, which could act as "sensors" of metabolic changes.

From this point, it is necessary to determine whether A2 receptors are indispensable parts of cardioprotection during remote conditioning. Interestingly, the activation of either A2a or A2b receptor alone was unable to decrease infarct size in the $CD73^{-/-}$ mice, which could not produce large amounts of adenosine (45). In this study, we showed that coadministration of remote conditioning and the selective A2a antagonist SCH or the selective A2b antagonist MRS led to the loss of infarct-limiting effect and cardiac functions. Meanwhile, the combination of the selective A2a agonist CGS and the selective A2b agonist BAY reduced infarct size and improved cardiac functions. These results clearly indicated that simultaneous stimulation of A2a and A2b ARs is critically important for the cardioprotection of remote conditioning, as revealed in the preliminary evidence (14, 46). But why both A2a and A2b receptors are required to produce cardioprotective effect? The most obvious reason is that both receptors are coupled to Gs protein and share the mutual downstream pathway. One possible answer may be that A2a receptor has high affinity and A2b receptor has low affinity of adenosine, which enables the graded escalation of inhibitory signals (13). More specifically, it is the accumulation of adenosine and stepwise recruitment of A2a and then of the A2b that guarantee the option of partially retarding immune cells to continue pathogen destruction but with less tissue damage (13). In this study, although cAMP expression was reduced by treatment with both A2 antagonists, A2a antagonist reduced it more than A2b antagonist (**Figure 7C**). The part of the reasons might be that A2a receptor signaling can contribute to A2b expression (47). In addition, A2a receptor may play a part in both transduction and reinforcement of the cardioprotective signal from A2b receptors (14). Our subsequent study should investigate these findings.

As mentioned above, remote conditioning inhibited the activation of NF-κB and lowered the levels of TNF-α and IL-1β, which is in line with the anti-inflammatory properties of A2 receptors. More generally, A2a receptor could produce a protective function through transforming macrophage form inflammatory to angiogenic phenotypes (48). It has been also shown that A2b cardioprotective effect may be bound up with the adjustment of TNF-α and neutrophil function (49). In this study, we demonstrated that remote conditioning induced the PKA activity, which is consistent with a rise in cAMP production. In fact, the activity of NF-κB is modulated by cAMP/PKA/NF-κB axis, in which PKA activation could inhibit the activation of NF-κB (50). Therefore, cAMP-dependent PKA phosphorylates and activates CREB, thereby inhibiting the activation of NF-κB

and reducing the production of TNF-α and IL-1β, which may be the mechanism beneath the cardioprotective effect of remote conditioning *via* A2a receptors.

In conclusion, we identified remote conditioning as a novel cardioprotective intervention in a murine model of AMI. This protection is induced, at least partially, by its anti-inflammatory actions in acute ischemic cardiac tissue damage. The signal transduction of remote conditioning from the trigger limb to heart involves the release of adenosine, which activated the synergistic reaction of A2a and A2b receptors, which are indispensable parts for cardioprotection of remote conditioning. Subsequently, A2 receptors activate cAMP/PKA/NF-κB axis to exert anti-inflammatory properties, which in turn contribute to cardioprotection. Our results established a new connection between remote tissue compression and cardiovascular diseases, which enhances our cognition about the role of tissue compression on RIC cardioprotection. These findings may provide a cue to developing the potential of remote conditioning as an immunomodulation procedure for cardioprotection and harnessing its effect toward a novel therapy.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Care and Use Committee of Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

SX, RG, HZ, and YG designed the study. SX and RG performed experiments. XB, XX, XFX, YL, and SL performed the measurements, collected, and analyzed the data. SX wrote the manuscript. All authors approved the final version of manuscript submitted.

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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.2021.723332/full#supplementary-material

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